

Toxicity of Neem (*Azadirachta indica*) Seed Cake to Larvae of the Mediterranean Fruit Fly, *Ceratitis capitata* (Diptera: Tephritidae), and Its Parasitoid, *Diachasmimorphalongicaudata* (Hymenoptera: Braconidae)

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TOXICITY OF NEEM (*AZADIRACHTA INDICA*) SEED CAKE TO LARVAE OF THE MEDITERRANEAN FRUIT FLY, *CERATITIS CAPITATA* (DIPTERA: TEPHRITIDAE), AND ITS PARASITOID, *DIACHASMIMORPHA LONGICAUDATA* (HYMENOPTERA: BRACONIDAE)

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ABSTRACT

The objective of this study was to evaluate the interaction of *Diachasmimorpha longicaudata* (Ashmead) parasitism and the toxicity of neem seed cake (NSC) on survival of *Ceratitis capitata* (Wiedemann), the Mediterranean fruit fly (medfly). Groups of 1600 - 1700 third instar *C. capitata* larvae were each wrapped in organdy cloth ("unit of parasitism") and exposed to approximately 500 couples of sexually mature *D. longicaudata* parasitoids (5-d old) for one hour. *C. capitata* larvae exposed or not to parasitism were transferred to plastic cups containing vermiculite with different proportions of neem seed cake (NSC): 0% NSC (control), 5% NSC, 10% NSC, 15% NSC, 20% NSC, 25% NSC, 30% NSC. Both NSC and the parasitism caused larval/pupal mortality and reduced the emergence of *C. capitata* flies. NSC affected parasitoid emergence negatively. The effect of parasitism coupled to NSC did not provide greater reduction in the medfly emergence than when parasitism was used alone. However, each of these 2 methods affect a different life stage of medfly, larvae and pupae, respectively, and their joint use may increase the probability of controlling medfly populations in field.

Key Words: *Ceratitis capitata*, biological control, integrated pest management, botanical insecticides

RESUMO

O objetivo deste trabalho foi avaliar a interação do parasitismo de *Diachasmimorpha longicaudata* (Ashmead) com a toxicidade da torta de nim sobre *C. capitata*. Larvas de 3o instar de *C. capitata* (Wiedemann) foram envolvidas em tecido tipo voil ("unidades de parasitismo") e expostas ao parasitismo, por uma hora, a aproximadamente 500 casais do parasitoide sexualmente maduros, com cinco dias de idade. As larvas, expostas ou não ao parasitismo, foram transferidas para copos descartáveis contendo vermiculita com diferentes proporções de torta de nim (0%, 5%, 10%, 15%, 20%, 25% e 30%). Tanto a torta de nim como o parasitoide *D. longicaudata* causaram mortalidade nas larvas e reduziram a emergência de *C. capitata*. A torta de nim afetou negativamente a emergência de *D. longicaudata*. O efeito do parasitismo associado ao da torta de nim não causou maior redução na emergência de *C. capitata* do que quando utilizados isoladamente. Entretanto, como atingem estágios diferentes de desenvolvimento da praga, larvas e pupas, respectivamente, o uso integrado destas técnicas pode aumentar a probabilidade de controle populacional de moscamede em campo.

Fruit flies (Diptera: Tephritidae) are major pests of fruits worldwide (Hickel 2002); their incidence increases the cost of production due to frequent applications of insecticides, directly reduces yields and limits exports due to quarantine restrictions (Nora et al. 2000; Bittencourt et al. 2006). The economic importance of tephritid flies

may vary according to country, region, host and time of year, and in some regions they may infest several species of fruit and compromise up to 100% of production (Zucchi 2007).

Traditionally, the control of flies has been done through bait sprays consisting of hydrolyzed protein mixed with an insecticide, and also by

the use of insecticide cover sprays (Nascimento & Carvalho 2000). However, based on consumer requirements, producers have adopted a series of measures to reduce use of chemical control. These measures include cultural practices, monitoring of fruit fly populations in orchards, fruit bagging, release of biological control agents, and application of selective chemical and/or alternative products such as botanicals (Lemos et al. 2002). Integrated pest management programs have encouraged the use of these practices, especially the use of natural enemies and environmental/cultural management measures that enhance natural enemy populations (Alvarenga et al. 2006).

The larval endoparasitoid of fruit flies *Diachasma mimorpha longicaudata* (Ashmead), introduced in Brazil in 1994 (Carvalho et al. 1999), locates the fruit fly third instars in the fruit through the vibrations produced by the third instar's mandibles (Lawrence 1981), oviposits in them, and completes its development in the medfly's pupal stage (Carvalho & Nascimento 2002). This parasitoid species has been intensely studied because it can be mass reared easily, has great foraging ability, mainly on fallen fruits, and attacks many fruit fly species around the world (Purcell et al. 1994; Sivinski et al. 1998).

Azadirachta indica A. Juss., popularly known as neem, has been studied for its diverse anti-insect pest properties, and for one of its components, azadirachtin, a botanical insecticide. Neem extracts can be as effective as other commercial insecticides (Prates et al. 2003), and also can be important in coping within insecticide resistance, because of the diverse modes of action of azadirachtin including antifeedant, oviposition repellent, inducer of egg sterility, reduced longevity and reduced fitness, and inhibitor of chitin biosynthesis (Jacobson 1989; Ascher 1993). Neem products have proven effective against the larvae and pupae of fruit flies (Stark et al. 1990; Di Ilio et al. 1999; Salles & Rech 1999; Singh 2003).

Because no single management method used as the sole means of medfly control is likely to provide adequate and durable control, the objectives of this study were (i) to evaluate the possibilities of combining the use of biological control by the parasitoid *D. longicaudata* with chemical control by means of the botanical insecticide, neem (neem seed cake used as a soil drench against pupating mature larvae and adults emergence), and (ii) to determine if these 2 methods used jointly are effective in controlling *Ceratitis capitata* (Wiedemann) larvae. The combination of these methods might lead to decreases in the use of conventional pesticides by farmers, which, in turn, would reduce environmental pollution and increase fruit quality and competitiveness in the market.

MATERIALS AND METHODS

The experiment was carried out using larvae of the fruit fly *C. capitata* and adults of the larval parasitoid *D. longicaudata* from colonies maintained at the Insect Rearing Laboratory of the State University of Montes Claros (UNIMONTES), Campus Janaúba-MG. The insects were kept under controlled temperature ($26 \pm 1^\circ\text{C}$), relative humidity ($65 \pm 10\%$) and at 14:10 h L:D.

Approximately 1600-1700 third instar *C. capitata* were placed in Petri dishes (10 cm diam) without a lid, and then the dish was wrapped with organdy cloth to simulate fruits infested with fruit flies larvae. Such enclosed groups of larvae were each called a "unit of parasitism" (UP). These units were placed face-down on the top of a screened rectangular cage (25 cm \times 20 cm) containing around 500 pairs of 5-d old sexually mature *D. longicaudata* parasitoids. The larvae within the UP were exposed to the parasitoids for 1 h. After exposure, the larvae were removed from the UP and transferred to plastic cups containing vermiculite with different proportions of neem seed cake (NSC) as follows: control (vermiculite only), 5% (5mL NSC plus 95mL vermiculite), 10% (10mL NSC plus 90mL vermiculite), 15% (15mL NSC plus 85mL vermiculite), 20% (20mL NSC plus 80mL vermiculite), 25% (25mL NSC plus 7mL vermiculite), and 30% (30mL NSC plus 70mL vermiculite).

Neem seed cake (NSC) is the by-product of oil extraction from neem seed, and contains around 1000 ppm or 0.1% of azadirachtin (http://www.natureneem.com/index_fichiers/Neem_Seed_cake.htm). To obtain the above proportions the solid NSC was mixed homogeneously with vermiculite, and if we consider that NSC contains 0.1% (w/w) of azadirachtin, then the treatments would have approximately 0%, 0.005%, 0.01%, 0.015%, 0.02%, 0.025% e 0.03% (w/w) of azadirachtin, respectively.

The cups containing the larvae were covered with organdy cloth secured with an elastic band and placed in a chamber set at $27 \pm 1^\circ\text{C}$ at 14:10 h L:D until pupation and adult emergence had occurred. Third instar larvae of *C. capitata* not exposed to *D. longicaudata* were also placed in separate plastic cups containing the same proportions of NSC in the vermiculite to serve as the non-parasitism controls.

The experiment was conducted in a completely randomized design with 2 factors, i.e., different proportions of NSC, and parasitized larvae and non-parasitized larvae. Each treatment, including 2 controls (parasitized and non-parasitized larvae at 0% NSC), had 4 replicates with 100 larvae either parasitized or not. Larval mortality and adult emergence of both medflies and parasitoids were recorded and analyzed. The percent parasitism was calculated based on Matrangola et al. (1998):

$$P\text{ (\%)} = \frac{\# \text{ emerged parasitoids} \times 100}{\# \text{ emerged flies} + \# \text{ emerged parasitoids}}$$

Fly emergence and larval fly mortality were subjected to factorial analysis of variance, and the means were compared by Scott-Knott at the 0.05 significance level. For both the parasitoid emergence and parasitism rate, we performed an analysis of variance, and the means were compared by Scott-Knott at the 0.05 significance level. All statistical analyses were performed by use of the statistical package, SISVAR (Ferreira 2000).

RESULTS

Both parasitization and neem significantly reduced the emergence of adult *C. capitata* ($P < 0.001$; $F = 8.45$) (Table 1). The harmful effect of NSC on *C. capitata* larvae was only observed on non-parasitized larvae, from which the decrease in emergence of flies was proportional to the increase of the NSC proportion in the vermiculite. The lowest emergence value (38.5%) was observed when non-parasitized larvae were kept on a substrate with 30% NSC. Except for the treatment with 5% NSC, all the other NSC treatments decreased significantly the number of emerged medflies in comparison to the untreated control (0% NSC). For parasitized medfly larvae, there were no significant effects of NSC on the emergence of medflies ($P = 0.51$; $F = 0.88$) (Table 1).

The use of parasitoids suppressed *C. capitata* emergence more than NSC did, because at all NSC proportions the mean numbers of emerged flies were significantly lower in the larvae exposed to parasitism than in those not exposed to parasitism (Table 1).

Larval mortality was significantly affected by parasitism ($P < 0.001$; $F = 976.89$) and by NSC ($P < 0.001$; $F = 10.58$) (Table 2). Regardless of the level of medfly larval exposure to NSC, parasitized larvae had higher mortality than non-parasitized ones. There was no significant interaction

between parasitism and NSC for larval mortality ($P = 0.08$; $F = 2.08$) (Table 2).

Regardless of whether larvae were exposed to parasitism or not, mean larval mortality was significantly higher in the treatments with NSC than in the control treatments, and mortality increased with increasing proportions of NSC in the vermiculite (Table 2). The highest larval mortality was observed with 30% NSC (70.8%), whereas the mean larval mortalities with 15%, 20% and 25% NSC were similar at these NSC concentrations, but significantly higher than at 5% and 10% NSC (Table 2).

Parasitoid emergence was negatively affected by exposure to NSC ($P < 0.001$, $F = 4.36$) (Table 3). All NSC proportions in the substrate reduced parasitoid emergence in comparison to the control. Five percent NSC in the substrate reduced the emergence 24.7% in comparison to the control, and 30% NSC completely prevented parasitoid emergence. NSC proportions in the substrate above 20% decreased *D. longicaudata* emergence more than 40% compared to the untreated control.

DISCUSSION

The results of this study show that NSC had a detrimental effect on survival of *C. capitata* third instar larvae and pupae and on adult emergence, regardless of whether or not the larvae were parasitized.

Neem products, such as the NSC, applied as soil drenches under the trees can be used to control fruit flies while they are immobilized in the soil in the pupal stage. This assertion is based on the pioneering work of Stark et al. (1990), who found that adult medfly emergence was inhibited after third instar *C. capitata* larvae had been exposed to a 14 ppm (0.0014%) ethanolic extract of azadirachtin.

In our studies NSC had adverse effects on *C. capitata* larvae caused by the active ingredient, azadirachtin, which is quite concentrated in NSC

TABLE 1. NUMBER OF EMERGED ADULTS (MEAN \pm SE) FROM *CERATITIS CAPITATA* LARVAE EITHER PARASITIZED OR NOT BY *DIACHASMIMORPHA LONGICAUDATA* AND HELD FOR PUPATION ON VERMICULITE SUBSTRATE MIXED WITH DIFFERENT PROPORTIONS OF NEEM SEED CAKE (NSC).

Proportion of NSC (%)	Parasitized larvae ¹	Non-parasitized larvae ¹	Mean
0	11.5 \pm 0.9 aA	78.8 \pm 3.3 dB	45.1
5	10.3 \pm 1.0 aA	75.5 \pm 3.2 dB	42.9
10	11.3 \pm 2.8 aA	63.8 \pm 7.3 cB	37.5
15	6.3 \pm 1.4 aA	68.8 \pm 1.0 cB	37.5
20	6.3 \pm 1.3 aA	58.0 \pm 1.9 bB	32.1
25	6.5 \pm 0.5 aA	52.8 \pm 3.5 bB	29.6
30	5.0 \pm 1.5 aA	38.5 \pm 3.3 aB	21.8
Mean	8.1	62.3	
CV	16.56		

¹Mean values followed by the same letter within a column (lower case) or row (upper case) are not significantly different ($P > 0.05$, Scott-Knott's test)

TABLE 2. NUMBER OF DEAD *CERATITIS CAPITATA* LARVAE (MEAN ± SE) EITHER PARASITIZED OR NOT BY *DIACHASMIMORPHA LONGICAUDATA* AND HELD FOR PUPATION ON VERMICULITE SUBSTRATE MIXED WITH DIFFERENT PROPORTIONS OF NEEM SEED CAKE (NSC).

Proportion of NSC (%)	Parasitized larvae	Non-parasitized larvae	Mean ¹
0	74.8 ± 3.6	19.0 ± 3.0	46.9 d
5	84.8 ± 2.7	20.5 ± 3.7	52.6 c
10	84.0 ± 3.1	29.8 ± 6.8	56.9 c
15	91.0 ± 0.8	26.8 ± 0.8	58.9 b
20	92.0 ± 1.5	32.5 ± 2.8	62.3 b
25	90.8 ± 1.6	37.0 ± 4.1	63.9 b
30	93.0 ± 1.2	48.5 ± 5.4	70.8 a
Mean1	87.2 A	30.6 B	
CV	11.51		

¹Mean values followed by the same letter within a column (lower case) or row (upper case) are not significantly different ($P > 0.05$, Scott-Knott's test)

(Aguiar-Menezes 2005). After neem seeds have been pressed to obtain oil, 90% of azadirachtin remains in the NSC (Martinez 2002); thus only 10% of the azadirachtin content is extracted with the oil (Ermel 1995). Moreover, the effect of NSC on *C. capitata* is enhanced by the extended period of exposure beginning in the larval third instar and lasting until adult emergence.

França et al. (2010) found that an increase in medfly emergence at higher concentrations of neem was related to the repellent effect of neem to the parasitoids when larvae were treated with neem (oil) before exposure to parasitoids. As a result, the reduced parasitism rate of larvae exposed to the highest concentrations of neem favored the development of fruit fly larvae. Unfortunately azadirachtin had no contact toxicity to *C. capitata* larvae, but was toxic by contact to *D. longicaudata*.

In our studies, the third trophic level, i.e., the *D. longicaudata* parasitoids, were sensitive to compounds present in NSC, given that adult parasitoid emergence was adversely affected by all NSC concentrations. Nevertheless Stark et al.

(1992), who used lower azadirachtin concentrations than in the present study with NSC, found no effect at the lowest concentrations. Insecticidal ingredients that act by contact are absorbed through the integument of the insect or enter via the spiracles (Aguiar-Menezes 2005).

In the present experiment the NSC caused mortality of *C. capitata* larvae/pupae and negatively affected the emergence of both medflies and *D. longicaudata*. The use of *D. longicaudata* also caused a significant reduction in the medfly emergence. However, the joint use of *D. longicaudata* with NSC did not provide greater reduction of medfly emergence than when only *D. longicaudata* parasitoids were used. Although NSC had detrimental effects on *D. longicaudata* parasitoids, NSC is a cheap product that is useful to control *C. capitata* in the larval/pupal stages when applied as soil drench under the fruit fly host trees. Besides, unlike most synthetic insecticides, NSC can be used more safely together with release of *D. longicaudata*, because NSC does not negatively affect the released adult parasitoids. Lower parasitoid emergence due to detrimental effects of neem on their larval stages could harm *D. longicaudata* establishment in the field either as a natural biological control or when applied in inoculative releases. On the other hand, if NSC is used with inundative *D. longicaudata* release programs, this combination may increase the probability of controlling *C. capitata* in field.

The results of this experiment show that the use of neem-based compounds is a promising tactic in programs of integrated pest management of *C. capitata*. Foliar spray of neem formulations retains its biological activity after 5-7days of exposure to UV radiation or even longer due to its systemic property (Schmutterer 1990), and when applied as NSC under fruit trees it seems logical that it would last much longer. However, studies are required to determine the persistence of the azadirachtin in NSC used as a soil drench,

TABLE 3. PERCENT EMERGENCE OF *DIACHASMIMORPHA LONGICAUDATA* (MEAN ± SE) FROM *CERATITIS CAPITATA* PARASITIZED LARVAE HELD FOR PUPATION ON VERMICULITE SUBSTRATE MIXED WITH DIFFERENT PROPORTIONS OF NEEM SEED CAKE (NSC).

Proportion of NSC (%)	Emergence (%)*
0	51.6 ± 6.3 b
5	26.9 ± 9.9 a
10	17.4 ± 5.3 a
15	22.8 ± 13.1a
20	10.6 ± 7.9 a
25	5.0 ± 5.0 a
30	0.0 ± 0.0 a
CV	85.80

*Mean values followed by the same letter within a column (lower case) or row (upper case) are not significantly different ($P > 0.05$, Scott-Knott's test)

as well as the effects of these compounds, under field conditions, on subsequent generations of the medflies and the parasitoid, *D. longicaudata*.

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