Approaches for assessing the impact of *Zea mays* (Poaceae) on the behavior of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and its parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae)

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Abstract

Plant derived volatiles are cues used widely that guide the behavior of plant associated insects, influencing both the ability of insects to locate host plants, as well as tritrophic interactions with predators or parasitoids. Therefore, an understanding of how volatiles impact a specific ecological system may aid the development of plants that are less attractive to pests or more amenable to biocontrol. Because each plant-insect interaction is different, it is important to develop bioassays to compare plants with different volatile profiles and assess their comparative attractiveness to specific insects. To this end, we developed a laboratory-based pair-wise choice assay to determine the oviposition preference of fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), a global crop pest, to maize, *Zea mays* L. (Poaceae) plants with different volatile profiles. An alternative greenhouse-based assay also was developed to assess the effect of different *Z. mays* plants on the oviposition behavior of *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae), a parasitoid wasp that can be used as a biocontrol agent for *S. frugiperda*. These bioassays are easily adaptable for use on a range of plant-insect interactions.

Key Words: bioassay; maize; oviposition; method; volatiles

Resumen

Los volátiles derivados de plantas son señales que se utilizan ampliamente para guiar el comportamiento de los insectos asociados a las plantas, lo que influye tanto en la capacidad de los insectos para localizar plantas hospederas como en las interacciones tritróficas con depredadores o parasitoides. Por lo tanto, la comprensión de cómo los volátiles impactan en un sistema ecológico específico puede ayudar al desarrollo de plantas que son menos atractivas para las plagas o más abierto para control biológico. Debido a que cada interacción planta-insecto es diferente, es importante desarrollar bioensayos para comparar plantas con diferentes perfiles volátiles y evaluar su atractivo comparativo para insectos específicos. Con este fin, desarrollamos un ensayo de elección por pares basado en laboratorio para determinar la preferencia de oviposición del gusano cogollero, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), una plaga mundial de cultivos, para el maíz, *Zea mays* L. (Poaceae), con plantas de diferentes perfiles volátiles. También, se desarrolló un ensayo alternativo en invernadero para evaluar el efecto de diferentes plantas de *Z. mays* sobre el comportamiento de oviposición de *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae), una avispa parasitoide que puede usarse como agente de control biológico de *S. frugiperda*. Estos bioensayos se adaptan fácilmente para su uso en un rango de interacciones planta-insecto.

Palabras Clave: bioensayo; maíz; oviposición; método; volátiles

Spodoptera frugiperda (J.E. Smith) (fall armyworm) (Lepidoptera: Noctuidae) is a neotropical insect pest that historically attacks row, vegetable, and turf crops from southern Argentina to southern Canada (Sparks 1979; Luttrell & Mink 1999; Braman et al. 2000; Nuessly et al. 2007; Souza et al. 2013). Within the last 5 yr, *S. frugiperda* has spread to other countries. In early 2016, it was discovered in western Africa (Goergen et al. 2016) and by late 2017, it had invaded most of sub-Saharan Africa causing significant yield losses in primarily smallholder *Zea mays* L. (Poaceae) (maize) farms (Abrahams et al. 2017). In 2018 it was found in Yemen and the state of Karnataka, India (Ganiger et al. 2018; Nagoshi et al. 2019), and by Dec 2018 was reported in Bangladesh, Sri Lanka, and Thailand. By Jun 2019, *S. frugiperda* was reported in Myanmar, China, Indonesia, Laos, Malaysia, Vietnam, Egypt, and the Republic of Korea. Japan reported its presence in Jul 2019, and *S. frugiperda* was officially reported in Australia and Mauritania in Feb and in Timor-Leste in Mar 2020, making it an agricultural pest of global significance.

Spodoptera frugiperda is a significant pest of *Z. mays*, feeding on different tissues throughout the plant's growth cycle. It attacks both field and sweet corn (Sparks 1979; Pair et al. 1986a). In whorl-stage plants, young larvae feed on the outer leaves and move into the whorl, subsequently damaging the emerging tassels. All larval stages can feed on the ear, with young larvae feeding on the silks and entering through the cob tip; older larvae generally enter through the husk (Nuessly & Webb 2017). Although *Bacillus thuringiensis* Berliner (Bacillaceae) (Bt) toxin containing varieties of field corn are well protected, non-

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transgenic commercial production of *Z. mays* sweet corn is managed by as many as 20 insecticide applications per season selected from over 30 labeled compounds (Kanissery et al. 2019). These applications are sprayed during both the vegetative and reproductive stages of the plants and include a variety of different modes of action.

One alternative to insecticidal management is the use of natural enemies that can be released throughout the season or maintained in agricultural habitats using conservation tactics (Lewis & Nordlund 1980). However, it is important to know which species are active in the habitat. Recently, surveys in southern Florida documented several important species including *Cotesia marginiventris* (Cresson) (Braconidae: Microgasterinae) (Meagher et al. 2016). This species has a wide geographic range, with records from 11 countries documented (Molina-Ochoa et al. 2003), and is successful in subtropical and warm temperate areas such as the southeastern USA (Ashley et al. 1982; Pair et al. 1986b; Riggin et al. 1992). A related species, *Cotesia icipe* (Fernandez-Triana & Fiaboe) (Hymenoptera: Braconidae) (Fiaboe et al. 2017), has moved from native *Spodoptera* spp. and is currently attacking *S. frugiperda* in eastern Africa (Sisay et al. 2018), whereas an unidentified *Cotesia* is attacking *S. frugiperda* in western Africa (Koffi et al. 2020).

Cotesia marginiventris is a solitary koinobiont endoparasitoid that attacks first and second instar larvae (Boling & Pitre 1970; Loke et al. 1983). After several host molts, adult wasps emerge from fourth instar larvae. Female wasps are attracted to host larvae by orienting to both larval frass and host-induced plant volatiles (Loke et al. 1983; Loke & Ashley 1984). A wide range of optimal and suboptimal noctuid lepidoptera can be used, but experience of females ovipositing on optimal host larvae increases their attraction and host-finding to these species (Tamò et al. 2006; Harris et al. 2012). Therefore, it appears that *C. marginiventris* may persist at low population densities on alternate hosts (Tingle et al. 1978; Johanowicz et al. 2002) and may increase its population when an optimal host such as *S. frugiperda* becomes abundant. This is helpful when conservation biological control tactics are used because the biological control agent does not need to be augmented. It should be noted that the identification of *C. marginiventris* may be questionable because several other very similar species have been reared from *S. frugiperda*, and the genus is undergoing reorganization (Michel-Salzat & Whitfield 2004). We are using the name *C. marginiventris* because of its long-time use in the literature in biological control of noctuid species.

Both *S. frugiperda* and *C. marginiventris* use plant derived volatile compounds as cues to locate suitable hosts. Gravid moths of *S. frugiperda* are attracted to *Z. mays* plants, yet they can distinguish plants that have been infested with *S. frugiperda* from uninfested plants, suggesting that they may perceive different herbivore induced volatiles (Signoretti et al. 2012). The herbivore induced volatiles produced by infested *Z. mays* also may be used as directional signals by *C. marginiventris* to aid in the location of their insect hosts (Turlings et al. 1990, 1991). Different varieties of *Z. mays* have been shown to produce different volatiles in response to herbivory by *S. frugiperda*, and to differ in their comparative effectiveness as locator signals for *C. marginiventris* (Fritzsche Hoballah et al. 2002; Block et al. 2018). Assays that may quantify the preference of both *S. frugiperda* and *C. marginiventris* for different *Z. mays* varieties or mutants in lab or greenhouse-based settings may be used to determine *Z. mays* genetic and chemical factors affecting insect attraction. In this study we describe approaches to quantify *S. frugiperda* and *C. marginiventris* oviposition preferences using pair-wise choice assays. The purpose of this study is to facilitate the use of these techniques by multiple researchers. The *Z. mays* inbred lines chosen for this study were B73, B104, and W22. All 3 inbred lines have complete genome sequence available (https://maizegdb.org/). Line B73 was the first *Z. mays* line to be sequenced, and this resulted in its extensive genetic and phenotypic characterization. Line W22 has a publicly available transposon generated mutant collection, and line B104 is one of the few inbred lines that is routinely transformed for gene overexpression and CRSPR/Cas9 gene editing studies. Therefore, these 2 lines are ideal choices for functional characterization of genes involved in volatile biosynthesis and regulation. Data from these assays have the potential to guide breeding of *Z. mays* lines that are less susceptible to *S. frugiperda*, both through stealth by being less attractive to gravid moths, and via better recruitment of natural enemies such as *C. marginiventris*.

Materials and Methods

MAINTENANCE OF *SPODOPTERA FRUGIPERDA* COLONIES

In order to perform bioassays with *S. frugiperda*, it is important to have a source of *S. frugiperda* eggs. These can be obtained either when needed from a commercial supplier, or reared in colony in the laboratory. *Spodoptera frugiperda* were reared from a starter colony from an insectary (Benzon Research, Carlisle, Pennsylvania, USA). The colony was initiated using larvae from 12 egg shipments (1,000 eggs per shipment) over a course of 6 mo to achieve as much heterogeneity as possible. An *S. frugiperda*-specific diet (Southland Products, Lake Village, Arkansas, USA) was used, and a 32-cell diet tray system with removable 4-cell lids (white trays, RT32W; lids, TRCV4; Frontier Agriculture Sciences, Newark, Delaware, USA) to rear larvae. Neonate larvae (morning after they emerged, < 24 h) were placed 1 per cell in the trays using a moistened camel-hair brush to guide them into cells with their silks. Lids were then placed on trays covering 4 cells, and trays placed in a stand-alone room held at 24 °C, RH 50%. About 17 d after neonate setup, pupae were pulled from diet cells using featherweight forceps. After pupae were removed from trays, they were sexed and held before being placed in adult cages. A screen cage system was used for egg production. Pupae (20 of each sex) were placed in pie pans filled with coarse vermiculate. Cylindrical screen cages (28 cm h, 21 cm d) were placed over top of the pans, and Bounty® paper towels (Procter and Gamble, Cincinnati, Ohio, USA) were stretched at the tops of the cages as an oviposition substrate and were secured with a rubber band. Emerged adults were supplied with a 20% (v/v) honey-sucrose solution that was placed in the cage in a small cup with a large cotton ball. Egg sheets were collected after 2 d and held for neonate emergence 3 d later.

LARVAL GROWTH BIOASSAYS

To determine if *S. frugiperda* larvae have differing abilities to grow on different varieties or mutant lines of *Z. mays*, a greenhouse based larval growth assay may be performed. We have used such assays successfully in several studies (Block et al. 2018, 2020). To perform these assays *Z. mays* plants were grown in the greenhouse in a soil made from 45% (v/v) Canadian peat, 20% (v/v) vermiculite medium, 20% (v/v) perlite coarse, and 15% (v/v) coir. The soil was mixed and provided by BWI Companies (Apopka, Florida, USA). The pH was adjusted to 5.5 to 6.5 with dolomitic lime and 1 mL of Osmocote (Scott's Miracle-Gro Co., Marysville, Ohio, USA), 15-9-12® slow release fertilizer per 7.6 L of soil was added before planting. The seeds were added at 3 per pot in a 11.4 cm pot and thinned to 2 plants per pot after germination. Plants were fertilized at every watering with a 20-20-20 solution of Peter's (ICL Specialty Fertilizers-North America, St. Louis, Missouri, USA) and a calcium and magnesium supplement. Plants were grown in greenhouses with 12 h supplemental lighting using a combination of

400 watt high pressure sodium and metal halide bulbs. The temperature range in the greenhouse was 25 to 40 °C.

Twelve 3-wk-old plants of a single *Z. mays* genotype were placed in each of 2 black windowless rearing and observation cages measuring 70 cm × 70 cm × 92 cm (BioQuip Products, Rancho Dominguez, California, USA). Plants were infested by gently placing 6 to 10 newly emerged *S. frugiperda* neonates into the central whorl of each plant using a paintbrush to move them during silking (ballooning). Care was taken to prevent neonates from moving away from the plants because at this life stage they can escape through the mesh of the cage.

To obtain larval growth curves, the larvae were removed from the plants using featherweight forceps and weighed daily from d 3 to 7 post infestation. As a precaution, due to aggressive and cannibalistic behavior, at days 5 to 7, larvae were collected in 50 mL Falcon® tubes (Corning Life Sciences, Tewksbury, Massachusetts, USA) and separated using Kimwipes® (Kimberly-Clark, Irving, Texas, USA). Larvae were collected from 1 cage at a time, weighed, and returned to the cage before collecting those from the next cage. The order in which the cages were sampled was randomized daily. Care was taken to prevent damage to the plants during collection of larvae. At the end of the time course the larvae were euthanized by overnight treatment at −20 °C.

OVIPOSITION CHOICE ASSAYS WITH *SPODOPTERA FRUGIPERDA*

To determine if *S. frugiperda* has a preference to oviposit on 1 type of *Z. mays* over another, an oviposition choice assay was performed with *Z. mays* lines that had different volatile (odor) profiles. The odor preference of many insects consists of a mix of innate and learned odors (Lewis & Takasu 1990; Gronenberg et al. 2014); therefore, it is important that moths used in these assays have exposure to all volatiles present in the assay. We developed a procedure for raising *S. frugiperda* on plant material rather than artificial insect diet to produce adult moths specifically for these assays. The following procedure was used (Fig. 1): the newly emerged neonates were placed in a small home-made container with a mesh lid filled with 10 cm long sections of *Z. mays* leaves from the varieties to be used in the oviposition studies. A piece of Kimwipe® was placed under the lid and the lid tightly sealed to prevent the neonates from escaping while allowing air flow into the container. Newly emerged neonates were added to the container for 3 consecutive d to compensate for the difference in emergence times between male and female adults. We did not mix larvae in a container that had more than 3 d difference in age because preliminary trials showed this led to increased cannibalism.

Once larvae reached the second instar they were transferred for rearing into 9.5 L Rubbermaid® food storage containers (Rubbermaid, Wooster, Ohio, USA) containing *Z. mays* stems and leaves from 2- to 5-wk-old plants. The containers were lined with paper towels and a wire mesh tray to keep the larvae elevated above their waste, and were outfitted with screen mesh lids to allow for respiration. Two to 3 times a wk, a selected number of larvae were transferred to new containers with fresh leaves, and when the larvae reached their maximum feeding stage additional fresh leaves were added as needed. Failure to transfer the larvae in preliminary trials led to fungal growth on leaf tissues, frass, and the paper towels. All plant material was collected and frozen at −20 °C overnight to euthanize larvae not transferred.

Once the larvae pupated, they were removed to open trays containing damp vermiculite. Pupae were sexed and then transferred to separate 9.5 L containers (as above) based on sex. Individuals with defective pupal formation were discarded at this stage. Each container consisted of a layer of vermiculite, misted daily with water to retain humidity, and a 29.6 mL plastic cup (Boardwalk/Essendant Co., Deerfield, Illinois, USA) containing a 20% (v/v) honey-sucrose solution with a cotton wick as a food source for the adult moths. Approximately 10 d later adult moths eclosed and were used for oviposition assays within 5 d. All rearing was accomplished at 25 °C under ambient light and humidity.

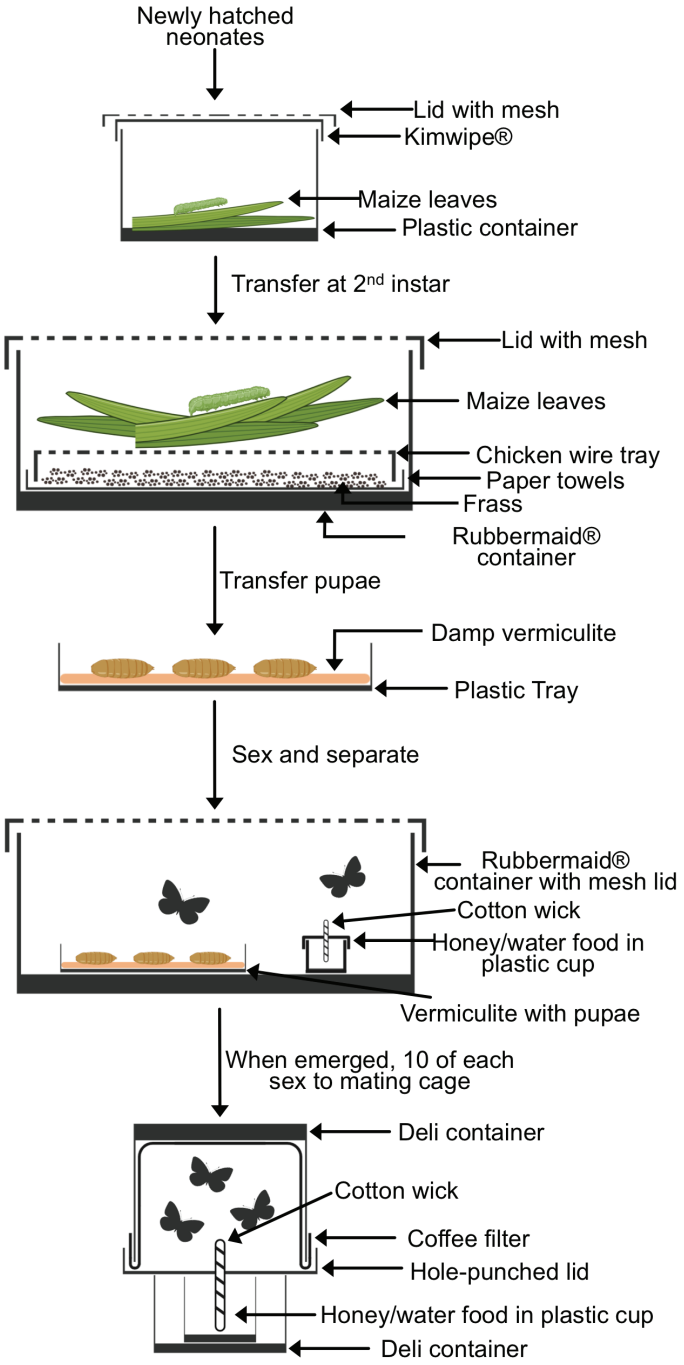

Fig. 1. *Spodopera frugiperda* rearing for use in oviposition assays.

To prepare an oviposition assay, equal numbers of male and female moths were placed into a mating apparatus, with an optimum number of 10 per sex and a minimum of 6. The apparatus consisted of an upside-down 473 mL deli container (Waddington North America, Chattanooga, Tennessee, USA) placed atop a 355 mL deli container (Genpak, Charlotte, North Carolina, USA). The 473 mL container was lined with a flat-bottomed coffee filter, and a 1 cm diam hole was punched

into the lid. A 29.6 mL plastic cup (Boardwalk/Essendant Co., Deerfield, Illinois, USA) containing 20% (v/v) honey-sucrose solution was placed inside the 355 mL container. A cotton wick was threaded through the hole in the lid, and the larger container was inverted over the 355 mL container such that the cotton wick was submerged into the honey water. Adults mated in the apparatus for 3 d. On the morning (9:00 A. M.) of the third d, the mating apparatus was transferred into a tent measuring 274 cm × 213 cm with a center height of 150 cm (Coleman Sundome Tent, B07ZHYS73W, Chicago, Illinois, USA) located in a climate-controlled room measuring 721 cm long × 325 cm wide × 217 cm high at 30 °C and 60% RH with external air exchange provided by a Panasonic Energy Recovery Ventilator (FV-04VE1, Newark, New Jersey, USA). The external air exchange is required to prevent excess accumulation of volatiles in the room that could interfere with the assay.

The *Z. mays* varieties to be tested in the oviposition assay were grown in the greenhouse under the conditions stated above until 1 mo old. When the moths were moved to the oviposition tent, 6 plants of each of the 2 *Z. mays* varieties at 2 plants per pot were placed in the tent using a randomized block design. Eight h later (5:00 P. M.), after the plants and insects have acclimatized to the new environment, the moths were released from the mating cage into the tent containing the plants, and all lights in the room were turned off leaving the oviposition assay to occur in total darkness. Darkness removed any effects of light on the movement and oviposition choice of the moths. Sixteen h later (9:00 A. M. the following d) the plants were removed from the tent and scored for oviposition. The oviposition success of each treatment can be assessed in a variety of ways including total number of eggs per plant per treatment. However, the number of eggs laid by each female was variable due to several factors including health, age, time after mating, and oviposition behavior such as egg mass size and laying prior to release (Meagher et al. 2011). Our preferred method of quantification, therefore, was to determine the total number of egg masses on each plant per variety, and then calculate the percentage of total egg masses on each variety for each assay. Occasionally the moths will have laid most of their eggs in the oviposition apparatus leading to 0 or low numbers of egg masses being observed on the plants. Generally, if fewer than 4 egg masses were observed, the experiment is discarded as failed. The oviposition assay was repeated 3 times to obtain robust data. For reference, Figure 2 shows a diagram to aid in the sexing of *S. frugiperda*, as well as representative images of *S. frugiperda* larvae, pupae, adults, and egg masses observed from the oviposition assay.

Larvae were reared on *Z. mays* inbred line B73 leaves and stems as described above, and 1-mo-old B73 plants were placed in the oviposition assay tent. The treatments used were B73 plants infested with 15 to 20 *S. frugiperda* neonates each and no treatment control of uninfested plants. A small paintbrush was used to transfer newly hatched neonates to the whorl of each infested plant treatment plant at 9:00 A. M. The neonates then were allowed to feed on the plants for 8 h prior to release of mated moths. Moths oviposited overnight (16 h), and the number of egg masses were counted the next morning.

REARING *COTESIA MARGINIVENTRIS*

Oviposition choice assays also may be performed with *C. marginiventris*; however, to our knowledge there is currently no commercial supplier of these insects. A method for rearing *C. marginiventris* in the laboratory was developed using known rearing methods for *C. marginiventris* and similar species (Allen 1958; Lewis & Burton 1970; Tillman & Scott 1997; Riddick 2004, 2007). The initial *C. marginiventris* insects to start the colony were obtained from field sites in Palm Beach County, Florida, USA, using the methods of Meagher et al. (2016). Briefly, sweet corn (*Z. mays*) plants exhibiting feeding injury in the leaf whorl directed

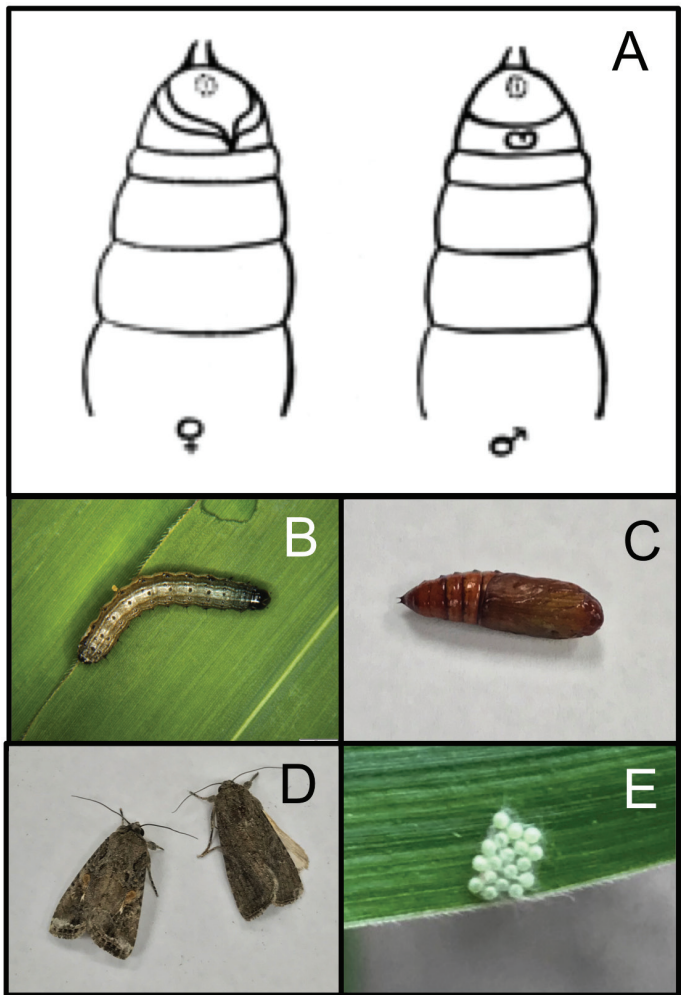

Fig. 2. *Spodoptera frugiperda* developmental stages. Line drawing of differences between male and female *S. frugiperda* pupae (A). Representative images of *S. frugiperda* larva (B), pupae (C), adults (D), and egg masses (E).

the search for *S. frugiperda* larvae. Larvae were pulled from the whorl and placed individually in 29.6 mL diet cups (Jet Plastica Industries, Hatfield, Pennsylvania, USA) with cut pieces of *Z. mays*. After returning from the field, larvae were identified and categorized based on size. Greenhouse-grown *Z. mays* variety 'Truckers Favorite' was added to cups that contained young larvae until they reached about the fourth instar; older larvae were placed in cups with artificial diet. Once the young larvae reached the fourth instar, they then were placed on artificial diet. Young larvae were initially placed on *Z. mays* tissue because parasitoid mortality was higher when they were placed directly onto artificial diet. Larvae were held in incubators at 23 °C, 70% RH, and 14:10 h (L:D) photoperiod.

Cotesia marginiventris were kept at 22 to 24 °C, RH 65 to 70%, and a photoperiod of 13:11 h (L:D). Adults were housed in a home-made rearing container (plastic framed box 20.5 × 20.5 × 20.5 cm), with organdy screen walls. Fabric walled cages are important because mating success for these wasps is higher on fabrics such as chiffon than on plastics or glass due to the importance of vibrational courtship mechanisms for this insect (Joyce et al. 2008). A cotton ball moistened with water and a cotton ball moistened with 20% (v/v) honey-sucrose solution were placed into individual 59.1 mL deli cups (Boardwalk/Essendant Co., Deerfield, Illinois, USA) and set in the container. Additional honey was applied to the sides of the cage, because feeding

with honey increases the female gravidity (Riddick 2007), and 4 to 5 Kimwipes® were placed in the cage to give the wasps a resting position. In a separate container, 50 to 100 *S. frugiperda* neonates were placed on *Z. mays* leaf sections as described above in the *S. frugiperda* oviposition section. After *S. frugiperda* larvae fed for 2 d, they and the leaf tissue they had been feeding on were placed in the cage containing the adult *C. marginiventris*. Fresh *Z. mays* leaves also were added to the cage for larval feeding. It was important that the leaves with *S. frugiperda* feeding damage and frass were present because these provide the olfactory cues which enabled the *C. marginiventris* to locate its *S. frugiperda* host. Two to 3 d later *S. frugiperda* larvae were removed from the *C. marginiventris* cage and placed 5 to 10 per well in diet trays. At this point a second set of *S. frugiperda* larvae were added to the *C. marginiventris* cage and the process was repeated. It was possible to use a third round of larvae, but with each additional round the percentage of parasitism decreased (Riddick 2004).

The trays were held at 25 to 26 °C, RH 40 to 45%, and a photoperiod of 13:11 h (L:D). Pupae begin to form 7 to 9 d post exposure and usually were found on the top of the cells. Adults emerged 4 to 5 d later with males typically emerging 1 d before the females; mating may occur within min of female emergence (Boling & Pitre 1970). Upon eclosion *C. marginiventris* adults were aspirated out with a mouth aspirator and placed in a clean cage. Parasitized *S. frugiperda* larvae grow at a significantly slower rate than their unparasitized conspecifics, and often will be consumed by unparasitized larvae before the *C. marginiventris* emergence if left in the same well. The visible growth difference between the 2, however, allows the easy removal and disposal of the unparasitized (larger) larvae within 4 to 5 d post exposure. An alternative to this is to place each larva into a separate well. Representative images of *C. marginiventris* developmental stages are shown in Figure 3.

PAIR-WISE ASSAYS FOR *COTESIA MARGINIVENTRIS* OVIPOSITION CHOICE ON DIFFERENT HOST PLANTS

An assay was developed for *C. marginiventris* to directly compare 2 plant varieties or lines that have potentially unique volatile profiles for their impact on the ability of *C. marginiventris* to locate its *S. frugiperda* host on each plant type. This oviposition assay started with 20 to 30 naïve adult *C. marginiventris* (not previously exposed to either *Z. mays* or *S. frugiperda*) being placed into the rearing container described above and allowed to mate for 2 d. Concurrently *S. frugiperda* neonates were placed into diet trays for 2 d. In the greenhouse, 2 pots of 2 to 3-wk-old plants with 2 plants per pot were selected for each of the 2 *Z. mays* varieties to be tested. The 4 pots then were placed using a randomized design in the previously described black, windowless rearing and observation cage. Eight of the 2-d-old *S. frugiperda* larvae were placed on each plant ensuring that the larvae were distributed evenly among the plant leaves. It was important that the larvae were on the leaves rather than in the whorl of the plant because the ability of wasps to parasitize the larvae in the whorls is limited (Loke et al. 1983). The larvae fed on the plants overnight and the *C. marginiventris* rearing container was moved to the greenhouse to acclimatize the wasps to the new environment. The next morning the wasps were released into the cage containing the infested plants. Five h later the larvae were removed from the plants and placed in diet trays, 1 larva per well. The trays were marked with the identity of the plant variety the larvae were feeding upon. Once the *C. marginiventris* pupae formed, the trays were scored for the number of non-parasitized *S. frugiperda* larvae, the number of parasitized *S. frugiperda* larvae (with visible *C. marginiventris* pupae in the wells), and the number of unparasitized or dead *S. frugiperda* (only those that died very shortly after removal from the plants). The percentage of parasitized larvae was determined for the *S. frugiperda* removed from each plant variety as the (number parasitized / [number parasitized + number not parasitized]) × 100. This assay was replicated 13 times. If an assay had only 1 to 4 parasitized larvae or many dead larvae, results for that assay were discarded. The number of total larvae retrieved from the plants often was lower

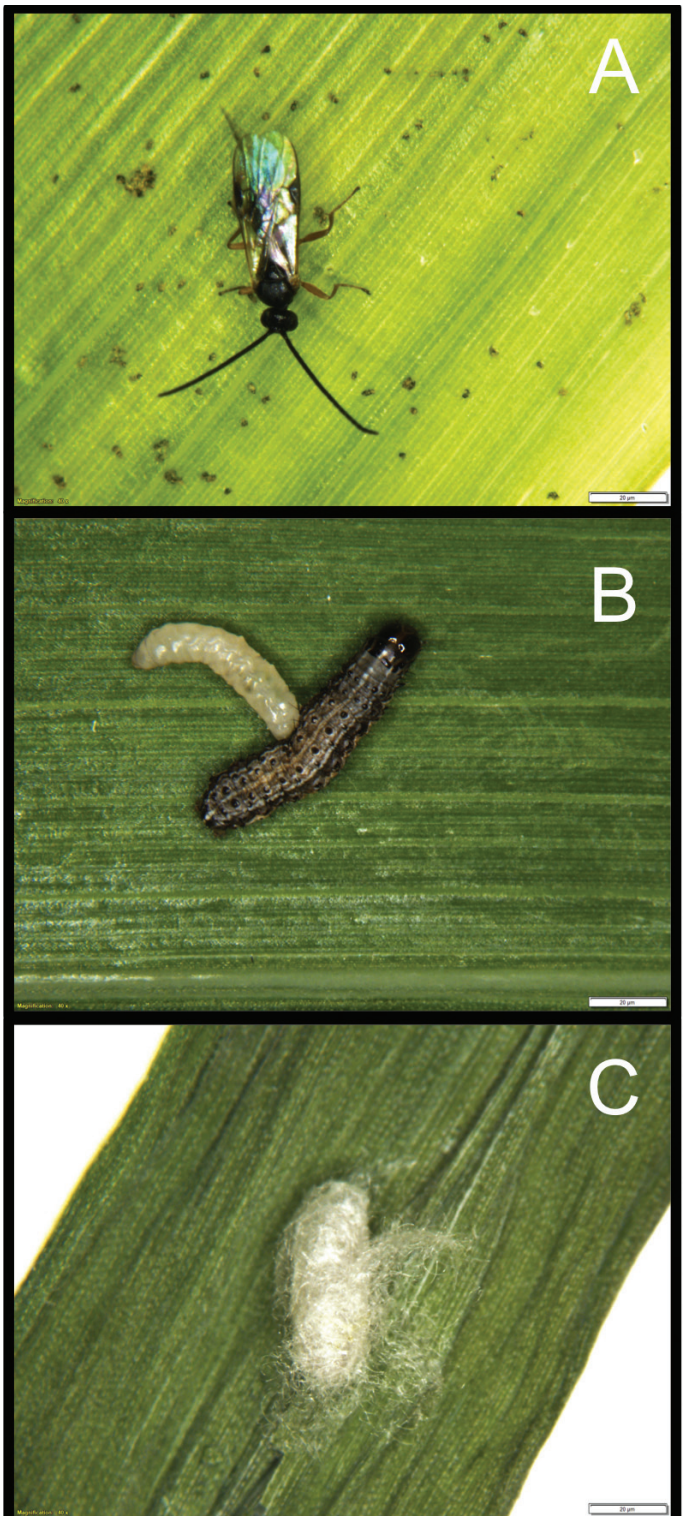

Fig. 3. Stages of *Cotesia marginiventris* development. Representative images of *C. marginiventris* adult (A), larva emerging from *S. frugiperda* host (B) and pupae (C) are shown. Size bars are 20 µm.

than the 32 larvae added to the plants because some larvae escaped the cage or were in the cage but not on a plant. These larvae were not included in the assay results.

STATISTICAL ANALYSIS

Statistical significance of treatments was determined by pair-wise *t*-tests with the difference considered significant if $P \leq 0.05$.

Results

LARVAL GROWTH RATES OF *SPODOPTERA FRUGIPERDA* ON *ZEA MAYS* INBRED B104

To test the variability of *S. frugiperda* larval growth rates, larval growth was measured in 2 independent greenhouse-based trials using the *Z. mays* inbred line B104. This inbred line was selected because it is one of the few inbred lines routinely used for *Z. mays* transformation, which makes it an important source for the development of targeted mutants. Therefore, this line is valuable for the functional characterization of defense genes. The data of larval weights from assays of larvae collected from *Z. mays* B104 inbred plants is in Supplementary Table S1. To calculate larval growth, the average larval weight at each time point was computed as well as the standard error of the mean. Some variation in overall growth weights and differences between treatments was observed between the 2 experiments, particularly at 6 d post infestation, likely due to differences in greenhouse temperature because the assays were performed on different wk of the yr (Fig. 4). In both experiments the number of larvae removed from the plants for weighing tended to decrease over time, likely due to larval mortality due to cannibalism and injury from other larvae during the experiment. The differences observed between the 2 independent experiments reinforce the importance of performing comparative analyses between different plant lines at the same time and the need to repeat these experiments 3 to 5 times to confirm that differences observed between different plant lines are robust and reproducible.

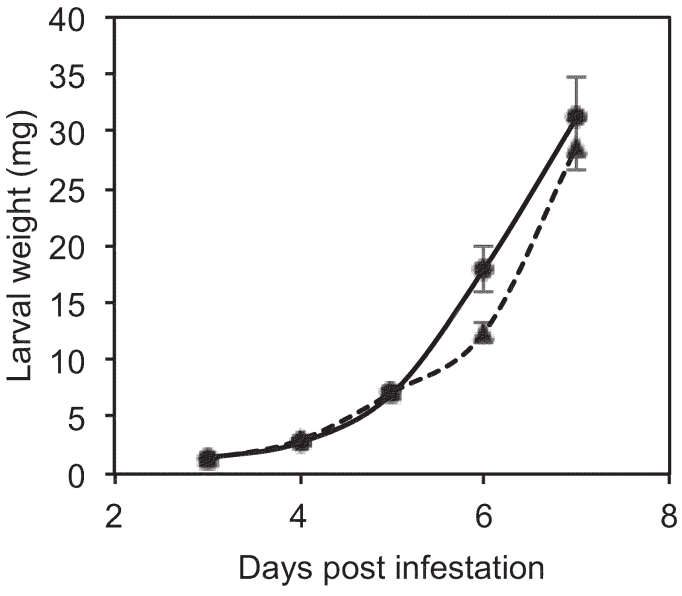

Fig. 4. Larval growth assays of *Spodoptera frugiperda*. Two independent larval growth assays for *S. frugiperda* on the *Zea mays* inbred line B104. Graph shows mean larval weights (± SE) from 3 to 7 d after infestation, *n* = (35–100). Because these growth assays were done at different times, they were not statistically compared.

THE IMPACT OF PRIOR *SPODOPTERA FRUGIPERDA* INFESTATION OF *ZEA MAYS* ON *S. FRUGIPERDA* OVIPOSITION PREFERENCE

To assess the effectiveness of our oviposition assay, we tested it with a treatment known to effect *S. frugiperda* attraction, which is that prior infestation with *S. frugiperda* larvae acts as an oviposition deterrent (Signoretti et al. 2012). This assay was performed using the *Z. mays* inbred line B73 (the first fully sequenced and most extensively characterized *Z. mays* inbred) with the moths having a direct choice between naïve (uninfested) plants and those infested with *S. frugiperda* neonates. The experiment was replicated 3 times (Fig. 5). In each of the trials the moths showed a higher number of egg masses on plants that were not infested compared to those that were, with 67, 97, and 70% of the egg masses on uninfested plants. There was variation both in the total number of egg masses and the number of plants containing egg masses in each experiment. In the first experiment 5 of the 6 uninfested plants contained egg masses ranging from 1 egg mass to 11 egg masses per plant. For the infested plants from the first experiment, only 3 of the 6 plants contained egg masses ranging from 1 to 7 egg masses per plant. In the second experiment, 2 uninfested plants in the same pot had egg masses, 17 and 21, respectively, whereas only 1 infested plant had a single egg mass. In the third experiment, 3 uninfested plants had from 1 to 4 egg masses, and a single infested plant had 3 egg masses. Despite this variation, when the data from the 3 experiments was combined, there was a statistically significant preference for oviposition on uninfested plants. These data show that this non-field-based oviposition assay may be effectively used to differentiate between attractive and non-attractive *S. frugiperda* host plants.

Cotesia marginiventris Displays No Significant Preference for Its *Spodoptera frugiperda* Hosts Feeding on *Zea mays* Inbred W22 Compared to Those on Inbred B104 in Oviposition Choice Assays

Previously, we have used an oviposition preference assay to successfully distinguish the oviposition preference of *C. marginiventris* for *S. frugiperda* feeding on *Z. mays* inbred line W22 when compared to those feeding on *Z. mays* inbred line B73 (Block et al. 2018). We wanted to test if this preference for W22 was widespread. Therefore, we assessed the oviposition choice of *C. marginiventris* on *S. frugiperda* feeding on W22 compared to those feeding on the *Z. mays* inbred line B104 using 13 independent pair-wise oviposition choice assays (Fig. 6). The percent parasitism for each of the lines ranged widely between the individual experiments (26–81% for W22 and 4–59% for B104), reinforcing the need for multiple replications to detect statistically significant trends. Data from Experiment 5 was discarded due to the high number of dead larvae (21). It was not easy to tell if the larvae that died shortly after removal from the plants were parasitized, but the results from this experiment were inconclusive. Data from Experiments 9, 10, 12, and 13 also were discarded due to less than 5 total larvae parasitized, indicating a low number of mated *C. marginiventris* females in the assay. The average percentage parasitization rate from each variety was calculated using the data from the remaining 8 experiments to be 48% for W22 and 33% for B104 ($P = 0.15$), suggesting that there was no significant difference in attractiveness between W22 and B104 for *C. marginiventris*. It is possible that repeating the experiment more times could give a slight significant difference; however, in the individual experiments no one variety showed a consistently increased number of parasitized larvae, supporting the finding that in contrast to the differences between inbreds W22 and B73, *C. marginiventris* does not have a strong preference for W22 over B104.

	Number of egg masses		
	Exp 1	Exp 2	Exp 3
NT-1	1	17	1
NT-2	1	21	2
NT-3	11	0	4
NT-4	0	0	0
NT-5	7	0	0
NT-6	6	0	0
FAW-1	1	0	0
FAW-2	5	1	3
FAW-3	7	0	0
FAW-4	0	0	0
FAW-5	0	0	0
FAW-6	0	0	0
Total NT	26	38	7
Total FAW	13	1	3
% NT	67	97	70
% FAW	33	3	30

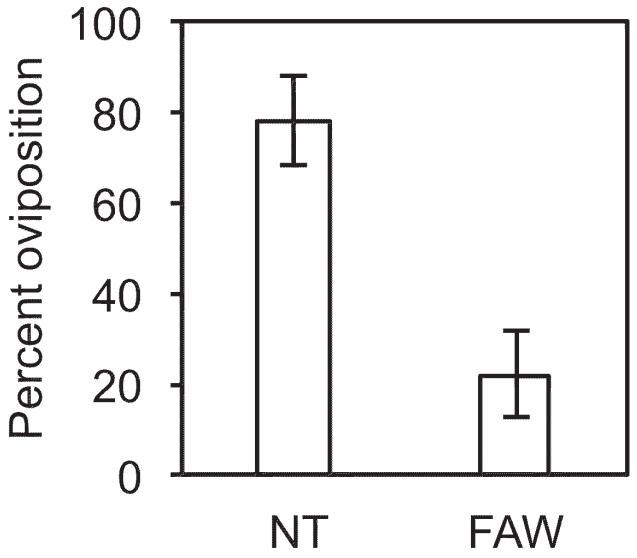

Fig. 5. *Spodoptera frugiperda* moths prefer to oviposit on uninfested *Zea mays* plants. To test the effect of prior infestation with *S. frugiperda* (FAW) compared to a non-treated (NT) control plant on *S. frugiperda* oviposition preference, a pair-wise oviposition assay was performed using 3 independent experiments (Experiments 1–3). In each experiment, 6 uninfested plants and 6 infested plant treatments were used, and egg masses on each plant counted (Table). The total number of egg masses on each treatment was determined and from these data the percent total oviposition (%NT and %infested plant) calculated. The graph shows the mean (± SE) percent parasitism for each treatment, and the treatments were statistically significantly different using a pair-wise *t*-test: $P \leq 0.05$; $n = 3$.

Exp	W22-P	W22-NP	W22-D	B104-P	B104-NP	B104-D	% W22-P	% B104-P
1	17	4	1	10	7	0	81	59
2	7	7	2	7	9	0	50	44
3	6	17	3	8	14	3	26	36
4	11	10	7	1	13	3	52	7
5	5	2	**16**	13	5	2	71	72
6	13	14	4	10	12	2	48	45
7	11	11	2	5	27	3	50	16
8	10	14	4	9	7	5	42	56
9	**2**	11	7	**2**	8	6	15	20
10	**1**	30	1	**1**	15	1	3	6
11	8	15	3	1	24	2	35	4
12	**1**	23	0	**2**	21	0	4	9
13	**2**	9	1	**1**	18	1	18	15

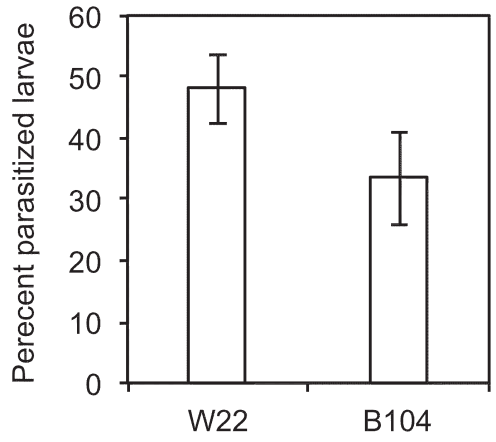

Fig. 6. *Cotesia marginiventris* wasps have a marginal preference to oviposit on *Spodoptera frugiperda* on W22 compared to B104 *Zea mays* inbred plants. To test the effect of different *Z. mays* varieties on oviposition preference of *C. marginiventris*, a pair-wise oviposition assay was performed using 13 independent experiments (Experiments 1–13). In each experiment, the number of *S. frugiperda* larvae recovered from B104 or W22 genotypes that were parasitized by *C. marginiventris* (P), not-parasitized (NP), or had died shortly after collection (D), and the percentage of larvae parasitized was calculated by (P/[P + NP]) × 100 for each plant variety (Table). Experiments that had less than 5 parasitized larvae or more than 15 dead (bold) were discarded. The graph shows mean (± SE) percentage parasitism for each treatment. The treatments were not significantly different using a pair-wise *t*-test with $P \leq 0.05$ and $n = 8$.

Discussion

This study showed that oviposition preference of *S. frugiperda* for uninfested over infested *Z. mays* plants may be observed using lab-based pair-wise oviposition assays. The trend in these assays was con-

sistent in individual experiments even though the total number of egg masses varied. Several herbivore induced volatiles have been identified in the *Z. mays* inbred B73 (Block et al. 2018). Therefore, one could envision using *Z. mays* mutants impaired in the production of specific volatiles (Richter et al. 2016) or chemical rescue techniques in conjunction with this oviposition assay to identify the specific volatiles or blends of volatiles able to repel *S. frugiperda*.

Our oviposition assays with *C. marginiventris* showed only a slight and not statistically significant oviposition preference for host *S. frugiperda* located on the *Z. mays* inbred W22 compared to those on inbred B104. These data suggest that the *C. marginiventris* use plant derived volatiles that are present at similar levels in W22 and B104 inbreds, thus leading them to not be able to distinguish between the 2 varieties. As *C. marginiventris* was shown previously to prefer W22 over the B73 inbred (Block et al. 2018), it would be interesting to perform this assay with pair-wise comparisons using a range of maize lines to perform correlation studies between the levels of specific volatiles and oviposition preferences of the *C. marginiventris*.

Therefore, the methods described here for greenhouse and lab-based oviposition choice assays involving *S. frugiperda* and *C. marginiventris* may be used to determine preferences between 2 different *Z. mays* lines. These assays may be coupled with headspace volatile analysis using gas-chromatography mass-spectrometry (GC-MS) methods (Carroll et al. 2006; Block et al. 2018) to identify volatiles that are important for host location. Ideally, they work in concert with other techniques to study phenotypes of the plants and the behavior of the insects. For example, one could envision complementary approaches of gas-chromatography-electroantennograms (GC-EAG) to determine which volatiles the insects can perceive (Ngumbi et al. 2010; Ortiz-Carreon et al. 2019), olfactometer assays to assess orientation cues using Y-tube or wind tunnel based approaches using whole plants, compound blends, or individual compounds (Fritzsche Hoballah et al. 2002; Fukushima et al. 2002; Signoretti et al. 2012), the greenhouse-based assays described here, and finally field-based oviposition assays (Degen et al. 2012).

The main advantages of the greenhouse and lab-based oviposition assays such as these are that they allow the testing of biological interactions in more controlled conditions than in the field. It is recommended to perform the replicates for the experiments by staggering production of the plants and insects such that new plants and insects are available each wk. In addition, for the *C. marginiventris* oviposition assays, the ability of the *S. frugiperda* to grow and survive on the different plant varieties could impact the development of the parasitoid (Ramirez-Romero et al. 2007). Therefore, it is suggested that an *S. frugiperda* growth assay, such as the one described here, be performed also to check for potential impacts on the assays that are not due to differential volatile production.

The methods described here are optimized for *S. frugiperda* and *C. marginiventris* on *Z. mays*, yet they may be adapted readily for use on other plant species that may be grown in the greenhouse and are host plants for *S. frugiperda*. They may be adapted also for use with other Noctuidae and their parasitoids, and as such for many plant insect or plant-insect-insect interactions. Furthermore, these assays may potentially be modified to assess the roles of individual compounds by using chemical rescue techniques in which dispensers are placed into or adjacent to the plants that release the test compound at a defined rate. This will allow the effect of the added compound on the oviposition preferences of the insect to be determined while maintaining the odor background of the plants. The assays also may be scaled up by using larger cages or spaces to test oviposition preferences where more than 2 plant lines are used. This is closer to field studies yet has the advantage of a controlled environment, and maintains the insects within that environment allowing for more reproducibility. The identification of volatiles that affect the behavior of pests and biocontrol agents such as *C. marginiventris* may provide important information to optimize breeding strategies of crops such that biocontrol is more effective while at the same time the plants are harder to locate or are even a deterrent to pests. Volatiles identified as attractants also may be used to increase the effectiveness and specificity of monitoring traps to aid integrated pest management.

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