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(–)-Germacrene D Increases Attraction and Oviposition by the Tobacco Budworm Moth *Heliothis virescens*

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Abstract

The sesquiterpene germacrene D (GD) activates a major type of olfactory receptor neuron on the antennae of the heliothine moths. In *Heliothis virescens* females, 80% of the recordings have shown activity of one neuron type responding with high sensitivity and selectivity to GD. With the aim of determining the behavioural significance of this sesquiterpene, we have used a two-choice wind-tunnel to study the preference of mated *H. virescens* females for host plants with and without (–)-GD added. Tobacco plants containing dispensers with low release rate of (–)-GD had a greater attractiveness than tobacco plants without this substance. In addition, a significant increase of oviposition was found on the plants with (–)-GD.

Introduction

Plants produce complex mixtures of volatile constituents, some of which are used as cues by insects to locate suitable hosts (Schoonhoven *et al*., 1998). Females of the polyphagous tobacco budworm moth, *Heliothis virescens* (F.) (Lepidoptera; Noctuidae; Heliothinae), display positive anemotaxis to host-plant volatiles (Tingle *et al*., 1990; Mitchell *et al*., 1991; Tingle and Mitchell, 1991), whereas caterpillar-induced plant volatiles, repel conspecific females (De Moraes *et al*., 2001). This species is found on a large number of economically important plants, such as cotton (*Gossypium hirsutum* L.), tobacco (*Nicotiana tabacum* L.), corn (*Zea mays* L.), tomato (*Lycopersicum esculentum* Mill.), sunflower (*Helianthus annuus* L.), as well as many wild plant species (Matthews, 1991). To determine which of the plant-produced compounds that are detected by insects, we used gas chromatography linked to single cell recordings (GC–SCR) from olfactory receptor neurons (ORNs) on the antennae of *H. virescens* (Røstelien *et al*., 2000a,b). At least 17 types of ORNs, each of them specialized for a few structurally similar plant compounds, have been found on the antennae of virgin females of the tobacco budworm moth. One type of neuron appearing in 80% of all recordings from ORNs ($n > 80$) responded with high sensitivity and selectivity to germacrene D (GD; for structures, see Figure 1) (Røstelien *et al*., 2000a). The large number of GD neurons on the antennae suggests that the compound is of particular importance in the selection of plants for nutrition and/or hosts for oviposition. The objective of the present study was to determine the role of GD, either as an attractant or a repellent, in host-plant location and oviposition by mated *H. virescens* females.

Material and methods

Insects

Heliothis virescens pupae were received weekly from a laboratory culture at Syngenta, Rosental, Switzerland. Pupae were sexed and kept in separate containers at constant conditions (22°C, 80–95% relative humidity, 14:10 h light: dark cycle, light phase starting at 8 p.m.). Every day, before onset of scotophase, the emerged females and males were placed in separate cylinders (diameter 10 cm, height 20 cm) with the tops covered by screens. The insects were provided with food (10% honey water) and pure water *ad libitum*. The following day, before onset of scotophase, adults were transferred to separate mating cylinders (one female and one male in each). Food and water were supplied and the insects were observed every 2 h under red light to confirm mating. The mated moths were sexed and the 3-day-old females were transferred to the wind-tunnel.

Plants

Seeds of tobacco were sown in the beginning of June and were grown indoors at room temperature and in natural

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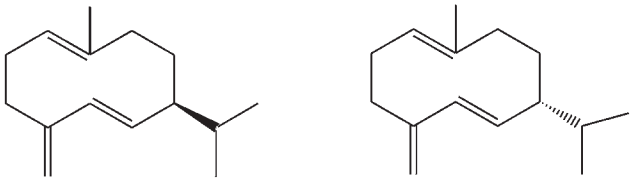

Figure 1 Structures of (–)-GD (left) and (+)-GD (right).

daylight. The plants were used for the behavioural experiments at flowering stage; some plants had a few seed capsules. The tobacco plants contained no detectable amounts (<0.1 ng/µl) of GD when the GD receptor neurons were stimulated with fresh materials from the plants. This was the reason for selecting the tobacco plants for the behavioural experiments.

Chemicals

(–)-GD was isolated from an ylang-ylang (*Cananga odorata* Hook) essential oil by MPLC as described previously (Røstelien *et al.*, 2000a). Chemical purity of the compound exceeded 99.2% estimated by gas chromatography using a DB-wax column (30 m, internal diameter 0.25 mm, film thickness 0.25 µm). The enantiomeric purity of 99.9% was determined by the use of a chiral column [25 m, internal diameter 0.25 mm, Heptakis (6-0-*t*-butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin (50% in OV1701)] (Schmidt *et al.*, 1998; König *et al.*, 1999).

The dispensers (red rubber tubes) were loaded with 500 µg of (–)-GD dissolved in 160 µl of hexane which was soaked into the walls from the inside (18 × 30 mm). The rubber was left in a hood at room temperature for 3 days to equilibrate the concentration inside the rubber and evaporate the hexane. The tube was cut in six pieces and the release rate of (–)-GD from all six parts was estimated by dynamic headspace (Porapak Q, 80–100 mesh, 99.999% nitrogen flow 20 ml/min) and gas chromatography (DB-wax column). The dispensers were used in the tests when (–)-GD was released within the range of 80–300 ng/h. This amount was in a range matching the sensitivity of the GD receptor neurons of *H. virescens* as measured by GC-SCR (Røstelien *et al.*, 2000a). Control dispensers were loaded with 160 µl of hexane only.

Two-choice wind-tunnel experiments

Behavioural tests were performed in a two-choice wind-tunnel (150 × 50 × 100 cm; Figure 2). The lower parts (50 cm height) of the two largest walls and the floor were made of Plexiglas, the upper parts of these walls as well as the end walls were made of wire mesh and the ceiling of black mosquito net. Mesh size was 1.5 mm at the side walls and 2.5 mm at the end walls. Air was sucked through the system by a tube (diameter 15.5 cm) connected to a fan. The inlet of the tube was placed in the middle of the tunnel, 40 cm above the floor. The air speed was ~15 cm/s, measured

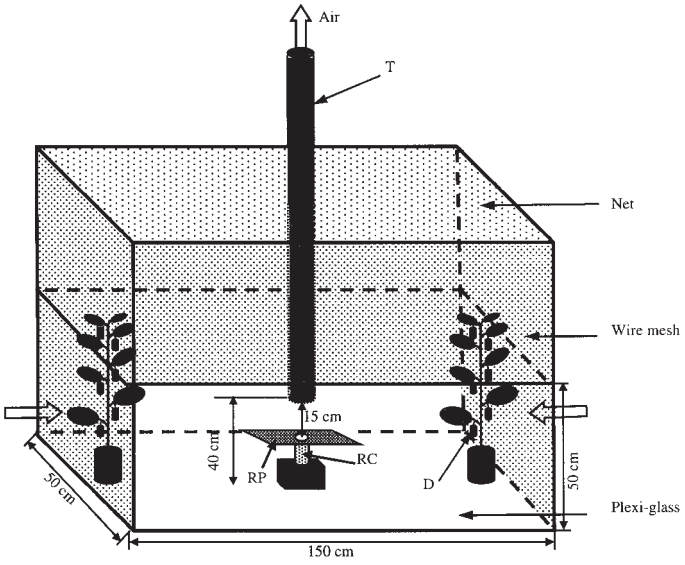

Figure 2 Scheme of the two-choice wind-tunnel consisting of Plexiglas, wire mesh and mosquito net. The airflow (indicated by arrows), created by a fan, was mainly drawn from the two ends of the tunnel containing the plants with the dispensers (D). The insects were introduced into the release cylinder (RC), from where they entered the release platform (RP), 15 cm below the inlet of the tube (T).

at the half distance between the tube and the end walls. The flow rates and the form of the plumes were estimated by introducing smoke obtained from $TiCl_4$ (titanium tetrachloride) into the wind-tunnel. The airflow was mainly directed from the two ends of the tunnel, with only weak flow from the sides. Two lamps (Osram Lumilux Plus Eco L58W/21-830) placed 130 cm above the wind-tunnel provided some background light in the room. To simulate dusk and provide uniform light allowing observation without red light, a tent surrounding the wind-tunnel was placed below the lamps.

Two plants with similar total leaf area were placed in the tunnel, one in each end, 90 cm from each other and at the same distance from the air tube. A new pair of plants was used for each experiment that started at 10.00 a.m. Six rubber dispensers were uniformly placed on each plant, one plant with (–)-GD dispensers and the other with control dispensers. Two mated females were introduced into a release cylinder (diameter 10 cm, height 15 cm, made of wire mesh) fastened to a foot (10 cm). The cylinder was placed in the centre of the tunnel 15 cm below the air-tube. On the top, the cylinder had a platform (20 × 30 cm) with a centred opening for releasing the insects. The females were acclimated to the test conditions for 10 min before release. The observation of the behaviour started when the females left the platform. After the experiments, the females and the plants were removed from the experimental room, the wind tunnel was cleaned with hexane and the air-fan was turned to maximum capacity overnight. During the next day of experiment, the positions of the two plants [(–)-GD and

control] were switched in the wind-tunnel. The data were taken in a protocol only from the females which demonstrated searching behaviour for a plant and/or visited both ends of the wind tunnel a few times. Females who stayed in a resting posture >20 min were not included.

The following experiments were conducted in the two-choice wind-tunnel with 3-day-old mated females.

Experiment 1: evaluation of the wind-tunnel

The preferences of the *H. virescens* females for one of the wind-tunnel ends, each containing a single tobacco plant, were evaluated by visually observing the behaviour for 1 h. The time (min) spent by the moths on each side of the tunnel was measured. Eighteen females were tested, of which six showed the searching behaviour.

Experiment 2: two-choice odour preference tests

The preference of the *H. virescens* females for a tobacco plant with dispensers releasing (–)-GD versus a plant with control dispensers was evaluated as in experiment 1. Twenty females were tested, of which eight showed the searching behaviour. In all but two experiments, only one of the two females introduced in the wind-tunnel showed active searching behaviour toward the plants and was included in the protocol. In the two other experiments, both females were included since both oriented toward the plants without interfering with each other.

Experiment 3: oviposition preference tests

The oviposition preference of *H. virescens* females for a tobacco plant with dispensers releasing (–)-GD versus a plant with control dispensers was evaluated by allowing one of the two females tested in experiment 2 to oviposit during the following scotophase. The number of eggs per plant was counted. Six experiments were run.

Data analysis

The data were calculated as percentages and analysed with the Wilcoxon matched pairs test (Sokal and Rohlf, 1995), using the computer program Statistica, for testing the preference for control plant versus control plant (experiment 1) and the preference for plant with GD versus control plant (experiments 2 and 3).

Results and discussion

The females demonstrated active searching behaviour for the tobacco with (–)-GD dispensers by performing zigzag flight upwind towards the plant. They hovered for 2–4 s at a distance of a few centimetres in front of the leaves with the dispensers. They then landed on the leaf or hovered in front of another leaf. After a while, some females flew away from the plant and landed on the tunnel wall for a short time. Then they relocated the air stream and repeated the behaviour of upwind flight and hovering in front of the GD-enriched plant. Altogether, the behavioural tests (experiment 2) showed that mated 3-day-old *H. virescens* females spent a significantly longer time (74 ± 12% SD of the observed time, $P < 0.02$) in the side of the wind-tunnel with the tobacco plant containing the (–)-GD-releasing dispensers, as compared to the side with the control plant (Figure 3). In contrast, the females did not show preferences when presented with plants without GD dispensers, as described in experiment 1 (55 ± 17% SD of the observed time, $P > 0.7$; Figure 3). The greater attractiveness of the tobacco plant with dispensers releasing (–)-GD also resulted in an increase of the oviposition on these plants in all six experiments (Table 1). Most of the eggs (79 ± 15% SD, $P < 0.03$) were found on the (–)-GD-enriched plants (Figure 4). The presented data show that (–)-GD at low release rate, added to tobacco plants containing no GD, increases the attraction of gravid females to the plants. Whether (–)-GD acts synergistically with the volatiles released by the tobacco plant or acts alone as an attractant, remains to be tested. In addition, it will be interesting to determine whether (–)-GD also stimulates oviposition or if the increased number of eggs on the plant was a result of increased attraction to the plant.

It is possible that (–)-GD is involved in one or in all of the three contexts: location of food source; finding a host plant for oviposition; and calling. The large number of very sensitive (–)-GD receptor neurons on the female antennae indicates that (–)-GD can be detected at large distances,

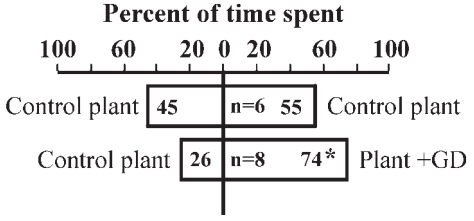

Figure 3 Flight responses of mated 3-day-old *H. virescens* females to (–)-GD, obtained in a two-choice wind-tunnel. Control plant represents a tobacco plant bearing dispensers loaded with evaporated hexane; plant + GD means a tobacco plant with dispensers releasing (–)-GD; the numbers (%) indicate average time spent by females at one side of the wind-tunnel during 1 h of observations; the values marked with asterisks differ significantly (Wilcoxon matched pairs test, $P < 0.02$); *n* represents number of females tested.

Table 1 Number of eggs laid by six *H. virescens* females on tobacco plants with GD dispensers and control plants in the six oviposition preference tests (experiment 3)

Female no.	GD plant	Control plant	Total
1	46	3	49
2	15	14	29
3	35	8	43
4	52	13	65
5	20	3	23
6	67	19	86

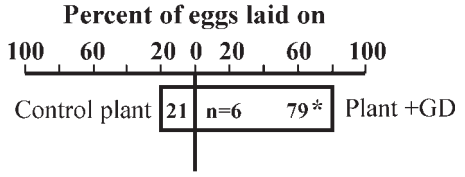

Figure 4 Oviposition responses of mated *H. virescens* females to (–)-GD in a two-choice test. Control plant represents a tobacco plant bearing dispensers loaded with evaporated hexane; plant +GD means tobacco plant with dispensers releasing (–)-GD; the numbers (%) indicate averages of eggs laid on indicated substrates; the values marked with asterisks differ significantly (Wilcoxon matched pairs test, $P < 0.03$); *n* represents number of females tested.

being important in host-plant location. Furthermore, the presence of a relatively large number of the same receptor neuron type on the male antennae (S. Ulland and H. Mustaparta, unpublished data) suggests that (–)-GD acts as a host-plant attractant for both sexes. This sesquiterpene is present in many of the host plant species, but not in all, as with the tobacco plants (Røstelien *et al.*, 2000a). Interestingly, it is the major component in leaf tissue and one of the numerous volatiles in the flower tissue of the host plant sunflower (Stranden *et al.*, 2002) (M. Ramirez and A.K. Borg-Karlson, unpublished data). These findings indicate that (–)-GD might be involved in location of plants for finding nectar, as well as host plants for mating and oviposition; this will be studied in future experiments.

GD is a chiral compound present in several plant families, including Asteraceae and Coniferaceae, host and non-host plants of *H. virescens*, respectively. As in sunflowers, GD is common in leaves and is also present in flowers of yarrow, pink (*Dianthus* spp.), mountain tobacco (*Arnica montana* L.), goldenrod (*Solidago virgaurea* L.), Canadian goldenrod (*Solidago canadensis* L.) and scented mayweed (*Matricaria recutita* L.) (Bülow, 1998) (J. Rohloff, personal communication). The content of GD varies among plant species, from pure (+)- or (–)-enantiomers (Figure. 1) to the racemate, controlled by separate synthases, as shown in Canadian goldenrod (Schmidt *et al.*, 1998). So far, it has been found that (–)-GD is the more common of the two enantiomers. Pure (–)-GD (>99%) is found in the needles of pine (*Pinus* L. spp.) (Røstelien *et al.*, 2000a) and in the leaves of certain chemotypes of yarrow, as well as in leaves and flower of sunflowers (Stranden *et al.*, 2002) (M. Ramirez and A.K. Borg-Karlson, unpublished data). The prominence of the (–)-enantiomer in the biological niche is also reflected in the evolved specificity of the ORNs in heliothine moths. According to GC–SCR, carried out in *H. virescens* as well as in *Helicoverpa armigera* (Hubner) and *Helicoverpa assulta* (Guenee), the ORNs responding to GD in these species belong to the same type, all showing highest sensitivity for the (–)-form (M. Stranden, A.-K. Borg-Karlson and H. Mustaparta, unpublished data). The dose–response curves of the ORNs show an effect of (–)-GD 10 times higher than that of (+)-GD (Stranden *et al.*, 2002). Thus, it is expected that both enantiomers of GD mediate the same kind of message to these moth species. Furthermore, one can assume that (–)-GD also acts as an attractant for the related species *H. armigera* and *H. assulta* when present in the host plants.

Since tobacco plants are well known hosts of *H. virescens*, the lack of GD in this species of plant, as shown in the GC–SCR studies of heliothine moths, seems puzzling. It means that the presence of GD may not be necessary for a plant to be used as a host by this species. Another possibility is that the content of GD varies among strains of tobacco, as shown for corn plants (M. Stranden and H. Mustaparta, unpublished data). On the other hand, in the numerous analyses of volatiles produced by tobacco plants, the presence of GD has not been reported (Andersen *et al.*, 1988; Loughrin *et al.*, 1990). Other volatile constituents may certainly play an important role in host location. Several components of plant volatiles—e.g. *E*-β-ocimene, *E*,*E*-α-farnesene, (+)-linalool—in addition to GD, all activating particular types of ORNs of heliothine moths, have been identified (Røstelien *et al.*, 2000b) (M. Stranden, A.-K. Borg-Karlson and H. Mustaparta, unpublished data). Obviously, a suitable host plant can be identified by the ratio of the different volatile compounds released.

In another lepidopteran species, the codling moth *Cydia pomonella* (L.) and in the two-spotted stinkbug *Perillus bioculatus* (F.), antennal responses to GD have recently been reported by the use of GC linked to electroantennogram recordings (GC–EAG) (Weissbecker *et al.*, 2000; Bäckman *et al.*, 2001; Bengtsson *et al.*, 2001). Behavioural responses to GD have previously been reported in only one lepidopteran species and two species of beetles. For females of the pickleworm moths (*Diaphania nitidalis* Stoll., Pyralidae), GD alone has been found to attract and increase oviposition, but less effectively than the naturally produced leaf blend of the host plant squash (*Cucurbita pepo* L.), containing 0.95 % of GD (Peterson *et al.*, 1994). Increased attraction to damaged, GD-enriched host plants (*Petasites paradoxus* Retz., *Adenostyles alliariae* Gouan.) has been found for the leaf beetle *Oreina cacaliae* (Schrank) (Kalberer *et al.*, 2001). In contrast, a masking effect of (–)-GD on host attraction is found for the cerambycid beetle *Monochamus alternatus* (Hope) (Yamasaki *et al.*, 1997). Another interesting finding is the presence of (–)-GD in volatiles collected from strawberry plants (*Fragaria ananassa* Duch), on which pheromone producing strawberry blossom weevils (*Anthonomus rubi* Herbst) were feeding (Innocenzi *et al.*, 2001). However, the behavioural effect of GD was not studied in this case.

In conclusion, by the use of the two-choice wind-tunnel we have demonstrated that (–)-GD, released at low doses from dispensers placed on tobacco plants, increased attraction of and oviposition by mated females of the tobacco budworm moth *H. virescens*. Thus, the behavioural

significance of the presence of a major olfactory receptor type with (–)-GD selectivity is demonstrated.

Acknowledgements

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