The Plant Sesquiterpene Germacrene D Specifically Activates a Major Type of Antennal Receptor Neuron of the Tobacco Budworm Moth Heliothis virescens

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Abstract
Plants release hundreds of volatiles that are important in interactions with insects or other organisms. However, knowledge is scarce as to which of the compounds are detected by the organism’s olfactory receptor neurons. In the present study, single receptor neurons on the antennae of the tobacco budworm moth, Heliothis virescens, were screened for their sensitivities to naturally produced plant volatiles by the use of gas chromatography linked to electrophysiological recordings from single cells (GC–SCR). Plant volatiles, collected by aeration of host and non-host plants, were tested on each receptor neuron via parallel GC-columns. Thus, simultaneous recordings of the gas chromatogram and the neuron responses to each component were obtained. One type of receptor neuron, appearing in 80% of all experiments, responded with high sensitivity and selectivity to one particular component, present in host as well as non-host mixtures. The component, identified as a sesquiterpene hydrocarbon by linked gas chromatography–mass spectrometry, was isolated from a sesquiterpene fraction of cubebe oil and identified by NMR as germacrene D. The purified compound was then re-tested via gas chromatography on the same receptor neuron type, verifying the identification. A weaker response to another sesquiterpene hydrocarbon was also recorded.

Introduction
Plants produce hundreds of secondary metabolites, including numerous volatiles, that are important in interactions with other organisms (Hartmann, 1996). Attraction of insects mediated by volatiles may in some cases be advantageous to the plant, e.g. for pollination, but may also be hazardous, when causing feeding of phytophagous larvae (Harborne, 1993; Bernays and Chapman, 1994). The polyphagous moth species, Heliothis virescens of the subfamily Heliotinae, uses a broad range of host plants, including sunflower, cotton, tomato and tobacco. The adult females select the host by laying eggs on the buds where the larvae develop and cause severe damage to the plant. The females probably use plant odours for host location, as indicated by wind-tunnel experiments demonstrating attraction, i.e. positive anemotaxis, to host plant volatiles (Tingle et al., 1990; Tingle and Mitchell, 1991). The importance of plant odours for this species is further indicated by the large number of olfactory sensilla on the female antenna and the large number of ordinary glomeruli in the antennal lobe of the brain (Almaas and Mustaparta, 1990; Christensen et al., 1995; Berg et al., 1998). They are apparently evolved for detection and processing of plant odour information.

Identification of plant volatiles that are recognized by insects or other organisms is a challenging task, considering the large number of components, often released in minute amounts. A method employed to identify naturally produced plant compounds detected by insect receptor neurons is gas chromatography linked to electrophysiological recordings from single receptor cells (GC–SCR). Several studies have demonstrated the value of this method for functional characterization of the olfactory receptor neuron types mediating plant odour information (Blight et al., 1995; Wibe and Mustaparta, 1996; Barata, 1997; Bichao et al., 1997; Røstelien et al., 1997; Wibe et al., 1997). In the pine weevil (Hylobius abietis) a relative large number of receptor neuron types have been classified, each responding specifically to one compound or a few structurally related compounds identified by linked gas chromatography–mass spectrometry (GC–MS) (Wibe et al., 1996, 1997). In a similar study of H. virescens, the receptor neurons were stimulated via a gas chromatograph with two columns installed, allowing each neuron to be tested with the same mixture separated in columns of different properties (Røstelien et al., 1997; T. Røstelien et al., submitted for publication). These results showed 12 types of receptor neurons, of which one type occurred frequently. The present paper presents the response characteristics of this particular neuron type, showing selectivity primarily for one compound, identified here as...
germacrene D (7-iso-propyl-10-methyl-4-methylene-cyclo-
dec-5,10-diene).

A part of this study has been reported previously in abstract form (Røstelien et al., 1997).

Material and methods

Insects and plants

Adult females of *H. virescens* used in the present study originated as pupae from a laboratory culture at Novartis Crop Protection, Rosental, Switzerland, and were kept as previously described (T. Røstelien et al., submitted for publication).

The host plant materials used were different strains of sunflower (*Helianthus annuus*), wild and cultivated tobacco plants (*Nicotiana tabacum*), tomato plants (*Lycopersicon esculentum*) and wild briar (*Rosa dumalis*). In addition, volatiles from other plant materials available in the laboratory were tested as non-host odours. These included samples obtained from spruce (*Picea abies*), juniper (*Juniperus communis*) (Wibe et al., 1996), and pine (*Pinus pinaster*) (Bichao et al., 1997). The test samples also included turpentine obtained during the commercial thermomechanical pulp process (TMP-turpentine) and a sesquiterpene fraction of the essential oil of cubebe pepper, the latter kindly provided by Dr Gerhard Schmaus, Dragoco, Holzminden, Germany.

Collection of naturally produced plant volatiles

The plant volatiles used for analyses in this study were the same mixtures as used by T. Røstelien et al. (submitted for publication). The mixtures were made according to selective responses primarily to one or two compounds when testing the neurons via the GC, with the various mixtures of naturally produced plant volatiles. The recordings were obtained at different antennal segments (proximally, nos 2–60), both at the lateral and medial sides of the antenna. The initial spontaneous activity varied from 2 to 10 spikes/s, sometimes showing a slight increase of firing rate after numerous stimulations. Most of the recordings from this neuron type showed spike activity from a single neuron (one spike amplitude), and all of them responded by excitation. They were characterized by a strong response to one particular component (I) eluted at

receptor neurons presented in the results, was isolated from a sesquiterpene fraction of cubebe oil containing <2% of this compound (Schmaus, 1988). A sample (2.5 g) of the fraction was separated from its oxygenated constituents by the use of medium pressure liquid chromatography (MPLC) (Jirón, 1996). The separation was performed on a column (500 × 25 mm i.d.) loaded with 110 ml of silica gel (MERCK, pore diameter 60 Å, particle size 35–60 mm). A solvent gradient of hexane/ethyl acetate, with an increase of polarity per volume solvent injected, was used as a mobile phase. This separation was followed by thin layer chromatography (TLC) using 10% ethyl acetate in hexane. The first 11 nonpolar fractions were combined and concentrated by careful evaporation of the solvent, resulting in 1.3 g of a sesquiterpene hydrocarbon mixture. This purified hydrocarbon fraction was effectively separated on 50 ml AgNO3-impregnated silica gel (5% w/w, Aldrich, column size 500 × 25 mm). The eluate was collected in tubes (150 × 10 ml) using a gradient of ethyl acetate in 100% hexane (500 ml) [(1.25% EtOAc (200 ml), 2.5 (100), 10 (100), 20 (100), 40 (100), 80 (100), 100 (100)]. Component I, which showed the highest activity in the electrophysiological experiments, was eluted in vials nos 118–123. By repeated chromatography (using the same conditions) of the fraction containing the active compound, ~15 mg of the 80% pure compound was obtained.

GC–MS and NMR analyses

The results of the separations were followed by GC–MS by using a Finnigan SSQ 7000 instrument in combination with a Varian 3400-GC, and a DB-wax or a DB-5 column (30 m, i.d. 0.25 mm, film thickness 0.25 µm). The structure of compound I was assigned using data from 1H- and 13C-NMR, DEPT, phase-sensitive COSY, HMQC, HMBC and NOESY spectra, which were measured on a Bruker DMX 500 spectrometer.

Results

The results presented here are based on recordings from 65 receptor neurons that were classified as one type, and represent 80% of all plant odour neurons (*n* = 80) characterized in *H. virescens* females. The classification was made according to selective responses primarily to one or two compounds when testing the neurons via the GC, with the various mixtures of naturally produced plant volatiles. The recordings were obtained at different antennal segments (proximally, nos 2–60), both at the lateral and medial sides of the antenna. The initial spontaneous activity varied from 2 to 10 spikes/s, sometimes showing a slight increase of firing rate after numerous stimulations. Most of the recordings from this neuron type showed spike activity from a single neuron (one spike amplitude), and all of them responded by excitation. They were characterized by a strong response to one particular component (I) eluted at
retention time 18.22 min for the non-polar (DB-5) column and at 14.71 min for the polar (DB-wax) column, as shown in Figure 1A,B. Strong responses at this retention time were obtained for volatiles collected from various plant materials of sunflower (Figure 1A–C), wild briar, spruce (Figure 1D) and juniper, as well as for volatiles of commercial TMP-turpentine and an essential oil fraction of the pepper plant. Volatiles obtained from wild as well as cultivated tobacco and tomato plants elicited weak responses. The neurons were further characterized by also responding to a second component (II) present in the volatiles of the non-host conifer materials, sawdust of spruce (Figure 1D), pine,
juniper and the turpentine extracts. This response appeared at retention time 9.55 min for the polar column (Figure 1D) and at 15.08 min for the non-polar column. A weak response to the high concentration of \( \alpha \)-pinene in these samples was also obtained.

In all experiments the response to both components (I and II) appeared as an increased firing rate, reaching a maximum (175 spikes/s for component I, shown in Figure 1E) at the peak concentration of the eluted compounds. The response showed a sharp decay followed by a slow decrease of firing rate that outlasted the GC-peak. The response significance of compound I was demonstrated further by dose–response characteristics. By injection of a sunflower mixture diluted in \( n \)-hexane (1:100, 1:1000 and 1:10000), weaker responses to the lower concentrations were obtained (10\(^{-4}\) dilution; \( \sim 0.1 \) ng of the active compound), shown for two concentrations in Figure 2. We found identical mass spectra for compound I in the various plant materials. The MS-library data suggested several sesquiterpene hydrocarbons as possible candidates, including \( \beta \)-cubebene and germacrene D (Figure 3). Different fractions of selective solutions from the cubebe oil were made and re-tested on the same neuron type, together with authentic samples of the possible sesquiterpene candidates. These tests eliminated several bicyclic and tricyclic compounds with a naphthalene skeleton (\( \alpha \)- and \( \lambda \)-muurolene; \( \alpha \)-, \( \delta \)- and \( \lambda \)-cadinene; \( \alpha \)-, \( \beta \)- and \( \lambda \)-ylangene; \( \alpha \)- and \( \beta \)-copaene; \( \alpha \)- and (–)\( \beta \)-cubebene; (–)\( \beta \)-elemene (Figure 3)). However, the stimulatory effect of component I was verified by the strong response of the purified cubebe oil fractions. The mass spectrum of component I in the fraction of highest purity (80%) was identical to the mass spectrum of component I of the various plant materials. The main peak of this fraction
elicited a strong response at the particular retention time when tested via the GC (Figure 4A,B). The mass spectral data obtained for the GC-peak II, were identical in all the non-host materials eliciting this particular response.

The chemical structure of component I in the purified fraction was identified as germacrene D. This structure was determined from the $^{1}H$- and $^{13}C$-NMR spectra (cf. Table 1). All chemical shifts were unambiguously assigned based on data from two-dimensional NMR spectra, and are also in accordance with data reported previously (Randriamiharisoa et al., 1986; Yamasaki et al., 1997). Other previously reported data (Kubeczka, 1979; Mori et al., 1990), showing a few chemical shift assignments that are not fully in accordance with our data, were not confirmed by the new...
two dimensional techniques. The less active component II was identified as a sesquiterpene hydrocarbon by GC–MS.

Discussion

The receptor neuron type described here, represented by 65 neurons out of 80 recorded, seems to be specialized for germacrene D, and activated more weakly by an unknown sesquiterpene hydrocarbon (Figure 1). The selective response to these components, out of hundreds present in the various host and non-host materials, indicates the significance of the compounds as biological signals for this neuron type. This is also supported by the high sensitivity and high maximum firing rate of the neurons to germacrene D. The high specificity was further demonstrated by there being no response to the structurally related sesquiterpenes tested as reference materials. Thus, these neurons show a degree of specialization corresponding to that of the pheromone receptor neurons in insects, including *H. virescens* (Berg et al., 1995). Like the pheromone receptor neurons, these germacrene D neurons also seem to respond more weakly to certain compounds of structural similarity, shown by the secondary response to compound II.

The identification of the SC-active peaks I and II in the gas chromatogram was facilitated using the two column types, which resulted in different retention times that corresponded in the GC–SCR and GC–MS experiments. The retention times and mass spectral data matched when comparing the isolated cubebe oil compound with component I of the various mixtures tested. Thus, the cubebe oil isolation product was identified as the active component (I). This was verified by the response measured when re-testing the isolated cubebe oil compound on the same type of receptor neuron. The chemical structure of the compound, determined by NMR, showed the properties of germacrene D with a large ten-carbon ring including two double bonds between carbons 1 and 10 and 5 and 6 (Table 1). The postulated biosynthesis of germacrene D in plants is conversion of farnesyl diphosphate (FDP), catalysed by a germacrene synthase (deKraker et al., 1998). A recombinant delta-selinensynthase isolated from grand fir has also been shown to produce germacrene D, as well as a number of other sesquiterpenes (Steele et al. 1998). Rearrangement of germacrene D can easily be performed in an acidic environment (Mori et al., 1990), and it is possible that molecules with a naphthalene skeleton, as the cadinene and copaene types, are present in the emitted blend of volatiles, partly as a result of rearrangements on the plant surface. These structural changes may have a biological significance, since heliothine females seem to prefer the young, nitrogen-rich

![Figure 4](image-url)
parts of a plant (Mitchell et al., 1991), which possibly emit greater amounts of germacrene D than older parts. Thus, germacrene D may be an important cue for eliciting oviposition behaviour.

The GC–SCR tests carried out in this investigation show that germacrene D is present in the blend released by many different plant species—hosts as well as the non-host conifers—and is detected by the moth receptor neurons. Its general presence in several essential oils (for example, of cubebe pepper and juniper fruits), as well as in volatiles released by sunflower, has been shown in previous studies (Thiery et al., 1990; Schmaus, 1988). The present study, demonstrating the high selectivity and sensitivity of a major receptor neuron type for germacrene D, suggests that germacrene D is an important signal for *H. virescens* in the interaction with the host plants. Recently, it has been shown that (−)-germacrene D acts as a masking substance of attractants for the cerambycid beetle, *Monochamus alternatus* (Yamasaki et al., 1997). Whether it acts as an attractant, feeding stimulant, oviposition stimulant or possibly inhibits these behaviours in the heliothinae moths is presently under investigation.

A large number of olfactory sensilla on the *H. virescens* female antenna, possessing receptor neurons that project into the ordinary glomeruli of the antennal lobe, appear to be involved in plant odour detection (Almaas and Mustaparta, 1990, 1991). If the number of germacrene D neurons recorded here is not favoured by size or location on the antennal segments, but reflects the relative abundance of olfactory neurons responding to this compound, other neuron types are present in a much smaller number. So far, 12 neuron types have been classified on the female antenna (unpublished data). It is likely that more types of olfactory neurons are present—consider, for example, the number of ordinary glomeruli (≈60; Berg et al., unpublished data) in the antennal lobe of the brain. Correspondence of receptor neuron types and number of glomerular units have been shown for the pheromone system in *H. virescens* males (Berg et al., 1998). Here the four units forming the macroglomerular complex (MGC), that is the first integration centre for pheromone information, each receives projections of one receptor neuron type. In vertebrates it is also suggested that there is a correspondence between the number of receptor neuron types (receptor protein types) and the number of glomeruli, either in the ratio 1:1 or 1:2 (Ngai et al., 1993; Ressler et al., 1994; Vassar et al., 1994; Mombaerts, 1999). Interestingly, results from ongoing studies using optical recordings show activity in a restricted area of the female antennal lobe during stimulation of the antenna with germacrene D originating from cubebe oil (unpublished).

In conclusion, a plant odour receptor neuron type on the antenna of *H. virescens* females is described, that responds with high sensitivity and selectivity to the sesquiterpene germacrene D. This plant odour receptor neuron displays a degree of specialization similar to the receptor neurons of the pheromone system of insects. The large number of germacrene D neurons observed in the present study, indicates the significance of germacrene D as a chemical cue in the interaction with host plants.

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### References


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