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Residual Toxicity and Biological Effects of Neem (*Azadirachta indica*) Oil Against Cotton Mealybug, *Phenacoccus solenopsis* Tinsley (Sternorrhyncha: Pseudococcidae)

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Abstract.- In the present study impact of different neem oil concentrations having been investigated on the development, longevity, fecundity and mortality of *Phenacoccus solenopsis* under ambient laboratory conditions using leaf and surface treatment bio-assay trials. Neem oil at 25000 and 30000 ppm on *Hibiscus* leaves killed 53% and 60% 2nd instar cotton mealybug nymphs, respectively after 24 h treatment which was significantly greater ($P<0.05$) than control. Neem oil at higher concentrations (20000, 25000 and 30000 ppm) adversely affected the duration for cocoon formation, pupal period, male longevity and shortened the male adult life duration and the longevity of mated and virgin females as compared with other treatments. However, neem oil concentrations did not have any significant effect on the oviposition and post-oviposition periods of females. Female fecundity and eggs per batch decreased by 73% and 50%, respectively when they were reared at 30000 ppm neem oil concentration as compared to those reared on untreated leaves. The detrimental effects of neem oil concentrations on cotton mealybug were sustained for three months under ambient laboratory conditions. The present work has shown that the neem oil solution can be stored up to three months under shade conditions without deteriorating its effectiveness.

Key words: Neem oil, oviposition, *Phenacoccus solenopsis* residual toxicity, *Azadirachta indica*.

INTRODUCTION

Cotton mealybug, *Phenacoccus solenopsis* Tinsley (Sternorrhyncha: Pseudococcidae) is a highly polyphagous pest of more than 154 plant species belonging to over 52 plant families (Arif *et al.*, 2009). It secretes large amounts of honey dew that promotes sooty mould development, which hinders normal photosynthesis process. *P. solenopsis* has become a major problem for cotton production which poses a considerable threat to cotton production worldwide. So far, it has been reported from 24 countries of five continents (Wang *et al.*, 2010). Mealybugs are sexually dimorphic, the female is wingless and does not experience complete metamorphosis whereas; males are winged and have complete metamorphosis (Anonymous, 2008). Control of *P. solenopsis* has largely relied on treatment with synthetic insecticides and crops may require several treatments to keep them free of *P. solenopsis* (Saeed *et al.*, 2007; Dhawan *et al.*, 2008). This over reliance on these toxic insecticides has led to increasing problems of biotype development, pest resurgence, destruction of natural enemies, ill effects on non target organisms and environmental pollution (Meyerdirk *et al.*, 1982; Mani and Krishnamoorthy, 1997; Campiche *et al.*, 2006; Peng *et al.*, 2010).

Considerable efforts are being made in various parts of the world to minimize the over use of traditional pesticides and increased use of Integrated Pest Management techniques emphasizing the joint use of biological and selective pesticides which are safe to the non target organisms and the environment.

Neem (*Azadirachta indica* A. Juss) is a multipurpose tree which is extensively grown in India and Pakistan region. Neem products such as leaf and bark extracts, neem seed kernel extract, neem oil and neem cake have been reported to be effective against over 200 species of insects, 3 mites and 5 nematodes and it is considered safe for human health and environment (Ascher, 1993; Raguraman and Singh, 1999; Ukeh *et al*., 2007).

Azadirachtin one of the most active triterpenoids found in neem seeds has been reported to alter insect behavior with its anti-feedant and

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repellent activities; modifies growth of insects by inhibiting the release of prothoracic hormones and has low mammalian toxicity (Schmutterer, 1990; Mordue and Blackwell, 1993; Ascher, 1993; Isman, 2006; Khattak and Rashid, 2006). The present study was carried out to evaluate the biological activities of neem oil on the development, longevity and fecundity of cotton mealybug and the feasibility of prolonged storage of neem oil for future use.

MATERIALS AND METHODS

Insect culture

The mass culture of mealybug, *Phenacoccus solenopsis* Tinsley derived from field collected individuals was maintained on pumpkin fruits (*Cucurbita moschata*) under laboratory conditions at 30±5°C with 50±10% R.H.

Neem oil preparation

As neem seeds are rich in azadirachtin (Schmutterer, 1990) were collected from the local farmers, shade dried and crude neem oil was expelled by crushing in double-screw oil expeller. The required concentrations were prepared in distilled water (v/v) by adding 1% of detergent to obtain 1 liter of solution (Musabyimana *et al.*, 2001). Warm water instead of ambient temperature water was used to obtain a good suspension. The prepared suspensions were tested for three consecutive months to test their shelf life. Six concentrations of neem oil *i.e.* 5000, 10000, 15000, 20000, 25000 and 30000 ppm were used to investigate their residual toxicity and growth regulating effects on cotton mealybug.

Bioassays

Toxic effect was assessed by using leaf dip and surface treatment bio-assay methods.

Residual contact

To study the residual effect of neem oil on cotton mealybug, fresh and young *Hibiscus* leaves were picked from the field, washed thoroughly with tap water and then completely air dried. These leaves were submersed in each neem oil concentration for 5 minutes and allowed to air dry for 30 minutes. Control leaves were dipped in distilled water only. Treated leaves were placed in plastic Petri dishes (9 cm). Counted number of 2nd instar nymphs of cotton mealybug was released in each treatment. Mortality of the nymphs in the Petri dishes was recorded after 24 and 48 hrs, respectively.

Insects survived after 48 hrs were kept separately in Petri dishes to investigate the sub lethal effects of neem oil on the test insect *i.e.* days to cocoon formation, pupal period (only for males), fecundity and total longevity of the insect. After emergence from their pupal cocoons the males were immediately transferred to another Petri dish for mating with the female of the same age and treatment for further reproduction if any.

Effects of neem oil on the fecundity and possibility of asexual reproduction were investigated in fecundity experiments. In these experiments, half of the females were kept virgin/ unmated for whole life to investigate the chances of parthenogenesis/asexual reproduction if any, whereas, rest of the females were offered newly emerged male for sexual reproduction. The pre-oviposition, oviposition and post-oviposition periods, and total eggs laid were recorded for mated females.

Contact effect

To investigate the contact action of neem oil on 2nd instar cotton mealybug, 9 cm Petri dishes were sprayed from all sides with a hand operated mist sprayer to the point of runoff, then left for 30 minutes to air dry. Thereafter, 2nd instar cotton mealybug nymphs were caged with the help of camel hair brush with one treated leaf. Cotton mealybug nymphs were removed after 48 hours, number of dead nymphs were counted and converted to percent mortality for each neem oil concentration.

Statistical analysis

The recorded data was subjected to analysis of variance (ANOVA) and the means were separated by applying the Least Significant Difference (LSD) test at $P \leq 5\%$ to determine which treatment levels differ significantly. All statistical analyses were carried out using computer software MSTATC.

RESULTS

Development and life span of P. solenopsis

Neem oil had significant effect on the days to cocoon formation ($P<0.05$), pupal period ($P<0.05$) and male adult longevity ($P<0.05$). The most effective concentration was 30000 ppm which delayed the male development and it required maximum duration (15 days) to reach to cocoon formation when fed on leaves treated with 3000 ppm neem oil in contrast to minimum (11.80 days) on untreated leaves (Table I). Higher concentrations had a significant effect on the male adult longevity and adult male had shortest life (1.13 days) when reared on 30000 ppm neem oil treated leaves. Neem oil at 5000 and 10000 ppm did not have any significant ($P>0.05$) effect on the male adult life. Neem oil at higher concentrations of 20000, 25000 and 30000 ppm also had a significant effect on the male adult life. Ingestion of leaves treated with 25000 and 30000 ppm neem oil had significant effect on the male total longevity. Higher concentrations had a significant effect on the total longevity of mated and unmated females. Virgin females had shortest total longevity of (42.11 and days 39.50) when reared on 25000 and 30000 ppm neem oil treated leaves. Neem oil at 15000 and 20000 ppm also had a significant reduction effect on the total longevity of virgin females. Females survived for an average of 47.56 and 44.08 days when reared on 15000 and 20000 ppm neem oil treated leaves. Neem oil at 5000 and 10000 ppm did not have any significant effect on the total longevity of virgin females ($F= 34.4039$; df=12; $P<0.05$). Females survived maximum (52.61 days) when reared on fresh/untreated leaves. Similarly neem oil at higher concentrations of 20000, 25000 and 30000 ppm respectively also had a significant effect ($F= 94.2544$; df=12; $P<0.05$) on the total longevity of mated females.

Fecundity of P. solenopsis

Pre-oviposition period was significantly longest (8.25 and 8.97 days) when cotton mealybug nymphs were fed on leaves treated with 25000 and 30000 ppm neem oil ($F= 5.1485$; df=12; $P<0.05$). The effect of neem oil at 5000, 10000, 15000 and 20000 ppm was statistically non-significant from the control. Neem oil had no significant effect on the oviposition ($F= 2.1570$; df=12; $P>0.05$) and post-oviposition periods ($F= 1.9374$; df=12; $P>0.05$) of adult females (Table II).

The most drastic effect of neem oil was observed on the fecundity of cotton mealybug ($F= 306.9621$; df=12; $P<0.05$). Results showed that the mean number of eggs was significantly higher (325.8) in non-treated leaves than those exposed to all tested concentrations of neem oil. Minimum number of 87.89 eggs was laid by female mealybugs when nymphs were fed on leaves treated with 30000 ppm neem oil. Females laid an average of 133.4 and 125.6 eggs when fed on leaves treated with 25000 and 20000 ppm neem oil being insignificantly different of each other. Females laid 229 eggs when reared on leaves treated with 5000 ppm neem oil (Table II).

Toxicity against cotton mealybug

Residual contact

Higher neem oil concentrations produced significantly ($P<0.05$) higher mortality of 2nd instar cotton mealybug, nymphs, compared to control (Fig. 1A). Among the concentrations tested neem oil at 20000, 25000 and 30000 ppm caused the highest mortality of cotton mealybug by consumption of treated leaves and differed significantly from the control. No significant differences were observed between lower concentrations of 5000 and 10000 ppm as compared to control. A slight increase in the toxicity of neem oil was observed after 48 hrs (Fig. 1B). Significantly highest (70%) numbers of mealybug nymphs were found dead after feeding on foliage treated with 30000 ppm neem oil and differed non significantly from 60 and 63.33% mortality in 20000 and 25000 ppm treated leaves. The effect of neem oil at 5000 ppm on the mortality of cotton mealybug was non significant ($P>0.05$) compared to control. Higher concentrations of 25000 and 30000 ppm were not only effective after 24 and 48 hrs but also remained toxic up to three months after the preparation of concentrations. The lower concentrations (15000 and 20000 ppm) also persisted for three months.

Table I.- Effect of neem oil on days (±SE) to cocoon formation, pupal period and longevity of male and female *Phenacoccus solenopsis*.

Neem oil (ppm)	Days to cocoon formation	Pupal period	Male Adult life	Male Total longevity	Unmated female longevity	Mated female longevity
5000	11.93 ± 0.06^{d}	7.06 ± 0.06^{a}	2.33 ± 0.12^{a}	21.33 ± 0.15^{a}	50.78 ± 2.04^{a}	41.81 ± 1.81^{a}
10000	12.00 ± 0.06^{d}	7.00 ± 0.06^{a}	2.26 ± 0.11^{ab}	21.27 ± 0.09^{a}	52.78 ± 2.22^{a}	41.39 ±1.85^{a}
15000	12.07 ± 0.06^{d}	7.00 ± 0.06^{a}	2.00 ± 0.11^{bc}	21.07 ± 0.11^{a}	47.56 ± 1.52^{b}	40.05 ± 1.50^{b}
20000	12.67 ± 0.12^{c}	5.86 ± 0.09^{b}	1.93± 0.06^{cd}	20.47 ± 0.16^{b}	44.08 ± 1.25^{c}	37.33 ± 1.82^{c}
25000	13.87 ± 0.13^{b}	4.46 ± 0.13^{c}	1.66 ± 0.12^{d}	20.00 ± 0.19^{b}	42.11 ± 1.42^{cd}	37.66 ± 2.01^{c}
30000	14.87 ± 0.16^{a}	4.00 ± 0.06^{d}	1.13 ± 0.09^{e}	20.00 ± 0.22^{b}	39.50 ± 1.91^{d}	37.11 ± 2.10^{c}
Control	11.80 ± 0.10^{d}	7.00 ± 0.06^{a}	2.33 ± 0.12^{a}	21.13 ± 0.15^{a}	52.61 ± 2.31^{a}	42.05 ± 2.08^{a}
LSD	0.3182	0.3182	0.4707	0.5927	2.780	0.7026

Means within columns followed by the same letter are not significantly different at 5% level of probability using LSD test.

Table II.- Effect of neem oil on the Pre-oviposition (±SE), Oviposition, Post-oviposition periods and Fecundity of *Phenacoccus solenopsis*.

Neem oil (ppm)	Pre-oviposition period in days	Oviposition period in days	Post-oviposition period in days	Total eggs laid	Eggs/laying
5000	5.89 ±0.23^{c}	9.11 ±1.78^{a}	3.11 ±0.85^{a}	229 ±27.65^{b}	37.28 ±3.27^{a}
10000	6.44 ±0.51^{c}	9.86 ±0.77^{a}	5.22 ±1.32^{a}	191.8 ±24.82^{c}	25.68 ±2.79^{bc}
15000	7.25 ±0.50^{bc}	9.00 ±1.72^{a}	3.78 ±1.01^{a}	151.0 ±14.20^{d}	24.51 ±2.75^{bc}
20000	7.11 ±0.59^{bc}	8.05 ±1.08^{a}	3.00 ±0.77^{a}	125.6 ±21.71^{e}	18.17 ±1.47^{cd}
25000	8.25 ±0.98^{ab}	8.19 ±1.36^{a}	3.72 ±1.07^{a}	133.4 ±20.98^{e}	24.29 ±2.23^{bc}
30000	8.97 ±0.46^{a}	7.58 ±1.02^{a}	3.11 ±1.06^{a}	87.89 ±16.89^{f}	15.48 ±1.37^{d}
Control	5.93 ±0.32^{c}	12.94 ±1.21^{a}	5.22 ±1.36^{a}	325.8 ±20.23^{a}	29.99 ±1.59^{ab}
LSD	1.412	3.775	2.166	14.03	7.819

Means within columns followed by the same letter are not significantly different at 5% level of probability using LSD test.

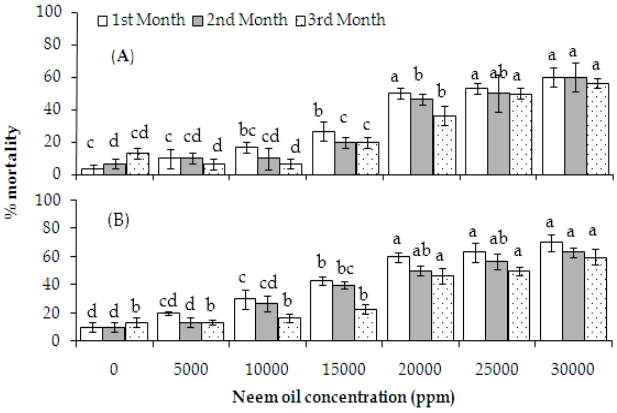

Fig. 1. Residual effect of neem oil on the percent mortality of 2nd instar nymphs of *P. solenopsis* reared on neem oil treated *Hibiscus* leaves. Means with different letter(s) in each treatment is significantly different (P < 0.05). A, 24 hours after treatment; B, 48 hours after treatment.

Contact effect

Toxicity of the neem oil to cotton mealybug nymphs increased according to concentration and exposure time. Neem oil at higher concentrations resulted into greater mortality of cotton mealybug nymphs when observed 24 and 48 hrs after treatment of arena (Fig. 2A). Neem oil at 30000 ppm was most toxic producing 63.33% mortality followed by at 25000 and 20000 ppm with 53.33 and 46.67% mortality. Neem oil at 5000 ppm could not produced significant (P>0.05) mortality of the test insect as compared to control.

More than 50% cotton mealybug nymphs were found dead after 48 hours in the arenas treated with 25000 and 30000 ppm neem oil concentrations when *P. solenopsis* was exposed to neem oil by contact action (Fig. 2B). Neem oil at 5000 ppm could not produce significant mortality of the test insect when observed after 48 hrs. The same

mortality trend persisted for three months after the preparations of the concentrations.

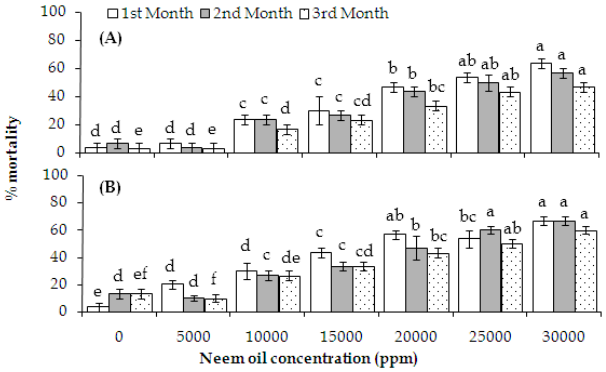

Fig. 2. Effect of neem oil on the percent mortality of *P. solenopsis* (Contact Effect). Means with different letter(s) in each treatment is significantly different (P < 0.05). A, 24 hours after treatment; B, 48 hours after treatment .

DISCUSSION

A variety of plant species carry chemical substances including terpenoids, alkaloids and phenolics etc. which may contribute to the protection of plants against herbivores (Lee *et al*., 2000). Neem extracts have a variety of behavioral and biological activities including insect repellent, anti-feedant, anti-oviposition and growth regulating properties against a wide range of insect pests and mites (Saxena, 1989).

Neem oil strongly affected the growth, survival and fecundity of cotton mealybug. Neem oil showed a higher mortality of cotton mealybug at higher concentrations of 25000 and 30000 ppm. The detrimental effects of neem oil were sustained up to three months which established its good shelf life properties. A consistent residual toxicity was also noticed in 3rd month after application which could be due to the active concentration of azadirachtin after three months of the preparation of solutions. Jenkins *et al*. (2003) also observed that neem kernel extract can be stored at high temperatures for at least five months without significant reduction in its efficacy. The efficacy of neem leaves and azadirachtin remained stable for 8 months of storage under ambient conditions against root-knot nematodes (Javed *et al*., 2007).

Neem oil at higher concentrations of 25000 and 30000 ppm were effective against cotton mealybug when they were exposed to treated leaves or when they were directly exposed to neem oil by direct contact in their tested arena. These observations are in agreement with Kavillieratos *et al*. (2007) reported that azadirachtin based insecticides can cause mortality to adults of *Sitophilus oryzae* and *Tribolium confusum* both by contact or ingestion methods reared on wheat and maize. Higher concentrations of neem oil also delayed the mealybug development and it required maximum developmental duration to reach the maturity. Neem products have been reported to have toxic and growth prolongation effects in insect pests depending on the concentrations and type of test insects (Ascher, 1993; Mordue and Blackwell, 1993; Hamd *et al*., 2005; Nathan *et al*., 2007). Kraiss and Cullen (2008) reported growth prolongation in *Aphis glycines* after treatment with neem derived chemicals.

Results of the present study indicate that neem oil at 25000 and 30000 ppm had a significant effect on the fecundity of cotton mealybug. Ingestion of leaves treated with 30000 ppm neem oil resulted to lowest number of total eggs laid by adult females, whereas, lower concentrations also significantly reduced the total number of eggs laid by cotton mealybug. These results also indicate that the most adverse effect of neem oil is on the fecundity of cotton mealybug. Irigaray *et al*. (2010) obtained similar results. They reported that Align (an insecticide containing azadirachtin) reduced the fecundity and fertility of European grape berry moth (*Lobesia botrana* Denis) adults treated with 1, 5 and 10 mgL^{-1}. Medina *et al*. (2003) also reported that when 3rd instar predatory larvae of *Chrysoperla carnea* were topically treated with azadirachtin, caused a negative effect on oviposition. A significant reduction (65%) in the fecundity of Brown citrus aphid (*Toxoptera citricida*) adults occurred when fed on seedlings treated with azadirachtin compared to that in the control (Tang *et al*., 2002). Results of the present study showed strong anti-ovipositional characteristics of neem oil on the exposed insects. Identical results were obtained by Tang *et al*. (2002) who noted that Neemix 4.5% (the neem commercial product)

reduced significantly the fecundity of citrus aphids. Azadirachtin affected the vitellogenin synthesis or its uptake by developing oocytes and resulted in drastic size reduction of follicles in treated females as compared with those in the controls (Medina *et al.*, 2004). Khaire *et al*. (1992) also reported that neem oil has an effect on the fecundity of pulse beetle, *Callosobruchus chinensis.*

Khan *et al*. (2007) reported reduced fecundity in *Bactrocera coccurbitae* and *Bactrocera dorsalis* after ingestion of neem incorporated with sugar solution. They determined that the decreased fecundity was due to the block of ovarian development. Vennila *et al*. (2010) in a study on the biology of *P. solenopsis* reported parthenogenesis (96.5%) in cotton mealybug. By contrast, in this study no virgin female was found depositing eggs at any concentration of neem oil including control. The reason for this unusual behavior of *P. solenopsis* is not clear. Neem oil at higher concentrations also had a significant effect on the total longevity of mated and unmated females. Dorn (1986) reported reduced longevity of *Oncopellus fasciatus* adults when azadirachtin was injected into newly emerged adults.

It can be concluded from the present findings that neem oil at higher concentrations of 25000 and 30000 ppm had significant effects on the biology and survival of cotton mealybug as insect mortality was significantly higher and prolonged developmental duration of survived nymphs. Another promising outcome from this study is that the neem oil solution can be stored up to three months under shade conditions without deteriorating its effectiveness.

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