



## Lethal and sublethal effects of neem on *Aphis gossypii* and *Cycloneda sanguinea* in watermelon

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**ABSTRACT.** The objective of this study was to evaluate the impact of oil extract of neem, *Azadirachta indica*, on the watermelon aphid *Aphis gossypii* and its natural enemy *Cycloneda sanguinea*. Toxicity bioassays were conducted with the commercial product DalNeem (1,475 g L<sup>-1</sup> azadirachtin) at 0.0037 µg a.i. mL<sup>-1</sup>, 0.0074 µg a.i. mL<sup>-1</sup> and 0.0148 µg a.i. mL<sup>-1</sup>, Malathion at 1 µg a.i. mL<sup>-1</sup> and distilled water as a control treatment. The products were sprayed to watermelon leaf discs, and the insects were exposed to the product residues. The instantaneous population growth rate of *A. gossypii* and the survival of *C. sanguinea* larvae exposed to the different treatments were calculated. A decrease in the instantaneous population growth rate of *A. gossypii* with increasing concentrations of neem was observed, and the aphids did not reproduce on the leaf discs treated with malathion during the first 24 hours of exposure due to its rapid lethal effect on adult insects. The larvae of the predator *C. sanguinea* exposed to malathion survived only for 24 hours. The survival of the predator exposed to different concentrations of neem was also significantly reduced compared to the predators exposed only to water. However, laboratory experiments may overestimate the effect of neem on predators because the individuals cannot employ escape behavior caused by neem repellency.

**Keywords:** *Citrullus lanatus*, *Azadirachta indica*, Coccinellidae, Aphididae.

### Efeito letal e subletal do nim em *Aphis gossypii* e *Cycloneda sanguinea* na cultura da melancia

**RESUMO.** O objetivo deste estudo foi avaliar o impacto de extrato oleoso de nim, *Azadirachta indica*, no afídeo *Aphis gossypii* e em seu inimigo natural *Cycloneda sanguinea*, na melancia. Ensaios de toxicidade foram conduzidos com o produto comercial (DalNeem®) (1.475 g L<sup>-1</sup> de azadirachtina) 0,0037 µg i.a. mL<sup>-1</sup>, 0,0074 µg i.a. mL<sup>-1</sup> and 0,0148 µg i.a. mL<sup>-1</sup>, Malathion 1 µg i.a. mL<sup>-1</sup>, e água destilada como tratamento controle. Os produtos foram aplicados em discos de folhas de melancia e os insetos foram expostos ao resíduo dos produtos. Foram calculados padrão de crescimento instantâneo da população de *A. gossypii* e sobrevivência das larvas de *C. sanguinea* expostos aos diferentes tratamentos. Houve uma diminuição no padrão de crescimento instantâneo da população de *A. gossypii* com aumento da concentração de nim. Afídeos não se reproduziram nos discos de folhas tratadas com malathion, devido ao rápido efeito letal em adultos, menor que 24 horas após a exposição. Larvas de *C. sanguinea* expostas ao malathion sobreviveram por 24 horas. Sobrevida do predador exposto a diferentes concentrações de nim também foi significativamente reduzida quando comparada a exposição à água. Entretanto, experimentos de laboratório podem ter superestimado o efeito do nim em predadores porque eles não mostraram comportamento de escape causado pela repelência do nim.

**Palavras-chave:** *Citrullus lanatus*, *Azadirachta indica*, Coccinellidae, Aphididae.

#### Introduction

Watermelon is a crop adapted to different growth and climate conditions in Brazil, including soil type, high temperatures, photo-oxidation, and water availability via irrigation. However, watermelon production is limited by the occurrence of disease and by the attack of insect pests that damage watermelon crops, leading to economic losses, such as the aphid

*Aphis gossypii* Glover, 1877 (Hemiptera: Aphididae) (BALDIN et al., 2009; PINTO et al., 2008). This insect causes direct damage by sucking the sap, which causes deformities of the leaves, twigs, branches and reduces the photosynthetic capacity of the attacked plants, as well as facilitating transmission of viruses of the Potyvirus genus (PINTO et al., 2008).

In the watermelon-production regions of Brazil,

the control of *A. gossypii* has been performed exclusively by the application of synthetic insecticides, the majority with a broad spectrum of action (MOREIRA et al., 2000). However, the constant use of these products may result in a series of problems, such as pest resistance to the insecticides, outbreaks of secondary pests, resurgence of pests, toxicity to the applicators and environmental and food contamination (CZEPACK et al., 2005). Additionally, non-target organisms may be negatively affected by the overuse of synthetic insecticides (AKTAR et al., 2009).

One of the main groups of natural enemies of pests associated with watermelon, particularly aphids, are insects of the family Coccinellidae (Coleoptera) (BREDA et al., 2011; SOUZA et al., 2012). Approximately 90% of the insects belonging to this group are considered beneficial because of their predatory activity, mainly on aphids (OLIVEIRA et al., 2006). Among these coccinellids, *Cycloneda sanguinea* (Linnaeus, 1763) (Coleoptera: Coccinellidae) is one of the most important species in Brazil, being found in many crops, preying on several species of aphids, including *A. gossypii* (BARROS et al., 2006; BOIÇA JUNIOR et al., 2004; COSME et al., 2007; ISIKBER, 2005; LEITE, et al., 2008; SCHUBER et al., 2012). Each larva of *C. sanguinea* may consume over 200 aphids/day (ISIKBER; COPLAND, 2001), and an adult may consume an average of 40 aphids/day (ISIKBER, 2005).

Due to environmental problems and the lack of successful pest control because of the exclusive use of synthetic insecticides, more efficient alternatives have been demanded by growers and consumers, and the use of botanical insecticides for pest control has therefore been studied within this context (AHMAD et al., 2003; BOIÇA JUNIOR et al., 2007; BREDA et al., 2011; ISMAN, 2006; MORDUE, 2004; ROEL et al., 2010; SILVA; MARTINEZ, 2004; SINGH et al., 2008; VENZON et al., 2007). Among such botanicals, neem, *Azadirachta indica* Juss., a plant of the family Meliaceae, has been used for more than two thousand years to control insects in some Asian countries, particularly in India, and the insecticidal activity of neem has been described for more than 400 species of insects (ALMEIDA et al., 2010). The main insecticide compound of most neem-based products is azadirachtin, a limonoid compound that affects insects by negatively interfering in their growth regulation, feeding activities, and reproduction (ANDRADE et al., 2012; JANINI, et al., 2011; MORDUE et al., 2010; PAVELA et al., 2004).

With the objective of assessing the feasibility of using neem to control one of the main pests of watermelon, the aphid *A. gossypii*, we evaluated the lethal and sublethal toxicity of oil extract of neem, *Azadirachta indica* Juss., on this pest and on one of the natural enemies frequently found in association with this aphid, the predator *C. sanguinea*.

## Material and methods

### Rearing of *A. gossypii* and *C. sanguinea*

Females of *A. gossypii* and adults of *C. sanguinea* were obtained from watermelon crops located in Gurupi, Tocantins, Brazil (11° 45'47 "S, 49°02'57"W). The aphids were reared on watermelon plants grown in plastic pots (3 L), kept inside wooden-frame cages (80 cm in length x 100 cm in height x 50 cm deep) covered with organza fabric. These cages were kept inside a greenhouse. To obtain the plants, seedlings of watermelon Crimson Sweet were grown in 200 mL plastic cups and transplanted one week after emergence to pots (3 L) containing a mixture of soil, sand and vermiculite substrate (1:2:1).

Adults of the predator *C. sanguinea* were reared in plastic pots (10 cm in diameter and a capacity of 500 mL) closed at the upper end with organza fabric attached using a gum-elastic. The predators were fed *A. gossypii* daily, and their eggs were removed using a moistened brush. The eggs were transferred to 200 mL plastic pots, sealed at the upper end with organza. At hatching, the larvae were individually transferred to other plastic pots (200 mL) and maintained until they reached the pupal stage. The feeding of the larvae was the same as for the adults. After emergence, the adults were distributed to new plastic pots of 500 mL for continued rearing. The pots with predators were maintained in a climate chamber regulated at  $27 \pm 2^\circ\text{C}$ ,  $70 \pm 10\%$  RH and a photoperiod of 12:12 (L:D)

### Bioassays

Experiments were performed according to the methodology adapted from Venzon et al. (2007) to evaluate the effect of neem on *A. gossypii* and its natural enemy, *C. sanguinea*. Toxicity bioassays were performed using different concentrations of a neem-based product, DalNeem (1,475 g L<sup>-1</sup> of azadirachtin), and comparing with the insecticide Malathion 500 EC (500 g L<sup>-1</sup> malathion) at the concentration recommended for watermelon aphid control (AGROFIT, 2010) and with distilled water. The respective formulations were tested on *A. gossypii* adults and on second-instar larvae of *C. sanguinea* obtained from the laboratory rearings.

The experimental design for both experiments was completely randomized, with nine replicates for *A. gossypii* and five for *C. sanguinea*. Watermelon leaves, obtained from plants grown free of pest infestation in a greenhouse, were dipped in the insecticide test solutions (products diluted in water) or water (control) for five seconds. For DalNeem, three concentrations were tested,  $0.0037 \mu\text{g a.i. mL}^{-1}$ ,  $0.0074 \mu\text{g a.i. mL}^{-1}$  and  $0.0148 \mu\text{g a.i. mL}^{-1}$ ; a concentration of  $1 \mu\text{g a.i. mL}^{-1}$  was tested for Malathion 500 EC (VENZON et al., 2007).

The leaves were left to dry for two hours. After this period, the petiole of each leaf was inserted into a hole in the lid of a glass tube containing 300 mL of water to maintain the turgor of the leaf until the end of the experiment. The hole was then sealed with moistened cotton. Each tube with a leaf was then placed inside a plastic pot (diameter 10 cm, 7 cm high) that was sealed with another plastic container of the same size, which was placed in an inverted position on the first container so that the edges of the two containers were held together. The base was removed from the bottom of the pot placed upside down, which was subsequently sealed with organza that was attached using a gum-elastic.

In the bioassay with *A. gossypii*, five adult females were transferred to each leaf. For the bioassay with *C. sanguinea*, one second-instar larva was released into each pot. Therefore, the experimental unit was represented by a pot containing one watermelon leaf with five *A. gossypii* adults or one *C. sanguinea* larva. The predator was fed ad libitum with a honey-sugar solution (10%) and adults of *A. gossypii*, which were added to the pot after the treatment applications.

For *A. gossypii*, the mortality of the adults, total number of nymphs produced by the females, and mortality of nymphs at 24 and 96h after treatment application were evaluated. In the experiment with *C. sanguinea*, each larva was followed from the second instar until its death or 240 hours. The evaluations were made at 8, 24, 48, 72, 96, 144, 192 and 240 hours after transfer of the larvae to the pots. Because 100% of the larva were alive after 240 hours in the control treatment, we did not correct the data for mortality (TAKAKURA, 2012).

#### Data analysis

To evaluate the product effects on the survival and reproduction of *A. gossypii*, for each neem concentration, the *A. gossypii* population growth rate was determined as the instantaneous rate of increase ( $r_i$ ) using the equation of Stark and Banks (1997):  $r_i = \ln(N_t/N_0)/t$ ,

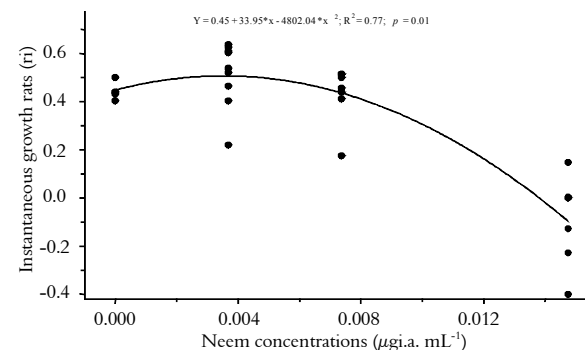
where:

$N_0$  is the initial number of individuals in the population and  $N_t$  is the number of individuals in the population at the end of time interval,  $t$  (four days). The duration ( $t$ ) of each experiment was six days. Positive values of  $r_i$  indicate a growing population,  $r_i = 0$  indicates a stable population; a negative  $r_i$  value indicates population decline, heading toward extinction (STARK; BANKS, 1997).

The survival data of *C. sanguinea* over time were analyzed using the PROC Lifetest from SAS Institute (2001). Survival curves for each treatment were compared using the Wilcoxon test of homogeneity at 5% probability (SAS INSTITUTE, 2001). PROC Lifetest uses a method called life table or actuarial estimates in which the entire observation period is divided into a series of time intervals, whereby the basic hypothesis is that censoring is distributed uniformly in each interval (TINAZZI et al., 2008).

#### Results and discussion

The instantaneous rate of population growth of *A. gossypii* decreased with increasing concentrations of neem and reached negative values at a concentration of  $0.0148 \mu\text{g a.i. mL}^{-1}$  (Figure 1). It was not possible to calculate this rate for the aphids exposed to malathion because all the individuals died within 24 hours.

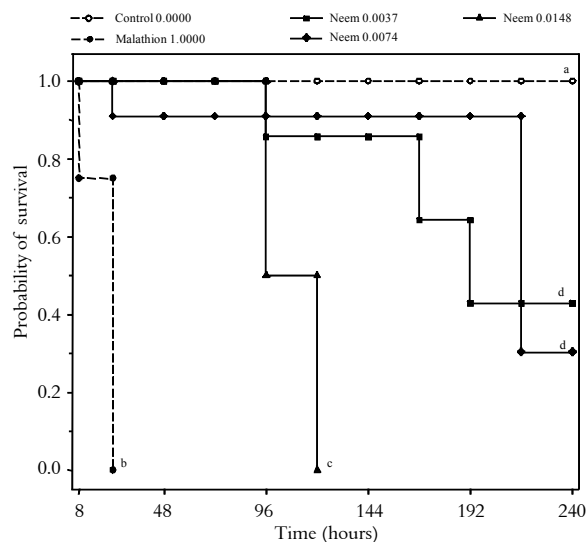


**Figure 1.** Instantaneous population growth rate ( $r_i$ ) of *A. gossypii* after four days of exposure to watermelon leaves treated with different concentrations of oil of neem.

For the predator *C. sanguinea*, significant differences in the survival curves of the larvae when they were exposed to different concentrations of neem, malathion, and water (control) were observed (Figure 2).

The survival of the larvae exposed to malathion was significantly reduced (24 hours max) compared to the larvae exposed to neem. At the highest concentration of neem tested ( $0.0148 \mu\text{g i.a. mL}^{-1}$ ), the survival of the larvae differed from the other

treatments, with the larvae living a maximum of 120 hours. Additionally, at the lower concentrations of the neem-based product (0.003688 and 0.007375  $\mu\text{g}$  i.a.  $\text{mL}^{-1}$ ), there was significant reduction in larval survival, which became more pronounced from 168 hours of exposure. At the highest concentration (0.0148  $\mu\text{g}$  i.a.  $\text{mL}^{-1}$ ), all the predatory larvae were dead at 120 hours of exposure.



**Figure 2.** Probability of survival of *C. sanguinea* exposed to watermelon leaves with *A. gossypii* and treated with different concentrations (in  $\mu\text{g}$  i.a.  $\text{mL}^{-1}$ ) of oil of neem, malathion and water (control) as a function of time. The lines followed by the same letter indicate no significant difference among them by the Wilcoxon test ( $p \geq 0.01$ ).

All the concentrations of neem and the insecticide malathion were toxic to the adults and nymphs of *A. gossypii* and to the predatory larvae of *C. sanguinea*. However, the toxicity level varied with the product and concentration. The neem-based product exerted its toxic effect slowly, in contrast to malathion, which exhibited a rapid killing effect on both species.

A reduction in the instantaneous growth rate of *A. gossypii* as a function of increasing concentrations of neem was observed. The instantaneous growth rate of *A. gossypii* was negative at the highest concentration of neem tested, indicating that the populations of this pest exposed to neem displayed an extinction tendency. Pinto et al. (2013), found that one application of neem and thiametoxan achieved similar control of cotton aphids at 72h after treatment followed by cotton seed oil. Santos et al. (2004) found that neem seed powder extracts negatively influenced the reproduction of *A. gossypii* and caused the mortality of nymphs. Studies by Venzon et al. (2007), which evaluated the effect of neem on the aphid *Myzus persicae* (Sulzer, 1776)

(Hemiptera: Aphididae) and on its predator *Eriopis connexa* (Germar, 1824) (Coleoptera: Coccinellidae), also found a negative correlation between the neem concentration and population growth rate of aphids. The authors also reported lethal and sublethal effects of neem on the predator.

Esparza-Diaz et al. (2010), found that the effect on mortality depends on the dose and type of *A. indica* extract; for *A. gossypii*, an oily extract of *A. indica* produced a lower mortality than a methanolic extract. The mortality caused by the methanolic and aqueous extracts also increased with the dose, though this did not occur for the hexanic and oily extracts, the effects of which decreased at the highest doses.

Malathion is a fast-acting insecticide that acts as an inhibitor of the enzyme acetyl cholinesterase, resulting in the accumulation of acetylcholine in the synaptic cleft and killing by the hyperexcitability of the central nervous system of the insect due to the continuous and uncontrolled transmission of nerve impulses (MAHAT et al., 2012). In contrast to malathion, neem action in insects is slow. Neem can stop the molting process, causing death of the insect during both the nymphal and larval stages or during the pupal period, because neem acts as a growth regulator, directly interfering with the balance of ecdysone and juvenile hormone during the synthesis of chitin and interfering during ecdysis and pupation (SINGH et al., 2008). Thus, an evaluation of acute toxicity only would not show the full insecticidal potential of neem. For this reason, we estimated the instantaneous rate of population increase ( $r_i$ ) of *A. gossypii* after exposure to neem residues; thus, we considered both the effects of neem on the survival and reproduction of the aphid after the period of time required for neem action. A negative value of the instantaneous rate of population increase ( $r_i$ ), indicating that the population is in decline, was obtained only at the maximum neem concentration tested.

Although the aphid population could be controlled using the highest neem concentration tested (0.0148  $\mu\text{g}$  i.a.  $\text{mL}^{-1}$ ), we also found that predator survival was reduced at this concentration compared to the lower concentrations tested. In addition, neem was toxic to *C. sanguinea* at the lower tested concentrations when compared to the control treatment. Similar results have been reported for other coccinellids (VENZON et al., 2007).

Breda et al. (2011), found that, despite the potential for use in the management of *A. gossypii*, the aqueous extract of neem showed significant toxicity to larval instars 1 and 4 of *C. sanguinea*, at the concentrations lethal and sublethal to the

aphid. Thus, care should be taken when using neem to control aphids, and it is important to monitor the pest by sampling to verify the actual need of application and the presence of predators. Moreover, it is important to note that the magnitude of the negative impact of neem on the predator in this study may be overestimated under the conditions of the experiment. The *C. sanguinea* larvae were continuously exposed to residues of the product because they were trapped in arenas that restricted their mobility. Thus, it becomes necessary to perform experiments using different exposure methods to simulate field conditions under which the predator has greater mobility and ability to escape to safe refuges free of insecticide. Cordeiro et al. (2010), found that a neem-based product (Neempro) caused irritability and repellency to larvae of the predator *Chrysoperla externa*. Indeed, this behavior may mitigate the negative effect of neem on predator populations under field conditions.

The watermelon crop has grown in economic importance in Brazil, and this has generated an increasing demand for areas of crops, thus increasing monoculture areas in some regions. The use of neem may be a favorable alternative to reduce the negative impacts of the exclusive use of chemical control, thereby avoiding such adverse problems as the appearance of insect resistance to the insecticide and the outbreak and resurgence of pests (NAUEN; ELBERT, 2003). The development of pest resistance using neem is minimized due to the different modes of action and the presence of azadirachtin and other active compounds in the formulated products (ASCHER, 1993).

It is important to note that the occurrence of the predator *C. sanguinea* is mainly in areas with large aphid infestations. Thus, the maintenance of small populations of aphids through the application of neem-based products early during infestations would prevent the attraction of the coccinellids that focus on aphids, which should help to minimize the effects of neem on these predators because they possibly avoid contact with these insecticides (AHMAD et al., 2003). Therefore, despite the advantages presented by the use of neem insecticides, field trials are still needed to assess the ultimate impact on the populations of natural enemies in watermelon crops.

## Conclusion

All concentrations of neem and the insecticide malathion were toxic to the adults and nymphs of *A. gossypii* and to the predatory larvae of *C. sanguinea*.

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