Phytochemistry 70 (2009) 751–758

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Repellent activity of alligator pepper, *Aframomum melegueta*, and ginger, *Zingiber officinale*, against the maize weevil, *Sitophilus zeamais*

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ARTICLE INFO

Article history:
Received 16 January 2009
Received in revised form 9 March 2009
Available online 22 April 2009

Keywords:
Aframomum melegueta
Zingiber officinale
Repellent
Sitophilus zeamais

ABSTRACT

The repellent activity of alligator pepper, *Aframomum melegueta*, and ginger, *Zingiber officinale* (Zingiberaceae), against the maize weevil, *Sitophilus zeamais* (Coleoptera: Curculionidae), was investigated in four-way olfactometer bioassays. Results showed that vacuum distilled *A. melegueta* and *Z. officinale* extracts were repellent towards adult *S. zeamais* both in the absence and the presence of maize, *Zea mays*, grains. Bioassay-guided liquid chromatographic fractionation of the distillates showed that fractions containing oxygenated compounds accounted for the repellent activity. Coupled gas chromatography–mass spectrometry (GC–MS), followed by GC peak enhancement and enantioselective GC using authentic compounds, identified 3 major compounds in the behaviourally active fractions of *A. melegueta* and *Z. officinale* to be (*S*)-2-heptanol, (*S*)-2-heptyl acetate and (*R*)-linalool in a ratio of 1:6:3, and 1,8-cineole, neral and geranial in a ratio of 5.48:1:2.13, respectively. The identification of these behaviourally active compounds provides the scientific basis for the observed repellent properties of *A. melegueta* and *Z. officinale*, and demonstrates the potential for their use in stored-product protection at the small-scale farmer level in Africa.

1. Introduction

The Zingiberaceae is a tropical monocotyledonous family comprising several species, of which many are known to produce essential oils, mainly in their seeds and rhizomes (Mabberley, 1987). Zingiberaceae species grow in damp, shaded parts of the lowland or on hill slopes, as scattered plants or thickets in West Africa, Asia and South America. The most widely distributed and commercially important of these species is *Aframomum melegueta* (Roscoe) K. Schum, a perennial herb which grows to about 1 m high. *A. melegueta* produces reddish-brown seeds, commonly referred to as alligator pepper and grains of paradise, which have a strong aromatic flavour and a pungent taste. These seeds are widely employed as spices and are also ingredients in numerous West African ethnomedical practices, as a remedy for a number of diseases such as constipation, rheumatic pains and fever (Ajaiyeoba and Ekundayo, 1999; Fernandez et al., 2006). A number of significant biological activities for *A. melegueta*, in particular anti-inflammatory, antioxidant and antitumour effects, have been reported elsewhere (Tjendraputra et al., 2001; Chung et al., 2001). Oloke and Kolawale (1988) studied the biological activities, including anti-microbial activity, of Nigerian-grown *A. melegueta* essential oil obtained by steam distillation, against a number of micro-organisms.

Ginger is the rhizome of *Zingiber officinale* Roscoe (Zingiberaceae), a herbaceous perennial species native of tropical Asia, where it is rarely found in the wild today. It is cultivated in most tropical countries, e.g. Australia, Brazil, China, Japan, Mexico, West Africa, the West Indies and parts of the United States. The taste and pungency of the harvested rhizome increase with growth and maturity. The completely unscraped West African variety is reported to have the highest essential oil content and the most pungent flavour (Langner et al., 1998; Singh et al., 2005). *Z. officinale* and its constituents have been reported to exhibit a wide range of pharmacological activities, e.g. antibacterial (Yamada et al., 1992), antioxidant (Jitoe et al., 1992; Kikuzaki and Nakatani, 1993), analgesic, anti-inflammatory, carminative, diuretic and stimulatory (Tanabe et al., 1993; Langner et al., 1998), and antifungal properties (Singh et al., 2005) attributed to its pungent principles (Yamahara et al., 1989). For centuries, *Z. officinale* has been known in Asia, Africa and other folk medicines as a most effective remedy for rheumatic diseases, respiratory diseases, loss of appetite, vomiting, nausea, and convulsion in children (Langner et al., 1998; Ali et al., 2008).

The protection of stored products against attack by pests is essential in many countries, particularly those suffering from

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doi:10.1016/j.phytochem.2009.03.012

inadequate storage facilities. Novel, environmentally compatible stored-product control agents are urgently needed to replace synthetic pesticides that are either not available for economic or regulatory reasons, or are ineffective, due to the increasing difficulty of managing pesticide resistance (Duke et al., 2003). Plant extracts and essential oils have potential for use in crop protection. They contain monoterpenoids, diterpenoids, sesquiterpenoids and other compounds that show ovicidal, larvicidal, repellent, deterrent, antifeedant and toxic effects in a wide range of insects (Pungitore et al., 2003; Liu and Ho, 1999; Isman, 2000, 2006). *A. melegueta* and *Z. officinale* extracts have not traditionally been used in stored-product protection against pests in Africa, but are available locally, and their volatile chemical constituents are known to be effective against other insect pests (Escoubas et al., 1995; Agarwal et al., 2001). Using the maize weevil, *Sitophilus zeamais* (Coleoptera: Curculionidae), which is a major pest of stored maize, wheat and rice in tropical and sub-tropical countries, as the target insect species, preliminary laboratory olfactometry bioassays showed that volatiles from *A. melegueta*, *Z. officinale* and *Piper guineense* were not only directly repellent, but also interfered with the attractiveness of maize, *Zea mays* (Ukeh, unpublished data). Thus, the aim of this study was to identify the volatile components of *A. melegueta* and *Z. officinale* responsible for repellency of *S. zeamais*, using bioassay-guided fractionation (Birkett et al., 2008) and coupled gas chromatography–mass spectrometry (GC–MS). Although steam distillation is normally used to obtain essential oil samples from aromatic plants, it was decided here to use the superior technique of vacuum distillation in order to minimise loss of thermally labile components that could potentially be exploited in pest control by direct release from crushed seed or rhizome material. Identification of repellent components from *A. melegueta* and *Z. officinale* would provide underpinning science for development of new, semiochemical-based strategies for *S. zeamais* control in storage conditions, particularly for use in small-holder farms.

2. Results

2.1. Olfactometer assays with A. melegueta and Z. officinale vacuum distillates

The vacuum distillate of *A. melegueta* showed significant repellency to both male and female *S. zeamais* weevils in the mean time spent in the test arm when tested in the absence of *Z. mays* when compared with the control arms (Fig. 1A; $P < 0.001$). In the presence of *Z. mays* grains, *A. melegueta* distillate was still repellent to the males ($P < 0.001$) and females (Fig. 1A; $P < 0.001$) when compared with the control arms. In the mean number of visits, both males ($\chi^2 = 14.53$, df = 3, $P = 0.002$) and females ($\chi^2 = 13.2$, df = 3, $P = 0.004$) significantly preferred control arms to *A. melegueta*. With *Z. mays*, males ($\chi^2 = 7.62$, df = 3, $P = 0.05$) and females ($\chi^2 = 11.68$, df = 3, $P = 0.009$) still showed preference for the control arms compared to *A. melegueta* in the mean number of visits (Fig. 1B).

For *Z. officinale,* the vacuum distillate showed significant ($P < 0.001$) repellent activity to both males and females when tested in the absence of *Z. mays*, and to males ($P < 0.001$) and females ($P < 0.001$) in combination with *Z. mays*, in the mean time spent in the arms (Fig. 2A). *Z. officinale* distillate also significantly reduced the mean number of visits by males ($\chi^2 = 12.5$, df = 3, $P = 0.006$) and females ($\chi^2 = 12.99$, df = 3, $P = 0.005$) to the test arm when tested alone, and males ($\chi^2 = 12.93$, df = 3, $P = 0.005$), females ($\chi^2 = 8.48$, df = 3, $P = 0.037$) in combination with *Z. mays* (Fig. 2B).

For fractions of *A. melegueta* and *Z. officinale* distillates, prepared by liquid chromatography over Florisil®, both male and female *S. zeamais* did not respond significantly to the hexane fractions in the mean time spent in the test arm (Fig. 1C, 2C) and in the mean number of visits (Fig. 1D, 2D) compared to the control arms. In contrast, the diethyl ether fractions of *A. melegueta* and *Z. officinale* vacuum distillates significantly influenced the behaviour of *S. zeamais*. In the mean time spent, males ($P = 0.006$) and females ($P = 0.002$) were significantly repelled by *A. melegueta* (Fig. 1C) when compared to control arms. Both sexes significantly preferred control arms to the test arm in the mean number of visits (Fig. 1D). With *Z. officinale*, males ($P = 0.005$), and females ($P = 0.004$) respectively were significantly repelled by the test arm when compared to the control arm in the mean time spent (Fig. 2C). In contrast, in the mean number of visits, behavioural responses from the females were not statistically different from the controls (Fig. 2D).

2.2. Chemical analysis of A. melegueta and Z. officinale vacuum distillates

GC–MS analysis of the vacuum distillates from *Z. officinale* (Table 1; Fig. 3A) and *A. melegueta* (Table 1; Fig. 3B) revealed the presence of 24 and 13 compounds respectively. GC–MS analysis of the behaviourally active Florisil® diethyl ether fractions of *A. melegueta* and *Z. officinale* vacuum distillates showed the presence of 3 major compounds from each extract. Compounds identified from *A. melegueta* were 2-heptanol, 2-heptyl acetate and linalool in a ratio of 1:6:3. GC Peak enhancement using enantioselective GC confirmed the stereochemistry of the chiral compounds from *A. melegueta* as (*S*)-2-heptanol, (*S*)-2-heptyl acetate and (*R*)-linalool. From *Z. officinale*, 1,8-cineole, neral and geranial were present in a ratio of 5.48:1:2.13.

2.3. Repellent activity of synthetic blends

The synthetic blends of the major components found in the behaviourally active *A. melegueta* and *Z. officinale* diethyl ether fractions prepared in their natural ratios, showed significant repellent activity against *S. zeamais*. In the mean time spent, both males and females were significantly repelled ($P < 0.001$) by *A. melegueta* synthetic blend tested individually (Fig. 1E), and males ($P = 0.002$), and females ($P = 0.022$) were significantly repelled by *A. melegueta* synthetic blend in combination with *Z. mays* when compared with the control arms (Fig. 1E). *S. zeamais* were also significantly repelled by *A. melegueta* synthetic blend in the mean number of visits to the test arm, when compared to the control arms (Fig. 1F). *Z. officinale* synthetic blend also elicited significant repellent activity ($P < 0.001$) against males and females when tested alone, and against males ($P < 0.001$), and females ($P = 0.008$) in combination with *Z. mays* in the mean time spent when compared with the control arms (Fig. 2E). Both sexes were significantly repelled by *Z. officinale* synthetic blend when tested alone, but only the females were repelled by the blend when tested in combination with *Z. mays* in the number of visits when compared to the control arms ($\chi^2 = 12.89$, df = 3, $P = 0.005$; Fig. 2F).

3. Discussion

This study showed that the volatile chemical components of *A. melegueta* and *Z. officinale,* isolated as vacuum distillates, consistently elicited repellent activity against adult *S. zeamais,* when tested individually and in combination with *Z. mays*. The results also suggest that the compounds responsible for the repellent activity of the vacuum distillates were present in the diethyl ether fractions isolated by liquid chromatography. For *A. melegueta,* the repellent activity was accounted for by a synthetic blend of (*S*)-2-heptanol, (*S*)-2-heptyl acetate and (*R*)-linalool, and for *Z. officinale*, by a synthetic blend of 1,8-cineole, neral and geranial. To

our knowledge, this is the first time (*S*)-2-heptyl acetate has been identified from *A. melegueta* seeds. The chemical composition of essential oils from the same plant or plant part could be due to the environment, developmental, genetic or some other factors. The yield and chemical composition of oils could also differ widely with the production technique, variety, cultivars or population,

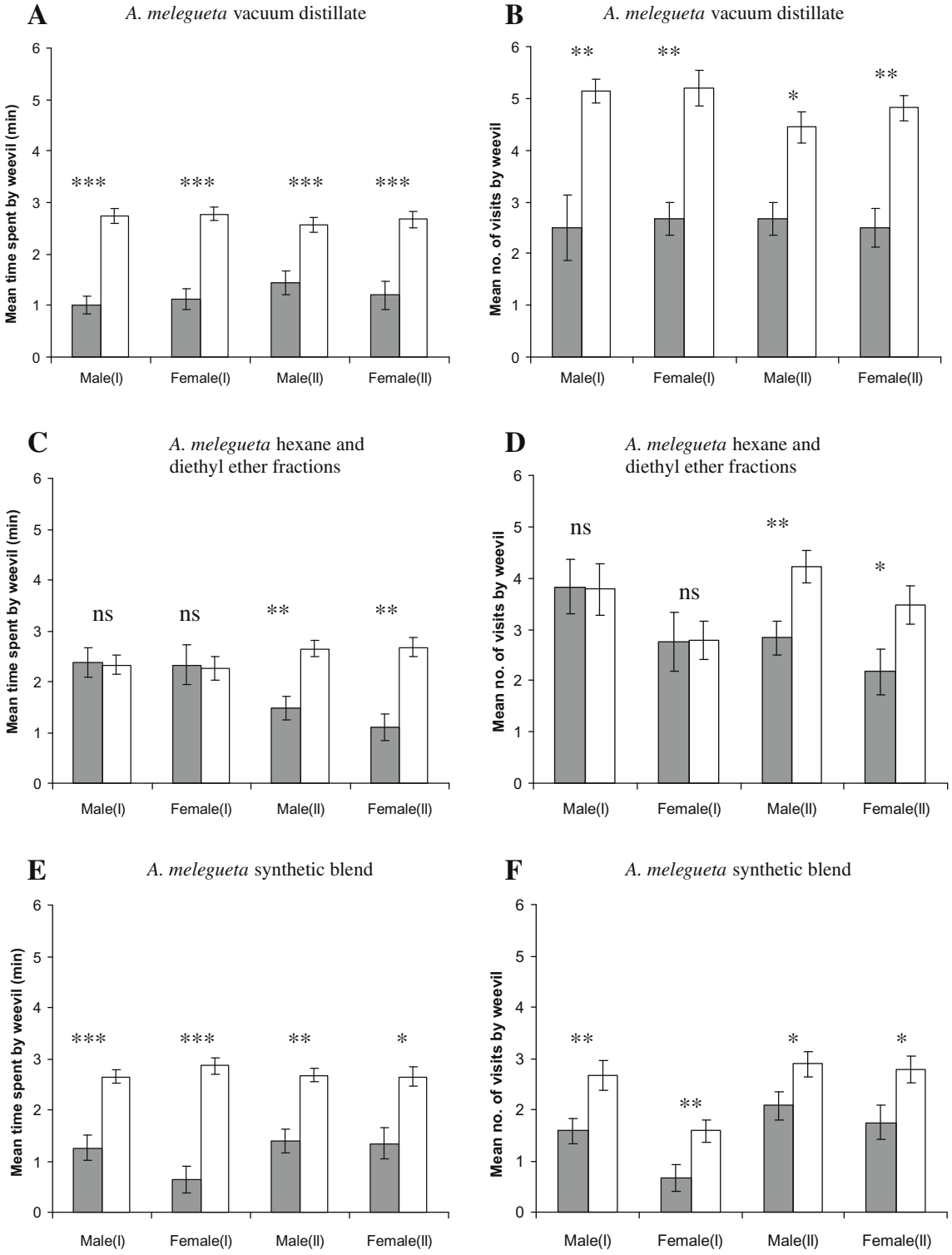

Fig. 1. Four-way olfactometer assay results with *Aframomum melegueta*. (A) Mean time spent in control and treatment arms by male and female *Sitophilus zeamais* in response to vacuum distillate (I), and in combination with maize, *Z. mays*, grains (II), (B) mean number of visits made by male and female *S. zeamais* in response to vacuum distillate, (C) mean time spent by *S. zeamais* in response to hexane (I) and diethyl ether (II) fractions, (D) mean number of visits made by *S. zeamais* in response to hexane (I) and diethyl ether (II) fractions, (E) mean time spent by *S. zeamais* in response to synthetic blend (I), and synthetic blend in combination with *Z. mays* grains (II) and (F) mean number of visits made by *S. zeamais* in response to synthetic blend (I), and synthetic blend in combination with *Z. mays* grains (II). Results are displayed as the mean ± SE of the 3 control arms, and the test arm. Statistical analysis was carried out as described in Section 4.6 ($P < 0.05$ = *, $P < 0.01$ = **, $P < 0.001$ = ***). Bars = standard errors of the means. $n = 12$ in all bioassays. ▩ = treatment; □ = control.

climatic and soil factors (Bailer et al., 2001; Singh et al., 2008). Thus, more work is needed to establish the generality of the present findings. Furthermore, further studies are required to examine the behaviour of mated males and females in response to the identified compounds.

The toxicity, fumigant and repellent effects of some of the volatile constituents found in this study have been demonstrated by other researchers. Five monoterpenoids, namely terpinen-4-ol, 1,8-cineole, linalool, *R*-(+)-limonene and geraniol have been reported to elicit direct toxicity and fumigant activity against

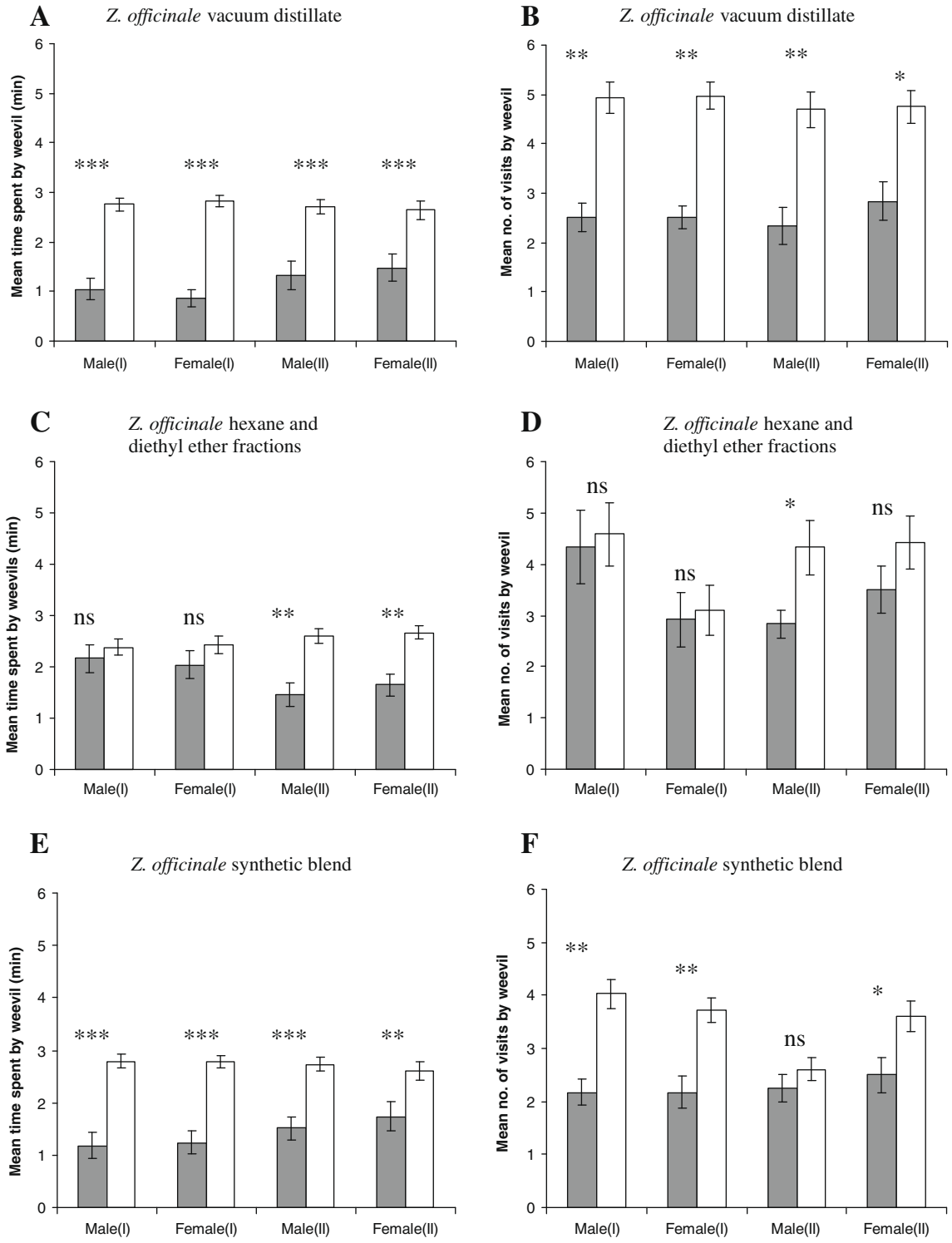

Fig. 2. Four-way olfactometer assay results with *Zingiber officinale*. (A) Mean time spent in control and treatment arms by male and female *Sitophilus zeamais* in response to vacuum distillate tested individually (I) and in combination with maize, *Z. mays*, grains (II), (B) mean number of visits made by male and female *S. zeamais* in response to vacuum distillate, (C) mean time spent by *S. zeamais* in response to hexane fraction (I) and diethyl ether (II) fractions of vacuum distillate, (D) mean number of visits made by *S. zeamais* in response to hexane (I) and diethyl ether (II) fractions, (E) mean time spent by *S. zeamais* in response to synthetic blend tested individually (I), and in combination with *Z. mays* grains (II), (F) mean number of visits made by *S. zeamais* in response to *A. melegueta* synthetic blend (I), and synthetic blend in combination with *Z. mays* grains (II). Results are displayed as the mean ± SE of the 3 control arms, and the test arm. Statistical analysis was carried out as described in Section 4.6 ($P < 0.05$ = *, $P < 0.01$ = **, $P < 0.001$ = ***). Bars = standard errors of the means. $n = 12$ in all bioassays. ▩ = treatment; □ = control.

Table 1
Compounds identified from coupled GC–MS analysis of vacuum distilled essential oils obtained from the rhizome of *Zingiber officinale* and *Aframomum melegueta* seeds.[a]

Peak no.	*Zingiber officinale*		*Aframomum melegueta*	
	Compound	% Peak area by GC	Compound	% Peak area by GC
1	Tricyclene	0.33	(*S*)-2-Heptanol[b]	2.78
2	α-Pinene	6.39	α-Pinene	0.19
3	Camphene	16.87	β-Pinene	0.64
4	6-Methyl-5-hepten-2-one	0.05	Myrcene	0.21
5	Sabinene	0.84	(*S*)-2-Heptyl acetate[b]	16.22
6	β-Pinene	0.25	(*E*)-Ocimene	23.22
7	Myrcene	3.2	Linalool oxide (furan)	0.59
8	3-Carene	0.71	(*R*)-Linalool[b]	8.68
9	1,8-Cineole[b]	22.63	(*E*)-4,8-Dimethyl-1,3,7-nonatriene	0.32
10	Terpinolene	0.66	No i.d.	1.18
11	(*R*)-Linalool	0.83	β-Caryophyllene	19.17
12	Borneol	0.63	Humulene	26.23
13	α-Terpineol	0.51	Germacrene-D	0.06
14	Neral[b]	5.03	Bisabolene	0.07
15	Geranial[b]	11.25		
16	Borneol acetate	0.15		
17	Cyclosativene	0.2		
18	Copaene	0.39		
19	α-Curcumene	1		
20	α-Muurolene	1.1		
21	Zingiberene	10.63		
22	(*E*,*E*)-α-Farnesene	3.69		
23	γ-Cadinene	3.21		
24	β-Cadinene	3.38		

[a] Peak nos. correlate to GC traces in Fig. 3.
[b] Compounds found in behaviourally active diethyl ether fractions and used in synthetic blends, following peak enhancement using GC and enantioselective GC.

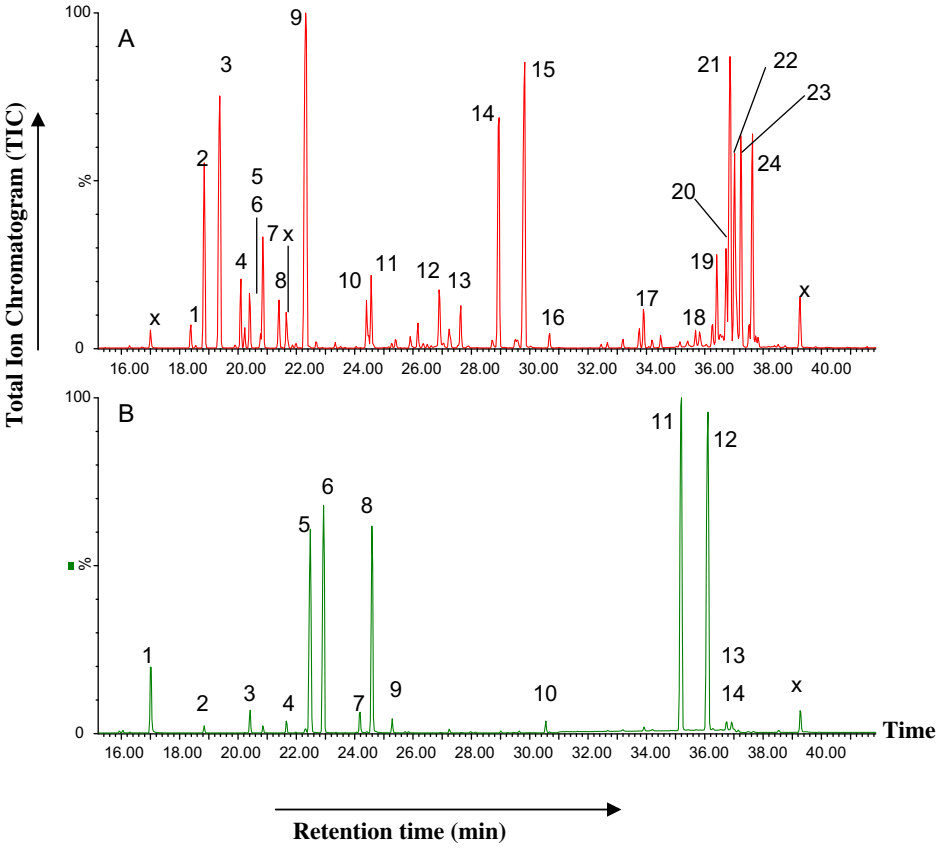

Fig. 3. Total ion chromatogram (TIC) obtained by coupled GC–MS analysis of vacuum distillates of: (A) *Zingiber officinale* and (B) *Aframomum melegueta*. Peak numbers correlate to compounds listed in Table 1. *X* = impurities (6.01% by GC for *Z. officinale*, 0.44% by GC for *A. melegueta*).

3-day-old eggs, third-instar larvae and pupae of the confused flour beetle, *Tribolium confusum* (Stamopoulos et al., 2007). Obeng-Ofori et al (1997) found 1,8-cineole to be highly repellent and toxic to *S. zeamais*, the granary weevil *S. granarius*, red flour beetle *T. castaneum* and larger grain borer *Prostephanus truncatus*. (*R*)-Linalool was identified in this study as a component of *A. melegueta* repellent

synthetic blend. Linalool is known to act as a reversible competitive inhibitor of acetylcholinesterase (Ryan and Byne, 1988). (*R*)-Linalool is a volatile oxygenated monoterpene compound widely found in higher plants, and repellent activity of this compound has been reported for a variety of insect species (e.g. Mauchline et al., 2008).

The results in this study show that multi-component blends found in *A. melegueta* and *Z. officinale* vacuum distillates cause repellency of *S. zeamais*. Single compounds were also tested, but were shown to be not significantly repellent (data not shown). Thus, the implication is that the blends, rather than specific components, could constitute control agents with sufficient broad spectrum of bioactivity to be deployed in stored-product pest control. The effectiveness of the blends is in agreement with Ndungu et al. (1995), who reported that the repellent action of the essential oil of the shrub, *Cleome monophylla* (Capparidaceae), against *S. zeamais* is an additive effect of the component compounds. Also, Bekele and Hassanali (2001) reported blend effects as responsible for bioactivity of the essential oil constituents of *Ocimum kilimandscharicum* and *O. kenyense* (Labiateae) against *S. zeamais* and the lesser grain borer, *Rhyzopertha dominica,* in Kenya. Tapondjou et al. (2005) attributed the repellent effects of crude oil extracts of *Cupressus sempervirens* (Cupressaceae) and *Eucalyptus saligna* (Myrtaceae) from the Western highlands of Cameroon against *S. zeamais* and *R. dominica* to an enhancing effect of some other minor constituents of the essential oils. Indeed, there is accumulating research evidence of the adaptive value of phytochemical diversity in ecological interactions among plants and their associated herbivores (Cates, 1996). Secondary plant metabolites are therefore recognised as important components of plant defence system against herbivores and pathogens, as well as shaping the diet of herbivores. Terpenoids are one of the most widespread and important classes of secondary metabolites, and can exert toxic, deterrent, antifeedant and repellent effects on insect herbivores. They are the dominant components of many natural volatile blends, and are responsible for many of the characteristic smells of plant oils, resins, fruits and flowers (Paré and Tumlinson, 1999). Terpenoid chemistry may vary among plants due to many factors which may include environmental and genetic influences (Langenheim, 1994; Wang and Lincoln, 2004). From an ecological and evolutionary perspective, *S. zeamais* has adapted to feed upon or colonise host plants, *i.e.* grasses, that produce and emit low levels of volatile secondary metabolites, and to avoid plants that produce high levels of such compounds, *e.g. A. melegueta* and *Z. officinale*. Detection of volatile compounds at a distance from these unsuitable host plants may serve as a mechanism by which *S. zeamais* is able to avoid plants that are unsuitable for feeding and colonisation (Pickett et al., 1998), *i.e.* avoidance of non-volatile toxic/antifeedant compounds (Escoubas et al., 1995; Agarwal et al., 2001).

The results obtained in the present study are encouraging, given the on-going global search for environmentally safe and non-toxic natural products for the protection of stored grains, and stress the importance of analysing plant oils or components in blends to elucidate their full potency for a given bioactivity. Furthermore, knowledge of underpinning science provides chemical markers for quality assurance. *A. melegueta* seed and *Z. officinale* rhizome material have the potential to be developed for use against *S. zeamais* infestation of stored maize at the small-holder farmer level, especially in the developing world. A major advantage of *A. melegueta* and *Z. officinale* materials is that they are not considered dangerous to human health and the environment (Isman, 2006), since the plants are edible and often incorporated as spices in the diet. Further studies are underway to evaluate the impact of *A. melegueta* seed and *Z. officinale* materials against *S. zeamais* under field storage conditions.

4. Materials and methods

4.1. Plant material collection

Matured rhizomes of *Z. officinale* and ripe fruits of *A. melegueta* were collected from fields around Akamkpa, while local yellow seeds were purchased from Akim foodstuff market in Calabar, all in Cross River State (situated between latitude 5°00′ and 5°15′ North and longitude 8°04′ and 8°25′ East) in southern Nigeria in December 2005. The identity of the repellent plant materials was confirmed in the Department of Crop Science, University of Calabar, Nigeria. The plant materials were dried in the shade for 3 days before transportation. In the laboratory, the repellent plant materials and maize seeds were preserved in the freezer at −20 °C until needed for experiments.

4.2. Insect culture

Sitophilus zeamais was obtained from stock culture maintained by Central Science Laboratory, Sand Hutton, York, United Kingdom and reared on untreated yellow *Z. mays* seeds, in a constant temperature and humidity room maintained at 25 °C, 65% relative humidity on a 12:12 darkness and light photoperiod until required.

4.3. Preparation of *A. melegueta* seed and *Z. officinale* rhizome vacuum distillates

A. melegueta seeds and partially dried *Z. officinale* rhizomes (both *ca.* 50 g) were ground/chopped into small pieces respectively, and extracted with distilled diethyl ether (50 ml) in an ultra-sonic bath at room temperature for 5 min. The contents were transferred to a round bottomed flask connected to a vacuum distillation apparatus equipped with a high vacuum pump (ES50 Vacuum Pump, Edwards, England), and distilled under a vacuum of <0.05 mm Hg for 24 h (Pickett and Griffiths, 1980). The distillates were removed, dried using anhydrous magnesium sulphate ($MgSO_4$), filtered and concentrated under a gentle stream of nitrogen to 4 ml for *A. melegueta* and 3 ml for *Z. officinale*. The vacuum distillates were stored in the freezer at −20 °C until needed for laboratory assays, liquid chromatography or chemical analysis.

4.4. Liquid column chromatography

Aliquots of the *A. melegueta* and *Z. officinale* vacuum distillates (1 ml) were concentrated carefully under a gentle stream of nitrogen to dryness, and immediately re-dissolved in distilled hexane (50 μl). The re-constituted extracts were subjected to small-scale liquid chromatography through Florisil® (100–200 mesh), as described in Birkett et al., 2008. Distilled hexane and diethyl ether were used sequentially as eluants, to obtain fractions containing non-polar and polar compounds, respectively. Samples were concentrated to their original volume (i.e. 1 ml) prior to use in behavioural assays.

4.5. Insect behaviour bioassays

All bioassays were carried out using 3-day-old virgin adult *S. zeamais* in a four-way airflow olfactometer as described in Pettersson (1970). The olfactometer consisted of three layers of 6 mm thick transparent Perspex screwed together forming a four-pointed star-shaped exposure chamber with four extended glass socket arms to which stimuli could be introduced. Air was drawn towards the centre of the olfactometer simultaneously from each arm at a rate of 200 ml min^{-1}, and drawn out at the rate of 800 ml min^{-1}. One arm of the olfactometer was used as a treatment arm and

the other three arms were used as control arms. One weevil was placed in the centre of the olfactometer, observed visually and its position recorded for 10 min. Each bioassay was replicated 12 times using a fresh insect, stimulus source and fresh olfactometer. Odour sources (10 μl) were tested for repellent activity against adult male and female *S. zeamais*, both in the absence and presence of *Z. mays* grains (2 g). Test odours comprised of: (i) *A. melegueta* and *Z. officinale* vacuum distillates, (ii) hexane and diethyl ether fractions of each vacuum distillate, prepared by liquid chromatography and (iii) synthetic blends of the components found in the behaviourally active diethyl ether fractions of the distillates. For *A. melegueta*, this comprised *(S)*-2-heptanol (0.1 mg/ml), *(S)*-2-heptyl acetate (0.6 mg/ml) and *(R)*-linalool (0.3 mg/ml) in a ratio of 1:6:3. For *Z. officinale*, this comprised 1,8-cineole (0.548 mg/ml), neral (0.1 mg/ml) and geranial (0.213 mg/ml) in a ratio of 5.48:1:2.13. In each bioassay, distilled solvent (10 μl) was used as control. The data recorded included (i) the time spent by the insect in the different areas of the olfactometer and (ii) the number of entries or visits into each area or odour zone.

4.6. Data analysis

The time spent in each olfactometer arm was tested using a one-way analysis of variance (ANOVA) followed by comparison of means by Tukey's 95% simultaneous confidence intervals (MINITAB 15 Statistical Software). For data on the number of visits, the null hypothesis was that weevils behave randomly and choose each olfactometer arm with a 25% frequency. The number of visits to the odour-treated arm was compared with the number of visits in control arms using a "global" χ^2 contingency table (Zar, 1999). Upon rejection of the hypothesis, data were analysed by targeted pairwise comparisons using a 2×2 χ^2 contingency table (Zar 1999).

4.7. Gas chromatography (GC) analysis of vacuum distillates and fractions

The chemical components of vacuum distillates of *A. melegueta* and *Z. officinale*, and behaviourally active chromatography fractions, were analysed using a 6890N gas chromatograph (GC) (Agilent Technologies) equipped with a split–splitless injector (230 °C) and flame ionization detector (FID). Hydrogen was the carrier gas. The GC was equipped with a HP-5 capillary column (30 m × 0.3 mm i.d., 0.25 μm film thickness). The oven temperature programme comprised of an initial temperature of 30 °C for 0.5 min, a rise to 150 °C at 5 °C/min, a hold at 150 °C (0.1 min), another rise to 250 °C at 10 °C/min and final hold at 250 °C for 45 min. Results were obtained with an enhanced integrator (HP Chemstation). The concentration of components in behaviourally active chromatography fractions was determined by using a multiple point external method.

The stereochemistry of identified chiral compounds was determined by enantioselective gas chromatography (GC) using authentic compounds using techniques similar to those described in Birkett et al. (2008). Briefly, enantioselective GC of behaviourally active *A. melegueta* and *Z. officinale* Florisil® diethyl ether fractions was performed on a 5890A GC equipped with a ß-cyclodextrin (Supelco beta-DEX™ 120; 30 m × 0.25 mm i.d., film thickness 0.25 μm) capillary column. Hydrogen was the carrier gas. The oven temperature programme comprised of an initial temperature of 40 °C for 1 min, then programmed to rise at 3 °C/min to 150 °C, and at 5 °C/min to 180 °C, and maintained at 180 °C for 15 min.

4.8. Coupled gas chromatography–mass spectrometry (GC–MS)

GC–MS analysis of *A. melegueta* and *Z. officinale* vacuum distillates, and of *A. melegueta* and *Z. officinale* diethyl ether fractions, was performed using a fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm, DB-5), attached to a cool on-column injector, which was directly coupled to a magnetic sector mass spectrometer (Autospec Ultima, Fisons Instruments, Manchester, UK). Ionization was by electron impact (70 eV, source temperature 250 °C). Helium was the carrier gas. The oven temperature was maintained at 30 °C for 5 min, and then programmed at 5 °C/min to 250 °C. Tentative identifications were made by comparison of spectra with mass spectral databases (NIST, 2005), and confirmed by peak enhancement on GC using authentic compounds (Pickett, 1990).

4.9. Chemicals

(*S*)- and (*R*)-2-Heptanol (>99%), hexane and diethyl ether, were purchased from Sigma–Aldrich (Gillingham, Kent, UK). (*S*)-2-Heptyl acetate (>99%) was synthesized from (*S*)-2-heptanol by acetylation using acetic anhydride in dry pyridine. (*R*)- and (*S*)-Linalool (99%) were acquired from Botanix Ltd. (Paddock Wood, Kent, UK). Citral (neral + geranial) (>95%) and 1,8-cineole (>99%) were purchased from Fluka (Germany).

Acknowledgements

This work was funded by Commonwealth Scholarship Commission in the UK to Donald Ukeh for PhD studies at the University of Aberdeen, under the Commonwealth Academic Scholarship and Fellowship Plan. The research was carried out in collaboration with the Biological Chemistry Department, Rothamsted Research, Harpenden, UK. The authors are grateful to Rod Weaver and Maureen Wakefield of Central Science Laboratory, Sand Hutton, York, United Kingdom for the supply of *Sitophilus zeamais*. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the United Kingdom.

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