

Lethal and sublethal effects of neem oil to the predatory mite *Proprioseiopsis neotropicus* (Acari: Phytoseiidae)

Toxicidad letal y subletal del aceite de nim sobre el ácaro depredador *Proprioseiopsis neotropicus* (Acari: Phytoseiidae)

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Abstract: Lethal and sublethal toxicity of the neem oil Bioneem® to the predatory mite *Proprioseiopsis neotropicus* (Acari: Phytoseiidae) were evaluated by combining lethal doses (LD) with population growth and biological parameter studies. Dose-mortality bioassays were conducted to estimate the lethal doses of the neem oil to *P. neotropicus* using probit analyses. Afterwards, the instantaneous rate of increase (r_i) was used to evaluate the sublethal effects of the neem oil to *P. neotropicus* based on reproduction and mortality data. The effects were further assessed by comparing some life history parameters of *P. neotropicus* exposed to a sublethal dose of neem oil. The dose of neem oil which kills 50% of the population (LD_{50}) of the predatory mite *P. neotropicus* was 7.5 $\mu\text{l}/\text{cm}^2$. The instantaneous rate of increase of the predatory mite *P. neotropicus* did not vary with increases of dosages of neem oil. Similarly, the LD_{25} of the neem oil did not affect biological parameters of the predatory mite. Based on our lethal and sublethal approach we conclude that the neem oil Bioneem® is generally selective to the predatory mite *P. neotropicus*.

Key words: Biological control. Natural enemy. Selectivity.

Resumen: Las toxicidades letal y subletal del aceite de nim, Bioneem®, sobre el ácaro depredador *Proprioseiopsis neotropicus* (Acari: Phytoseiidae) fueron evaluadas mediante la combinación de dosis letales (DL), el crecimiento demográfico y estudios de los parámetros biológicos. Se llevaron a cabo bioensayos de dosis-mortalidad para estimar las dosis letales del aceite de nim sobre *P. neotropicus* mediante análisis de probit. Después, la tasa instantánea de crecimiento poblacional (r_i) se utilizó para evaluar los efectos subletales del aceite de nim a *P. neotropicus* basado en la reproducción y datos de mortalidad. Además, los efectos fueron evaluados mediante la comparación de algunos parámetros de la historia de vida de *P. neotropicus* expuestos a una dosis subletal de aceite de nim. La dosis de aceite de nim que mata al 50% de la población (DL_{50}) del ácaro depredador *P. neotropicus* fue estimada en 7,5 $\mu\text{l}/\text{cm}^2$. La tasa instantánea de crecimiento poblacional del ácaro depredador *P. neotropicus* no varió con el aumento de las dosis de aceite de nim. De manera similar, la dosis subletal de aceite de nim no afectó los parámetros biológicos del ácaro depredador. Basado en nuestro enfoque letal y subletal llegamos a la conclusión de que el aceite de nim Bioneem® es generalmente selectivo para el ácaro depredador *P. neotropicus*.

Palabras clave: Control biológico. Enemigo natural. Selectividad.

Introduction

Many studies have focused on lethal effects of pesticides on target and non-target arthropod species. Only few studies have attempted to understand the side effects of chronic exposure to pesticides on reproduction patterns of natural enemies, which may lead to disruption in population dynamics and influence the impact of pest species on crops. Lethal concentrations or doses are widely used to assess the toxicity of pesticides to arthropods (Stark and Banks 2003; Desneux *et al.* 2007), but they evaluate only mortality as a toxicity parameter. Arthropod populations exposed to pesticides can be negatively affected by sublethal effects ranging from reduction in oviposition rates, survival and growth rate to impaired foraging behavior and interferences on biological aspects (Stark and Banks 2003; Teodoro *et al.* 2005, 2009; Desneux *et al.* 2007; Alzoubi and Cobanoglu 2008; Guedes *et al.* 2009). Lethal doses combined with growth rates or biological studies have been used to evaluate the toxicity of pesticides to arthropods as they allow a more complete toxicological assessment of such compounds by integrating both lethal and sublethal effects (Teodoro *et al.* 2005; Desneux *et al.* 2007; Guedes *et al.* 2009; Hamed *et al.* 2010). The instantaneous

rate of increase (r_i) is a measure of population growth over a given period of time and it has been successfully used to estimate population increase of arthropods (Walthall and Stark 1997; Stark and Banks 2003). The r_i is closely correlated with the intrinsic growth rate (r_m) with the main advantage that r_i discards the need of establishment of life table studies (Walthall and Stark 1997). Positive r_i -values indicate population increase, negative r_i -values indicate decrease, and $r_i = 0$ indicates a stable population (Walthall and Stark 1997).

Neem (*Azadirachta indica* A. Juss) based pesticides have been proven to be efficient in controlling a range of agricultural pests and they have been recognized as relatively non-toxic to natural enemies (Gonçalves *et al.* 2001; Venzon *et al.* 2005, 2008; Isman 2006; Andrade *et al.* 2012). These botanical pesticides contain azadirachtin, a limonoid triterpene, the main insecticidal component which leads to feeding inhibition, disruption of immature development, lower fecundity and fertility of adults, behavior alterations, cell anomalies and mortality in eggs, larvae and adult stages (Mordue (Luntz) *et al.* 2005). Although considered generally selective for several natural enemies, some investigations have also shown side effects of neem pesticides on non-target species (Castagnoli *et al.* 2002; Cordeiro *et al.* 2010).

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Naturally occurring predatory mites of the family Phytoseiidae are key natural enemies of pest mites (McMurtry and Croft 1997) and they are subjected to the same pesticides exposure in the field as target pest species. However, few comprehensive studies have addressed the lethal and sublethal effects of botanical pesticides to natural enemies (Venzon *et al.* 2007; Cordeiro *et al.* 2010). *Proprioseiopsis neotropicus* (Ehara, 1966) (Acari: Phytoseiidae) is a generalist predatory mite found inhabiting natural vegetation and crop fields in Brazil, Colombia and Ecuador (Moraes *et al.* 2004; Mineiro *et al.* 2009). Although no study has specifically addressed the role of *P. neotropicus* in biological control, it is expected that this predatory mite may contribute in the natural regulation of pest mite populations due to its generalist feeding habit and wide distribution. Here, we aimed at assessing the lethal and sublethal effects of a neem based pesticide to the predatory mite *P. neotropicus* by combining lethal dose estimates with demographic and biological studies.

Materials and methods

Stock cultures of the predatory mite *P. neotropicus* were established from mites collected from *Acacia mangium* Willd 1806 plants maintained free from pesticides in São Luís, Maranhão state, Brazil. The predatory mite was reared indoors at uncontrolled temperature, humidity and photoperiod. The mites were confined to a plastic arena (5 cm diameter) floating in distilled water within an open Petri dish (10 cm diameter x 1.5 cm depth). Each arena was centrally perforated by a pin attached to the bottom of the Petri dish by a silicon-based glue. Pollen of castor bean (*Ricinus communis* L. 1753) was provided as food source every other day.

All experiments were conducted under laboratory conditions (27 ± 5 °C, 10h L: 14 h D photoperiod, and 60 ± 10% relative humidity). Dose-mortality bioassays were carried out to determine the lethal doses of the neem oil Bioneem® (Bioneem, Araçuaí - MG, Brazil) to adult females of *P. neotropicus*, at the beginning of their reproductive period (7-8 days old). The neem oil was sprayed through a Potter tower (Burkard, Rickmansworth, UK) in plastic arenas (5 cm diameter) at 0.34 bar (34 kPa) pressure with a 2.3 ml spray aliquot which resulted in a residue of 1.7 ± 0.30 mg/cm² of the pesticide in accordance with recommendations of the International Organization for Biological Control of Noxious Animals and Plants/ West Palearctic Regional Section (IOBC/WPRS) (Hassan *et al.* 1994). Control arenas were sprayed with distilled water, since distilled water was used to dilute the neem oil. Sprayed arenas were dried in open air for 1 h before 5 adult females (7-8 days old) of *P. neotropicus* were placed on them. Five replicates (arenas) for each dose were used. The arenas were placed to float in Petri dishes in the laboratory as described for rearing colonies of the predator. The doses were selected after preliminary tests with broad dosage range allowing selection of lower (the highest neem oil dose unable to kill *P. neotropicus*) and upper (the smallest neem oil dose able to kill 100% of *P. neotropicus*) mortality responses. The doses used ranged from 1.7 to 10.1 µl of the neem oil/ cm², which represents 1 to 5.9 folds the dosage recommended for field applications. Mite mortality was assessed after 72 h exposure and the mites were considered dead if unable to move for a distance at least equal to their body length. Dosage-mortality curves were estimated by probit analyses using PROC PROBIT procedure (SAS Institute 2002).

The instantaneous rate of increase (r_i) was used to evaluate the sublethal effects of the neem oil to the predatory mite *P. neotropicus* based on reproduction and mortality data. This index is calculated using the equation $r_i = [\ln(N_f/N_0)] / \Delta t$, where N_f is the final number of live mites (including eggs and immatures), N_0 is the initial number of mites, and Δt is the interval (7 days) elapsed between start and end of the bioassay (Walthall and Stark 1997). Five adult females of *P. neotropicus* as previously described were placed on arenas sprayed with increasing lethal doses (LD₅, LD₁₀, LD₂₅, LD₅₀, LD₉₅, LD₉₉) of neem oil, which were based on the dosage-mortality curves previously obtained. Control arenas (LD₀) were sprayed only with distilled water. Five replicates for each dose were used. A male predator was added to each arena and replaced whenever it died. One-way ANOVA was conducted to assess the effects of increasing lethal doses of neem oil on the instantaneous rate of increase of *P. neotropicus*.

Sublethal effects of the neem oil was further assessed by comparing some biological parameters of the predatory mite *P. neotropicus* exposed to the LD₂₅ of neem oil and LD₀ (control). Females and males of *P. neotropicus* were confined to arenas for 12 hours to obtain eggs of similar age. Arenas (5 cm diameter) were sprayed through a Potter tower with the LD₂₅ of neem oil, i.e. 5.7 µl of neem oil/cm² or distilled water (LD₀ - control) at the same conditions described above. Preliminary observations showed that eggs that had contact with LD₂₅-sprayed arenas did not hatch; therefore we decided to transfer newly hatched larvae to arenas. Larvae were individually placed on arenas and observed twice a day (8 a.m. and 16 p.m.) until reaching adulthood. Newly molted females and males were observed once a day (8 a.m.) until their death. One male of the stock culture was added to each arena containing a newly molted female and the periods of preoviposition, oviposition, postoviposition, eggs/female and eggs/female/day were recorded. Castor bean pollen was supplied every other day as food source on a small piece of cover glass placed on the arena to avoid direct contact with the neem oil. Cotton threads underneath a small plastic sheet (1.0 x 0.5 cm) served as oviposition place. The periods of immature development, preoviposition, oviposition, postoviposition, eggs/ female and eggs/ female/ day of the predatory mite *P. neotropicus* exposed to the LD₂₅ of neem oil and the control (LD₀) were compared by t tests.

Results

Lethal doses (LD₅, LD₁₀, LD₂₅, LD₅₀, LD₉₅, and LD₉₉) of neem oil, which were estimated based on dose-mortality bioassays, are shown in the Table 1. Doses of neem oil below the maximum recommended dosage for field applications (i.e., 15 ml of neem oil/ L of distilled water or 1.7 µl of neem oil/cm²) did not kill the predatory mite *P. neotropicus*. The completely elimination of *P. neotropicus* populations was achieved only in dosages 10 fold higher than the recommended dosage for field applications (Table 1).

Reproductive side effects caused by chronical exposure to the neem oil were assessed by calculating the instantaneous rate of increase (r_i) of *P. neotropicus* populations. r_i values were not affected by increases on the neem oil doses ($F_{4,20} = 1.80$, $P = 0.16$, Table 2). Positive values of r_i indicate population growth of *P. neotropicus* exposed up to the LD₅₀, i.e., 7.5 µl of neem oil/cm² ($r_i = 0.2 \pm 0.03$ / day). Predator extinction ($N_f = 0$) took place only after exposure to the LD₉₅ (14.4 µl/

Table 1. Toxicity of the neem oil Bioneem® to the predatory mite *Proprioseiopsis neotropicus* ($n=150$, $\chi^2=0.42$, $P=0.81$). LDs were estimated based on dose-mortality bioassays using probit analyses. CI denotes confidence interval.

Slope \pm SE	LD ₅ [*]	LD ₁₀	LD ₂₅	LD ₅₀	LD ₉₅	LD ₉₉
	(95% CI)	(95% CI)	(95% CI)	(95% CI)	(95% CI)	(95% CI)
5.78 \pm 1.21	3.9 (2.6-4.7)	4.5 (3.3-5.3)	5.7 (4.8-6.5)	7.5 (6.6-8.9)	14.4 (11.2-25.4)	18.9 (13.7-40.1)

* Represents lethal dose in μl of neem oil/ cm^2 .

cm^2) of neem oil onwards. Comparisons among biological parameters of individuals non-exposed and exposed to a sub-lethal dose (LD₂₅) were also performed. The duration of the periods of larva ($df=107$, $t=1.80$, $P=0.07$), protonymph ($df=77$, $t=0.40$, $P=0.68$), deutonymph ($df=66$, $t=0.42$, $P=0.67$), preoviposition ($df=18$, $t=0.11$, $P=0.90$), oviposition ($df=18$, $t=0.79$, $P=0.43$), postoviposition ($df=6$, $t=0.75$, $P=0.47$), number of eggs/female ($df=20$, $t=1.14$, $P=0.26$) and number of eggs/female/day ($df=18$, $t=0.60$, $P=0.55$) of *P. neotropicus* were not affected by the sublethal dose of the neem oil (Table 3).

Discussion

The combination of dose-response with population growth and biological parameter bioassays were used to evaluate the toxicity of the neem oil Bioneem® to the predatory mite *P. neotropicus*. This predatory mite showed highly tolerant to action of the neem oil. Predatory mites of the family Phytoseiidae, as *P. neotropicus*, are important natural enemies of pest mites, but their efficiency is contingent on a combination of a myriad of factors, including botanical pesticide use. Neem based pesticides have been used against a variety of agricultural pests and have been broadly recognized as selective to non-target natural enemies (Gonçalves *et al.* 2001; Venzon *et al.* 2005, 2008; Isman 2006; Andrade *et al.* 2012), although very few studies have addressed the potential side-effects of these botanical insecticides (Castagnoli *et al.* 2002; Cordeiro *et al.* 2010).

Toxicity of pesticides to arthropods has been widely evaluated by comparing the lethal doses of these compounds (Stark and Banks 2003; Desneux *et al.* 2007). As observed in previous studies using neem based pesticides (Cote *et al.* 2002), our results for mortality show that the dosage of Bioneem® (1.7 $\mu\text{l}/\text{cm}^2$) recommended for field applications was unable to kill *P. neotropicus*, suggesting that neem oil might have considerable selectivity to this predator. The estimated LD₅₀ of the neem oil for *P. neotropicus* was over four times higher than the dose recommended for field application and the completely extinction occurred only after exposures to the LD₉₉ (18.9 $\mu\text{l}/\text{cm}^2$) of neem oil, which represent situations rather unlikely to happen in any crop system. However, such

analysis might be done with considerable cautions. Since the major effects of neem-based insecticides normally take a while to occur, e.g. the field degradation of azadirachtin varies from one week to three months (Stark and Walter 1995; Sundaram 1996; Mordue (Luntz) *et al.* 2005), this lower toxicity could be due to the fact that 72 h exposure would be insufficient to evaluate the real side effects of neem oil to *P. neotropicus* and to compute all the effects derived from the non-lethal exposures, which may prevail over lethal ones under field conditions. Additionally, we assessed only one potential exposure pathway (residual bioassay) and the possibility of *P. neotropicus* being more severely affected by other exposure pathways (e.g. ingestion of preys that have been exposed to neem or direct exposure through spraying) can not be ruled out.

Organisms exposed to pesticides are known to suffer with sub-lethal effects (Stark and Banks 2003; Desneux *et al.* 2007; Teodoro *et al.* 2005, 2009; Guedes *et al.* 2009) that are, therefore, not apparent on mortality bioassays. Here, the combination of lethal doses estimates with studies of sub-lethal effects on growth rate and some life history parameters allowed a more accurate evaluation of the impact of neem oil to the predatory mite *P. neotropicus*. The instantaneous rate of increase (r_i) of *P. neotropicus*, which was evaluated over a period of 7 days and reflects sub-lethal effects on population growth, did not respond to increases on lethal doses up the LD₅₀ (7.5 μl of neem oil/ cm^2) of neem oil (Table 2). This reinforces the assumption of selectivity of the neem oil to this natural enemy. Additionally, all of these populations presented similar positive values of r_i , indicating population growth of *P. neotropicus* and confirming low toxicity of neem oil to this natural enemy. Predator extinction took place only after exposures to the LD₉₅ of neem oil onwards. Additional sublethal studies evaluating some biological parameters of the predatory mite *P. neotropicus* exposed to LD₂₅ of neem oil and LD₀ (control) were in line with population growth results (Table 3). The LD₂₅, which was over three times higher than the recommended field concentration of the neem oil, did not affect the biological parameters of the predatory mite (Table 3). However, it is important to stress that eggs of *P. neotropicus* that had contact with LD₂₅ - sprayed arenas did not hatch, suggesting ovicidal activity of the neem oil. More

Table 2. Instantaneous rate of increase (r_i) of the predatory mite *Proprioseiopsis neotropicus* exposed to increasing lethal doses of the neem oil Bioneem® (in $\mu\text{l}/\text{cm}^2$). Positive values of r_i indicate that population growths of *P. neotropicus* exposed up to the neem oil LD₅₀ (7.5 μl of neem oil/ cm^2). Means followed by the same letters were not significantly different ($P > 0.05$) with one-way Anova. Means \pm SD are shown.

	LD ₀ Control	LD ₅ 3.9	LD ₁₀ 4.5	LD ₂₅ 5.7	LD ₅₀ 7.5
$r_i \pm$ SD	0.30 \pm 0.02a	0.22 \pm 0.08a	0.21 \pm 0.09a	0.29 \pm 0.06a	0.25 \pm 0.03a

Table 3. Durations of larva, protonymph, deutonymph, preoviposition, oviposition, postoviposition, number of eggs/ female and number of eggs/ female/ day of the predatory mite *Proprioseiopsis neotropicus* exposed to the LD₂₅ of neem oil Bioneen® in comparison to control (LD₀). Means ± SD are given. n = number of replicates. Means within each row followed by the same letters were not significantly different (P > 0.05) with Student's t test.

Biological parameters	LD ₀ (Control)		LD ₂₅	
	Duration (days) ± SD	n [§]	Duration (days) ± SD	n
Larva (developmental time)	0.54 ± 0.32a	78	0.43 ± 0.20a	31
Protonymph (developmental time)	0.97 ± 0.55a	60	1.04 ± 0.68a	19
Deutonymph (developmental time)	0.98 ± 0.52a	50	0.92 ± 0.60a	18
Preoviposition period	3.78 ± 2.32a	14	3.66 ± 1.36a	6
Oviposition period	15.5 ± 10.33a	14	11.16 ± 12.95a	6
Postoviposition period	3.20 ± 2.77a	5	6.00 ± 7.81a	5
Eggs/ female	12.66 ± 16.68a	14	5.68 ± 10.50a	6
Eggs/ female/ day	1.21 ± 0.46a	14	1.36 ± 0.58a	6

studies are necessary to shed light on the mechanisms underlying the ovicidal activity of neem oil.

Several studies report that botanical pesticides like the neem oil are efficient against pest mites and have low toxicity to natural enemies (e.g. Brito *et al.* 2006). Indeed, population growth and biological parameters of the predatory mite *P. neotropicus* were not affected by both increasing doses and the LD₂₅ of the neem oil in this study, indicating no potential impairments of the biological control provided by this natural enemy. Therefore, it is expected that neem oil would not significantly affect populations of the predatory mite *P. neotropicus*. Likewise, Venzon *et al.* (2005) did not find negative effects of concentrations up to 0.1 g a.i./l of neem on survival of the predatory mite *Iphiseiodes zuluagai* Denmark & Muma, 1972 (Acari: Phytoseiidae). Further studies focusing on behavioral responses of *P. neotropicus* treated with neem oil will contribute to a better understanding of the sublethal effects caused by this botanical pesticide. Based on our combined lethal and sublethal approach we conclude that the neem oil Bioneen® is generally selective to the generalist predatory mite *P. neotropicus*.

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