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INHIBITION OF OVIPOSITION BY NEEM EXTRACT: A BEHAVIORAL PERSPECTIVE FOR THE CONTROL OF THE MEDITERRANEAN FRUIT FLY (DIPTERA: TEPHRITIDAE)

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Abstract

The behavioral inhibitory effect of methanol extracts from neem leaves (*Azadirachta indica* A. Juss) at different concentrations (0, 10,000, 18,000, 32,000 and 56,000 ppm) was evaluated using naïve and experienced medflies (*Ceratitis capitata* (Wied.)) ovipositing on the fruits of grape cv. 'Itália'. The grapes were immersed in the specific treatments and were exposed for 24 h to 3 pairs of female and male medflies, both naïve and experienced, in a choice test. At concentrations ≥ 18,000 ppm, the extract that was applied to the grapes inhibited oviposition. The previous experience with treated grapes did not affect the response of the medflies. This study is the first step toward the application of the behavior control of the medflies as a tool in grape vineyards. The potential for using oviposition inhibitory in behavioral control of *C. capitata* are discussed.

Key Words: neem; limonoids; azadirachtin; fruit fly; insect learning; *Vitis vinifera*

Resumo

Avaliou-se o efeito da inibição para oviposição do extrato metanólico de folhas de nim (*Azadirachta indica* A. Juss.) em diferentes concentrações (0; 10.000; 18.000; 32.000 e 56.000 ppm) para *Ceratitis capitata* (Wied.), com e sem experiência, em bagas de uva cv. Itália. Para tanto, bagas de uva foram imersas nos tratamentos e expostas por 24 h para três casais, com e sem experiência, em teste com chance de escolha. O extrato provocou inibição da oviposição de *C. capitata* quando pulverizados em bagas nas concentrações ≥ 18.000 ppm em experimento com chance de escolha. A experiência recente de *C. capitata* não alterou a sua resposta em relação a bagas de uva tratadas com extrato de folhas em metanol em condição de livre escolha. O papel da inibição de oviposição e do aprendizado para *C. capitata* são discutidos. A utilização de inibidores de oviposição como controle comportamental de *C. capitata* é discutida. Esse estudo representa o primeiro passo para utilização do controle comportamental de *C. capitata* em parreirais de uva.

The São Francisco River Valley (SFRV) in northeastern Brazil produces 95% of Brazil's grape exports. Three decades ago, Malavasi et al. (1980) reported low numbers of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) in the region. During this period, the fly's host plants were still dispersed over the holdings of small growers. With the expansion of the fruit-growing areas, an explosive increase occurred in the medfly populations on the farms (Haji et al., 2005). Until 1997, *Anastrepha* spp. were more abundant than the medfly. Subsequently, however, the medfly has become the dominant pest in rural areas (Haji et al. 2005). In this region, the primary hosts of *C. capitata* are *Malphigia glabra* L. (acerola, Barbados cherry), *Psidium guajava* L. (guava) and *Mangifera indica* L. (mango). These crops are cultivated along the irrigated perimeters near the grape-growing areas. After sexual maturity, the adults disperse through the grape orchards, and the females lay eggs in the grapes. This behavior explains the pattern of the concentration of damage to the crop and the capture of medflies in the traps located on the borders of the vineyards. Despite the evidence that the medfly is beginning to colonize the grape cultivars in the SFRV, it is still believed that the grape vineyards do not support resident medfly populations because of the low suitability of the grapevines as hosts.

The behavior and ecology of *C. capitata* in grape orchards allow the application of a push-pull control strategy. This approach uses derivatives of neem (Azadirachta indica A. Juss) to in-

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hibit oviposition ("the push") and other strategies ("the pull"), as McPhail traps, bait stations and/or primary hosts as trap plants. Unfortunately, the push-pull technique is not suitable for species with elevated growth rates. In addition, adaptive learning by the insects could interfere with the effectiveness of the control method (Cook et al. 2007).

The medfly populations in the SFRV do not reside in the vineyards. This fact implies that the populations to be controlled are small and that the risk of adaptive learning by the insects is relatively low. In the past, learning has been observed to alter the oviposition behavior of insects (Cunningham et al. 1998; Rojas and Wyatt 1999). The comprehension of its role is essential for the adequate evaluation of repellents and deterrents, consisting in one of the main factors from the effective development of behavioral manipulation methods by the use of these substance groups on the field (Liu & Liu 2006). Investigations examining non-host plants or extracts have demonstrated that the insects' experiences [eg. *Pluttella xylostella* (L.)] may induce oviposition on non-host plants or on host plants treated with extracts from non-host plants (Liu et al. 2005; Liu & Liu 2006; Zhang et al. 2007; Wang et al. 2008).

In this study, we tested the hypothesis that a methanol extract of neem leaves applied to grapes could inhibit the oviposition of *C. capitata* in an experimental choice paradigm. In addition, we investigated the influence of learning (experience) on medfly oviposition. This study is the first step toward the application of the push-pull strategy as a tool in the integrated management of the medfly in grape vineyards in the northeast of Brazil.

Materials and Methods

Extracts

Azadirachta indica leaves were collected from trees, and were dehydrated in an oven at 40 °C for 48 to 96 h. Next, they were ground to powder in a knife mill. To prepare the extract, a sample of 200 g of powder was divided equally among five Soxhlet extractors. A filter paper cartridge containing 40 g of powder was placed in each extractor with 300 mL of solvent. Hexane was the first solvent used. After 24 h of reflux, the hexane was removed and dichloromethane added. After 12 h of reflux, the dichloromethane was removed and methanol was added. The sample was kept under reflux with the methanol for 13 h. These reflux times were chosen to ensure that the extraction was complete in each case. The completion of the extraction could be verified by observing that the solvent became colorless after a sufficiently long exposure to the sample. This change indicated that the extraction had reached its limit. The extracts were concentrated in a rotary evaporator at 40 °C at low pressure using a water column. After this procedure, the extracts were placed in glass flasks in a laminar flow cabinet until the solvents evaporated completely.

The leaves are the structure (matter excels) more abundant in the neem plant for the production of extracts. Besides it, the most common limonoids with higher activity over insects found in the neem plant are polar compounds (azadirachtin, salanin and 3- tigloylazadirachtol). That is the reason why the leaf extract in methanol was chosen for the bioassay. The determination of the azadirachtin levels from the leaf extract in methanol was performed according to Forim et al. (2010).

Bioassays

A bioassay was performed using a choice test to evaluate the inhibition of oviposition induced by the neem leaf extract in methanol at different concentrations (0, 10,000, 18,000, 32,000 and 56,000 ppm). Plastic cages each 13 cm diam ×16 cm high were used. On the lid, an opening 6 cm in diameter covered with anti-aphid netting allowed aeration. The insects were introduced through a different opening in the side of the cage. Three pairs of medflies (5-d-old) were placed in a cage with deionized water and an artificial diet (1 part hydrolyzed protein to 3 parts sugar), which were offered to the insects ad libitum. Grapes cv. 'Italia' were immersed for 5 s in one of the neem treatments or in deionized water (the control). After the immersion, the excess moisture from the grapes was removed by placing the grapes on paper towels for 10 min. Next, 2 grapes were placed on the top of each cage at equidistant points. After 24 h, the grapes were removed and the number of eggs, punctures and eggs per puncture were recorded for each grape. The 5 treatments followed a completely randomized design with 15 replicates per treatment.

A second bioassay was conducted to evaluate the females' recent learning experiences (i.e., their habituation to the treatment after recent experience). On the d after the grapes had been removed, new grapes (treated and control) were exposed to the same medfly females, which were 6 d old. These grapes were exposed for 24 h, and the same parameters previously cited were undertaken. The 5 treatments formed a completely randomized design with 15 replicates per treatment.

Statistical analysis

To study the inhibition of oviposition, the Deterrence Index was applied (DI) (Lin et al. 1990). The index is calculated using the formula DI = 2G/(G+P), where G = % eggs (or punctures) in the

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treated grape and P = % eggs (or punctures) in the control. Based on the DI and on the standard deviation values obtained, the Classification Intervals (CI) for the means of the treatments were estimated by the formula:

$$CI = 1 \pm t_{(n-1;\alpha=0.05)} \times \frac{SD}{\sqrt{n}},$$

where t = the value of the Student's t corresponding to a probability of 5%, SD = standard deviation, and n = number of replicates. An extract was considered to have no effect if the estimated DI value was inside the CI. The extract was considered to have an inhibitory effect if the DI value was less than the lower bound of the CI. The extract was considered to have a stimulating effect if the DI value was greater than the upper bound of the CI. The number of eggs per puncture and the percentage of punctures on the different eggs classes (0 to 5; 6 to 10; 11 to 15; and above 15 eggs) found for naïve females and for females with previous experience in the choice test were compared using the Student t-test ($\alpha = 0.05$).

Results

An inhibitory effect was observed at concentrations greater than or equal to 18,000 ppm (1.8%) of the neem leaf methanol extract. This effect was found both for females that had previously laid eggs in treated grapes (females with recent experience) and for naïve females (Table 1). For the naïve females, at concentrations equal to or higher than 18,000 ppm, the number of eggs laid per grape was less than 25% of the total number of eggs laid (treatment plus control) (23.10%, 23.19% and 21.77% at concentrations of 18,000, 32,000 and 56,000 ppm, respectively). At these concentrations, the total amount of eggs laid in the respective control conditions was more than 75% of the summed amount of eggs laid in the treatment and control conditions (Table 1). The same behavior was observed for the experienced females. The concentrations of 18,000, 32,000 and 56,000 ppm were responsible for inducing oviposition inhibition, regardless of female experience (Table 1). A reduction in the number of punctures per grape was observed for both naïve and experienced females at all of the concentrations (Table 2). Therefore, the extract was characterized as an inhibitor of oviposition for the medfly in both cases (Table 1 and Table 2). No significant differences among treatments in the number of eggs per puncture were observed. The experience of the flies did not change their oviposition behavior, as the oviposition patterns of experienced and naïve females were similar. There was also no significant difference on the frequency of punctures (%) in the range of eggs laid per puncture (0 to 5; 6 to 10; 11 to 15; > 15 eggs).

Discussion

In general, we suggest that four mechanisms are involved in the inhibition of the oviposition of fruit flies: the repellent effect, locomotor stimulation, suppressor effects and/or deterrent effects. The data presented in this study revealed small

Table 1. Percentages of eggs laid by naïve and experienced medflies in grapes and oviposition deterrence index (DI ± SE) in the free-choice test.

Experience	Concentration[1] Extract	Concentration[1] Aza[2]	Eggs (%)	Deterrence Index (M ± SE)	Classification Interval	Classification[3]
Naïve	10,000	0.0060	33.16	0.66 ± 0.17	(0.64; 1.36)	Null
	Control	—	66.84			
	18,000	0.0108	23.10	0.46 ± 0.11	(0.76; 1.24)	Inhibitor
	Control	—	76.90			
	32,000	0.0192	23.19	0.46 ± 0.10	(0.78; 1.22)	Inhibitor
	Control	—	76.81			
	56,000	0.0336	21.77	0.44 ± 0.11	(0.76; 1.24)	Inhibitor
	Control	—	78.23			
Experienced	10,000	0.0060	38.09	0.76 ± 0.12	(0.75; 1.25)	Null
	Control	—	61.91			
	18,000	0.0108	26.18	0.52 ± 0.10	(0.79; 1.21)	Inhibitor
	Control	—	73.82			
	32,000	0.0192	27.33	0.55 ± 0.12	(0.74; 1.26)	Inhibitor
	Control	—	72.67			
	56,000	0.0336	25.39	0.51 ± 0.11	(0.77; 1.23)	Inhibitor
	Control	—	74.61			

[1]Concentration: ppm [2]Aza: Azadirachtin; [3]Classification where Null: included in the classification interval (CI < DI < CI); Inhibitor: DI < CI; and Stimulant: DI > CI.

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Table 2. Percentages of punctures made by naïve and experienced medflies in grapes and oviposition deterrence index (DI ± SE) in the free-choice test.

Experience	Concentration[1] Extract	Concentration[1] Aza[2]	Punctures (%)	Deterrence Index (M ± SE)	Classification Interval	Classification[3]
Naïve	10,000	0.0060	32.72	0.65 ± 0.13	(0.71; 1.29)	Inhibitor
	Control	—	67.28			
	18,000	0.0108	29.98	0.60 ± 0.12	(0.75; 1.25)	Inhibitor
	Control	—	70.02			
	32,000	0.0192	23.59	0.47 ± 0.10	(0.78; 1.22)	Inhibitor
	Control	—	76.41			
	56,000	0.0336	24.06	0.48 ± 0.10	(0.78; 1.22)	Inhibitor
	Control	—	75.94			
Experienced	10,000	0.0060	38.82	0.78 ± 0.10	(0.79; 1.21)	Inhibitor
	Control	—	61.18			
	18,000	0.0108	27.66	0.55 ± 0.08	(0.83; 1.17)	Inhibitor
	Control	—	72.34			
	32,000	0.0192	27.22	0.54 ± 0.12	(0.75; 1.25)	Inhibitor
	Control	—	72.78			
	56,000	0.0336	25.22	0.50 ± 0.10	(0.79; 1.21)	Inhibitor
	Control	—	74.78			

[1]Concentration: ppm [2]Aza: Azadirachtin; [3]Classification where Null: included in the classification interval (CI < DI < CI); Inhibitor: DI < CI; and Stimulant: DI > CI.

numbers of eggs and punctures in treated grapes. The estimated values of the deterrence index suggest that oviposition was inhibited. The number of eggs per puncture in the treated grapes and in the control was not significantly different. Punctures with fewer eggs (especially punctures having between 0 and 5 eggs) were not more frequent in treated grapes. This result contradicts the hypothesis that the extract had a deterrent effect on *C. capitata* oviposition. In other words, after the introduction of the ovipositor, the presence of the extract on the surface of the grape did not affect the medfly's oviposition behavior. The inhibition of oviposition resulted from the interaction or isolated action of the *A. indica* leaf extract as repellent, locomotor stimulus and/or suppressor. The negative stimulus may have facilitated one or more of the following behavioral tendencies: it may have induced the females to move away from the treated grape (repellent), it may have induced the females to move and disperse more quickly in the presence of the neem extract (locomotor stimulus) and it may have inhibited the initial penetration of the ovipositor (suppressor stimulus). The findings of this study can be extended to other fruit fly species. Initially, Singh & Srivastava (1983) demonstrated that neem seed extract inhibits the oviposition of *Bactrocera cucurbitae* (Coquillett) and *Bactrocera dorsalis* (Hendel) when sprayed over *Momordica charantia* L. and *Psidium guajava* L. fruits, respectively. In another study (Chen et al. 1996) of *B. dorsalis* on guava fruits treated with neem seed extracts, the oviposition of the females was inhibited. In addition, fewer females were found on fruits treated with neem extracts. This last-mentioned finding suggests that the *A. indica* extracts acted as a repellent and/or as a locomotor stimulus. The results reported by Valencia-Botín et al. (2004) also suggest that the neem extract's property of repelling insects is the main factor responsible for the smaller numbers of *Anastrepha ludens* (Loew) eggs in oranges sprayed with neem aqueous extract (5%) and neem oil (Neemix® 4.5%).

For the control of fruit flies in the field, the repellent effect, locomotor stimulus and suppressor effects are more useful than the deterrence of oviposition. The first three mechanisms inhibit the introduction of the ovipositor by the medfly and, consequently, reduce the number of punctures and the number of eggs laid, as verified by this study. The females produce damage directly by penetrating the grapes with the ovipositor. By doing so, they allow the entrance of microorganisms and induce deformations, rottenness and fruit fall. To support the adoption of the push-pull strategy, such mechanisms must be functional to stimulate the dispersion of the insects in the area and attract them easily to a single site of control. In the SFRV in Northeastern Brazil, the neem extract could help to protect the vineyards by preventing the entrance and dispersion of the medfly.

The results of this study are consistent with the findings of Chen et al. (1996), which indicated that the ovipository inhibition of *B. dorsalis* females on guava fruits treated with *A. indica* extracts was not affected by previous experience. The authors verified that the inhibition of oviposition persisted after exposing the guava fruits for 7 d, replacing them each 24 h. Investigations of the

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significance of preimaginal experience with oviposition-repellent or deterrent allelochemicals are notably rare. Previous studies have demonstrated that the moth *P. xylostella*, unlike *C. capitata* (present study) or *B. dorsalis* (Chen et al. 1996), has the ability to use recent experience (Liu et al. 2005; Liu and Liu 2006; Zhang et al. 2007; Wang et al. 2008). The influence of learning in *C. capitata* has already been demonstrated in the case of the search for hosts. The preimaginal experience of the immature fly allows the choice of hosts to be made according to that experience (Prokopy et al. 1989). Other studies have shown that, after a female has landed on a host fruit, the acceptance or rejection of the fruit by the fly is conditioned by the recent oviposition experience of the adult (Cooley et al. 1986; Papaj et al. 1987).

This study has shown that, under laboratory conditions, a methanol extract of neem leaves applied to grapes at concentrations higher than 18,000 ppm can inhibit the oviposition of *C. capitata* if the insect is allowed to choose between treated and nontreated substrates. The experience of *C. capitata* did not affect its responses to grapes treated with the methanol extract. The inhibition of the ovipository behavior of the insect is highly valuable in pest management. Because a simple puncture represents damage to the fruit, a reduction of the number of punctures makes the use of the neem leaf extract in methanol a promising approach to the management of the medfly. It is also important to note that the flies did not become habituated to the extract at different concentrations. The use of extracts is promising because the experience of the females with treated fruits did not affect their ovipository behavior. Investigations examining medfly control in grape-growing areas are still rare. The use of synthetic pesticides in toxic baits is not possible because the registered toxic baits in Brazil produce spots on the grapes. The application of pesticides to an entire area poses a high risk to the workers' health. In addition, pesticides may increase the incidence of such secondary pests as the whitefly *Bemisia tabaci* B-biotype (Gennadius) and may disturb populations of the natural enemies of pests; also, pesticides may leave residues in the fruits and prevent their sale on international markets.

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