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Insect growth regulator effects of azadirachtin and neem oil on survivorship, development and fecundity of *Aphis glycines* (Homoptera: Aphididae) and its predator, *Harmonia axyridis* (Coleoptera: Coccinellidae)

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Abstract

BACKGROUND: *Aphis glycines* Matsumura, an invasive insect pest in North American soybeans, is fed upon by a key biological control agent, *Harmonia axyridis* Pallas. Although biological control is preferentially relied upon to suppress insect pests in organic agriculture, approved insecticides, such as neem, are periodically utilized to reduce damaging pest populations. The authors evaluated direct spray treatments of two neem formulations, azadirachtin and neem seed oil, under controlled conditions for effects on survivorship, development time and fecundity in *A. glycines* and *H. axyridis*.

RESULTS: Both azadirachtin and neem seed oil significantly increased aphid nymphal mortality (80 and 77% respectively) while significantly increasing development time of those surviving to adulthood. First-instar *H. axyridis* survival to adulthood was also significantly reduced by both neem formulations, while only azadirachtin reduced third-instar survivorship. Azadirachtin increased *H. axyridis* development time to adult when applied to both instars, while neem oil only increased time to adult when applied to first instar. Neither neem formulation affected the fecundity of either insect.

CONCLUSIONS: Results are discussed within the context of future laboratory and field studies aimed at clarifying if neem-derived insecticides can be effectively integrated with biological control for soybean aphid management in organic soybeans.

Keywords: organic agriculture; neem; soybeans; *Aphis glycines*; *Harmonia axyridis*; insect growth regulator

1 INTRODUCTION

1.1 Soybean aphid and multicolored Asian lady beetle

The soybean aphid, *Aphis glycines* Matsumura, is an exotic pest in the United States. First detected in Wisconsin in 2000 and rapidly spreading to 21 states and three Canadian provinces, the aphid has become established as an economically important pest of soybeans, *Glycine max* (L.) Merr.[1] Soybean aphid is native to the temperate regions of Asia, where it is suppressed by numerous parasitoids and predators.[2] In China, coccinellids are thought to play a key role in suppression of soybean aphid populations owing to high predation rates and abundance in soybean fields.[3] The multicolored Asian lady beetle, *Harmonia axyridis* Pallas, is one of the most abundant of these coccinellids in both the USA and China.[4] Although *H. axyridis* provides a suppressive effect on soybean aphid populations in soybean fields, aphid outbreaks may still cause economic damage, even in the aphid's native range.[5]

Harmonia axyridis only responds reproductively after aphid prey density exceeds 100 per plant.[6] At these densities, *H. axyridis* actively begins to suppress the aphid population. However, the timing of this suppressive effect may not reduce soybean aphid densities to a level that would prevent economic yield loss. Periodic soybean aphid outbreaks require growers to employ pest suppressive tactics, such as foliar insecticides, to prevent or minimize economic yield loss.[5,7]

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1.2 Arthropod pest management in organic agriculture

Arthropod pest management in organic farming emphasizes a systems approach,[8,9] using multiple and varied tactics to prevent damaging pest levels and minimize the need for curative measures.[10] Tactics including, but not limited to, pest-resistant or -tolerant crop plant selection, crop rotation, soil management and biological control are incorporated into the organic cropping system design.[10,11] Only when a combination of these tactics has failed to keep pests below economically damaging levels can National Organic Program (NOP)-approved insecticides be utilized.[9,11] Because of reliance on multiple arthropod pest management tactics, it is important to ensure that insecticide use is compatible with other strategies and minimizes non-target effects on key biological control agents.

1.3 Neem-derived insecticides

Seed extracts of the neem tree, *Azadirachta indica* A Juss, have been used for centuries as a botanical insecticide.[12] Neem-derived insecticides are approved for use in organic agriculture.[13] Depending on target insect species and life stage, effects of neem include antifeedant behavior, adult sterility and insect growth regulation. Antifeedant effects vary among species, but insect growth disruption and adult sterility are more consistent.[14] Neem-derived insecticides have been shown to be effective against hundreds of pest species including members of Diptera, Lepidoptera and Hemiptera, which includes aphids.[12] Azadirachtin is considered the main biologically active component of neem-derived insecticides, although there are several other limonoids present in complete neem seed oil that show similar insecticidal properties.

Both purified azadirachtin and complete neem oil have been proven effective in causing aphid mortality. Brown citrus aphids, *Toxoptera citricida* (Kirklady), were fed citrus leaves dipped in azadirachtin, resulting in increased adult and nymph mortality, reduced number of nymphal molts and decreased adult fecundity at all tested concentrations.[15] Spraying azadirachtin onto *T. citricida* on potted citrus plants also significantly reduced aphids, while the water-treated control population increased. Systemic applications of azadirachtin to rape plants, *Brassica napus* subsp. *napus* L., significantly reduced cabbage aphid, *Brevicoryne brassicae* (L.), nymphal longevity, increased nymphal mortality and reduced adult fecundity.[16] There is some evidence that complete neem seed oil may be more effective than purified azadirachtin. Neem seed oil caused 62% more pea aphid, *Acyrthosiphon pisum* Harris, nymphal mortality than purified azadirachtin when aphid nymphs were placed on treated plants.[17] The authors attributed this result to increased penetration of the insect cuticle by the oil and unique components of complete neem oil which affect insecticide efficacy. Additionally, a formulation containing complete neem oil was shown to be more effective than an azadirachtin formulation in causing adult mortality when treating fruit flies, *Ceratitus capitata* (Wiedemann).[18]

Neem-derived insecticides are regarded as generally compatible with insect natural enemy conservation.[10,19] Adult green lacewings, *Chrysoperla carnea* (Stephens), were provided azadirachtin-treated water with no effect on mortality or fertility of the insect.[20] Although there was a significant reduction in *C. carnea* fecundity, the effect was reversible upon discontinued ingestion of azadirachtin. When three braconid parasitoids of tephritid fruit flies were monitored for emergence from azadirachtin-treated fly puparia, no adverse effects were noted.[21] Likewise, azadirachtin had little impact on an aphidiid parasitoid, *Lysiphlebus testaceipes* (Cresson), of brown citrus aphid.[15] However, among the Coccinellidae, azadirachtin caused delayed pupation, wing deformation and creation of pupa–adult intermediates in *Coccinella septempunctata* L.[22] Conversely, *H. axyridis* adults were unaffected by low concentrations of azadirachtin used as a fungicide.[23]

These conflicting reports highlight the importance of testing neem-derived insecticides against not only the target pest insect but also key biological control agents. While neem-derived insecticides are commonly used in organic farming,[24] critical information is lacking on their efficacy against soybean aphid and potential non-target effects on coccinellid predators which may negatively affect biological control. In a previous study, a direct spray application of azadirachtin was found to be innocuous to multiple life stages of *A. glycines* and *H. axyridis* when the insects were monitored for mortality for a short term (72 h) post-treatment.[25] However, it has previously been shown that, when neem extracts were applied topically with a microsyringe to aphids, it took as many as 9 days for insect growth regulating effects to be detected.[26] Similar contact toxicity may likewise be detected with a direct spray to the insects. These effects may be expressed as immature insects attempt to molt into later stages of their life cycle. Furthermore, azadirachtin may be expected to have an effect on fecundity rather than mortality when treating adult stages of insects.[27]

1.4 Objectives

The objectives of this study were to assess the long-term effects of a direct spray application of an azadirachtin commercial insecticide formulation and complete neem seed oil against multiple life stages of *A. glycines* and *H. axyridis*. Mortality, development time and fecundity of survivors were recorded in order to address possible differential insect growth regulator effects by insect species and life stage.

2 MATERIALS AND METHODS

2.1 *Aphis glycines* colony and bioassay test units

An *A. glycines* colony was founded from individuals collected in summer 2005 from soybeans at Arlington Agricultural Research Station, University of Wisconsin-Madison, Arlington, WI. Aphids were maintained on soybean plants (variety 'Vinton 81'). Soybean plants were grown four plants per 15 cm pot in a 1:1 mix of 3M Metro Mix (Conrad Fafard, Inc., Agawam, MA) and sphagnum peat moss, with a pinch of *Bradyrhizobium japonicum* (Nitrogin, Inc., Brookfield, WI) inoculum added per pot. Plants were grown in a greenhouse, supplemented with grow lights to attain a 16:8 h light:dark photoperiod. Plants were fertilized weekly using 20-9-20 water-soluble fertilizer (Technigro, Bellevue, WA) and irrigated as needed. As aphid-infested plants began to decline, leaves were excised and placed on healthy plants. Once the aphids had migrated to healthy plants, dead plant material was removed.

Aphid adults were randomly collected from the colony using a fine-tip, No. 1 camel hair brush and placed on an excised aphid-free soybean leaflet, selected from the upper portion of an uninfested plant, with the underside of the leaf facing upwards, in a plastic petri dish (100×15 mm). The petiole of the leaflet was wrapped in moistened cotton and kept moist until the leaflet was discarded. Petri dishes were kept in a temperature-controlled chamber at $22 \pm 1\,^{\circ}C$ with a 16:8 h light:dark photoperiod. Nymphs produced by these adults were removed daily and combined randomly into groups of ten, and each group was placed on a soybean leaflet in a petri dish unit, identically to the adults. If the desired stage for experimentation was nymph, these groups were then used as the test unit for the bioassay. Thus, the bioassay was carried out on first instars. If the desired stage was cohort adults, these groups were raised to adults, being provided fresh leaflets every 2 days. On the day of the adult aphid bioassay, the groups of F1 adults were transferred to new petri dishes with fresh leaflets. Test units were kept in a temperature-controlled chamber under the aforementioned conditions until the time of spraying.

2.2 *Harmonia axyridis* colony and bioassay test units

The *H. axyridis* colony was founded from adults collected in the summer of 2005 at Arlington Agricultural Research Station, University of Wisconsin, Arlington, WI. Beetles were held in $61 \times 45 \times 47$ cm screened cages in a greenhouse supplemented with grow lights to attain a 16:8 h light:dark photoperiod. Cages also contained soybean plants infested with soybean aphids. Prior to experimentation, beetles were removed from cages and grouped into male/female pairs in petri dishes (100×15 mm). Dishes were kept in growth chambers at $25 \pm 1\,^{\circ}C$ and 16:8 h light:dark photoperiod. These beetles were provided an *ad libitum* supply of soybean aphid and frozen *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs (Beneficial Insectary, Redding, CA).

Petri dishes containing the male and female pairs were checked daily for egg masses. Eggs were transferred to a new petri dish (100×15 mm) and kept under the aforementioned conditions. Eggs were allowed to hatch, and larvae were reared in fresh petri dishes to the desired developmental stage on a diet of frozen *E. kuehniella* eggs.

The experiment was conducted on first and third instars, pupae and adults. Larvae and pupae were sprayed 24 ± 4 h after molting. Adults were sprayed 5–7 days after molting. Fifteen individuals of the larval stages or ten individuals of pupal or adult (1:1 sex ratio) stages were considered a replication. Pupae were removed from the substrate on which they had formed by using a razor blade. Replicate groups of larvae, pupae or adults were then placed into plastic petri dish bottoms (100×15 mm) for treatment application. Test units were kept in a temperature-controlled chamber at $25 \pm 1\,^{\circ}C$ and 16:8 h light:dark photoperiod until time of treatment.

2.3 Treatments

2.3.1 Neem-derived insecticides

Treatments included azadirachtin $45\,g\,L^{-1}$ EC (Neemix® 4.5 EC; Certis USA, Columbia, MD; 0.511 L ha^{-1} = 23 g AI ha^{-1}), neem seed oil (Ahimsa Botanicals, Bloomington, MN; 1% v:v) emulsified with organic castile soap (Dr Bronner's Soaps, Menomonee Falls, WI; 0.1% v:v) and a deionized water-only control. Neemix was applied at the highest labeled field rate for aphids on a legume crop. A 1% solution of neem oil was chosen because this concentration had been found to significantly reduce cotton aphid, *Aphis gossypii* Glover, populations under field conditions.[28] A carrier volume equivalent to 281 L ha^{-1} (deionized water) was used to determine the ratio of insecticide to water needed to prepare treatments for laboratory bioassay. This application volume is similar to the range recommended to achieve optimal soybean canopy deposition with insecticides under commercial field conditions.[29]

2.3.2 Laboratory bioassay

The experiment for each developmental stage of each insect consisted of the three direct spray treatments in a completely randomized block design with six replications through time. A Potter precision laboratory spray tower (Burkard Scientific Ltd, Uxbridge, UK) was used to deliver the insecticide treatment to each test unit. The total volume of carrier plus insecticide was converted from L ha^{-1} into g cm^{-2} to give a measurable unit for determining treatment rate on the test unit.[30] The spray tower was calibrated between each treatment spray, within each replicate, by manipulating spray pressure and weighing sprayed petri dishes until the target deposition was reached.[30] Aliquots of 5 mL were used consistently, and spray

pressure was adjusted between 54 and 80 kPa. Test units were also weighed after treatment to record precise deposition.

Treated *A. glycines* nymphs and adults were allowed to dry for 30 min, uncovered, before being transferred using a fine, No. 1 camel hair brush to individual petri dishes (60 × 15 mm) with an excised soybean leaf set up in the same manner as the test units. They were then covered and returned to the temperature-controlled chamber at 22 ± 1 °C and 16:8 h light:dark photoperiod.

Treated *H. axyridis* larvae and adults were transferred immediately after treatment using a fine, No. 1 camel hair brush to individual covered petri dishes (60 × 15 mm) lined with filter paper and provided frozen *E. kuehniella* eggs *ad libitum*. Pupae were allowed to dry uncovered for 30 min before being transferred to fresh petri dishes (100 × 15 mm) and covered. All were returned to the temperature-controlled chamber at 25 ± 1 °C and 16:8 h light:dark photoperiod.

2.4 Assessment

Aphis glycines nymphs were checked every 24 h post-treatment, and developmental stage was recorded. Instar was determined by checking for shed exuviae. Recording continued for each of the four nymphal instars until each aphid became a reproducing parthenogenic female adult or died. Adult aphids were checked every 24 h post-treatment for 10 days. Nymph and adult aphids were considered dead when they failed to respond to gentle prodding from a No. 1 camel hair brush. Nymphs produced by treated aphid adults were recorded and removed from the test unit at each time check.

Harmonia axyridis larvae were checked every 24 h, and developmental stage or mortality was recorded until they each became an adult. Mortality was determined by failure to respond to prodding by a No. 1 camel hair brush. Instars were determined according to previously published methods.[31,32] Pupae were checked every 24 h until molting to an adult. Pupal mortality was determined if adult molt failed to occur in 7 days.[33] Adults were checked every 24 h for 14 days for mortality or egg masses. All egg masses produced were removed and placed in fresh Petri dishes, labeled and kept until eclosion. Percentage eclosion was recorded for each mass.

2.5 Statistical analysis

For nymph and adult *A. glycines,* ending proportion mortality was calculated by dividing the number of individuals that died during the experiment by the total number of individuals that were alive or dead at the end of the experiment in each treatment replicate. Unaccounted-for escapees (individuals that were unable to be located in a dish) were disregarded, as their ending state could not be determined. Proportion means were arcsine square root transformed and compared using analysis of variance with replication and treatment as random and fixed factors respectively (ANOVA) (Proc Mixed).[34]

A Kaplan–Meir curve was used to estimate the time it took for treated aphid nymph survivors to become adults, where the event is adulthood. Resulting curves were compared using a log-rank test, with chi-square analysis ($P < 0.05$).[35] For surviving treated aphid adults, the mean number of nymphs produced per adult per day (fecundity) and longevity (days) of adults within the 10 day observation time period of the study were analyzed using an ANOVA.

For *H. axyridis*, ending state of each individual of each treated instar (first and third) was marked as either dead or adult. The resulting proportion that had become adults was compared between treatments for each of the instars using chi-square analysis (Proc Freq).[34] Pairwise comparisons were then made between all combinations of treatments using chi-square analysis. Resulting *P*-values were Bonferonni corrected to account for multiple comparisons. An ANOVA was performed to compare treatments by the time it took for individual larvae to become adults. A separate test was carried out for each instar. The mean proportion mortality of pupae for each treatment replicate was arcsine square root transformed and treatments compared using ANOVA. Mean numbers of eggs produced by each female adult per day and mean percentage eclosion rate for each egg mass were analyzed using ANOVA.

For *A. glycines* and *H. axyridis*, ANOVA means were separated using Tukey's test ($P < 0.05$; Proc Means),[34] as appropriate after a significant *F*-test.

3 RESULTS

3.1 *Aphis glycines*

Mortality of *A. glycines* nymphs was significantly different between neem-derived treatments and the water-only control ($F = 80.18$; $df_{2,10}$; $P < 0.0001$). There was no significant difference in nymphal mortality caused by either neem insecticide (Fig. 1). There was an overall significant difference between treatments for the time it took surviving aphid nymphs to become adults. Pairwise comparisons between

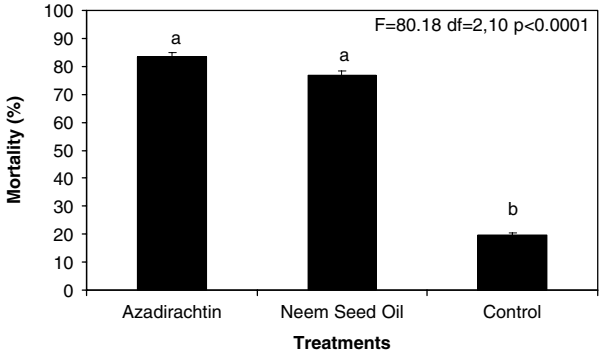

Figure 1. Final *Aphis glycines* nymphal mortality for azadirachtin ($n = 50$), neem oil ($n = 49$) and a water-only control ($n = 46$). Treatment means sharing a letter are not significantly different from each other (Tukey's at $P < 0.05$).

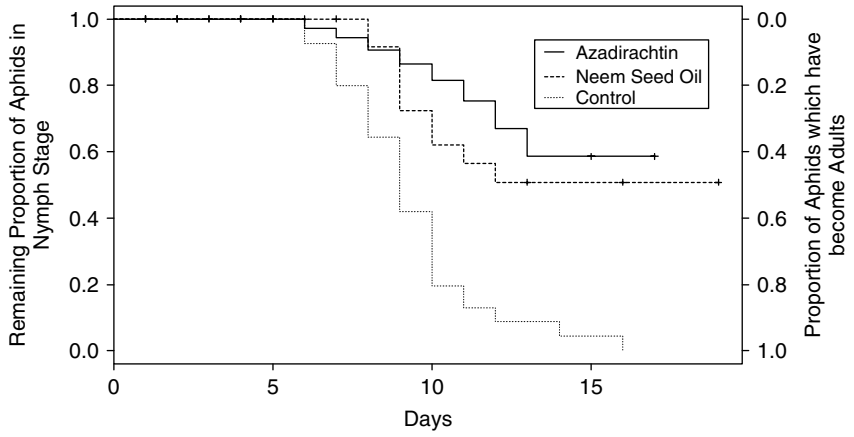

Figure 2. The proportion of *Aphis glycines* nymphal survivors that became adults during post-treatment evaluation. Curves were compared using a chi-square analysis ($\chi^2 = 32.3$; df = 2; $P < 0.0001$).

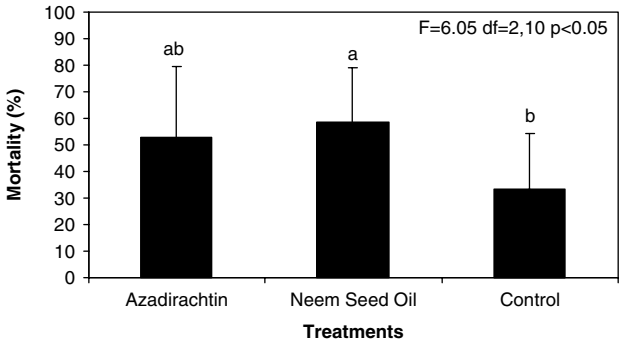

Figure 3. Final *Aphis glycines* adult mortality for azadirachtin ($n = 52$), neem oil ($n = 56$) and a water-only control ($n = 50$). Treatment means sharing a letter are not significantly different from each other (Tukey's at $P < 0.05$).

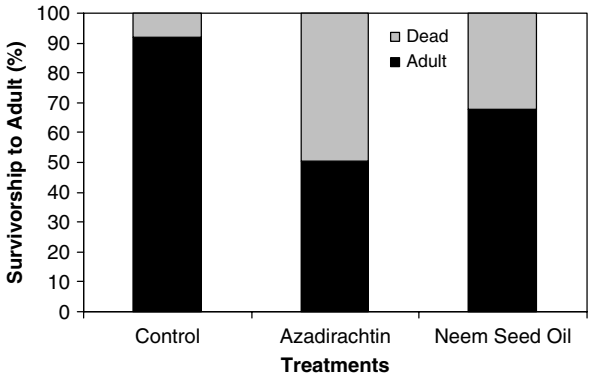

Figure 4. Percentage of first-instar *Harmonia axyridis* larvae that were either adult or dead at the conclusion of evaluation after treatment with azadirachtin ($n = 89$), neem oil ($n = 83$) or water control ($n = 89$). Treatments were compared using chi-square analysis ($\chi^2 = 37.11$; df = 2; $P < 0.0001$).

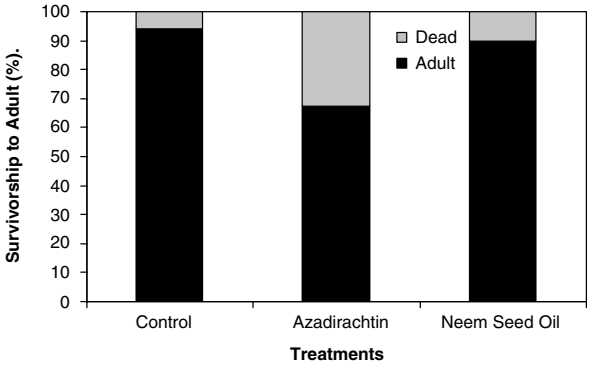

Figure 5. Percentage of third-instar *Harmonia axyridis* larvae that were either adult or dead at the conclusion of evaluation after treatment with azadirachtin ($n = 86$), neem oil ($n = 87$) or water control ($n = 83$). Treatments were compared using chi-square analysis ($\chi^2 = 25.21$; df = 2; $P < 0.0001$).

treatments showed that aphid nymphs in the control group took significantly less time to become adults than those treated with azadirachtin ($\chi^2 = 24.6$; df = 1; $P < 0.0001$) and neem oil ($\chi^2 = 15.7$; df = 1; $P < 0.0001$); however, there was no difference ($P = 0.45$) in time to adulthood between azadirachtin and neem oil treatments (Fig. 2).

For *A. glycines* adults, overall mortality effects were also significant ($F = 6.05$; $df_{2,10}$; $P = 0.019$). Mortality caused by neem oil was significantly higher that of the water-only control; however, azadirachtin did not differ from the control and the two neem-derived treatments were not statistically different from each other (Fig. 3). There were no significant differences in adult *A. glycines* fecundity ($P = 0.50$) or longevity ($P = 0.41$) when comparing those that had been treated with azadirachtin, neem oil or a water-only control.

3.2 Harmonia axyridis

The number of *H. axyridis* first (Fig. 4) and third (Fig. 5) instars that survived to adulthood varied significantly between treatments. For first-instar pairwise comparisons, survivorship to adult was significantly reduced by azadirachtin ($\chi^2 = 18.9$; df_1; $P = 0.002$) and neem oil ($\chi^2 = 16.1$; df_1; $P = 0.002$) compared with the water control; however, there was no difference between the two neem-derived treatments ($P = 0.0624$). For third-instar pairwise comparisons, survivorship to adult was significantly reduced by azadirachtin compared with neem oil ($\chi^2 = 12.7$; df_1; $P = 0.008$) and the water

Table 1. Mean time (days ± SE) for *Harmonia axyridis* larvae to develop to adult post-treatment[a]

	H. axyridis developmental stages	
Treatment	First instar	Third instar
Water control	12.97 (±0.17) a	9.82 (±0.18) a
Neem oil	13.65 (±0.21) b	10.01 (±0.18) a
Azadirachtin (Neemix 4.5®)	14.14 (±0.24) b	11.63 (±0.22) b

[a] Means within a column followed by the same letter are not significantly different from each other (Tukey's at $P < 0.05$).

control ($\chi^2 = 18.9$; df_1; $P = 0.002$) and there was no significant difference between neem oil and the water control ($P = 0.30$). Mean development time to adulthood was significantly affected by treatment for first ($F = 8.19$; $df = 2, 176$; $P = 0.0004$) and third ($F = 23.4$; $df = 2, 206$; $P < 0.0001$) instars. The time it took first instars to become adults was significantly increased by azadirachtin and neem oil treatments compared with the water control (Table 1). For third instars, only azadirachtin significantly increased time to adulthood, and there was no difference between neem oil and the water control (Table 1).

Mortality of *H. axyridis* pupae ranged between 0 and 30%, with no significant differences between treatments ($P = 0.44$). There was no adult mortality in the study.

Mean number of eggs oviposited per female per day ranged between 16.46 and 18.32, with no significant differences between treatments ($P = 0.25$). Subsequent mean egg eclosion rates ranged between 56.8 and 61%, with no significant treatment differences ($P = 0.2133$).

4 DISCUSSION

4.1 Aphis glycines

Both neem-derived insecticides in the present study significantly increased *A. glycines* nymphal mortality. Comparison of results with previous work suggests variability in neem-derived insecticide toxicity among aphid species. Results of the present study established a 20% survival rate to adulthood of first-instar *A. glycines* nymphs treated with azadirachtin, compared with a 33% rate of survival to adult observed for first-instar cow pea aphids, *Aphis craccivora* Koch, placed on broad bean, *Vicia faba* L., seedlings previously treated with azadirachtin.[36] Furthermore, 0% of brown citrus aphid nymphs survived to adult when fed seedlings previously dipped in azadirachtin.[15] In a study recording the effects of complete neem seed oil on aphid mortality 9 days after a topical treatment, the neem oil concentration required to attain 50% mortality in second instars of six different aphid species ranged between 1.66 and 5.30%.[26] By contrast, the present study attained an *A. glycines* nymphal mortality of 77% with a 1% neem oil concentration. While the previous study recorded aphid mortality at a 9 day endpoint, this study required 23 days until the last nymph had either reached adulthood or died. Thus, an extension of the evaluation period may elucidate greater similarity in neem insecticide efficacy between species.

Compared with a water control, *A. glycines* nymphs that survived to adulthood spent significantly more time in an immature state after treatment with the neem-derived insecticides (Fig. 2). Similarly, first-instar *A. craccivora* took more than twice as long to develop into adults than the control when treated with azadirachtin.[36] The present results suggest possible long-term effects on *A. glycines* populations by extending the non-reproductive phase of individual aphids, thus slowing population growth.

When applied to adult *A. glycines*, both neem-derived insecticides had limited impact on mortality (Fig. 3) compared with these same treatments applied to first-instar nymphs (Fig. 1). Similarly, no significant differences were observed for adult survival of the currant lettuce aphid, *Nasonovia ribis-nigri* Mosley, green peach aphid, *Myzus persicae* Sulzer, or strawberry aphid, *Chaetonsiphon fragaefolii* Cockerell, after placement on leaves previously treated with either azadirachtin, neem oil or a water-only control.[26] Conceivably, differential susceptibility of *A. glycines* by life stage (immature versus adult) to azadirachtin and neem oil in the present study can be attributed to neem mode of action as an insect growth regulator.[27]

Although adult sterility is one of the more consistent effects of neem-derived insecticides on a range of insect species,[14] there was no effect on the fecundity of *A. glycines* adults treated with either neem preparation in the present study. Conversely, brown citrus aphid adults that were fed seedlings previously dipped in azadirachtin were found to produce 65% fewer offspring than the control group.[15] A significant reduction in fecundity was also observed for cabbage aphid fed rape plants grown hydroponically in an azadirachtin solution.[16] This discrepancy between the present study and previous studies may be explained by differences in routes of exposure of the target insect to the test substance. In a route of exposure comparison study, nymphs of three aphid species were either treated with a direct spray of an azadirachtin formulation before being placed on a host plant or sprayed while on the host plant.[37] Aphids sprayed while on the plant showed a significant reduction in fecundity compared with those sprayed previous to placement, suggesting a significant effect of route of exposure to neem. Methods exposing *A. glycines* to topical, ingestion and residual exposure routes were beyond the scope of this study, which was designed to compare effects of two different neem preparations applied by a direct spray method to a specific pest/predator combination previously untested in this manner. However, future work comparing residual contact and ingestion variables with a direct spray treatment may elicit greater effect of neem-derived insecticides on *A. glycines* reproductive capability.

4.2 Harmonia axyridis

The present laboratory bioassay demonstrates potential non-target effects of neem treatments on *H. axyridis* larval survival and development time. Compared with a water control, both neem treatments significantly reduced first-instar survival to adult, while only azadirachtin significantly decreased survivorship of third instars to adult. Between the azadirachtin treatment groups, first instar appears more susceptible than third instar (Figs 4 and 5). Among other coccinellids, mortality rates for second-instar *Hippodamia variegata* Goeze treated with azadirachtin and neem seed powder mixed with water were increased 40 and 27% respectively, compared with a water control.[38] Similarly, only 42% of first-instar *Coccinella undecimpunctata* L. treated with a 1% neem oil concentration (equivalent to the present study) survived to adulthood.[39]

For development time, azadirachtin-treated survivors of both instars required significantly more time than a water control to become adults, while neem oil only extended time to adult eclosion when applied to first instars (Table 1). This delayed development to adult and apparently greater susceptibility of early instars are similar to effects of both neem treatments on first-instar *A. glycines* nymphs (Fig. 2).

The authors detected no significant effect of either neem treatment against *H. axyridis* pupae. This result differs from a study in which significantly greater *H. variegata* pupal mortality was observed after treatment with azadirachtin and neem seed extract, 38 and 29% mortality respectively, compared with a water control.[38] Interestingly, these authors found both neem treatments to cause less mortality to the larval stage of *H. variegata* than observed in the present study of *H. axyridis*, yet they found greater pupal mortality. The pupal stage of another natural enemy, *C. carnea*, although not a coccinellid, also sustained no harmful effects after being topically treated with azadirachtin.[40] The authors surmise that this result may be due to the protective role of the pupal cocoon. Although *H. axyridis* pupae do not have the same silken cocoon as *C. carnea*, the pupal casing may be playing a similar protective role. Differential susceptibility by species is also a possible explanation for these differences, highlighting the importance of testing individual species across developmental stages to assess pesticide impact on non-target organisms desired in insect biological control programs.[41]

Results of the present study revealed no significant differences in azadirachtin or neem oil direct spray contact effects on *H. axyridis* fecundity and eclosion rate of eggs oviposited by treated adults. Alternatively, azadirachtin was sprayed onto potted pea plants, *Pisum sativum* L., containing pea aphids and *Coccinella septempunctata* L. (Coccinellidae) adults.[42] At 100 ppm azadirachtin concentration, *C. septempunctata* oviposition was significantly reduced compared with the control group, and, at 600 ppm, oviposition was prevented entirely. The authors refer to their method as treating a microcosm,[43] or a controlled ecological model, which allowed them to expose *C. septempunctata* to all three routes of neem exposure – contact, residual and ingestion (via neem-treated aphid prey) – in an effort to mimic the level of exposure of insects in an agricultural setting. As previously discussed under *A. glycines* adult sterility effects, this type of experimental design would be a logical next step for further elucidation of insect growth regulator effects on *H. axyridis*, particularly when combined with field studies comparing azadirachtin and neem oil treatment effects on survivorship, development and fecundity for both species.

4.3 Formulations of neem

The differential effects of neem-derived insecticides, inter- and intraspecifically, have been discussed by other authors as potentially attributable to differences in the bioactivity of the neem or neem formulation. Azadirachtin and other chemicals in neem tree raw materials may vary depending on geographic origin and yearly variations in environmental growing conditions.[44] Additionally, there is evidence that the method of azadirachtin extraction affects the bioefficacy of the insecticide formulation and thus may vary considerably between manufacturers.[45] This may explain insecticidal differences observed between Neemix® treatment in the present study and azadirachtin treatments used by other researchers. Owing to this level of variance in bioactivity of neem-derived insecticides, caution is necessary in making assumptions about the effects of different neem-derived insecticides.

5 CONCLUSION

The results suggest that neem-derived insecticides may be useful for soybean aphid management in organic soybeans. Although mortality effects are not immediate,[25] neem insecticides ultimately cause high *A. glycines* nymph mortality and delay development time to adult for nymph survivors. Moreover, non-target effects of azadirachtin and neem oil were only observed for immature *H. axyridis* life stages. Future laboratory study is warranted to assess whether a combination of direct spray contact, residual contact and ingestion will amplify insect growth regulator effects reported for both species in the present study. In addition, field experiments testing azadirachtin and neem oil in small plots prior to large-scale application in organic soybeans are recommended to determine the relative efficacy of these botanical insecticides against soybean aphid, and to modify application methods, timing and frequency as appropriate to minimize non-target effects on *H. axyridis*.

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