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Olfactory cues of mahogany trees to female *Hypsipyla robusta*

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Abstract

The mahogany shoot borer, *Hypsipyla robusta* (Moore) (Lepidoptera Pyralidae), is a serious pest insect in the tropical forests of Africa, Asia and Australia. This insect causes multiple branching of young shoots in indigenous mahogany plantations. Gravid insects are attracted to oviposition sites by volatile organic compounds (VOCs) released by their hosts. Therefore, in this study, we aimed to (i) identify and characterise the VOCs released by the shoots of *Entandrophragma* and *Khaya* mahogany and (ii) to determine the electrophysiologically active VOCs which could influence the olfactory response of *H. robusta*. Volatile samples were collected from shoots of *Entandrophragma angolense* (Welwitsch) de Candolle, *Entandrophragma utile* (Dawe et Sprague) Sprague, *Khaya anthotheca* (Welwitsch) de Candolle and *Khaya ivorensis* Chevalier by closed-loop-stripping-analysis. The VOCs were identified by gas-chromatography mass-spectrometry (GC-MS) and characterised by comparing their retention times with those of authentic standards. For the first time, 29 VOCs were characterised as typical of the four mahogany species studied. The VOCs included alcohols, aldehydes, alkanes, alkenes, esters, ketones, monoterpenes, alcohol sesquiterpenes and sesquiterpenes. The majority were esters (10) and sesquiterpenes (8). GC-MS/electroantennographic detection experiments revealed antennal responses of the female moth to (*Z*)-*β*-ocimene, (*Z*)-3-hexen-1-yl acetate, hexan-1-ol, nonanal, (*Z*)-3-hexen-1-yl butanoate, 2-ethyl hexan-1-ol, decanal, *β*-caryophyllene, (*Z*)-3-hexen-1-yl hexanoate and germacrene D. Dose-response experiments with three of the compounds revealed antennal responses at concentrations of 10^{-7} to 10^{-2}. We therefore suggest that these compounds are olfactory cues of female *H. robusta* and could be used in behaviour-based control of *H. robusta*.

Key words: volatile organic compounds, antennally-active compound, *Entandrophragma* spp., *Khaya* spp., insect-plant interactions, electroantennography, GC-MS/EAD, EAG, CLSA.

Introduction

Mahogany trees belong to the Meliaceae family and represent one of the most economically important tree species in the world (Ofori *et al*., 2007; Lopes *et al*., 2008). Mahoganies indigenous to Africa comprise several species. In Ghana, the most common ones include *Entandrophragma angolense* (Welwitsch) de Candolle, *Entandrophragma utile* (Dawe et Sprague) Sprague, *Khaya anthotheca* (Welwitsch) de Candolle and *Khaya ivorensis* Chevalier (Irvine, 1961; Louppe *et al*., 2008). These species are susceptible to the mahogany shoot borer, *Hypsipyla robusta* (Moore) (Lepidoptera Pyralidae) attack. In the neotropics, *Swietenia macrophylla* (King) is indigenous and susceptible to *Hypsipyla grandella* (Zeller) (Lepidoptera Pyralidae), a moth closely related to *H. robusta*. *Toona ciliata* (Roemer), another Meliaceae tree, is indigenous to tropical Australia and Asia and is also susceptible to *H. robusta* (Newton *et al*., 1993; Cunningham and Floyd, 2004).

H. robusta is capable of attacking mahogany trees at any age; however damage on young trees causes higher economic losses. The incidence of *H. robusta* attack occurs mainly during rainy seasons. The life cycle requires 25-55 days and there are several overlapping generations ranging between 6 to 9 in a year (Wagner *et al*., 2008). This prolific insect causes devastating damage, making it one of the major causes of failure of mahogany plantations (Newton *et al*., 1993; Nair, 2007). In fact, attempts to plant indigenous mahoganies in large plantations in Africa, Asia, and tropical Australia have failed because of attacks by *H. robusta* (Cunningham and Floyd, 2004; Cunningham *et al*., 2005; Nair, 2007; Ofori *et al*., 2007; Opuni-Frimpong *et al*., 2008a). The female lays about 200-450 eggs over a period of five to eight days singly on soft shoots and the larvae bore into succulent parts of the shoots and feed (Griffiths, 2001). Larvae form tunnels from top to bottom within 1-2 months and terminal shoots are killed in the process. The destruction of the terminal shoots leads to the development of lateral shoots and hence a bushy and crooked tree is formed. The growth of the tree is retarded and the desired tall, straight and economically valuable bole form is subsequently not achieved (Nair, 2007; Ofori *et al*., 2007; Wagner *et al*., 2008). Sometimes eggs are also laid on the compound leaves and larvae can bore into the leaf veins (Griffiths, 2001).

Several different groups of volatile organic compounds (VOCs) are released under different conditions by plants. Injured plants release specific blends of VOCs that differ from those of undamaged plants, resulting from the interaction with abiotic factors such as mechanical wounding (Howe, 2004) and biotic factors such as herbivores (Spiteller and Boland, 2003; Merkx-Jacques and Bede, 2004). Some VOCs released by undamaged plants are used as cues by insect herbivores to orientate to their specific hosts (Soares *et al*., 2003; Rodriguez-Saona *et al*., 2006; Midega *et al*., 2011; Paiva *et al*., 2011). It is also known that, the attraction of gravid female herbivores to suitable host plants is

mediated largely by olfactory cues in many insect species (Hern and Dorn, 2004; Bichão *et al*., 2005; Tasin *et al*., 2006; Piñero *et al*., 2008; Thakeow *et al*., 2008; Alhmedi *et al*., 2010). To date, there is no known evidence of studies on the chemoecological interaction between *H. robusta* and its hosts. However, few data are already reported for *H. grandella*. For instance, it is known that *H. grandella* responds to *β*-caryophyllene which is released by *S. macrophylla* (Soares *et al*., 2003).

In our previous study, we examined the relative susceptibility of four mahogany species indigenous to Africa, *E. angolense*, *E. utile*, *K. anthotheca*, and *K. ivorensis*, to the attack of *H. robusta* in a field study in Ghana (Opuni-Frimpong *et al*., 2008b). Our previous results showed that *E. utile* was the least damaged species, followed by *E. angolense* and *K. ivorensis*, while *K. anthotheca* was the most injured one. However, the role of mahogany volatile compounds in the attraction of *H. robusta* has not been investigated so far. Therefore, a chemical ecological study was undertaken aiming to identify the specific VOCs released by the shoots of the four mahogany species previously studied, and to characterise those detected by *H. robusta* as olfactory cues.

Materials and methods

Plant materials

The mahogany trees, *E. angolense*, *E. utile*, *K. anthotheca* and *K. ivorensis* were selected from two mahogany plantations located in the Ashanti region of Ghana (6°37'26.00"N, 1°15'22.04"W and 6°41'02.19"N, 1°37'29.38"W). These plantations are located within the moist semi-deciduous forest, which is the most extensive type of forest in Ghana (Hall and Swaine, 1981; Wagner *et al*., 2008). Trees in this forest type include *Entandrophragma* and *Khaya* species (Taylor, 1960; Hawthorne, 1990). Annual rainfall in the area is about 1500 mm with a dry season from December through March. The major rainfall seasons are in May/June, and September/October with temperatures between 10 °C and 35 °C. Only coppices between 1.5 and 2 m tall with bole diameters between 3 and 8 cm were used in our experiments. In each plantation, 5 trees for each species were randomly selected, giving us a total of 10 trees each of *E. angolense*, *E. utile*, *K. anthotheca* and *K. ivorensis* for the two plantations. The selected trees had fresh leaves and were tagged for volatile sample collection from their shoots. Volatiles were sampled directly in the plantations during the rainy season in May and June parallel to the active oviposition period of *H. robusta* females.

Volatile samples collection

Samples for GC-MS and GC-MS/electroantennographic detection (GC-MS/EAD) analyses were collected from the upper shoot (20-30 cm) of each sampled tree bearing ca. 4-5 compound leaves. Shoots were carefully enclosed within a polyester oven bag (Melitta®-Toppits®, Minden, Germany), such that no mechanical damage was caused. Volatiles were collected using the closed-loop-stripping-analysis (CLSA) method (Boland *et al*., 1984). A 12 V vacuum pump (DC12/16FK type, Fürgut, Tannheim, Germany) circulated air within the oven bag to an adsorbent trap loaded with 1.5 mg charcoal (CLSA-Filter, Daumazan sur Arize, France). The pump was powered by a 6 V rechargeable battery (Conrad Electronic GmbH, Hirschau, Germany) and the air flow was 1 L/min. Sampling was performed for 3 hours. Altogether, 40 volatile samples were collected, 10 from each mahogany species. As negative control, we collected air samples from empty oven bags placed within the two plantations. Negative control samples were collected at the same time as the mahogany volatiles.

The volatile samples were eluted from the charcoal filters into 1 mL-glass vials (Chemic ALS, Rutigliano, Italy) using 100 µL of a mixture consisting of methylene chloride (two parts) and methanol (one part), both solvents were SupraSolv® quality (Merck/VWR, Darmstadt, Germany). After elution, the CLSA filters were cleaned using a set of solvents at different polarity (methylene chloride, methanol and acetone) to remove all possible volatile traces from the filters and to avoid contamination of subsequent samples. The activated charcoal filters were then heated at 70 °C for one hour in an oven before reusing. The eluted volatile samples were stored in an ultra-low temperature freezer at −80 °C until GC-MS and GC-MS/EAD analyses began.

Standard compounds

The following authentic standard compounds with the given purity were obtained from commercial sources for the purpose of comparing their retention times and indices with volatile compounds we collected from our plant samples: hexan-1-ol (≥99% purity, Sigma-Aldrich, Steinheim, Germany), 1-octen-3-ol (>98% purity, Merck, Darmstadt, Germany), 2-ethyl hexan-1-ol (>99% purity, Merck), nonanal (>98% purity, Merck), decanal (≥ 98% purity, Sigma-Aldrich), (*Z*)-3-hexen-1-yl acetate (≥98% purity, Sigma-Aldrich), (*Z*)-3-hexen-1-yl butanoate (≥98% purity, Sigma-Aldrich), (*E*)-2-hexen-1-yl butanoate (96% purity, Aldrich, Steinheim, Germany), methyl salicylate (≥99% purity, Sigma-Aldrich), (*Z*)-3-hexen-1-yl 3-methyl butanoate (97% purity, Aldrich), (*Z*)-3-hexen-1-yl hexanoate (98% purity, Aldrich), (*Z*)-3-hexen-1-yl benzoate (≥97% purity, Sigma-Aldrich), linalool (97% purity, Merck), (*E*)-nerolidol (90% purity, Aldrich), *α*-cubebene (≥97.0% purity, Fluka, Germany), *α*-copaene (≥90% purity, Aldrich), *β*-caryophyllene (≥98.5% purity, Fluka), *α*-humulene (98% purity, Fluka), farnesene (mixture of isomers, Sigma-Aldrich).

GC-MS analysis

All volatile samples collected were analysed in a network GC system (6890N, Agilent Technologies, Santa Clara, USA) fitted with a mass selective detector - MS (5973 Network, Agilent Technologies). The GC-MS had a non-polar HP-5MS column (Agilent Technologies). This column was 30 m long and had an internal diameter of 0.25 mm with 0.25 µm film thickness. One µL of the volatile samples was injected at a time into the GC in the pulsed splitless mode when the injector tem-

perature was 250 °C. The carrier gas used was helium flowing at a rate of 1 mL/min and at an average velocity of 36 cm/sec. The initial temperature of the oven was set at 50 °C and held for 1.5 min. It was then heated at a rate of 7.50 °C/min until it reached 200 °C. This final temperature was held for 5 min. The total run time was 26.50 min. The mass range of the mass spectra was 20 to 345.

The volatile compounds were initially tentatively identified by comparing their mass spectra with those in the database of NIST 11 (Gaithersburg, MD, USA) and Wiley 7N (John Wiley, NY, USA) by using Enhanced ChemStation (ver. E.02.02.1431, Agilent Technologies). The identity of 19 compounds (see table 1) was then confirmed by comparing their mass spectra and retention times with those of the authentic standard compounds at a concentration of 10^{-4} (100 ng/µl). The linear retention indices of the compounds were calculated for HP-5MS and INNOWAX columns using the retention times of *n*-alkane series (C_{10}-C_{23}) as reference compounds (van den Dool and Kratz, 1963). Linear retention indices were also compared with those already published in literature.

Insect collection

Larvae of *H. robusta* were collected from infested mahogany trees adjacent to the two plantations. Several *H. robusta*-infected shoots of *Entandrophragma* and *Khaya* mahoganies were dissected and the larvae were removed. The larvae collected were reared on wheat media following the protocol used in Couilloud and Guiol (1980). On the emergence of adults, the antennae of 2-4 day old females were excised and used for electrophysiological experiments.

GC-MS/EAD experiments

Electrophysiological experiments were performed to determine the antennal responses of 10 adult females of *H. robusta* to volatile samples collected from *E. angolense*, *E. utile*, *K. anthotheca* and *K. ivorensis*. The experiments were carried out using a GC system (HP 6890N, Agilent Technologies) coupled in parallel to a mass spectrometer (MS 5973 Network, Agilent Technologies) and to an electroantennographic detector (EAD) in a setup as described in Weissbecker *et al.* (2004). The GC had an INNOWAX polar column (Agilent Technologies), 30 m long, with an internal diameter of 0.25 mm and 0.25 µm film thickness. A carefully excised antenna from a female *H. robusta* was placed on an antenna holder (Färbert *et al*., 1997), ensuring that the two ends of the antenna were immersed in a Ringer solution that was adapted to insect hemolymph electrolyte concentration (Kaissling and Thorson, 1980). EAG signals were amplified by a factor of 100 and recorded with the Agilent ChemStation software. Humidified air at room temperature was admixed to the GC effluent and supplied to the antenna at a flow rate of 20 L/h. The experiments were performed by injecting 1 µL of VOC solution from each of the four species of mahogany into the GC in the pulsed splitless mode when the injector was at 250 °C. The carrier gas for the VOCs was helium which was flowing at a constant rate of 1 mL/min. The oven was at an initial temperature of 50 °C. This initial temperature was held for 1.5 min and rose by 7.5 °C/min until a temperature of 200 °C was reached. This final temperature was held for 5 min. EAG signals were recorded in parallel to the GC-MS chromatograms. Compounds were tentatively identified by comparing their mass spectra to those in the database base of NIST 11 (Gaithersburg, MD, USA) and Wiley 7N (John Wiley, NY, USA) using Enhanced ChemStation (ver. E.02.02.1431, Agilent Technologies). The interpretation was confirmed by matching the mass spectra and retention times with those of authentic standards on the INNOWAX column.

Dose-response experiments

To determine the threshold concentrations at which females of *H. robusta* are able to detect mahogany host volatiles, we performed electroantennography (EAG) experiments with three compounds that showed high responses in the GC-MS/EAD experiments. Thus, hexan-1-ol (99%, Aldrich), nonanal (>98%, Merck) and 2-ethyl hexan-1-ol (>99%, Merck) were puffed at different concentrations over the antennae. Authentic standard compounds were diluted into six different concentrations from 10^{-2} to 10^{-7} (10 mg/g - 0.1 µg/g) (w/w) in paraffin oil (Uvasol®, spectrosc. qual., high visc., Merck/VWR). Pure paraffin oil was used as negative control. Pieces of filter paper (Schleicher and Schuell, Dassel, Germany) cut to 1.5-2 cm^2 were soaked with 60 µL of the standard dilution or paraffin oil only and inserted into 10 mL glass syringes (Poulten and Graf GmbH, Wertheim, Germany). The syringe was then flushed with synthetic air and after a short equilibrium time a stimulus was supplied. Since these compounds were puffed in the gas phase, their absolute concentrations in air were proportional to their respective vapour pressures of 1.22 mbar, 0.65 mbar and 0.22 mbar at 21 °C (Yaws, 2007) and their dilution factor in paraffin oil. Therefore, the respective concentrations in terms of parts per trillion (ppt), parts per billion (ppb) or parts per million (ppm) puffed were; hexan-1-ol: 10^{-7} = 122 ppt; 10^{-6} = 1.22 ppb; 10^{-5} = 0.0122 ppm; 10^{-4} = 0.122 ppm; 10^{-3} = 1.22 ppm; 10^{-2} = 12.2 ppm; nonanal: 10^{-7} = 65 ppt; 10^{-6} = 0.65 ppb; 10^{-5} = 6.5 ppb; 10^{-4} = 65 ppb; 10^{-3} = 0.65 ppm; 10^{-2} = 6.5 ppm and 2-ethyl hexan-1-ol: 10^{-7} = 22 ppt; 10^{-6} = 0.22 ppb; 10^{-5} = 2.2 ppb; 10^{-4} = 22 ppb; 10^{-3} = 0.22 ppm; 10^{-2} = 2.2 ppm.

Reproducibility was achieved by always puffing 5 mL of syringe headspace over the antenna after a standard resting time of 2 min. Each set of experiments always started with the lowest concentration. Each sample was puffed three times and the mean EAG amplitude constituted the experimental unit used for a dose-response curves. Three females of *H. robusta* were tested for a total of 9 electroantennograms for each concentration.

Statistical analysis

The mean amount of each compound in the four mahogany species was estimated by the mean peak area (± SE) of the 10 replicates and expressed in relative per-

cent, taking the amount in *E. angolense* as 100% for each compound. To determine differences in the mean amounts, we performed an analysis of variance (ANOVA) followed by the Tukey's honestly significant difference (Tukey HSD) test (IBM SPSS, version 20; Armonk, NY, USA). To determine the threshold concentrations at which females of *H. robusta* are able to detect mahogany host volatiles, we performed Kruskal-Wallis tests followed by Mann-Whitney U tests (IBM SPSS). The Mann-Whitney U test was also performed to determine differences in the response to compounds at different concentration levels.

Table 1. VOCs from the shoots of *E. angolense*, *E. utile*, *K. anthotheca* and *K. ivorensis* collected by CLSA over a period of 3 hours and characterised by GC-MS (HP-5MS non-polar column and INNOWAX polar column).

No	Compound	LRI on HP-5MS	R-LRI non-polar	LRI on INNOWAX	R-LRI polar	Relative amount in percent TIC†			
						E. angolense	*E. utile*	*K. anthotheca*	*K. ivorensis*
Alcohols									
1	hexan-1-ol[a]	880	879	1322	1325	100 ± 94	77 ± 58	4 ± 1	2 ± 2
2	1-octen-3-ol[a]	982	980	1417	1423	100 ± 54	10 ± 6	31 ± 10	47 ± 25
3	2-ethyl hexan-1-ol[a]	1031	1030	1458	1446	100 ± 47	57 ± 25	46 ± 14	24 ± 8
Aldehydes									
4	nonanal[a]	1105	1106	1376	1378	100 ± 19	72 ± 12	62 ± 19	48 ± 21
5	decanal[a]	1205	1207	1481	1484	100 ± 18	76 ± 22	59 ± 19	45 ± 18
Alkanes									
6	1,1-dimethyl-3-methylene-2-vinylcyclohexane[c]	1117	-	n.d.	-	100 ± 37	179 ± 62	53 ± 17	31 ± 17
Alkenes									
7	(*E,E*)-2,6-dimethyl-1,3,5,7-octatetraene[b]	1132	1130	1187	-	100 ± 64	84 ± 23	41 ± 13	18 ± 13
Esters									
8	(*Z*)-3-hexen-1-yl acetate[a]	1010	1009	1293	1305	100 ± 53	341 ± 169	296 ± 94	15 ± 9
9	methyl benzoate[b]	1099	1099	1601	1635	100 ± 88	4 ± 2	5 ± 2	22 ± 21
10	(*Z*)-3-hexen-1-yl butanoate[a]	1185	1186	1438	1448	100 ± 66	37 ± 18	175 ± 55	229 ± 83
11	(*E*)-2-hexen-1-yl butanoate[a]	1193	1195	1450	1466	100 ± 78	11 ± 9	2 ± 1	0
12	methyl salicylate[a]	1198	1197	1781	1785	100 ± 31	459 ± 228	92 ± 29	77 ± 24
13	(*Z*)-3-hexen-1-yl 3-methyl butanoate[a]	1236	1237	1465	1477	100 ± 64	32 ± 12	100 ± 32	107 ± 34
14	(*Z*)-3-hexen-1-yl (*E*)-2-butenoate[c]	1235	-	1576	-	100 ± 57	56 ± 22	451 ± 143	180 ± 72
15	(*E*)-3-hexen-1-yl (*E*)-2-ethyl-2- butenoate[c]	1324	-	1643	-	100 ± 44	32 ± 6	69 ± 22	25 ± 5
16	(*Z*)-3-hexen-1-yl hexanoate[a]	1380	1381	1630	1643	100 ± 100	206 ± 130	493 ± 156	561 ± 322
17	(*Z*)-3-hexen-1-yl benzoate[a]	1576	1571	2093	2119	100 ± 76	92 ± 33	124 ± 39	16 ± 5
Ketones									
18	(*Z*)-geranyl acetone[b]	1455	1455	1827	-	100 ± 22	135 ± 84	47 ± 15	46 ± 13
Monoterpenes									
19	(*Z*)-*β*-ocimene[b]	1049	1049	1223	1234	100 ± 60	41 ± 8	9 ± 3	16 ± 6
Alcohol sesquiterpenes									
20	linalool[a]	1101	1103	1514	1522	100 ± 51	120 ± 60	88 ± 28	131 ± 82
21	(*E*)-nerolidol[a]	1566	1568	2054	2050	100 ± 31	257 ± 76	211 ± 67	119 ± 51
Sesquiterpenes									
22	*α*-cubebene[a]	1354	1357	1463	1465	100 ± 58	54 ± 36	181 ± 57	223 ± 104
23	*α*-copaene[a]	1382	1385	1482	1488	100 ± 25	15 ± 10	120 ± 38	40 ± 9
24	*β* -elemene[b]	1397	1399	1577	1570	**100 ± 44 a**	**12 ± 3 b**	**46 ± 14 a**	**26 ± 5 a**
25	*β*-caryophyllene[a]	1430	1433	1587	1594	100 ± 38	11 ± 5	75 ± 24	50 ± 26
26	*α*-humulene[a]	1465	1468	1659	1663	100 ± 40	6 ± 4	84 ± 27	72 ± 22
27	germacrene D[b]	1492	1492	1698	1705	100 ± 30	44 ± 16	109 ± 34	121 ± 41
28	*α*-farnesene[a]	1512	1512	1723	1725	100 ± 75	78 ± 16	33 ± 10	355 ± 186
29	*δ*-cadinene[b]	1533	1532	1749	1773	**100 ± 37 a**	**15 ± 10 b**	**45 ± 14 a**	**43 ± 15 a**

TIC = Total ion chromatogram; LRI = Linear retention index; R-LRI = reference linear retention index; R-LRI is retention index already published in peer-reviewed journals and listed on NIST webbook and Pherobase; Means of *β*-elemene and *δ*-cadinene with different letters (in bold fonts) indicate statistical differences based on Tukey's HSD at $p \leq 0.05$. There were no statistical differences in the mean amounts of all the other compounds.

† The estimated amount in percent of each compound in the four mahogany species is the mean peak area of 10 replicates; [a] compound confirmed by authentic standard compound; [b] compound confirmed by comparing LRI with already published LRI in peer-review journals and listed on NIST webbook and Pherobase; [c] compound tentatively identified by Enhanced ChemStation software version D.02.00.275 and NIST mass spectra library; n.d.: not detected on respective column.

Table 2. VOCs from *E. angolense*, *E. utile*, *K. anthotheca* and *K. ivorensis* that elicited consistent antennal responses indicated by (+) when tested with antennae of females of *H. robusta* in GC-MS/EAD experiments on INNOWAX polar column; (-) indicates inconsistent antennal responses.

No.	Compound	LRI	R-LRI	*E. angolense*	*E. utile*	*K. anthotheca*	*K. ivorensis*
1	(*Z*)-*β*-ocimene	1223	1234	-	+	+	+
2	(*Z*)-3-hexen-1-yl acetate	1293	1305	-	+	+	-
3	hexan-1-ol	1322	1325	+	+	+	-
4	nonanal	1376	1378	+	+	+	+
5	(*Z*)-3-hexen-1-yl butanoate	1438	1448	+	-	+	-
6	2-ethyl hexan-1-ol	1458	1446	+	+	-	-
7	decanal	1481	1484	+	-	+	+
8	*β*-caryophyllene	1587	1594	+	+	-	+
9	(*Z*)-3-hexen-1-yl hexanoate	1630	1643	-	-	+	-
10	germacrene D	1698	1705	-	-	+	-

LRI = Linear retention index on INNOWAX column; R-LRI = Reference linear retention index; R-LRI is a retention index already published in peer-reviewed journals and listed on NIST webbook and Pherobase.

Results

Volatile organic compounds identified

Among the 40 volatile samples, we characterised a total of 29 VOCs in the shoots of *E. angolense*, *E. utile*, *K. anthotheca* and *K. ivorensis*. They belonged to 9 chemical classes and consisted of 3 alcohols, 2 aldehydes, 1 alkane, 1 alkene, 10 esters, 1 ketone, 1 monoterpene, 2 alcohol sesquiterpenes and 8 sesquiterpenes (table 1).

All the 29 compounds were present in the two *Entandrophragma* species. Among the *Khaya* species, all the 29 compounds were also detected in *K. anthotheca.* With the exception of (*E*)-2-hexen-1-yl butanoate, all other compounds were also detected in *K. ivorensis*. Statistical analysis revealed that, the mean amount of *β*-elemene in *E. angolense* was significantly higher than that in *E. utile*. There was no significant difference between the mean amount of *β*-elemene in *E. angolense* and that in the two *Khaya* species. Likewise, the mean amount of *δ*-cadinene in *E. angolense* was significantly higher than that in *E. utile* but there was no significant difference between the mean amount of *δ*-cadinene in *E. angolense* and that in the two *Khaya* species. No statistical differences were found in the amounts of the other compounds among the four mahogany species (table 1).

GC-MS/EAD experiments

Antennae of females of *H. robusta* responded to stimuli from 10 of the 29 compounds identified in the GC-MS analysis. The 10 antennally active compounds were (*Z*)-*β*-ocimene, (*Z*)-3-hexen-1-yl acetate, hexan-1-ol, nonanal, (*Z*)-3-hexen-1-yl butanoate, 2-ethyl hexan-1-ol, decanal, *β*-caryophyllene, (*Z*)-3-hexen-1-yl hexanoate and germacrene D (table 2).

Out of the 10 antennally active compounds, 8 elicited reproducible antennal responses in at least two of the four mahogany species. Nonanal elicited consistent antennal responses in all the four mahogany species. (*Z*)-*β*-ocimene, hexan-1-ol, decanal and *β*-caryophyllene elicited consistent antennal responses in three of the four mahogany species. (*Z*)-3-Hexen-1-yl acetate, (*Z*)-3-hexen-1-yl butanoate and 2-ethyl hexan-1-ol elicited consistent antennal responses in two mahogany species whiles (*Z*)-3-hexen-1-yl hexanoate and germacrene D elicited consistent antennal responses in at least one species of the four mahogany species (figure 1).

Dose-response experiments

The dose-response experiments were conducted with females of *H. robusta*, using three compounds (hexan-1-ol, nonanal and 2-ethyl hexan-1-ol) which elicited strong EAD responses. The EAG dose response profiles showed that hexan-1-ol elicited electrical responses ranging from 0.37 ± 0.03 mV (mean ± SE) to 3.05 ± 0.02 mV. For nonanal and 2-ethyl-hexan-1-ol, the electrical responses ranged from 0.41 ± 0.01 mV to 2.40 ± 0.02 mV and 0.34 ± 0.01 mV to 2.04 ± 0.01 mV, respectively (figure 2).

In all the EAG experiments, the antennal responses to the test compounds showed significant differences from the response to the paraffin oil control (hexan-1-ol: χ^2 = 19.32, d.f. = 6, p = 0.004; nonanal: χ^2 = 19.63, d.f. = 6, p = 0.003; 2-ethyl hexan-1-ol: χ^2 = 19.17, d.f. = 6, p = 0.004) (figure 2A - 2C). Mann-Whitney U tests showing the statistical differences are presented in table 3. The antennal responses of the three compounds at the six different concentrations 10^{-7} – 10^{-2} (0.1 µg/g to 10 mg/g) were also compared (figure 2D). The first difference in the response to the three compounds appeared at the concentration of 10^{-6} where the response to 2-ethyl hexan-1-ol was significantly less than the other two. The same trend was observed at 10^{-5} and 10^{-3}. At 10^{-4} and 10^{-2} the response of the female antennae differed statistically among all the three chemicals tested. The Mann-Whitney U test results showing statistical difference or otherwise between the three compounds at the different concentrations are presented in table 4.

Discussion

In our previous study we demonstrated that *H. robusta* causes different degrees of damage to the four mahogany species investigated (Opuni-Frimpong *et al*., 2008b). We therefore wished to analyse the volatile compounds released by each species. Our results showed that the

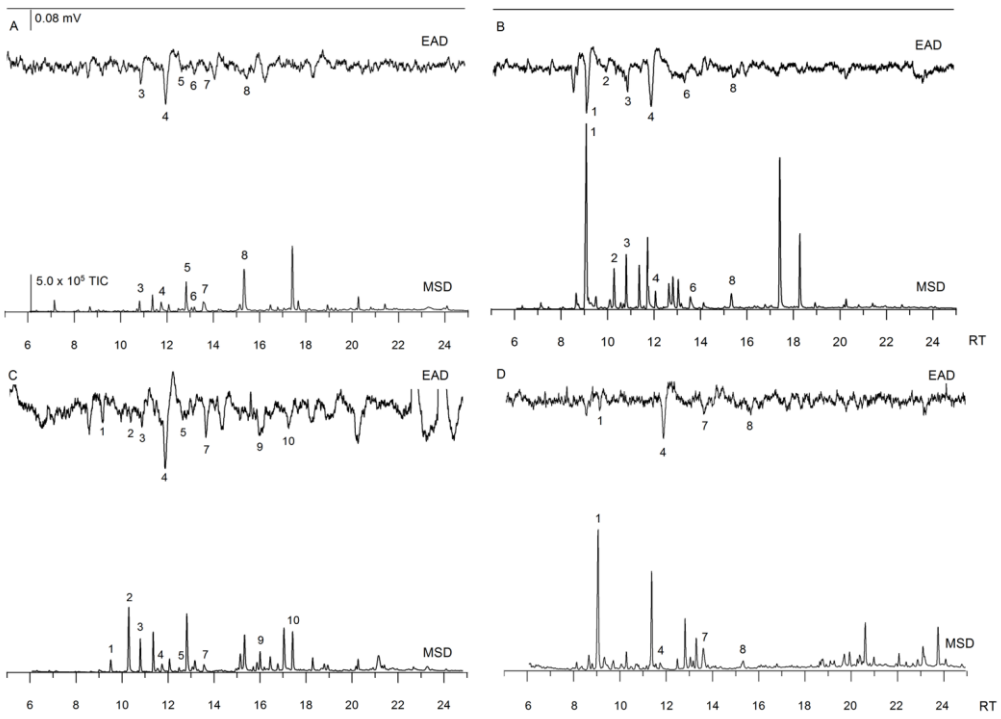

Figure 1. Coupled GC-MS/EAD chromatograms of female *H. robusta* response to volatiles from (A) *E. angolense*, (B) *E. utile*, (C) *K. anthotheca* and (D) *K. ivorensis*. 1: (*Z*)-*β*-ocimene; 2: (*Z*)-3-hexen-1-yl acetate; 3: hexan-1-ol; 4: nonanal; 5: (*Z*)-3-hexen-1-yl butanoate; 6: 2-ethyl hexan-1-ol; 7: decanal; 8: *β*-caryophyllene; 9: (*Z*)-3-hexen-1-yl hexanoate; 10: germacrene D. Upper traces (EAD) are electroantennograms of the responses of female *H. robusta* antennae to corresponding gas-chromatographic profiles denoted by MSD (lower traces). EAD: electroantennographic detector; MSD: mass selective detector; RT: retention time.

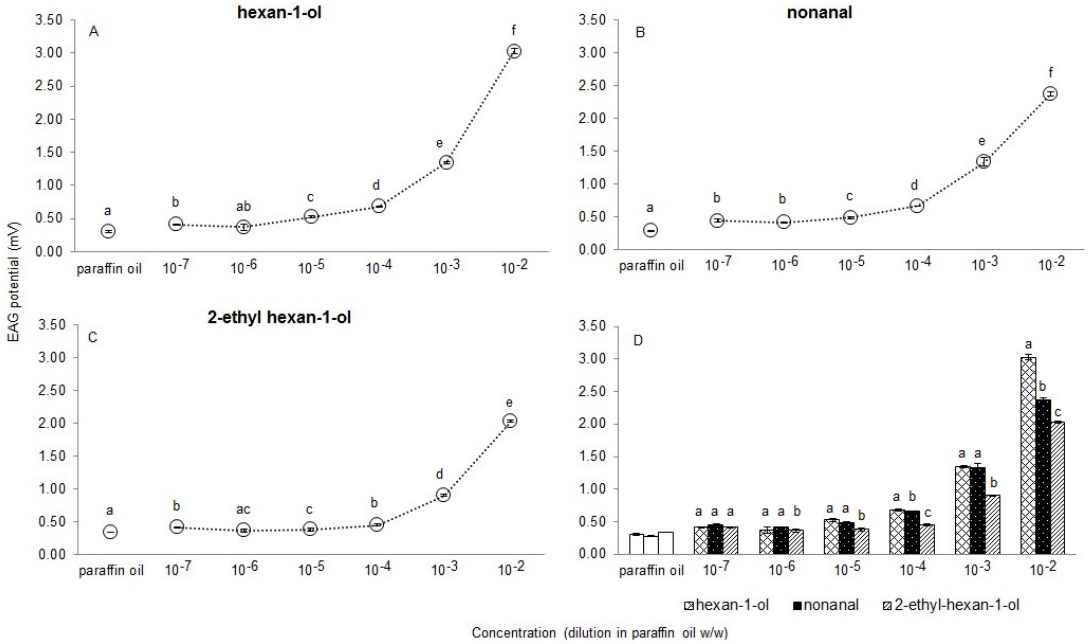

Figure 2. Dose-response curves of *H. robusta* females to paraffin oil and different doses (concentrations 10^{-7} to 10^{-2}) of (A) hexan-1-ol, (B) nonanal, (C) 2-ethyl-hexan-1-ol diluted in paraffin oil w/w. (D): a comparison of the antennal responses (mean ± standard deviation) to hexan-1-ol, nonanal and 2-ethyl-hexan-1-ol at each concentration. Different letters indicate statistical differences in the antennal responses at the respective concentrations based on Mann-Whitney U tests at $p \leq 0.05$. The absolute concentrations of hexan-1-ol, nonanal and 2-ethyl hexan-1-ol in the gas phase based on their vapour pressures of 1.22 mbar, 0.65 mbar and 0.22 mbar respectively at 21 °C and the dilution factor in paraffin oil are the following: hexen-1-ol: 10^{-7} = 122 ppt; 10^{-6} = 1.22 ppb; 10^{-5} = 0.0122 ppm; 10^{-4} = 0.122 ppm; 10^{-3} = 1.22 ppm; 10^{-2} = 12.2 ppm; nonanal: 10^{-7} = 65 ppt; 10^{-6} = 0.65 ppb; 10^{-5} = 6.5 ppb; 10^{-4} = 65 ppb; 10^{-3} = 0.65 ppm; 10^{-2} = 6.5 ppm; 2-ethyl hexan-1-ol: 10^{-7} = 22 ppt; 10^{-6} = 0.22 ppb; 10^{-5} = 2.2 ppb; 10^{-4} = 22 ppb; 10^{-3} = 0.22 ppm; 10^{-2} = 2.2 ppm. (ppt = parts per trillion; ppb = parts per billion; ppm = parts per million).

Table 3. Mann-Whitney U statistics of the antennal responses of female *H. robusta* comparing paraffin oil and hexan-1-ol, nonanal and 2-ethyl hexan-1-ol at concentration levels of 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2}.

Paraffin oil vs.	hexan-1-ol		nonanal		2-ethyl hexan-1-ol	
test compounds 10^{-7} – 10^{-2}	*U*	*p*	*U*	*p*	*U*	*p*
paraffin oil vs. 10^{-7} (0.1 µg/g)	0.00	0.050 *	0.00	0.046 **	0.00	0.046 **
paraffin oil vs. 10^{-6} (1 µg/g)	1.00	0.127, n.s.	0.00	0.043 **	1.00	0.105, n.s.
paraffin oil vs. 10^{-5} (10 µg/g)	0.00	0.050 *	0.00	0.043 **	0.00	0.046 **
paraffin oil vs. 10^{-4} (0.1 mg/g)	0.00	0.050 *	0.00	0.043 **	0.00	0.046 **
paraffin oil vs. 10^{-3} (1 mg/g)	0.00	0.046 **	0.00	0.046 **	0.00	0.046 **
paraffin oil vs. 10^{-2} (10 mg/g)	0.00	0.046 **	0.00	0.046 **	0.00	0.046 **

No statistical difference (n.s.), statistical significance at $p = 0.05$ (*), statistical significance at $p < 0.05$ (**).

Table 4. Mann-Whitney U statistics of the antennal responses of female *H. robusta* comparing hexan-1-ol, nonanal and 2-ethyl hexan-1-ol at each concentration levels.

	10^{-7} (0.1 µg/g)		10^{-6} (1 µg/g)		10^{-5} (10 µg/g)		10^{-4} (0.1 mg/g)		10^{-3} (1 mg/g)		10^{-2} (10 mg/g)	
	U	*p*	*U*	*p*	*U*	*p*	*U*	*p*	*U*	*p*	*U*	*p*
hexan-1-ol vs. nonanal	0.50	0.077 n.s.	1.00	0.105 n.s.	0.50	0.072 n.s.	0.00	0.046 **	4.00	0.817 n.s.	0.00	0.046 **
hexan-1-ol vs. 2-ethyl hexan-1-ol	2.00	0.261 n.s.	3.00	0.513 n.s.	0.00	0.050 *	0.00	0.050 *	0.00	0.046 **	0.00	0.046 **
nonanal vs. 2-ethyl hexan-1-ol	1.50	0.184 n.s.	0.00	0.046 **	0.00	0.046 **	0.00	0.046 **	0.00	0.050 *	0.00	0.050 *

No statistical difference (n.s.), statistical significance at $p = 0.05$ (*), statistical significance at $p < 0.05$ (**).

VOC composition of *Entandrophragma* and *Khaya* mahoganies are similar. The only two differences observed were that (*E*)-2-hexen-1-yl butanoate was not detected in *K. ivorensis* and a significant difference was observed only in the amounts of the sesquiterpenes, *β*-elemene and *δ*-cadinene. The amounts of these two sesquiterpenes were significantly lower in *E. utile* than the other three species. Our previous study showed that *E. utile* is the least susceptible mahogany species to *H. robusta* (Opuni-Frimpong *et al.,* 2008b). It is possible that, the amount of *β*-elemene and *δ*-cadinene in the volatile blends of the mahogany trees may influence the susceptibility of the trees. However, these two compounds did not elicit antennal responses in our experiments.

Many sesquiterpenes are released by undamaged plant material as baseline level of volatile metabolites and are known to play important biological roles in insect-plant interactions (Paré and Tumlinson, 1999; Das *et al*., 2013). In the mahogany species *S. macrophylla*, the sesquiterpenes (*α*-copaene, *β*-elemene, *β*-caryophyllene and germacrene D) were detected as part of the essential oils collected from fresh terminal shoots, mature and senescent leaves (Soares *et al*., 2003). All of these sesquiterpenes were also characterised in all the mahogany species we studied. In fact, most of the sesquiterpenes (*α*-cubebene, *α*-copaene, *β*-elemene, *β*-caryophyllene, *α*-humulene, germacrene D and *δ*-cadinene) we characterised were also previously found in *Cedrela odorata* L., *Cedrela fissilis* Vellozo and *T. ciliata* which also belong to the Meliaceae family (Maia *et al*., 2000). Once again, *α*-cubebene, *α*-copaene, *β*-caryophyllene, *α*-humulene and germacrene D have been characterised in *Guarea macrophylla* Vahl (Lago *et al*., 2006) another member of the Meliaceae family.

It is interesting to note that, out of the 8 sesquiterpenes we characterised in the four mahogany species, 2 showed antennal activity in GC-MS/EAD experiments. These were *β*-caryophyllene and germacrene D. An earlier study indicated that *β*-caryophyllene plays a major role in the attraction of *H. grandella* to *S. macrophylla*; it was suggested that germacrene D could also be an attractant to *H. grandella* although they could not show antennal activity for germacrene D (Soares *et al.*, 2003). In our experiments, we confirmed that both compounds are antennally active and therefore could play important olfactory roles in both *Hypsipyla* species. That *H. grandella* and *H. robusta* could be influenced by the same sesquiterpene compounds is not surprising because these species are closely related. Both *β*-caryophyllene and germacrene D have been found to influence insect-plant interactions in many other ecological systems (Mozuraitis *et al.*, 2002; Rasmann *et al*., 2005; Köllner *et al*., 2008; Ibanez *et al*., 2010; Hare, 2011; Xiao *et al*., 2012).

Six-carbon (C_6) aldehydes, alcohols and their esters are called green leaf volatiles. They are released in high amounts from wounded leaves by autolytic oxidative breakdown of membrane lipids (Paré and Tumlinson, 1999; Matsui *et al*., 2012). In our study, we did not find typical volatiles of damaged leaves such as (*Z*)-3-hexen-1-al and (*Z*)-3-hexen-1-ol but we found the corresponding ester, (*Z*)-3-hexen-1-yl acetate, which is the acety-

lated form of (*Z*)-3-hexen-1-ol. Moreover, 5 other esters [(*Z*)-3-hexen-1-yl butanoate, (*Z*)-3-hexen-1-yl 3-methyl butanoate, (*Z*)-3-hexen-1-yl (*E*)-2-butenoate, (*Z*)-3-hexen-1-yl hexanoate and (*Z*)-3-hexen-1-yl benzoate] were found, derived clearly from (*Z*)-3-hexen-1-ol. The compound, (*E*)-2-hexen-1-yl butanoate, comes from the esterification of (*E*)-2-hexen-1-al which is a result of the isomerization of (*Z*)-3-hexen-1-al (Matsui *et al*., 2012). Therefore, our finding suggests that the sampling method we adopted did not strongly interfere with the volatile profile of the intact mahogany trees. Three esters [(*Z*)-3-hexen-1-yl acetate, (*Z*)-3-hexen-1-yl butanoate and (*Z*)-3-hexen-1-yl hexanoate] showed antennal activity with *H. robusta*. Among these, it has been revealed in previous studies that (*Z*)-3-hexen-1-yl acetate in combination with other green leaf volatiles is attractive to insect pests such as *Cydia molesta* (Busck) (Lepidoptera Tortricidae) (Natale *et al*., 2003) and *Grapholitha molesta* (Busck) (Lepidoptera Tortricidae) (Lu *et al*., 2010) and (Z)-3-hexen-1-yl hexanoate is known to stimulate attraction in the moth, *Cydia pomonella* L. (Lepidoptera Tortricidae) (Yang *et al.*, 2004).

Three alcohols (hexan-1-ol, 1-octen-3-ol and 2-ethyl hexan-1-ol) were detected in all the volatile samples we collected. Among them, hexan-1-ol and 2-ethyl hexan-1-ol were antennally active. Hexan-1-ol has been previously found to be an attractant of *Plutella xylostella* L. (Lepidopetera Plutellidae) (Reddy and Guerrero, 2000). It has also been suggested that hexan-1-ol is involved in the volatile blends used by female insects for orientation towards oviposition sites in many other insect species (Groot *et al*., 1999). Among aldehydes, we detected nonanal and decanal and both were antennally active. Nonanal is known to be an attractant for oviposition in *Epiphyas postvittana* (Walker) (Lepidoptera Tortricidae) (Suckling *et al*., 1996) and decanal has also been found to mediate attraction of the moth *Argyresthia conjugella* (Zeller) (Lepidoptera Yponomeutidae) (Bengtsson *et al*., 2006).

All the previous findings suggest that the antennally active compounds we identified for *H. robusta* could play important roles in the olfactory response of this species to *Entandrophragma* and *Khaya* mahoganies. They may elicit behavioural responses as single compounds as well as in combination (Bruce *et al*., 2005; Bruce and Pickett, 2011). It is well known that antennal responses to volatiles depend on both the sensitivity of olfactory receptors of the insect under study and the concentration of each compound in the air running over the antennae (Visser, 1979; Schütz *et al*., 1996). The response amplitudes of our EAD experiment presented in figure 1 therefore depended on the physiology of each antenna and the concentration of the antennally active compounds in the difference species studied.

The dose-response experiments have revealed that, hexan-1-ol, nonanal and 2-ethyl hexan-1-ol elicit antennal responses already at a low concentration of 10^{-7} (122 ppt, 65 ppt and 22 ppt respectively) and that, these responses were also significantly different from the paraffin oil control (figure 2A-2C and table 3). The concentration of 10^{-7} could therefore be the threshold for females of *H. robusta* to detect the presence of these compounds in mahogany trees. However, in two cases (hexan-1-ol and 2-ethyl hexan-1-ol) no statistical differences were found between the antennal responses to paraffin oil and the test compounds at the concentration of 10^{-6}, therefore a threshold concentration of 10^{-5} could be established for those two compounds. Our study also showed that, the females of *H. robusta* react differently to the three test compounds only at a relatively high concentration (figure 2D and table 4), hexan-1-ol being the most perceived.

Conclusion

To the best of our knowledge, this study presents the first record of VOCs produced by the shoots of *E. angolense*, *E. utile*, *K. anthotheca* and *K. ivorensis*. Altogether 29 compounds were characterised in all four species with the exception of (*E*)-2-hexen-1-yl butanoate which was not present in *K. ivorensis*. We have also showed for the first time that (*Z*)-*β*-ocimene, (*Z*)-3-hexen-1-yl acetate, hexan-1-ol, nonanal, (*Z*)-3-hexen-1-yl butanoate, 2-ethyl-hexan-1-ol, decanal, *β*-caryophyllene, (*Z*)-3-hexen-1-yl hexanoate and germacrene D are electrophysiologically active compounds to *H. robusta*. These compounds could be important olfactory cues for *H. robusta* and may be involved in its host recognition. Of the ten antennally active compounds, (*Z*)-3-hexen-1-yl acetate, hexan-1-ol, nonanal, decanal, *β*-caryophyllene, (*Z*)-3-hexen-1-yl hexanoate and germacrene D are already known as attractants of some other moth species to their host plants. Our findings contribute to the search for a reason for the discrimination between mahogany species by *H. robusta* and breaks the ground for further studies in this direction.

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