Antifeedant and Toxic Effects of a Neem (*Azadirachta indica* A. Juss)-Based Formulation Neemix® Against *Nezara viridula* (L.) (Hemiptera: Pentatomidae)[1]

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Abstract The antifeedant activity of Neemix 4.5 EC, a commercial formulation of azadirachtin from the neem tree (*Azadirachta indica* A. Juss), was tested against adult *Nezara viridula* (L.) in the laboratory using a cowpea pod-dip method. A toxicity assay was conducted by dipping fourth-instar nymphs. Feeding by adults was significantly reduced in treated pods compared with controls, based on counts of salivary deposits on pod surfaces, inside pod walls and on seeds. The antifeedant effect of azadirachtin was significantly greater on pods treated with 5% aqueous solution than on those treated with 0.5%, indicating that the antifeedant activity was related to concentration. Bugs were initially repelled by Neemix before approaching treated pods to feed. The LC_{50} for nymphs was 61% (27450 ppm azadirachtin) at 2 d and ranged from 1.8 to 6.2% (810 to 2790 ppm) at 5 d post-treatment, which indicated that neem was slow acting. Sublethal treatment of nymphs disrupted molting and caused morphological defects in adults. Development time to adulthood also was prolonged, and longevity of females was reduced by neem treatments. Azadirachtin may provide an effective component of a comprehensive management program for *N. viridula*.

Key Words Antifeedant effects, toxicity, longevity, azadirachtin, neem, Neemix®, *Nezara viridula*, cowpea

The neem tree, *Azadirachta indica* A. Juss, has been recognized for many years as a potential source of natural products for insect control (Schmutterer 1988, 1990, Isman et al. 1991, National Research Council 1992, Ascher 1993, Mordue and Blackwell 1993). Extracts from the leaves and seeds have activity against over 200 species of phytophagous insects from several orders (Schmutterer 1990, Singh and Saxana 1999). The most active constituent of neem is the tetranortriterpenoid compound azadirachtin. The diverse biological effects of neem on insects include repellence, feeding deterrence and growth disruption (Schmutterer 1990, National Research Council 1992, Ascher 1993, Mordue and Blackwell 1993). The intensity of these effects depends on the concentration of azadirachtin and the species of insect tested (Schmutterer 1988, 1990).

The efficacy of azadirachtin, as well as its mode of action, has been extensively demonstrated in insects with chewing mouthparts such as Lepidoptera and Orthop-

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tera (Ladd et al. 1978, Ladd 1981, Schmutterer 1990, Isman 1993, Aerts and Mordue 1997, AliNiazee et al. 1997, Ilio et al. 1999, Joshi and Lockwood 2000). However, fewer studies have focused on insects with piercing-sucking mouthparts such as the hemipterans (van den Heyde et al. 1984, Schmutterer 1990). Saxena and Khan (1985) reported that phloem feeding by *Nephotettix virescens* (Distant) was significantly reduced on rice plants treated with various concentrations of neem oil. Disruption of normal feeding by neem extracts also was reported for *Phenacoccus manihoti* Grantz on cassava leaves (Mourier 1997) and *Nezara viridula* (L.) on pecan nuts (Seymour et al. 1995). Topical application of neem leaf solution to fifth-instar coffee bugs, *Antestiopsis orbitalis bechuana* (Kirkadly), resulted in death or deformed adults (Leuschner 1972). Growth disruption and impaired fertility or reduced reproduction caused by neem extracts also were reported for the milkweed bug, *Oncopeltus fasciatus* Dallas and Dysdercus spp. (Redfern et al. 1981, Dorn 1986, Schmutterer 1990).

Nezara viridula is a cosmopolitan sucking insect pest of several agricultural and horticultural crops, including cowpea, throughout the tropics and temperate regions of the world (Todd 1989, Panizzi 1997). The objectives of the current studies were to assess the antifeedant and toxic effects of azadirachtin, the active material in a commercial formulation Neemix 4.5 EC, on *N. viridula.*

Materials and Methods

Insects. *Nezara viridula* colonies were established from adults and nymphs collected from collard (*Brassica oleracea* L.) and green bean (*Phaseolus vulgaris* [L.]) fields in Charleston, SC. Insects were reared in the laboratory at 24 ± 0.5°C room temperature, 55 to 65% RH, and a photoperiod of 14:10 (L:D) h. Adults were maintained in metal screen cages (36 × 36 × 36 cm) and strips of paper towel hung from the sides of the cages, provided oviposition sites. Eggs were collected from the paper strips, allowed to hatch in Petri dishes (10 × 1.5 cm) and nymphs were reared to adulthood in plastic cages (19 by 13.5 cm) after the second instar molt. Bugs were fed green beans and sunflower (*Helianthus annuus* [L.]) seeds, with corn (*Zea mays* L.) and shelled peanut (*Arachis hypogaea* L.) provided as supplements. Water was provided by a moistened wick in a plastic cup of water.

Neem compound. Azadirachtin was supplied by Certis USA (Columbia, MD) as a commercial formulation, Neemix®, containing 4.5% azadirachtin. The formulation was diluted with distilled water to prepare different concentrations of azadirachtin for the tests.

Antifeedant activity. The antifeedant effect of azadirachtin was evaluated by counting and recording numbers of feeding punctures made by adult *N. viridula* on treated and control cowpea pods in "no-choice" laboratory tests. Pods were obtained from field-caged cowpea to insure that pods were not damaged prior to the tests. Two concentrations of azadirachtin (0.5 and 5%) were prepared for the antifeedant tests. Pods were treated by dipping them for 30 s at each concentration. Pods dipped in distilled water served as the control. After treatment, pods were allowed to air dry for about 1 h then presented to adult *N. viridula* for 48 h. Bugs were starved for 24 h before the test. Only adults older than 2 d were used. Simmons and Yeargan (1988) reported that feeding activity in the green stink bug, *Acrosternum hilare* (Say), increased after the second day post-molt, reaching a maximum at 6 d. *Nezara viridula* were placed singly into plastic crispers (11 × 8.5 cm) containing a treated or control

pod. Twenty *N. viridula* (10 males and 10 females) were used for each treatment and three replications were made. The experiment was repeated two times, June 1999 and June 2000. The tests were conducted at 24 ± 0.5°C room temperature, 55 to 65% RH, and a photoperiod of 14:10 (L:D) h.

Observations were made of feeding behavior of bugs to monitor feeding inhibition (AliNiazee et al. 1997). Feeding damage to pods was determined by the acid fuscin test (Bowling 1979, 1980). Stained pods were rinsed in water and examined for cone-shaped salivary deposits or stylet sheaths that indicated feeding. Feeding punctures on the outer surface of pods, inner walls and on the developing seeds were counted.

Data were analyzed using analysis of variance (ANOVA) and the means were separated by Fisher least significant difference (LSD) test at $P < 0.05$ (SAS Institute 1996). The count data of feeding punctures were log transformed (log × + 0.1) to normalize data (Gomez and Gomez 1984) before analysis.

Toxicity assay. Azadirachtin concentrations of 0.1, 0.5, 1.0, 5.0, and 20.0% were used for the test. One day post-molt fourth stage nymphs of *N. viridula* were dipped singly for 1 s in each azadirachtin concentration. Nymphs dipped in distilled water served as controls. Ten nymphs were treated at each concentration and three replications were made. Fresh Neemix solutions were prepared for each replication. After treatment, nymphs were left to dry for about 20 minutes on paper towels then held in plastic containers (19 × 13.5 cm), and although mortality data were taken at 2 and 5 d (when most bugs were ready to molt to fifth instars) after treatments, observations were made until they died. Bugs were fed green beans and water was provided by a wick in a plastic container of water.

Time required for molting to adult and any deformities were noted. Also, longevity and fecundity of surviving females were recorded. Fecundity was determined by holding individual females with untreated single males from the colony in plastic cages (11 by 8.5 cm). Dead males were replaced until the female died. The entire experiment was repeated twice during June through October 2000 and 2001.

Concentration-mortality data were analyzed by probit analysis using POLO-PC (LeOra Software 1987) to calculate lethal median concentration (LC_{50}) values, concentration-response lines, and chi-square goodness of fit. Control mortality did not exceed 3% in either year: POLO-PC uses Abbott's (1925) formula to correct for control mortality (Abbott 1925). Data on sublethal effects of azadirachtin on insects were analyzed by one-way ANOVA, and treatment means were separated using LSD test at $P < 0.05$ (SAS Institute 1996). Percentage data were transformed to arcsine square root values to stabilize error variances (Gomez and Gomez 1984) before analyses.

Results

Antifeedant activity. There was no significant ($P > 0.05$) interaction between sex of bug and azadirachtin concentration with respect to feeding activity of *N. viridula* in either 1999 or 2000. Also, males and females did not differ significantly ($P > 0.05$) in their feeding preference for treated and control pods in both years. Bugs showed an initial negative feeding response by hanging on the lid of the container before feeding on treated pods.

Treatment with neem significantly ($P < 0.05$) reduced pod-feeding by *N. viridula* compared with controls in 1999 and 2000 (Fig. 1). In both years, mean numbers of

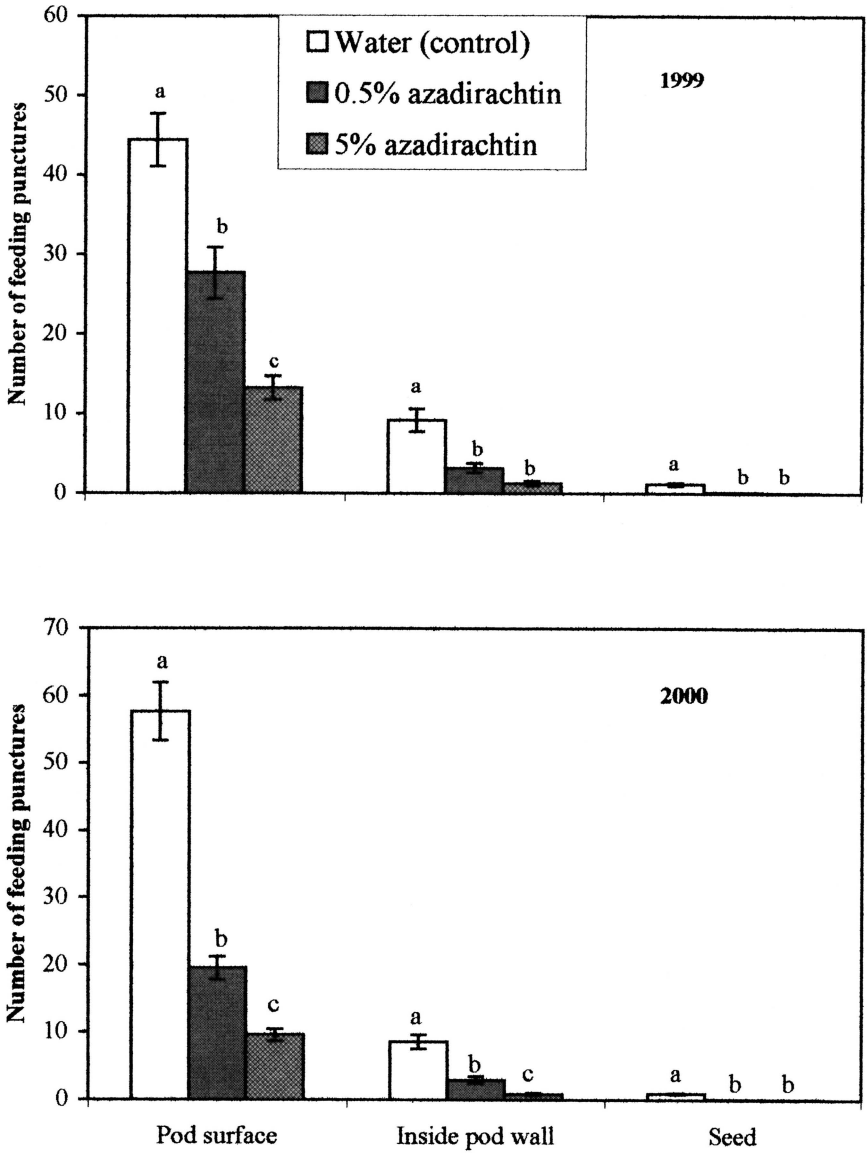

Fig. 1. Effect of azadirachtin on cowpea pod-feeding by *Nezara viridula* (L.). Bugs were allowed to feed for 48 h on pods that had been dipped in azadirachtin solutions or distilled water for 30 s. Each treatment was replicated 3 times. Bars (mean ± SE) within each pod parameter with different letters are significantly different ($P < 0.05$, LSD test), n = 60.

feeding punctures on pod surfaces were significantly ($P < 0.05$) fewer at 5% than at 0.5% azadirachtin concentration, which showed that the antifeedant activity depends upon concentration. However, number of feeding punctures on the inside pod surface and seed did not differ significantly ($P > 0.05$) between 5% and 0.5% azadirachtin concentrations in 1999. In 2000, feeding punctures of bugs on the inside pod surface were significantly ($P < 0.05$) fewer at 5% than at 0.5% azadirachtin. As in 1999, there were no significant ($P > 0.05$) differences in the number of feeding punctures on seeds treated with azadirachtin at 5% and 0.5% in 2000. Significantly ($P < 0.05$) more feeding punctures were recorded on the pod surfaces than on the inside pod walls or on the seeds in 1999 and 2000. Thus, not all the pod-feeding affected the developing seeds. Mean numbers of feeding punctures on pods were pod surfaces > inside pod walls > seeds.

Toxicity assay. Mortality of nymphs of *N. viridula* 5 d after dipping in Neemix solutions was concentration-dependent and differed significantly ($P < 0.05$) among treatments, with mean mortality ranging from 17% to 87% from the lowest and highest concentrations, respectively (Fig. 2). In 2000, the LC_{50} (and 95% CL) for 5 d mortality of nymphs was 1.8% (0.83 to 4.18) (Fig. 2). The LC_{50} (61.0%) for 2 d mortality was higher than at 5 d, indicating that azadirachtin acted slowly. The LC_{50} for 5 d mortality corresponds to 810 ppm azadirachtin and that for 2 d to 27,450 ppm azadirachtin. In 2001, the LC_{50} (and 95% CL) of azadirachtin for 5 d mortality was 6.2% (2.75 to 25.38) (Fig. 2). For 2 d mortality the LC_{50} was 100%. These LC_{50}s correspond to 2,790 ppm and 45,000 ppm azadirachtin, respectively. The LC_{50} values for the 5 d mortalities in 2000 and 2001 were not significantly ($P > 0.05$) different from each other due to overlap of the 95% confidence limits.

Sublethal effects of azadirachtin. In 2000, the molting process of nymphs was disrupted at the two highest concentrations (5 and 20%) (Table 1). Nymphs were weakened by the second day after treatment and died without molting. Most of the deaths (Fig. 2) at the lower concentrations resulted from failure to molt. Significantly ($P < 0.05$) fewer nymphs molted to fifth instars, and percent emergence to adults also was significantly ($P < 0.05$) lower in all azadirachtin treatments compared with controls. Moreover, time required for development to adulthood was significantly ($P < 0.05$) longer in azadirachtin treatments. Additionally, a significantly ($P < 0.05$) greater percentage of adults from the azadirachtin treatments were deformed compared with those in the control (Table 1). Adult deformities consisted of twisted wings and scutellum that folded up toward the anterior of the insect (Fig. 3). Longevity of females from Neemix treatments was significantly ($P < 0.05$) reduced compared with that in the control. Also, females from neem treatments did not produce eggs and lived for 7 d compared with those in the control that lived for 32 d.

The results of 2001 were similar to those of 2000. No molting to adults occurred when nymphs were treated at Neemix concentrations higher than 0.5% (Table 2). Deformed adults resulted from nymphs treated at 0.5% and lower concentrations. In addition, development time of these adults was significantly ($P < 0.05$) longer compared with that in the control group. Longevity and fecundity of treated females were significantly ($P < 0.05$) reduced.

Discussion

Azadirachtin, the active compound, in Neemix had antifeedant activity against adult *N. viridula* based on feeding punctures on treated cowpea pods compared with

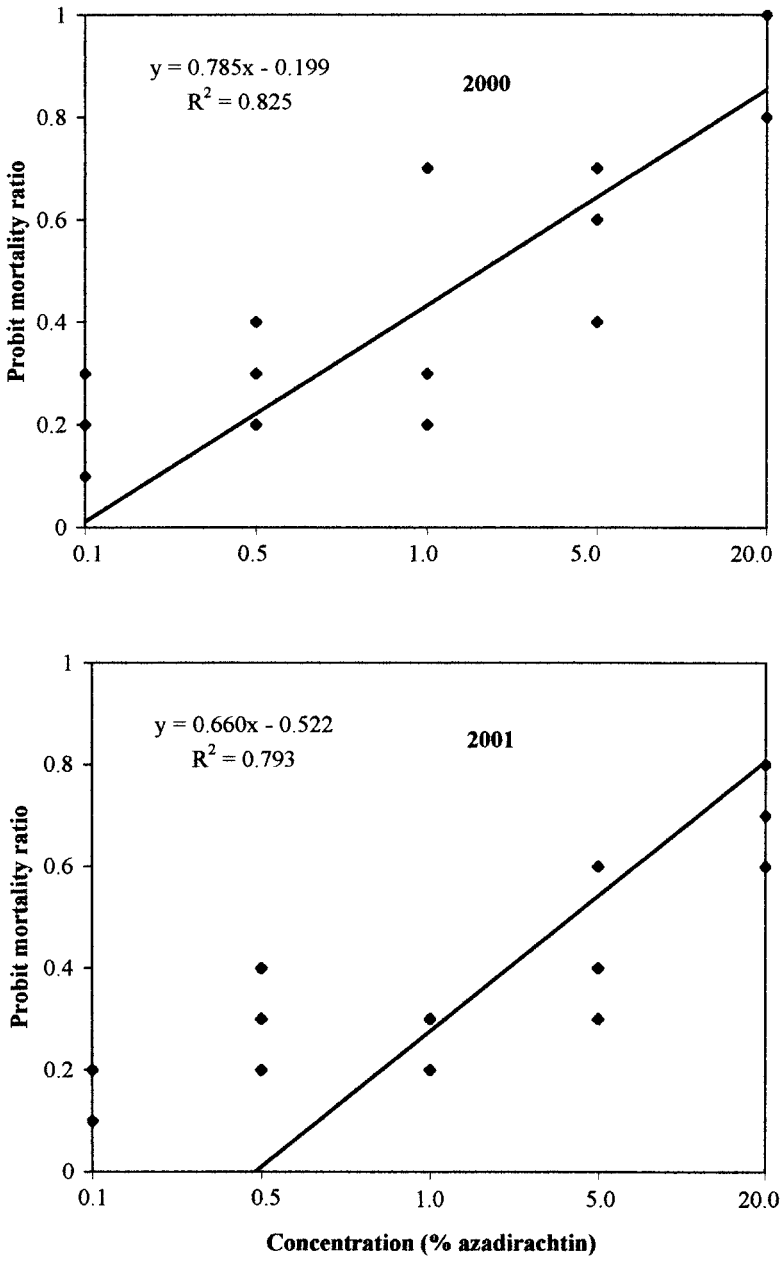

Fig. 2. Mortality of *Nezara viridula* (L.) 5 d after treatment of fourth instars with different concentrations of azadirachtin by dipping for 1 s; 10 nymphs were used per treatment and replicated 3 times.

Table 1. Development, longevity and fecundity of *N. viridula* after exposure to azadirachtin, 2000

*Conc. (%)	% molts to 5th instar	% molted to adults	Time (d) to adulthood	% malformed adults	Longevity** (d)	# eggs per female
Control	90.0 ± 5.8a	76.7 ± 12.0a	13.1 ± 2.6b	4.8 ± 4.7b	32.3 ± 6.8a	123.7 ± 12.4
0.1	50.0 ± 5.8b	23.3 ± 8.8b	15.5 ± 3.3a	83.3 ± 16.7a	7.0 ± 1.2b	0.0 ± 0.0
0.5	33.3 ± 13.3bc	6.7 ± 3.3bc	15.9 ± 3.0a	100.0 ± 0.0a	–	–
1.0	13.3 ± 3.3c	0.0 ± 0.0c	–	–	–	–
5.0	0.0 ± 0.0d	–	–	–	–	–
20.0	0.0 ± 0.0d	–	–	–	–	–

* Concentration of formulated Neemix containing 4.5% azadirachtin active ingredient. Fourth instars of *N. viridula* were dipped in aqueous neem concentrations for 1 s; 10 nymphs were used per concentration and replicated 3 times. Nymphs treated with 5 and 20% azadirachtin died as fourth instars.

** Females only.

Dash indicates all insects died.

Means (±SE) within a column followed by different letters are significantly different ($P < 0.05$, LSD test).

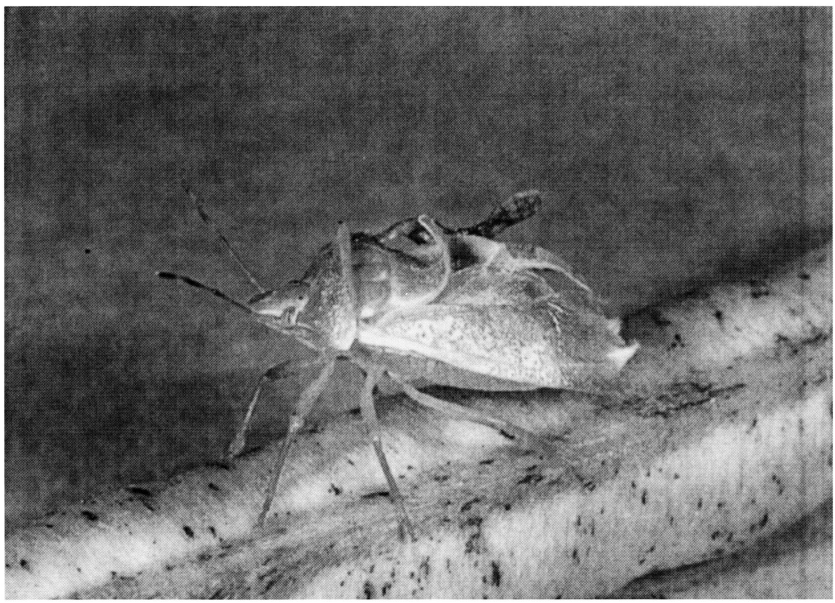

Fig. 3. Morphological defects in *Nezara viridula* (L.) resulting from azadirachtin treatments.

controls. Seymour et al. (1995) reported that treating pecan nuts with neem seed extract significantly decreased the number of *N. viridula* feeding sites compared with the control. In the present study, the antifeedant effect of neem on *N. viridula* feeding depended on concentration; significantly more feeding punctures were recorded on pods treated at 0.5% than at 5% neem. Seymour et al. (1995), however, did not find

Table 2. Development, longevity and fecundity of *N. viridula* after exposure to azadirachtin, 2001

*Conc. (%)	% molts to 5th instar	% molted to adults	Time (d) to adulthood	% malformed adults	Longevity** (d)	# eggs per female
Control	96.7 ± 3.3a	83.3 ± 3.3a	13.2 ± 1.1b	3.7 ± 3.7b	27.1 ± 3.4a	65.6 ± 11.1a
0.1	70.0 ± 15.3b	36.7 ± 3.3b	16.2 ± 0.5a	52.8 ± 12.1a	12.0 ± 2.6b	13.0 ± 13.0b
0.5	26.7 ± 6.7c	16.7 ± 6.7c	17.3 ± 1.4a	88.9 ± 11.1a	8.3 ± 3.2b	0.0 ± 0.0b
1.0	10.0 ± 5.8cd	0.0 ± 0.0d	–	–	–	–
5.0	0.0 ± 0.0d	–	–	–	–	–
20.0	0.0 ± 0.0d	–	–	–	–	–

* Concentration of formulated Neemix containing 4.5% azadirachtin active ingredient. Fourth instars of *N. viridula* were dipped in aqueous neem concentrations for 1 s; 10 nymphs were used per concentration and replicated 3 times. Nymphs treated with 5 and 20% azadirachtin died as fourth instars.

** Females only.

Dash indicates all insects died.

Means (±SE) within a column followed by different letters are significantly different ($P < 0.05$, LSD test).

significant differences in the feeding frequency of *N. viridula* on pecan nuts that were treated with different neem concentrations. Neem concentrations used in the present test were similar to those of Seymour et al. (1995). According to Schmutterer (1990), insects are able to differentiate between treated and untreated parts of their host plant, therefore an even coverage of neem-based materials is required for it to be effective. In addition, the effectiveness of neem on an insect is influenced by the host plant (Schmutterer 1988, 1990, Lowery et al. 1993). Other workers (Saxena et al. 1981, Schmutterer 1990, Isman 1993, Mordue and Blackwell 1993, Smirle and Wei 1996, AliNiazee et al. 1997, Murugan et al. 1998) have reported that the antifeedant activity of neem depends on its concentration as in the present study.

The higher number of feeding punctures on the pod surface than on either the inside pod wall or the seed in all the treatments was probably due to probing effects of bugs before actual feeding (Saxena and Khan 1985). Passerini and Hill (1993) described three modes of rejection of neem-treated plants in the sahelian grasshoper, *Kraussaria angulifera* (Krauss) as palpation, biting and nibbling. They explained that biting and nibbling on treated millet did not necessarily constitute a meal. In the present study, it is also likely that neem repelled *N. viridula* because bugs initially showed negative feeding response before starting to feed on treated pods. This same behavior was reported by Jackai et al. (1992) for *Clavigralla tomentosicollis* Stål. on cowpea. Male and female *N. viridula* exhibited similar feeding patterns on cowpea pods as Bowling (1980) reported for the species in soybean.

Neem treatment of fourth instars caused toxic and growth-disruptive effects. However, there was no quick knockdown effect (Schmutterer 1990) as shown by the higher LC_{50} values for 2 d mortality compared with 5 d in the present study. Smirle and Wei (1996) reported a higher LC_{50} for neem oil at 3 d than at 7 d after treatment in the pear sawfly, *Caliroa cerasi* L. Despite the slow speed of kill, the debilitating effects of neem treatment have been reported to reduce insects' capacity to harm crops several days before death (Schmutterer 1990, Jackai et al. 1992). In the present study some of the nymphs that died were weakened 2 d after treatment and stopped feeding a few days before death.

Higher neem concentrations suppressed molting of *N. viridula* as was also dem-

onstrated in *O. fasciatus* (Redfern et al. 1981, Dorn et al. 1986). Lower concentrations affected ecdysis and resulted in death or malformation of nymphs and adults. Similar effects of neem were reported for other sucking bugs such as *A. orbitalis bechuana* (Leuschner 1972), *O. fasciatus* (Redfern et al. 1981, Dorn et al. 1986) and *Leptocorisa oratorius* Fabricius (van den Heyde et al. 1984). Fewer *N. viridula* adults emerged in neem treatments, and nymphal development time was prolonged, as observed in studies with *C. tomentosicollis* (Jackai et al. 1992) and *Dysdercus koenigii* F. (Koul 1984a). Additionally, fecundity of females was adversely affected after neem treatments, resulting in fewer or no eggs produced compared with those from controls (Tables 1, 2). Koul (1984b) observed sterility effects of neem in *D. koenigii,* and Schmutterer (1990) reported that females of *D. fasciatus* and *O. fasciatus* derived from topically treated nymphs produced fewer eggs than untreated bugs. The growth inhibitory effects of neem in this study could be attributed to azadirachtin, which is known to interfere with the neuroendocrine control of molting and reproduction in insects (Schmutterer 1988, 1990, Ascher 1993, Mordue and Blackwell 1993, Aerts and Mordue 1997).

In conclusion, laboratory studies showed that azadirachtin was an antifeedant to adult *N. viridula,* reduced damage to cowpea pods and seeds, and was toxic to nymphs. Azadirachtin caused nymphal mortality and reduced longevity and fecundity in adults. Thus, Neemix or some other neem formulation may play a role in preventing damage by *N. viridula* and possibly other sucking bug pests of cowpea.

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