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## RESEARCH

# Do *Sesamia nonagrioides* (Lepidoptera; Noctuidae) Gravid Females Discriminate Between Bt or Multivitamin Corn Varieties? Role of Olfactory and Visual Cues

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**ABSTRACT.** The Mediterranean corn borer, *Sesamia nonagrioides* Lefèbvre, is a key pest of corn and a main target of *Bacillus thuringiensis* (Bt) corn in Northeast Spain. Trends for future biotechnology crops indicate that Bt, non-Bt, and stacked corn varieties with metabolic pathways for vitamin-increased traits could coexist in same region. Knowledge of the oviposition response of gravid females of *S. nonagrioides* to these different varieties could be extremely important for managing strategies aimed for delaying resistance development. In dual-choice assays, we examined the host preference of gravid females of *S. nonagrioides* for four corn varieties: a new transgenic corn with increased vitamin levels, its near isogenic counterpart (M37W), a Bt corn plant, and its near isogenic counterpart. Olfactory cues were the predominant ones when gravid females looked for a suitable host to lay eggs, and no synergistic effects were observed when both visual and olfactory cues were present. When the plant was visible, the females preferred the odors emitted by the nontransgenic to its multivitamin transgenic counterpart and when they only could detect the volatiles they also preferred the nontransgenic M37W variety to the Bt corn variety. If gravid females are less attracted to corn with an increased level of vitamins, this could impact insect resistance management and the value of refuge plants, if such traits are stacked with an insect resistance trait.

**Key Words:** agricultural entomology, behavior

Genetically modified (GM) organisms are those that have been modified by the application of recombinant DNA technology or genetic engineering, a technique used to alter a living organism's genetic material. With the rapid advances in biotechnology, a number of GM or transgenic crops carrying novel traits have been developed and released for commercial agriculture production. These include pest-resistant cotton, corn, canola (mainly Bt or *Bacillus thuringiensis*), glyphosate herbicide-resistant soybean and cotton and viral disease-resistant potatoes, papaya, and squash FAO (2011). These characteristics have brought several positive farm impacts, such as the decrease in the use of insecticides and herbicides to control corn borers and weeds, leading to a reduction in the contamination of the air, groundwater, and soil that improves the health and safety of farm workers, it has also resulted in savings in energy, equipment, machinery, and human labor (Batista and Oliveira 2009).

In addition, various transgenic crops under development, that have not yet been commercially released, have traits for biofortification, phytoremediation, and production of pharmaceuticals, such as rice or corn with a high level of carotenoid for production of vitamin A (e.g., golden rice) and bananas with vaccines FAO (2011). Commercial cultivation of transgenic crops started in the early 1990s. The choice of GM crops varies among the developing countries, with insect-resistant cotton being the most important commercially produced transgenic crop in Asian and African countries, while herbicide-resistant soybean followed by insect-resistant corn is predominant in South America (FAO 2012).

In 1997, the European Union authorized the cultivation of transgenic plants expressing Bt and in the following year, Bt corn began to be cultivated in Spain. Since then, the cultivated area has increased from 20,000 hectares in 1998 to 137,000 hectares in 2013; and Bt corn production in Spain accounts for 92% of the European Union total (Riesgo 2013). The initial Bt corn hybrids were developed to control the European corn borer, *Ostrinia nubilalis* (Hübner), but they were also found to be efficient for controlling the Mediterranean corn borer *Sesamia nonagrioides* (Lefèbvre) (Eizaguirre et al. 2005). Regional

yield rates of Bt corn are variable and related to corn borer damage (Gomez-Barbero and Rodriguez-Cerezo 2006). Riesgo (2013) indicated that in Aragon and Catalonia, the two regions in Spain suffering the highest pressure from corn borers, the yield of Bt corn varieties was 10% and 8% higher, respectively, than the yield of non-Bt varieties.

Apart from the herbicide resistance/tolerance and insect resistance, new GM corn approaches have aimed to ensure the food safety of millions of people. Naqvi et al. (2009) developed an elite inbred South African transgenic corn plant (MV), not yet commercialized, in which the levels of three vitamins,  $\beta$ -carotene, ascorbate, and folate increased. The transgenic kernels contained 169 times the normal amount of  $\beta$ -carotene, 6 times the normal amount of ascorbate, and twice the normal amount of folate.

Lepidopteran females use host plant quality to make decisions about whether to lay eggs. Some studies have shown that, when confronted with an array of potential hosts, females display a hierarchy in their preferences, laying more eggs on their most preferred plant, fewer eggs on their next preferred plant, and so on (Thompson and Pellmyr 1991, Awmack and Leather 2002). These preferences are mainly related to the characteristics of the host plant, such as volatile and nutritional chemistry and morphology.

The role of vision in insect interactions with host plants has received relatively little attention because it is assumed that the chemical cues are the ultimate sensory determinants of host findings (Reeves 2011). However, several studies have shown that vision can be even more important than olfaction, particularly in diurnal insects (Aluja and Prokopy 1993, Harris et al. 1993, Withers and Harris 1996, Patt and Sétamou 2007, Reeves and Lorch 2009) and that some nocturnal moths use visual and odor cues during host finding (Rojas and Wyatt 1999) or to elicit feeding behavior (Raguso and Willis 2002). Furthermore, many insect orders use their excellent night vision to discriminate colors, orient themselves using faint celestial cues, fly unimpeded through a complicated habitat, and navigate (Warrant and Dacke 2011). On the other hand, Ramaswamy (1988) and Balkenius et al. (2006) give several examples in which nocturnal moths do not use vision during

host location. Therefore, the use of vision in nocturnal moths during host location is still unclear, and its role probably varies among species.

The Mediterranean corn borer *S. nonagrioides* is a major pest of corn crops around the Mediterranean region. It is an oligophagous species with a fairly wide range of host plants, mainly of the Poaceae family, and the corn plant is its major host (Glaser et al. 2013, Lopez et al. 2013, Dimotsiou et al. 2014). Lopez et al. (1999) noticed that *S. nonagrioides* females mated before flying to localize a suitable host to lay eggs, whereas Robert and Frerot (1998) found that gravid females are not affected by the presence of conspecific eggs. Konstantopoulou et al. (2002, 2004) studied the corn chemical cues that stimulate oviposition in gravid females and reported a significant difference in the female choice and oviposition response between the hybrids tested.

Jaenike (1990) signaled that females have evolved to select those host plants that maximize larval survival and growth, so gravid females of *S. nonagrioides* would be expected to choose for oviposition those plants with most nutritional benefits, such as the transgenic corn plant with increased vitamin content developed by Naqvi et al. (2009). Gravid females would also be expected to discriminate between Bt and non-Bt plants, although some studies have shown that ovipositing females of different moths cannot discriminate between Bt plants and their isogenic counterpart (Hellmich et al. 1999, Torres and Ruberson 2006, Van den Berg and Van Wyk 2007). In the region where this study was carried out, non-Bt and Bt corn fields currently coexist and one should not rule out future planting of new varieties of multivitamin corn with very different characteristics, among which gravid females of *S. nonagrioides* may choose the most suitable ones for oviposition.

The aim of this study was to determine whether gravid females of *S. nonagrioides* could discriminate between transgenic multivitamin or Bt corn plants versus their respective near isogenic counterparts, based on the emission of chemical odor cues in dual-choice olfactometer assays. It also considered the role of vision in the host location process of gravid females.

## Materials and Methods

**Insects.** A laboratory culture was established with *S. nonagrioides* larvae collected from corn fields in the Lleida area (northeast Spain). Larvae were fed on a semiartificial diet at 25°C and a photoperiod of 16:8 (L:D) h, and the culture was renewed every three or four generations with larvae or pupae from the field (Eizaguirre and Albajes 1992). For oviposition, single adult couples resulting from the laboratory culture were caged with a five-leaf corn plant. Laid eggs were maintained on the plant till the day before hatching (larval capsule visible). Then, the eggs and a piece of leaf were transferred to a transparent cylindrical container (3.5 cm in diameter by 2 cm in height) with 1 cm<sup>3</sup> of diet. Seven days later, the larvae were separated and reared individually in a similar container until pupation. Pupae were sexed by external morphological differences and the resulting adults were separated by sex and maintained at 20°C and a photoperiod of 16:8 (L:D) h. For mating, newly emerged males and females were transferred to a plastic container (50 × 50 × 50 cm) at a ratio of 1♀:2♂. The adults were not given food. After 24 h, the females were collected and used for dual-choice bioassays. After the experiments, mated females were then dissected to confirm the presence in the bursa copulatrix of spermatophores, which indicate successful mating.

**Plants.** Corn, *Zea mays*, seeds from four corn varieties tested, Bt corn DKC 6667 and its near isogenic counterpart DKC 6666 (Delkab, Monsanto, St Louis, Missouri, USA), the transgenic multivitamin MV, not yet commercial, and its near isogenic counterpart M37W, were sowed in regular potting soil in plastic pots (diameter 8 cm, depth 9 cm). The plants were kept in a greenhouse under a photoperiod of 16:8 (L:D) h at 25 ± 2°C and 70% relative humidity (RH). Between 15 and 20 d after planting, seedlings with four to five leaves were used for the experiments.

**Host Plant Preference and Vision Role in the Host Location.** A Y-tube olfactometer was used to test the olfactory and visual responses of 24-h-old adult mated females of *S. nonagrioides* to the odors emitted by the different corn plant varieties.

The Y-tube olfactometer consisted of three glass chambers (diameter 20 cm, length 34 cm) linked by glass tubes (diameter 10 cm, length 30 cm): one chamber was a reservoir, for the test insects and the other two were the experimental chambers for the plants (Fig. 1A). The tubes diverged in a “V” form at an angle of 45°. The dimensions of the tubes and reservoirs offered the insects sufficient room to fly freely inside the olfactometer. Each of the experimental chambers had an inlet fan (3 cm diameter) 28 cm from the base to introduce the air in the olfactometer, and the reservoir chamber had two exhaust fans at a similar height to extract the air and produce an air stream from the experimental chambers to the reservoir chamber. The inlet fans and the exhaust fans worked concomitantly to provide an air speed of 0.22 m/s. The olfactometer was located in a climate room under a photoperiod of 16:8 (L:D) h at 25 ± 5°C and 65 ± 5% RH, illuminated by red light of 4 lux of intensity that allows observation without disrupting insect behavior (Sans et al. 1997).

For each experiment, a group of 3–4 gravid females was placed in the reservoir chamber and a pot with a plant of the different varieties was placed in each experimental chamber, in paired tests. This low number of females in each experiment allowed them to be controlled individually. The time given to the insects to respond to the corn plant varieties was the first 3 h after the onset of the scotophase, since during this time gravid females of *S. nonagrioides* seek host plants (preliminary experiments). When a female arrived at a plant, it was gently removed from the olfactometer. After 3 h, the number of females that reached each of the two varieties of the experiment was recorded. Each experiment was repeated until at least 40 insects responded. Only the females that performed the oviposition behavior (oriented flight to the odor source, sweeping the plant with the ovipositor) and laid eggs were taken into account for the statistical analysis. Females that showed calling behavior were not considered and were removed from the olfactometer. After each experiment, the corn plant varieties in the chambers were exchanged to avoid a positional bias, and the order of treatments was randomized. The olfactometer was thoroughly cleaned with ethanol after each session.

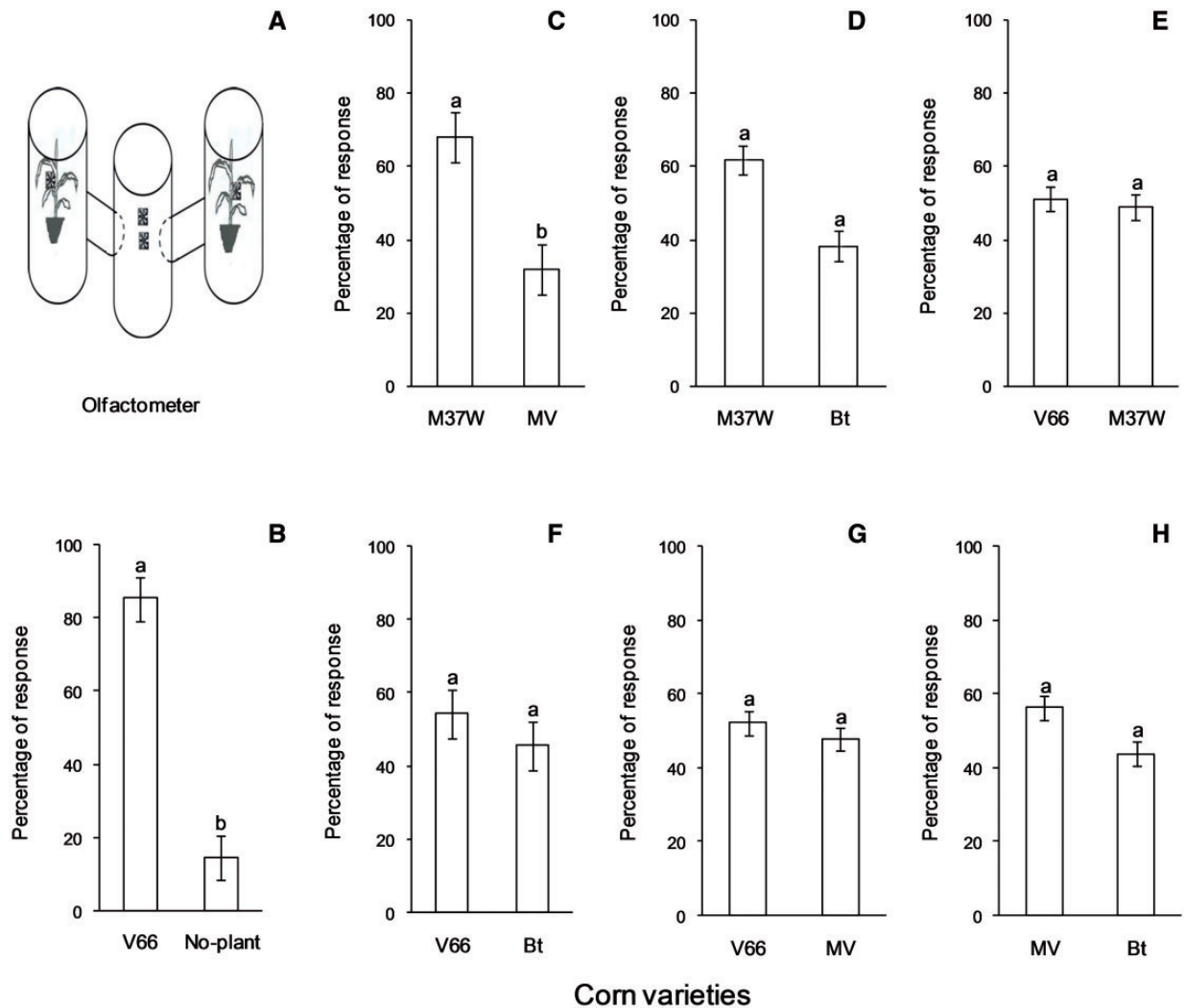
To evaluate only olfactory cues, eliminating the role of vision at the host location by the gravid females, the same paired experiments with the four corn varieties were performed but the corn plants were hidden behind a black screen perforated with holes (a circle of 10 cm diameter with 100 holes of 0.5 mm diameter) to allow the insects to perceive the plant volatiles without the vision cues (Fig. 2A). Results obtained when the females could see the plants were compared with results obtained when the females could not see the plants but perceived the plant volatiles through the holes in the screen.

**Statistical Analysis.** All statistical analyses were done using JMP Version 8 (2008). For all comparisons, the level of  $P \leq 0.05$  was considered significant. All data obtained from the dual-choice assays were subjected to a binomial test (Zar 1996), considering that females showed no preference for either olfactometer arm (variety) (50:50 response). Responses were converted to percentages for presentation.

## Results

### Host Plant Preference

**Olfactory Plus Vision Cues.** Figure 1 shows the response of the gravid females of *S. nonagrioides* to the different corn varieties in the paired experiments. A scheme of the olfactometer with the visible plants is represented in Fig. 1A. As expected, most females chose the chamber with the plant when the alternative chamber had no plant (Fig. 1B, control conditions). Gravid females preferred the nontransformed M37W variety to its transgenic multivitamin counterpart (M37W vs. MV) ( $P = 0.009$ ) (Fig. 1C). However, they did not discriminate between nontransformed M37W and Bt plants (MV vs. V67) ( $P = 0.11$ ) (Fig. 1D); nontransgenic DKC 6666 and M37W (V66 vs. M37W) ( $P = 0.47$ ) (Fig. 1E); Bt corn and its isogenic non-Bt variety (V66 vs. V67) (Fig. 1F) ( $P = 0.25$ ); non-Bt DKC 6666 and the multivitamin variety (V66 vs. MV) ( $P = 0.44$ ) (Fig. 1G); or the multivitamin variety and the Bt variety (MV vs. V67) ( $P = 0.23$ ) (Fig. 1H).



**Fig. 1.** Response of gravid *S. nonagrioides* females to different (GM or not) corn varieties in a dual-choice olfactometer. Varieties tested were a Bt corn, DKC 6667 (Bt), its isogenic counterpart, DKC6666 (V66), a transgenic multivitamin corn (MV), and its isogenic counterpart (M37W).  $N = 40$  for each dual-choice experiment. Bars with different letters indicate different responses to the two varieties compared, error bars represent  $\pm$  SE (binomial test,  $P < 0.05$ ).

**Only Olfactory Cues.** Figure 2 shows the response of the gravid females of *S. nonagrioides* to the different corn volatiles (nonvisible plants) in the paired experiments. A scheme of the olfactometer with the hidden plants behind a screen is represented in Fig. 2A. As in the previous experiment, most females chose the chamber with the plant when the alternative chamber had no plant (Fig. 2B, control conditions). Some differences from the previous results were found when the gravid females of *S. nonagrioides* were exposed to the odors emitted by the corn plant varieties hidden behind a perforated black plastic screen. Gravid females preferred the M37W variety to its transgenic counterpart or multivitamin (M37W vs. MV) ( $P = 0.02$ ) (Fig. 1C) and to the Bt corn variety (M37W vs. V67) ( $P = 0.02$ ) (Fig. 1D). However, they did not discriminate between nontransgenic M37W and DKC 6666 varieties (M37W vs. V66) ( $P = 0.16$ ) (Fig. 1E); non-Bt and Bt corn (V66 vs. V67) ( $P = 0.42$ ) (Fig. 1F); multivitamin and nontransgenic DKC 6666 (MV vs. V66) ( $P = 0.3$ ) (Fig. 1G); and multivitamin and Bt corn (MV vs. V67) ( $P = 0.5$ ) (Fig. 1H).

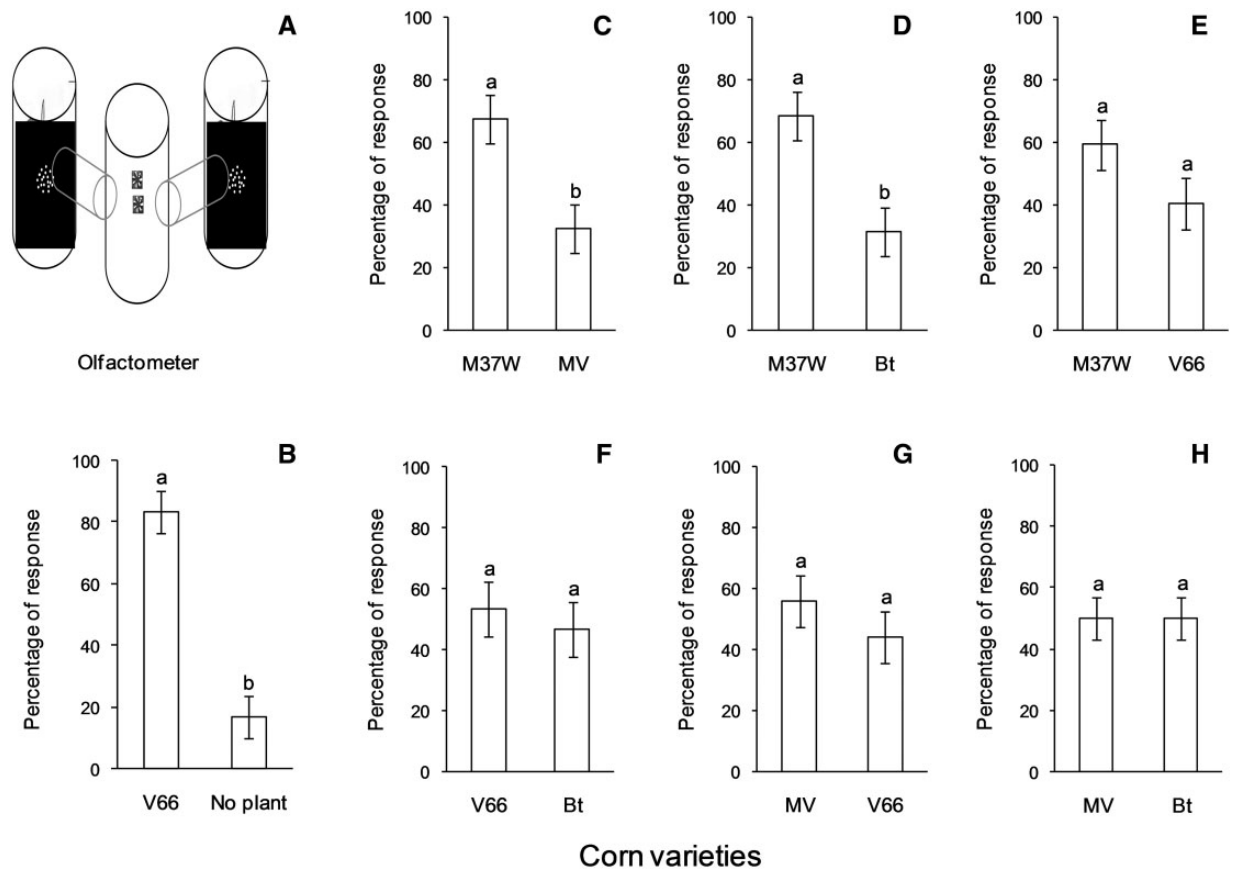
## Discussion

The host selection process in insects can be divided into two steps. First, insects “choose” their host from a distance using olfactory and visual cues and then they “select” their host only after contact when

gustatory cues are employed (Bruce et al. 2005). In this study, we tested the importance of the olfactory and visual cues at a short distance employing a dual-choice olfactometer to compare the response to four corn varieties, two of them widely grown in Spain (DKC 6666 and DKC 6667), and the remaining two corresponding to a new multivitamin transgenic corn and its isogenic counterpart (M37W).

When the plant was not visible *S. nonagrioides* gravid females showed a preference for the odor of the corn plant over the clean air, confirming their response to the host plant volatiles. Moreover, gravid females responded in a similar way to the plant when they could see it and when they could only smell it, suggesting that the olfactory cues are the predominant ones for localizing and choosing the plant. Konstantopoulou et al. (2002) confirmed the importance of volatile cues for the gravid females of *S. nonagrioides* but they also found synergistic or additive responses when both visual and odor cues were present that are not evident in the present results. Other nocturnal moth species in which it has been reported that olfactory cues play a more important role than visual cues in the host plant finding process are *Chilo suppressalis* (Walker) (Hou et al. 2010) and *Plutella xylostella* L. (Couty et al. 2006). In contrast, Calatayud et al. (2008) indicated that the females of *Busseola fusca* (Fuller) were not able to recognize the volatiles produced by the different plant species. Warrant and Dacke (2011) stated that in nocturnal insects, the role of vision is related to navigation and orientation in the night.





**Fig. 2.** Response of gravid *S. nonagrioides* females to the volatiles (they could not see the plant) of different (GM or not) corn varieties in a dual-choice olfactometer. The varieties tested were a Bt corn, DKC 6667 (Bt), its isogenic counterpart, DKC6666 (V66), a transgenic multivitamin corn (MV), and its isogenic counterpart (M37W).  $N=40$  for each dual-choice experiment. Bars with different letters indicate different responses to the two varieties compared, error bars represent  $\pm$  SE (binomial test,  $P < 0.05$ ).

Pickett et al. (2012) reported that insects possess a highly sensitive olfactory system with the capacity even to select different varieties from their host plant. On the other hand, Konstantopoulou et al. (2002) found that different genotypes of corn plants affected *S. nonagrioides* oviposition differentially. Jaenike (1990) suggested that gravid females oviposit on plants that maximize the fitness of their progeny, so we hypothesized that the multivitamin corn would be most preferred because of the increased multivitamin while the Bt corn would be less preferred due to the presence of toxic substances. However, the results of this study demonstrated that females of *S. nonagrioides* preferred the odors emitted by the nontransgenic variety to those of its multivitamin transgenic counterpart when the plant was visible and when it was not visible; and they also preferred the M37W variety to the Bt corn variety when they could only detect the volatiles without seeing the plant. It cannot be ruled out that the differences on the response of gravid females to the varieties are due to natural or genetic differences between the GM plant and its near isogenic. Differences on the response might also be due to unintended effects of the genetic transformation not related to the traits introduced in the transformed varieties, as the pleiotropic effects found by Saxena and Stotzky 2001. Although it is a general assumption that an increase in vitamin in diets favors the development of the insect, Goggin et al. (2010) warn of some adverse effects of the high vitamin C content on development, which can lead gravid females to avoid these diets. Gravid females did not discriminate significantly between the varieties in the remaining paired comparisons. Although Lei et al. (2009) demonstrated that the females of *Liriomyza trifolii* (Burgess) preferred non-Bt cotton for ovipositing, Obonyo et al. (2008), Van den Berg and Van Wyk (2007), Torres and Ruberson (2006), and Hellmich et al. (1999) signaled that lepidopteran females such as *O. nubilalis*, *Heliothis virescens* (Fabricius), *Helicoverpa zea* (Boddie), *Sesamia calamistis*, and *Chilo partellus*

cannot discriminate between transgenic varieties and their isogenic conventional cultivars.

The trends for the next generation of biotech crops indicate that future stacks are likely to involve not only multiple pest resistance but also the combination of these traits with engineered metabolic pathways and simultaneous introductions of multiple pathways through metabolic engineering (e.g., pathways for beta carotene, ascorbate, folate, and vitamin E synthesis) (Halpin 2005, Naqvi et al. 2009, Que et al. 2010, ISAAA 2014). The possibility that gravid females of *S. nonagrioides* are less attracted to corn with an increased level of vitamins, as suggested by this study, could have important unintended consequences for insect resistance management, especially if this vitamin trait is stacked with an insect resistance trait. In this case, the stacked GM plant could be less attractive to gravid females than the refuge plants, which would increase the value of refuge plants (i.e., more production of insect-trait susceptible insects) for managing resistance. Therefore, it is necessary to study the response of gravid females to the new gene stacked varieties.

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