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Tomato-produced 7-epizingiberene and *R*-curcumene act as repellents to whiteflies

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ABSTRACT

How whiteflies (*Bemisia tabaci*) make the choice for a host plant prior to landing, is not precisely known. Here we investigated whether they respond to specific volatiles of tomato. Zingiberene and curcumene were purified from *Solanum habrochaites* (PI127826), characterised by NMR and X-ray analysis and identified as 7-epizingiberene and *R*-curcumene. In contrast, oil from *Zingiber officinalis* contained the stereoisomers zingiberene and *S*-curcumene, respectively. Using a combination of free-choice bio-assays and electroantennography, 7-epizingiberene and its dehydrogenated derivative *R*-curcumene were shown to be active as semiochemicals to *B. tabaci* adults, whereas the stereoisomers from ginger were not. In addition, *R*-curcumene elicited the strongest electroantennographic response. Bio-assays showed that a cultivated tomato could be made less attractive to *B. tabaci* than its neighbouring siblings by the addition of the tomato stereoisomer 7-epizingiberene or its derivative *R*-curcumene. These sesquiterpenes apparently repel adult whiteflies prior to landing, presumably because it informs them that after landing they, or their offspring, may be exposed to higher and lethal concentrations of the same compounds.

1. Introduction

By deciding which host plant to select for laying their eggs, flying adults of polyphagous insects determine a great deal of the developmental success of their relatively less mobile offspring. Here, we study these decisions for the case of the sweetpotato whitefly, *Bemisia tabaci* Gennadius, a polyphagous insect, first described as a plant pest in 1889 and continuing to be a threat to various horticultural and agricultural crops worldwide, mostly due to vectoring various devastating plant viruses (Jones, 2003). Prior to landing on a plant these insects may use a combination of vision and olfaction, to scan for cues that inform them on plant quality (Visser, 1988). Post-landing, they may also use gustatory or other sensory and metabolic cues to perceive host quality and to decide whether or not to oviposit. For *B. tabaci* post-landing behaviour consists of labial dabbing, probing and, finally, feeding, whereas pre-landing responses to colour, especially yellow-green (wavelength 500–600 nm), have been described by van Lenteren and Noldus (1990). Recently, we found that, in addition to visual cues, whiteflies use semiochemicals for selecting a host plant from a distance. They display avoidance behaviour to a selection of tomato terpenes, in particular the sesquiterpenes zingiberene and curcumene (Bleeker et al., 2009). Tomato zingiberene is known to be toxic and to have a negative effect on whitefly feeding and oviposition (Freitas et al., 2002; Muigai et al., 2002); ginger oil, which contains mostly zingiberene, has a similar impact (Zhang et al., 2004). However, the repellency in response to these compounds has not been elucidated so far.

In this study we isolated and purified zingiberene and curcumene focussing on the effect of these sesquiterpenes on the initial decision making process of whiteflies and addressed the following questions: (a) whether *B. tabaci* antennae can perceive these volatiles; (b) how specific perception is for different stereoisomers purified from tomato and ginger and; (c) how different stereoisomers influence whiteflies in deciding to land on a plant.

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2. Results

When given a choice between cultivated tomato (*Solanum lycopersicum* cv. Moneymaker) and wild tomato *Solanum habrochaites* PI127826, more than 90% of the whiteflies choose cultivated tomato (Fig. 1a; $P < 0.000$). However, in a no-choice assay, approximately 60% of the whiteflies will still land on the wild tomato (Fig. 1b), which is not significantly different from the numbers found on the cultivated tomato ($P = 0.267$). On the wild tomato, all whiteflies subsequently die (Fig. 1c), whereas >75% will survive on the cultivated tomato indicating its suitability as a host (Fig. 1c).

Since the wild tomato PI127826 contains large amount of zingiberene and because zingiberene acts as repellent to whiteflies (Bleeker et al., 2009), we decided to investigate the role of zingiberene in the initial choice behaviour of whiteflies in more detail. To this end, we first purified it from ginger (*Zinger officinalis*) oil, which is very rich in zingiberene (Antonious and Kochhar, 2003) and from leaves and stem material of the wild tomato PI127826 (Supplementary information). To determine the structure of the tomato and ginger zingiberene, both compounds were subjected to NMR analysis. Comparison of the NMR-spectra with those given by Breeden and Coates (1994) gave no absolute certainty about the structure. However, both zingiberenes could be purified by crystallisation of the Diels–Alder adduct, which made it possible to obtain unambiguous structural evidence of stereochemistry at carbons 4 and 7 by X-ray analysis (Supplementary information). It substantiated that *S. habrochaites* contained 7-epizingiberene (Breeden and Coates, 1994) whereas ginger oil contained its diastereomer zingiberene. When exposed to air, GC–MS analyses showed isolated zingiberene to spontaneously converted to curcumene (data not shown). Indeed, controlled dehydrogenation of both 7-epizingiberene and zingiberene resulted in pure *R*- and *S*-curcumene, respectively (Supplementary information).

Next, we assayed host preference in bio-assays with purified 7-epizingiberene or zingiberene applied to one of four different *S. lycopersicum* cv. Moneymaker plants of the same age, size and colour. As expected, in the absence of zingiberene the four tomatoes were equally attractive to *B. tabaci* (data not shown). Next, zingiberene (10 μg) was administered on paper cards to one of the four plants in the experimental setup. Zingiberene purified from ginger oil did not appear to make plants repellent to *B. tabaci* as the percentage of recaptured *B. tabaci* on each of the four tomato plants did not deviate significantly from an equal distribution (Fig. 2a; $P = 0.521$). Applied at the same concentration, 7-epizingiberene purified from tomato was very effective in reducing the number of whitefly visitations (Fig. 2c). The percentage of whiteflies visiting the plant with 7-epizingiberene decreased significantly ($P < 0.001$), whereas the three control plants harboured increased numbers of *B. tabaci*, as expected, although not in equal numbers. Both curcumenes were subsequently tested in free-choice bio-assays to ascertain their effect on repellence as well. Clearly, *B. tabaci* was only repelled by *R*-curcumene (Fig. 2b and d; $P = 0.475$ and $P < 0.001$, respectively), the derivative of tomato 7-epizingiberene.

To assess whether the four compounds used in the bio-assays are actually perceived by whitefly antennae, we employed electroantennography (Thakeow et al., 2008) on whitefly antennae by puffing a dilution of the selected purified compounds over an isolated antenna. Although each of the two stereoisoforms of zingiberene, as well as each of the two enantiomers of curcumene, appeared to elicit an electrical response in the antennae of whiteflies, *R*-curcumene consistently elicited the highest effect (Fig. 3a). For that reason, *R*-curcumene was diluted further. Even at higher dilution (10^{-4}) whitefly antennae still responded to the presence of this compound (Fig. 3b).

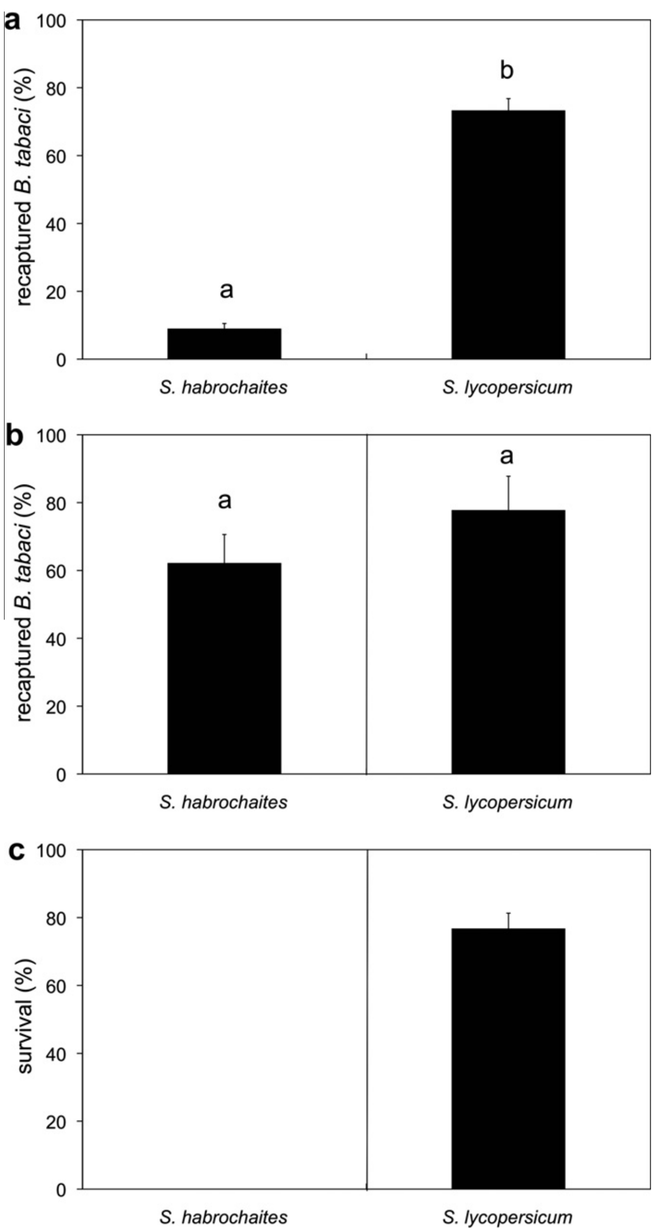

Fig. 1. Repellence and toxicity of PI127826. *B. tabaci* (a) preference 2 h after release (as % of released *B. tabaci*, $n = 100$) in a choice assay on tomato plants; (b) presence on tomato leaf (as % of total released, $n = 100$) in a no-choice assay (after 2 h); (c) survival on tomato leaflet (as % of total released, $n = 50$) in a no-choice assay (after 48 h). Bars represent mean values of 5–8 replications (+SE). Different letters represent statistically significant differences. Tomato plants used; *S. habrochaites* (PI127826) and *S. lycopersicum* (cv. Moneymaker).

3. Discussion

When given no other option, whiteflies will find and settle on a host displaying toxic properties, even if chances for survival are poor, indicating that visual cues dominate the response under the conditions of this assay (Fig. 1b and c). Since visual and odour cues can interact when processed in the insect brain (Balkenius et al., 2009), it is important to perform olfactory preference tests against the same visual background. Our host-choice assays show that, when given the choice between wild and cultivated tomato the initial decision of most whiteflies is to settle on the cultivated tomato (Fig. 1a). This is most likely due to the presence of zingiberene and curcumene in the headspace of PI127826 (52.1 and

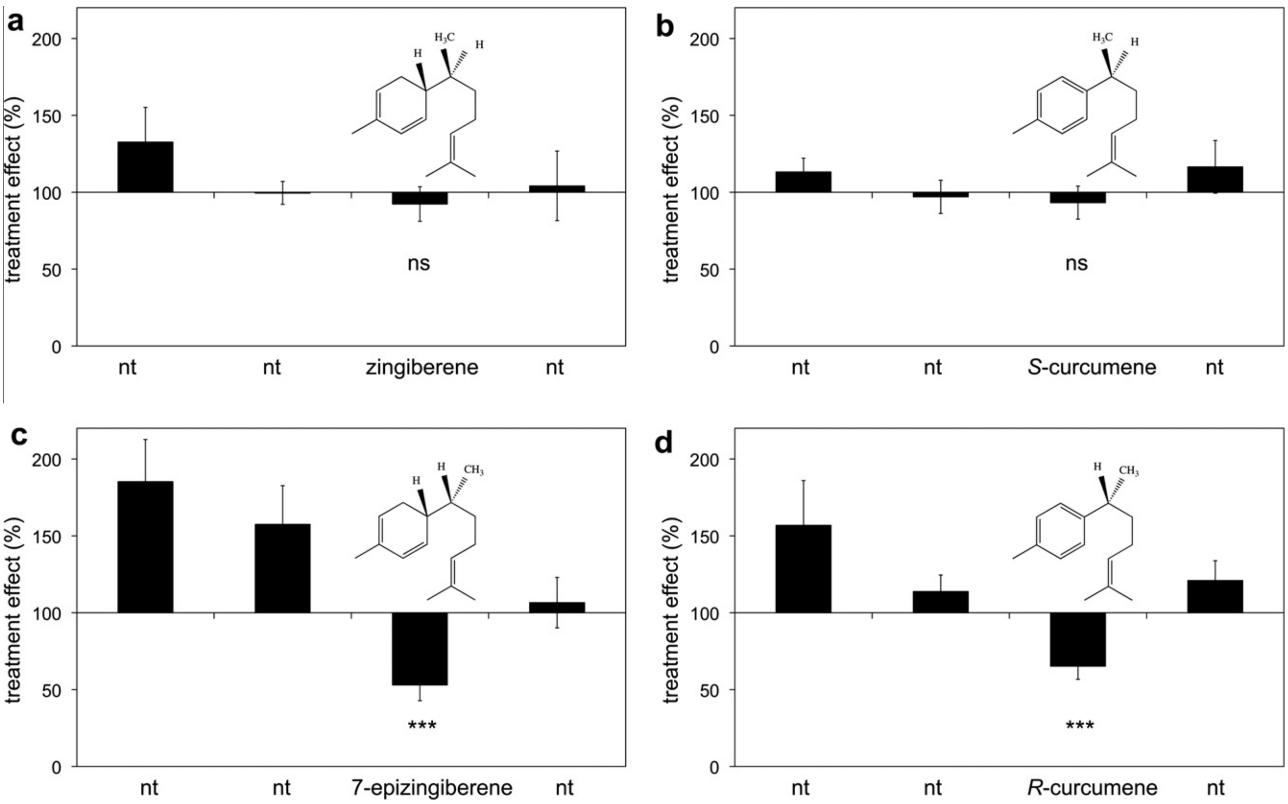

Fig. 2. Stereoisomer specific compounds can act as repellent to whiteflies. Treatment effect of the addition of 10 μg of (a) *Z. officinalis* zingiberene (b) *Z. officinalis* *S*-curcumene, (c) *S. habrochaites* (PI127826) 7-epizingiberene (d) *S. habrochaites* *R*-curcumene (PI127826) added to one plant in a setup of four *S. lycopersicum* plants (expressed as % of control setup with four untreated plants). Bars represent mean values of eight replications (+SE). nt: Plants in setup to which no volatiles were added. ns: Non-significant effect, ***: $P < 0.001$.

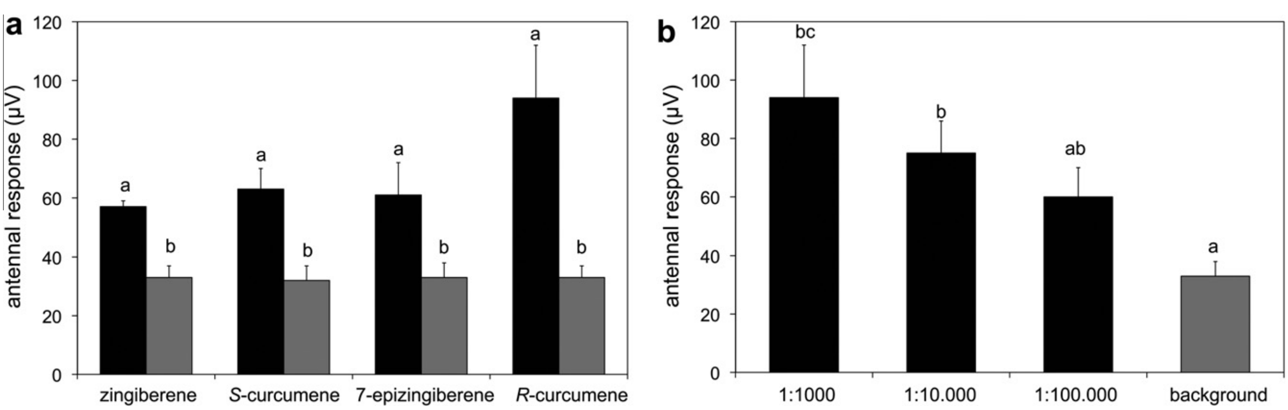

Fig. 3. Electroantennographic responses of isolated *B. tabaci* antennae to purified compounds. EAG reponse to (a) 10^{-4} dilution of zingiberene, 7-epizingiberene, *S*-curcumene and *R*-curcumene and (b) to a 10^{-4}–10^{-6} dilution range of *R*-curcumene. Bars represent the mean values of ⩾5 replications + SE. Different letters represent statistically significant differences. Black bars represent responses to the application of odour standards of purified compounds in paraffin oil. Grey bars represent responses to the application of paraffin oil only (background control).

28.2 μg 24 h^{-1} g^{-1}, respectively), which act as repellents to whiteflies (Bleeker et al., 2009). Moreover, against a background of identical visual cues, specific sesquiterpenes repel whiteflies from a plant which it would otherwise choose as a host (Fig. 2). Applying 10 μg of sesquiterpenoids in the bioassay should be within the range of sesuiterpenes emitted by the wild tomato plants, although it is not possible to measure the exact concentration in the experimental setup. Our results prompted the question why and how whiteflies are repelled by these compounds. In general, volatile compounds released by plants may help phytophagous arthropods to find a suitable host plant (e.g. Webster et al., 2008), or to avoid an unsuitable one (e.g. Cook et al., 2007). Hence, by their association with food quality of the host plant, the presence of toxic plant compounds or even the danger from predators inhabiting the plant, these compounds may convey information, and thus act as genuine semiochemicals. The arthropods may either learn this association first and upon subsequent perception of these semiochemicals, they may respond in a way that promotes their reproductive success (adaptive learning; Egas and Sabelis, 2001; Nomikou et al., 2003; van Wijk et al., 2008). Alternatively, they do not require a learning experience and respond innately, as a consequence of natural selection that acted many generations on the reproductive performance resulting from their olfactory and behavioural response.

Although we do not wish to exclude other possible functions of 7-epizingiberene and *R*-curcumene, such as a role in masking attractive plant odours or signaling negative plant qualities other than those directly related to the compounds themselves, the currently available evidence prompts us to hypothesise that whiteflies (*B. tabaci*) are repelled at a distance from the tomato plant by the sesquiterpenes *R*-curcumene and 7-epizingiberene, because it signals the presence of these compounds in toxic concentrations in the plant. When given a choice between cultivated tomato and the wild tomato, *S. habrochaites* (PI127826), whiteflies prefer not to land first on the latter (Fig. 1a). In line with our hypothesis, this specific wild tomato contains toxic levels of 7-epizingiberene in its trichomes (Supplementary information, Weston et al., 1989). When given no other choice than PI127826 leaves as a source of food, *B. tabaci* will still settle on these leaves in absence of an alternative (Fig. 1b). However, we showed that it will not survive there (Fig. 1c) in agreement with earlier reports on the toxic effects of, as we now know, 7-epizingiberene (Weston et al., 1989; Maluf et al., 2001; Eigenbrode et al., 1994; Freitas et al., 2002; Muigai et al., 2002; de Azevedo et al., 2003). At a distance from the tomato plant, this volatile compound (or its dehydrogenated form *R*-curcumene) is present at low concentrations and, because it is not toxic at these concentrations, it can provide information on the presence of that compound at higher concentrations near or at the source. Thus, the compound perceived at a distance provides reliable information on the presence of toxic concentrations of a (related) compound on this plant suggesting that is why it may trigger avoidance behaviour of the whiteflies.

The repellent effect of zingiberene was shown to depend on stereochemistry. Each of the two stereo-isoforms of zingiberene has toxic effects on whiteflies and other herbivores after they come into contact with these compounds (Eigenbrode et al., 1994; Freitas et al., 2002; Zhang et al., 2004; Weston et al., 1989). However, whiteflies can detect the presence of 7-epizingiberene (and *R*-curcumene) prior to contact with the host plant and modify their choice behaviour accordingly (Fig. 2). Since our whiteflies were reared on cucumber, which does not contain any form of zingiberene or curcumene, their response to 7-epizingiberene and *R*-curcumene must be innate. Such an innate response to zingiberene is apparently absent in whiteflies despite its severe toxicity. This phenomenon of differential responses to stereo-isoforms is well known from electroantennography with insects. Not only do many plant metabolites exist in different stereo-isoforms with distinct properties, they also occur in different plant species and their effects on insect receptors and neuronal discrimination between enantiomers of volatile compounds differ with the insect species, as shown for beetles (Wadhams et al., 1982), moths (Stranden et al., 2002) and weevils (Wibe et al., 1998).

Despite antennal responses to all four sesquiterpenes (Fig 3a), whiteflies appear to be able to specifically differentiate between the stereoisomers when locating tomato plants since only 7-epizingiberene and *R*-curcumene were repellent (Fig. 2). Because zingiberene converts to curcumene in contact with air (Chen and Ho, 1988), it might well be the compound that *B. tabaci* detects most frequently at larger distances from the tomato plant. Also, *R*-curcumene elicited a response in the antennae when more diluted (Fig. 3b), suggesting that whiteflies can detect this compound at a greater distance from the plant. Moreover, the response at a dilution of 10^{-4} was only slightly lower than at 10^{-3}. This extended range of detection points at the potential relevance of this particular compound for long-range orientation and host–plant finding. Hence, *R*-curcumene might be the most effective compound to ‘push’ whiteflies away from the tomato crop and to be used in combination with an attractant to lure this pest to a plant or site where it can be killed (so called push–pull strategies for pest control; Cook et al., 2007; Hassalani et al., 2008).

4. Concluding remarks

Besides dominant visual cues, *B. tabaci* makes use of volatile semiochemicals prior to choosing a host. Only tomato-derived 7-epizingiberene and *R*-curcumene prompted a modified behaviour in whiteflies under free-choice conditions, although their respective stereoisomers, zingiberene and *S*-curcumene, from ginger elicited a similar response in antennae, as determined by electroantennography. Thus 7-epizingiberene and *R*-curcumene most likely signal the presence of a hostile environment on the site of landing.

5. Experimental

5.1. General experimental procedures

A detailed description of the isolation of zingiberene, 7-epizingiberene, (*S*)-curcumene and (*R*)-curcumene can be found in Supplementary information. In short; 7-epizingiberene **2** was isolated from leaf and stem material of *S. habrochaites* (PI127826) by hexane extraction of tissue ground in liquid nitrogen. The crude tomato plant extract was filtered with pentane over a plug of silica gel from which almost pure (>90%) 7-epizingiberene was obtained. This was dissolved in 10 ml tetrahydrofuran (THF) to which 4-phenyl 1,2,4-trizoline-3,5-dione (PTAD; Fig. 4) was added dropwise over 10 min. The reaction mixture was concentrated and purified by flash chromatography to give a 4:1 mixture of isomers **1a** and **1b** (Fig. 4). After base hydrolysis, 7-epizingiberene was dehydrogenated by heating in benzene with palladium on carbon, as described by Breeden and Coates (1994) to obtain (*R*)-curcumene **3**. This (*R*)-curcumene possessed 10–20% of a double bond isomer, most probably **4** (see Supplementary information for NMR data). The Diels–Alder adduct **1a** could be purified by crystallisation, which enabled X-ray analysis for unambiguous structural elucidation of stereochemistry at carbons 4 and 7 (Fig. 4; CCDC 782570 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html). Zingiberene was isolated from ginger oil (Natura Sanat B.V., The Netherlands) as described by Millar (1998). Ginger oil was kugelrohr distilled and reacted with PTAD as described above. The reaction mixture was concentrated and purified to give a colourless oil consisting of a 4:1 mixture of diastereoisomers. After base hydrolysis, pure zingiberene was obtained and after dehydrogenation and purification (*S*)-curcumene was obtained.

5.2. Biological materials

Tomato plants (*S. lycopersicum*, *S. habrochaites*) were grown in a greenhouse compartment under controlled conditions (22–25 °C, 16/8 h photoperiod at 500 $\mu E\,m^{-2}\,s^{-1}$). A population of *B. tabaci* (biotype Q) was collected from tomato in a greenhouse in Santa María del Águila (Almería province, Spain) and reared in a climatised chamber (Snijders, Tilburg; 28 °C, 16 h light 150 $\mu E\,m^{-2}\,s^{-1}$, RH 75%) on cucumber. For bio-assays adult whiteflies, males and mated females alike, were randomly taken from the population reared in the laboratory.

5.3. Bio-assays

No-choice and choice assays were carried out in a mesh cage (1 m × 1 m × 1 m) inside a closed greenhouse compartment

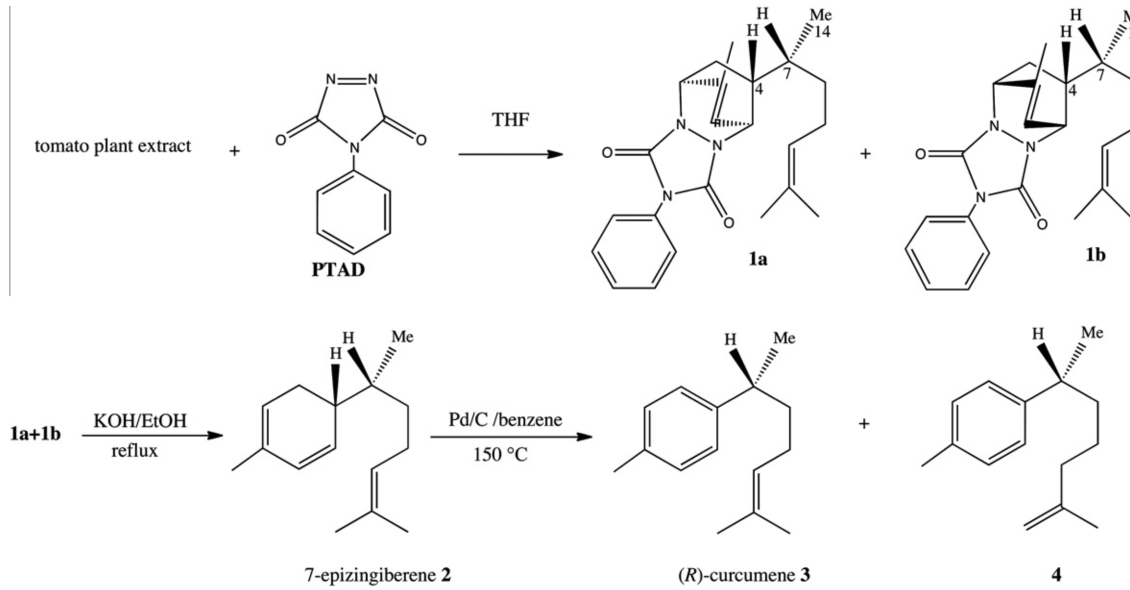

Fig. 4. Schematic representation of 7-epizingiberene purification.

(28 °C, RH 65%), with two potted, three-week old plants (either *S. lycopersicum* cv. Moneymaker, or *S. habrochaites* PI127826) placed in the middle of the cage. A total of 100 whiteflies was released in the cage. After 2 h the number of whiteflies settled on the plants was recorded. The test was repeated with two plants, now 1 *S. lycopersicum* C32 and 1 *S. habrochaites* PI127826 again with 100 whiteflies released in the mesh cage that were recaptured after 2 h.

Toxicity assays were performed in 0.5 l glass jars closed with cheesecloth inside a climatised chamber. The fourth fully expanded leaflet of a three-week old C32 or PI127826 was placed at the bottom with the petiole wrapped in wet cotton wool covered with aluminium foil. Carefully, 50 whiteflies were released in the jar and survival was scored after 48 h.

Free choice experiments with *B. tabaci* were carried out in the same greenhouse compartment, as described in detail by Bleeker et al. (2009). In short, four potted, three-week old, tomato plants (*S. lycopersicum* cv. Moneymaker) were placed in a square arrangement with each plant at a distance of 75 cm from its neighbour in a plastic-covered wooden tray filled with potting soil. The tray was positioned in the centre of the greenhouse compartment. A total of 150 adult whiteflies were captured from the rearing described above, placed at 4 °C for 2 min and then released in the middle of the setup. After 10 and 20 min from release the whiteflies were recaptured and the number of whiteflies on each plant was recorded. To test the effect of a compound on repellence, 10 μg of a purified sesquiterpene was applied to 10 filter paper discs (Whatman, 25 mm diameter) and attached to 1 of the 4 plants in the setup. The position of the treated tomato was randomised. Five minutes after placing the volatiles on the plant, whiteflies were released. Discs with volatiles were always added to the same plant, whereas the other plants received paper discs without the compound under investigation. Prior to each assay with volatiles, the choice experiment was done with the four plants untreated, in the same spatial position, to establish a control value under the same background, environmental conditions and arrangement. For statistical comparison the non-treated setup was compared to the treated setup. In the figures, the background attraction of each plant in the untreated setup was set to 100%. Each experiment was repeated eight times with new, inexperienced whiteflies and with the same plants in altered positions. For each sesquiterpene a new set of tomato plants was used.

5.4. Electroantennographical assays

B. tabaci antennae were carefully dissected and used to record electroantennographical (EAG) responses. EAGs were measured by manual injection of the compound into a humidified stream of air passing over the excised antenna mounted onto an antenna holder housed in a polytetrafluoroethylene (Teflon) cell (flow rate 250 ml/min). Within this holder, the ends of the excised antennae contacted a hemolymph electrolyte Ringer solution that provided electrical contact to a pair of Ag/AgCl-electrodes (Thakeow et al., 2008). The EAG potentials were amplified by a factor 100 using a high impedance amplifier with a built-in low pass filter, set to a cut-off frequency of 1 Hz. The amplified and filtered signal was digitised using a 35900E A/D-converter (Agilent) and recorded by the GC ChemStation software (Agilent) (Weißbecker et al., 2004). Odour standards were produced from a dilution series of the respective purified compounds in paraffin oil (Uvasol quality, Merck/VWR). Small pieces of filter paper (2 cm^2; Schleicher and Schuell, Dassel, Germany) were soaked with 100 μl of the standard dilution or paraffin oil only (control). The filter paper was put into a 10 ml glass syringe (Poulten and Graf GmbH, Wertheim, Germany). Inside the air volume of the syringe, the odourant accumulated at a concentration that depends on the concentration of the substance in the solution and its vapour pressure as predicted by Henry's law. A reproducible stimulus could be supplied by puffing 5 ml of air from the syringe over the antenna (Schütz et al., 1999). Dilutions of the compounds were measured at least five times for their impact on the response of at least five different whiteflies. Though purified, we cannot exclude that compounds present in trace amounts affected the AEG analysis. However, the whitefly antenna in the experimental system could not be kept stable for a period long enough to perform GC-AEG against a background of plant headspace odour.

5.5. Statistical analyses

The effect of addition of sesquiterpenes on response was tested as the ratio between control and untreated by ANOVA using a randomised complete block design, fitted including the effects of plant and position and their possible interaction. Statistical analyses for other choice and non-choice bio-assays and for the effect of sesquiterpenes in the electroantennographical assays were carried out using ANOVA. Values with $P < 0.05$ were regarded as significant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.10.014.

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