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Behavioural Responses of *Tribolium castaneum* (Herbst) to Volatiles Identified from Dry Cocoa Beans

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Abstract: New hybrid types of cocoa beans are attractive to insects in storage, however some of the insects feed little, if at all, on these beans compared to those of the traditional type (mixed genotypes). Based on a sniffing test using GCMS, differences in flavour volatiles in these types of beans have been determined and from these, six major volatiles of cocoa beans were selected for olfactometric analysis using a Pettersson olfactometer to determine which of them contributed to the attraction of *Tribolium castaneum* (Herbst) to the cocoa beans. The behaviour of *Tribolium* was affected by dose of 2-phenyl ethanol, acetophenone, 3-methyl butyraldehyde, ethyl butyrate, ethyl 3-hydroxybutyrate and butyl 2-methacrylate. Compared to beans of the new hybrid varieties, beans of the traditional type cocoa contained less 3-methylbutyraldehyde but more ethyl butyrate and acetophenone. In future breeding programmes, reducing the amount of acetophenone and ethyl butyrate but increasing the amount of 3-methylbutyraldehyde in cocoa beans may deter *Tribolium* from feeding on beans in storage.

Key words: Cocoa beans, *Tribolium*, 2-phenyl ethanol, acetophenone, 3-methyl butyraldehyde, ethyl butyrate, ethyl 3-hydroxybutyrate and butyl 2-methacrylate

INTRODUCTION

Stored products such as cocoa beans are vulnerable to infestation by numerous species of insects and mites, many of which can cause serious nutritional damage and economic loss. Early detection is essential, yet these pests of stored products tend to be elusive and their presence is often not obvious until the infestation has become well established (Stubbs *et al*., 1985). Identification of volatiles emitted by cocoa beans that attract these pests could lead to the development of a lure in a monitoring system.

Alternative hybrids were developed to augment the Series II hybrids (Adu-Ampomah and Sersah, 1988) being grown by farmers. The increasing production of the alternative hybrids over the years has shown that their yields are relatively higher than most of the traditional Amelonado and Local Trinitario cocoa (Jonfia-Essien, 2006). In Ghana, dry cocoa beans in storage were monitored for insect pests from 1995 to 2000 and *Lasioderma serricorne*, *Tribolium castaneum*, *Cadra cautella* (*Ephestia cautella*) and *Corcyra cephalonica* were among the eleven species identified as most important (Jonfia-Essien, 2004). Recently, it has been observed that insects are more attracted to dry cocoa beans of new hybrid varieties than to those of the traditional type, however they do not feed on the new hybrids but cause severe damage to the latter (Jonfia-Essien, 2006). Insect infestation of the beans begins from the time they are placed on mats for drying. The contamination of dry beans of new hybrid varieties by high numbers of insects is a nuisance.

Dry cocoa beans have a characteristic aroma and it is possible that the level of insect infestations during storage is related to chemical signals from the beans. *Tribolium castaneum* (Herbst) is one of the major pests that is attracted to dry cocoa beans and contributes significantly to the damage inflicted on the beans by stored product pests (Jonfia-Essien, 2001). This paper reports flavour volatiles found in dry cocoa beans and the response of *Tribolium* adults to some of these volatiles.

MATERIALS AND METHODS

Materials: Dry cocoa beans of four new hybrid varieties (designated as HV_1, HV_2, HV_3 and HV_4) and a traditional type (TV, composed of mixed genotypes) (Table 1), were collected from the Cocoa Research Institute of Ghana. Adults of *Tribolium castaneum* were collected from the insectaries at the Research Department of the Quality Control Division (Cocobod), Ghana.

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Table 1: Genotypes and yield of the hybrid varieties and the traditional type cocoa beans

Cocoa type	Hybrid variety	Genotype (♀×♂)
Alternative hybrids	HV_1	SC5 × K5
	HV_2	APA5 × K5
	HV_3	SPA10 × P30
	HV_4	EET399 × P30
Series 2 hybrids	TV	T85/799 × PA7
(Traditional type)		T85/799 × T79/501
		T85/799 × Amelonado
		T 63/971 × Amelonado
		T60/887 × Amelonado

Sniffing test for flavour volatiles: Volatile compounds from dry cocoa beans were collected on a Tenax trap (SGE Scientific, UK) by passing air through the beans in a sample bottle at a flow rate of 50 mL min^{-1} for 15 min. Using a CHISA injector at 240°C, the volatiles were thermally desorbed for 10 min into an HP 5890 series II GC (Hewlett-Packard, Avondale, PA) equipped with a BP-1 column of 25 m length, 0.22 mm i.d. and 1.00 μm phase thickness. Forty cm of the column was submerged in liquid nitrogen during the injection to focus the volatile compounds on the column. Helium was used as carrier gas at 20 psi. The oven was kept at 30°C initially and then increased to 220 at 4°C min^{-1}. The GC was attached to a Fison MD 800 Mass Spectrometer with ionisation at 70 eV. The spectra were recorded from 25 to 250 Da and Mass Spectrometer data were analysed using MassLynx software.

The column outlet was connected to a sniffing port. Sniffing of the chromatogram, which took a total of 28 min, was divided into two parts. Each of eight trained panellists participated in the sniffing of both parts but during two distinct sessions to avoid lassitude. Elution of aroma relevant flavouring was recorded on a time-odour chart. The eight individual aromagrams of the samples were summed to produce a master aromagram. The time of the peaks and the description of flavour volatiles were put on a Microsoft excel spreadsheet and aligned. Compounds that were detected several times by the panellists were identified from the time of the peak and the computer library of volatiles.

Olfactometric analysis: A four-arm olfactometer supplied by Rothamsted Research, Harpenden, UK was modified and used. It was designed for investigating olfactory responses of small insects placed in the central chamber in which they can walk around freely (Pettersson, 1970). The equipment produced four distinct odour fields, one in each quadrant of the chamber.

Preliminary runs with the olfactometer were carried out to establish a reliable method of operation. Each of the four quadrants was fitted with a 30 mL plastic vial (Sterilin, Scientific Laboratory Supplies Limited, UK). The base of each vial was cut off to create a chamber for the introduction of volatiles. Both ends were plugged with Suba•Seals (Sigma-Aldrich, UK) and the outermost Suba•Seal was attached to a 0.2 μm sterile-EO filter (Sigma-Aldrich, UK). The filter purified the ambient air as it was pulled through the vial into the olfactometer and allowed the unidirectional flow of air into the olfactometer. The Suba•Seal at the inward end was attached to the olfactometer using 1000 μL and 100 μL pipette tips (Sarsted Limited, UK). All four quadrants were made airtight using a thin smear of Vaseline on the openings where the pipette tips were fitted. The experiments were performed in a controlled environment maintained at 30±2°C.

For each run with the olfactometer, a single insect was dropped through the hole in the middle of the top of the olfactometer into the arena or bioassay area, the tubing replaced and the suction pump switched on. Airflow was maintained at 400 mL min^{-1} by regulating a Platon Air/Luft flow meter placed between the olfactometer and a Capex L2XPC X75-002/4 suction pump No. DN 8210. The pump sucked air out through the central hole, drawing it equally through the four arms. The pump, flow meter and the olfactometer were connected to each other by Fisherbrand silicon tubing with a bore size of 6.5 mm and wall thickness of 1.5 mm. Cold light was supplied from a $KL1500_{LCD}$ Schott illuminator. The insect was allowed about 5 min (accommodation period) to acclimatize to the olfactometer environment after which a video camera positioned directly above the bioassay area was used to record its behaviour or movements for 20 min. The videocassettes were reviewed and the time spent in each quadrant by the insect was recorded. After each run, the olfactometer was cleaned with methanol and the air in the system was purged by switching on the suction pump for 15 min.

The solvent methanol was tested against a blank to determine if it was responsible for any attraction of the insects to the volatile compounds. Pure standards of the volatile compounds under investigation were prepared by diluting the solutions with a known volume of HPLC analytical methanol to 12.5, 25, 50 and 100 μl mL^{-1}. Using separate 1 mL syringes, 20 μL of the prepared volatile solution (test sample) of known concentration and 20 μL of the methanol (solvent) were injected simultaneously into the 30 mL vials through their respective Suba•Seals.

Responses of *Tribolium* to volatile compounds: To determine the responses of *Tribolium* to the six main volatile compounds (2-phenyl ethanol, acetophenone, 3-methyl butyraldehyde, ethyl butyrate, ethyl 3-hydroxybutyrate and butyl 2-methacrylate) detected in

Table 2: Significant flavour volatiles detected by panellists

Significant odourant	Odour quality (description)	Frequency	Detection (%)
Methyl acetate	Fruity, Chocolate	5	63
2-Methyl propanal	Fruity, Roasty	6	75
3-Methyl butanal (3-methylbutyraldehyde)	Choking, Pungent	7	88
2-Methyl butanal (2-methylbutyraldehyde)	Choking, Chocolate	5	63
1-Butene, 1-methoxy	Fruity, Chocolate	4	50
2-Methyl, methyl ester	Fruity, Sweet	4	50
1-Butanol, 3-methyl acetate	Fruity, Banana	5	63
Hexanedinitrile, 2-methyl	Spicy, Gallic	5	63
N,N-diethyl formamide	Roasty, Fishy, Acidic	5	63
Butanoic acid, ethyl ester (ethyl butyrate)	Fruity, Sweet	8	100
Methyl 2-methyl butyrate	Sweet, Fruity	5	63
2-Methylbutyl ester	Fruity, Sweet	8	100
Methyl 2-methyl butyrate	Sweet, Fruity	5	63
Pentanoic acid, 2-methylbutyl ester	Fruity, Sweet	8	100
Ethyl 2-hydroxybutyrate	Sweet	4	50
2-Butanol, 3-methyl-, acetate	Fruity, Banana	8	100
2-Methyl propanoic acid,	Sweet, Fruity	5	63
2-Pentanol acetate	Fruity, Banana	5	63
Decanone	Fruity, Orange, Oily	4	50
1,3-Dimethyl, benzene	Smokey	6	75
1-Butanol, 2-methyl, acetate	Fruity, Banana	5	63
Acetophenone	Sweet, Pungent	8	100
Dimethyl disulfide	Spicy, Strong onion-like	6	75
Phenethyl alcohol	Sweet, Spicy	7	88
Methyl disulfide	Spicy, Strong onion-like	7	88
Butanoic acid, 3-hydroxy ethyl ester	Fruity	8	100

the cocoa beans, a series of olfactometry experiments was carried out. In each experiment, the solvent methanol was used as a control in one or more arms of the olfactometer. The treatments were run in each arm of the olfactometer to remove any positional effects and three replicate runs were made. The volatiles at different concentrations were compared with methanol as the control in a randomized complete block design and in total, three experiments were carried out (Table 2).

Responses of *Tribolium* to dry cocoa beans: To determine the responses of *Tribolium* to dry cocoa beans, two experiments were performed. In the first experiment, the 30 mL chambers with Suba•Seals were filled with 5 g of cocoa beans. In each run there were four different varieties of beans (selected from HV_1, HV_2, HV_3, HV_4 and TV) connected to the arms of the olfactometer.

In the second experiment, 1000 mL bottles fitted with two-hole rubber stoppers (No. 29; Fisher Scientific, UK) were used but all other equipment remained the same as described above. Each bottle was filled with 400 g of dry beans and in each run there were four different varieties of beans (selected from HV_1, HV_2, HV_3, HV_4 and TV) connected to the arms of the olfactometer.

In both experiments, each bean variety was run in each arm of the olfactometer to remove any positional effects and three replicate runs were made in a randomized complete block design.

Statistical analysis: In each experiment, the solvent methanol was used as a control in one or more arms of the olfactometer, the treatments were run in each arm of the olfactometer to remove any positional effects and three replicate runs were made. The volatiles at different concentrations were compared with methanol as the control in a randomized complete block design. Also the data generated from the experiments involving dry cocoa beans were subjected to multivariance analysis using a randomised complete block design. Genstat 8th edition 2005 was used for the analysis of variance. Means were calculated from data generated and then converted into percentages for graphical presentation but the raw data were converted to logarithms before subjecting to statistical analysis due to the wide range between the figures. The parameters were considered in the analysis of variance at probability levels $p<0.001$ and $p>0.05$. Standard deviation was calculated using the logarithmic figures for the error bars on the graphs.

RESULTS

Many volatiles were liberated from beans of the traditional type cocoa (Fig. 1) and the panellists recorded 87 aroma relevant flavour volatiles on a time-odour chart. On the basis of 50% detection, only 26 odour-active volatiles were considered as significant odourants in the dry beans (Table 3). Identification of compounds on the GCMS revealed that the flavour volatiles detected by all the panellists were the fruity and sweet smelling ethyl butyrate, 2-methylbutyl ester and pentanoic acid, 2-methyl ester, the fruity and banana smelling 2-butanol, 3 methyl acetate, the fruity smelling butanoic acid, 3-hydroxy ethyl

Table 3: Treatments involving flavour volatiles in the olfactometer experiments

	Treatments					
Experiment	Volatiles	Concentration (μl mL^{-1})	Duration of exposure (min)	Olfactometer position	Replicates	Total runs
1	AP, PA	12.5, 25, 50, 100	10, 20, 30, 40	Q1, Q2, Q3, Q4	3	374
2	MT, BK	-	20	Q1, Q2, Q3, Q4	3	24
3	AP, PA, EB, MB, EH, BM	12.5, 25, 50, 100	20	Q1, Q2, Q3, Q4	3	288

AP: Acetophenone, PA: 2-Phenyl Ethanol, EB: Ethyl Butyrate, MB: 3-Methyl Butyraldehyde, EH: Ethyl 3-Hydroxybutyrate, BM: Butyl 2-Methacrylate, MT: Methanol, BK: Blank, Q: Quadrant

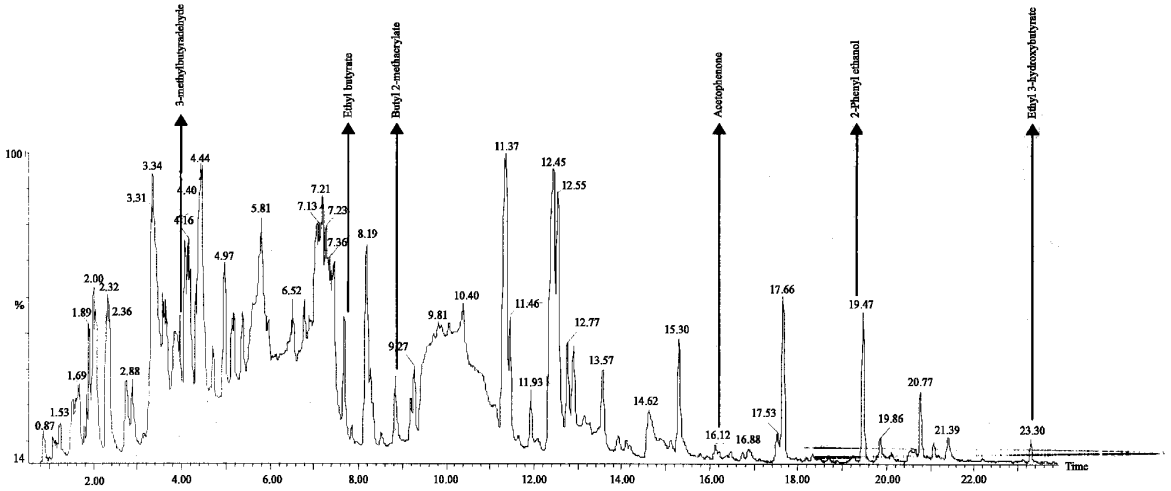

Fig. 1: Chromatogram of volatiles in dry beans of traditional type cocoa showing significant odourants

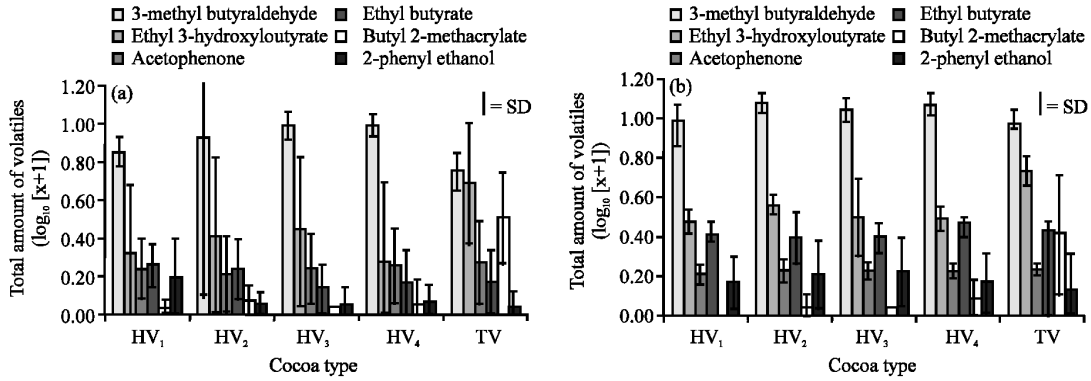

Fig. 2: (a) Percentage of the total amount of selected volatiles in TV, HV_1, HV_2, HV_3 and HV_4 cocoa beans (b) Percentage of the total amount of selected volatiles in TV, HV_1, HV_2, HV_3 and HV_4 cocoa beans repeated after 20 months of storage

ester and the sweet, pungent smelling acetophenone. The chromatograms for all the panellists were very similar in profile. In all, nine odour-active flavour volatiles scored 90 to 100% detection on the time-odour chart but, based on availability, standards of only six of the compounds were used for the olfactometer analysis.

The total amount of volatiles in the beans of the new hybrid varieties and the traditional type was linear across a range of an optimum value ($p>0.05$), however there were significant differences between the relative amounts of different flavour volatiles ($p<0.001$) in all the types of cocoa beans analysed.

The traditional type cocoa beans contained less 3-methylbutyraldehyde but more ethyl butyrate and acetophenone compared to the new hybrid varieties (Fig. 2a). A similar trend was observed when the analysis was repeated after beans of the same genotypes had been stored for twenty months (Fig. 2b). The feeding

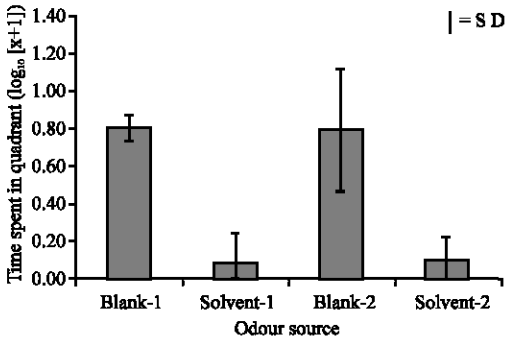

Fig. 3: Response of *Tribolium castaneum* to methanol solvent

preference of *Tribolium* for the traditional type cocoa beans, which resulted in severe damage as compared with the beans of the new hybrid varieties, was associated with differences in the amounts of 3-methylbutyraldehyde, ethyl butyrate and acetophenone in the beans ($p<0.001$).

Tribolium adults spent more time at the blank chamber (Fig. 3) than at the solvent odour field. They responded positively to acetophenone (Fig. 4a), phenyl ethanol (Fig. 4b) and ethyl butyrate (Fig. 4c) at 50 µl mL^{-1}. They spent significant time ($p<0.001$) at the quadrant containing the three volatiles but were not attracted to the volatiles at 12.5, 25 and 100 µl mL^{-1}. However, they also responded positively to 3-methyl butyraldehyde (Fig. 4d)

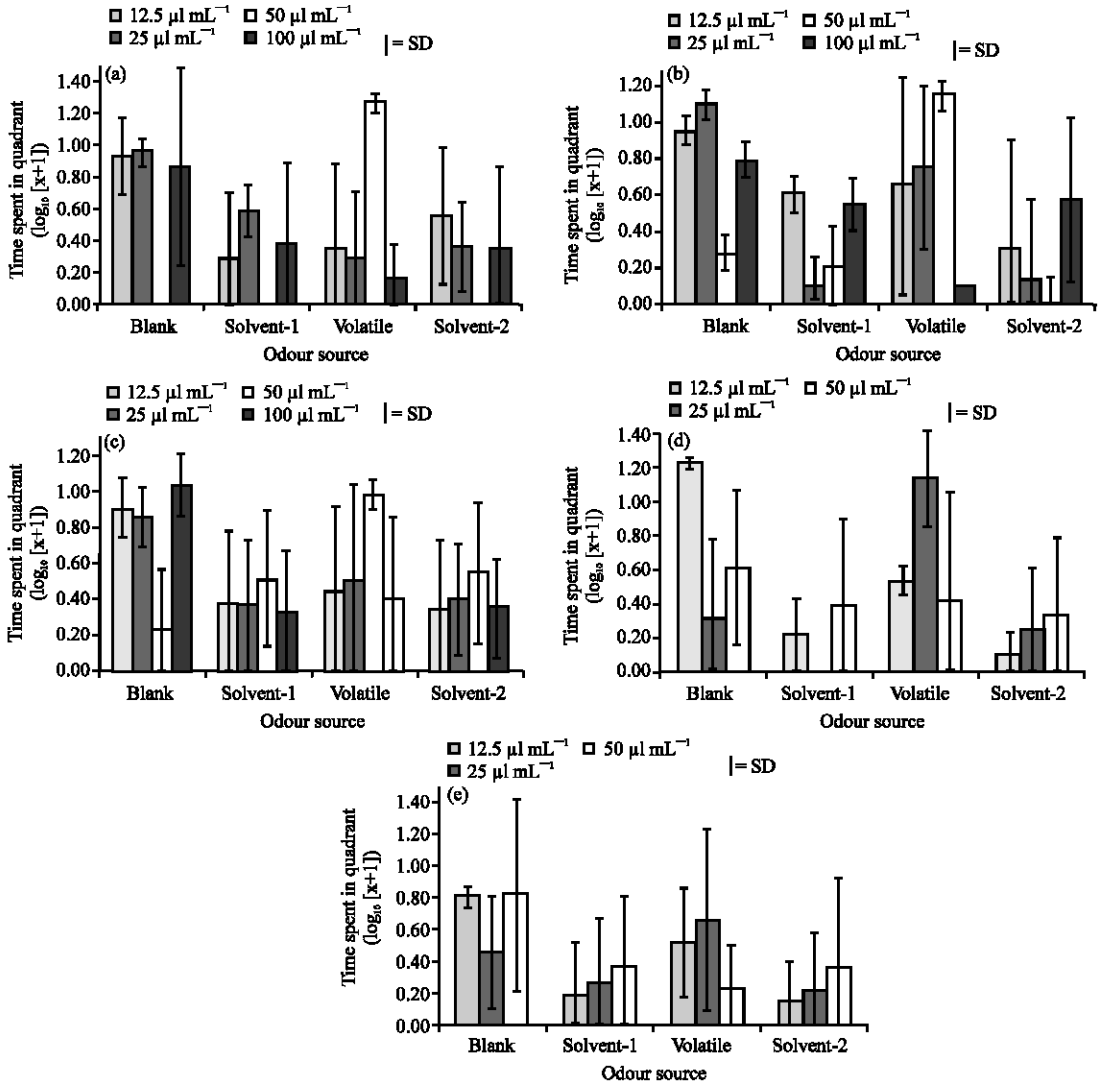

Fig. 4: (a) Response of *Tribolium castaneum* to acetophenone (b) Response of *Tribolium castaneum* to phenyl ethanol (c) Response of *Tribolium castaneum* to ethyl butyrate (d) Response of *Tribolium castaneum* to 3-methyl butyraldehyde (e) Response of *Tribolium castaneum* to butyl methacrylate

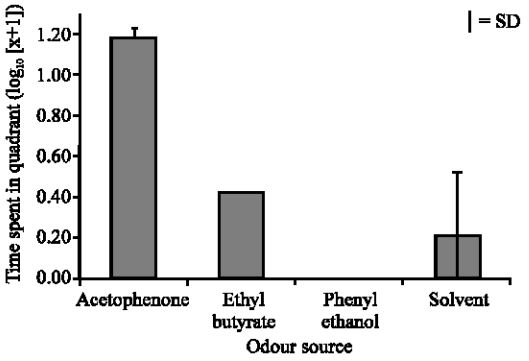

Fig. 5: Response of *Tribolium castaneum* to acetophenone, ethyl butyrate and phenyl alcohol at 50 μl mL^{-1}

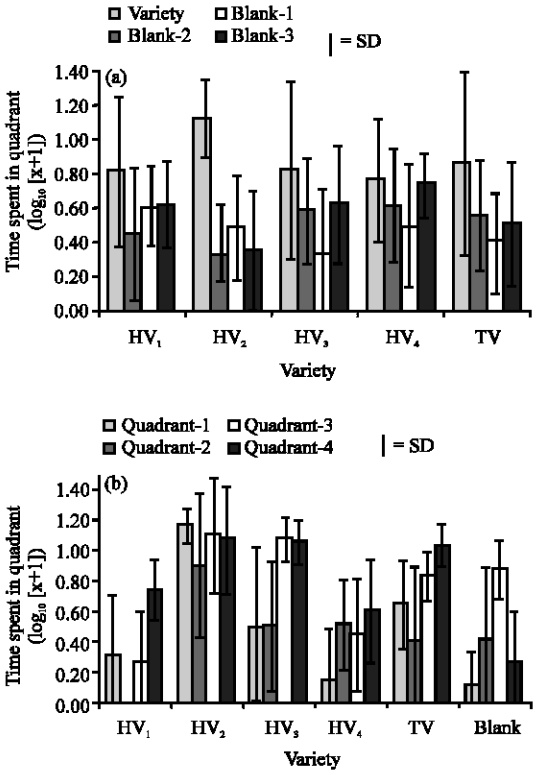

Fig. 6: (a) Attraction of *Tribolium castaneum* to beans of TV, HV_1, HV_2, HV_3 and HV_4 (b) Preferential attractiveness of *Tribolium castaneum* to beans of TV, HV_1, HV_2, HV_3 and HV_4

and butyl methacrylate (Fig. 4e) at 25 μl mL^{-1} with significant time ($p<0.001$) at the quadrant containing the two volatiles at this concentration, but were not attracted to the volatiles at 12.5, 50 and 100 μl mL^{-1} .

Although attracted to acetophenone, ethyl butyrate and phenyl alcohol, they spent more time ($p<0.001$) at the acetophenone chamber than the other two odour fields when the three volatiles were offered at the same time (Fig. 5).

Dry cocoa beans of the selected hybrid varieties and the traditional type were attractive ($p<0.001$) to *Tribolium* (Fig. 6a) when offered singly. There was preferential attraction ($p<0.001$) to some of the cocoa types when four were offered at the same time (Fig. 6b), with a higher preference for HV_2 compared to all other cocoa types ($p<0.001$), followed by HV_3 and then TV. HV_1 was the least preferred among the cocoa types.

DISCUSSION

Insects can perceive many volatiles that are not detected by the human nose, however, the use of the sniffing technique (Pollien *et al*., 1997) has been shown by Al Abassi *et al*. (1998) to be valuable for the study of volatiles responsible for attraction between ladybird adults. In the absence of electroantennography, the sniffing technique was used in the present study. The typical aroma of cocoa was mainly formed by some chemical reactions when the samples were subjected to high temperature in the GCMS. The liberation of the specific flavour volatiles, which already existed in the cocoa beans in the form of precursors, could be attributed to the high temperatures (Sakharov and Ardila, 1999; Wollgast and Anklam, 2000). The individual volatiles have a characteristic retention time, which accounts for different peaks occurring at different times in succession on the chromatogram.

The discrimination of *Tribolium* against the methanol solvent ($p<0.001$) used in diluting the standard volatile compounds is an indication that the methanol solvent was not responsible for attraction of the insects to any of the volatile compounds.

The relative amounts of flavour volatiles in all of the types of cocoa beans tested may be responsible for the feeding preference of the insects. The insects may have been prevented from feeding by the high amount of 3-methylbutyraldehyde in the beans of the new hybrid varieties. The amounts of ethyl butyrate and acetophenone may have been enough to cause attraction to the beans but insufficient to overcome the inhibition of feeding. This would explain why some insect pests of dry cocoa beans are attracted to beans of the new hybrid varieties in storage but do not feed on them.

The differences in behavioural response to the two-fold differences in the concentrations of compounds presented to the insects in the olfactometer bioassays could be attributed to the preferential attraction of the insects to volatiles. The observed responses imply that

the behaviour of the insect is affected by the concentration gradient of the volatile compounds. These findings are similar to those of Pike *et al.* (1994) who demonstrated that the attraction of *Sitophilus zeamais* Motsch. to maize volatiles was concentration dependent. This is important information for effective control and monitoring of insect pests. The quantitative change in volatile chemicals emitted by plants is known to play a major role in host location cues in some hymenopteran parasitoids of chewing insects, as well as certain predatory mites (Steinberg *et al*., 1993; Tumlinson *et al*., 1993; Takabayashi *et al*., 1994).

The volatiles tested acted as olfactory cues and such cues are known to be important in host plant location by other insects such as pollen beetles (Free and Williams, 1978; Blight and Smart, 1999). Electrophysiological responses from the antennae of pollen beetles were elicited by some floral volatiles in coupled gas chromatography-electroantennography studies (Blight *et al*., 1995).

The observed responses of *T. castaneum* to the flavour volatiles suggest that the odour of a host comprises a blend of many volatiles but that only some serve as olfactory cues. In a laboratory test using a two-arm olfactometer, Velemir and Pettersson (2003) concluded that adult *Coccinella septempunctata* were attracted to specific volatiles, thereby confirming observations in the field. Attraction of maize weevils *Sitophilus zeamais* Motsch. to trapped maize volatiles was also shown. Nonanal and hexanoic acid were identified as the major active components of cassava and maize volatiles, respectively (Pike *et al*., 1994).

Showing greater preference for acetophenone ($p<0.001$), *T. castaneum* adults clearly discriminated between the different volatiles when they were offered at the same time.

This is in agreement with the searching behaviour of *Coccinella septempunctata* investigated by Velemir *et al.* (2001) and supported by the results of Obata (1986) who showed the significant role of aphid-induced plant volatiles in food searching behaviour of *C. septempunctata.*

It is clear that *Tribolium* adults have different preferences for the cocoa types. This study confirms the observed high attraction to beans of most hybrid varieties in storage compared to the traditional type and also the significant damage ($p<0.001$) caused to beans of the traditional type but absence of feeding on those of the hybrid varieties.

The main conclusion from this study is that many volatile compounds were detected from cocoa beans but only some of them, notably acetophenone, phenyl ethanol and ethyl butyrate, affected the behaviour of *T. castaneum.* Although the behavioural attraction of *T. castaneum* to stored cocoa beans has been little studied, this study has shown that odour cues may play a role. All the cocoa types were attractive to the insects but the insects had feeding preference for beans of the traditional type. This may be due to the variation in the relative amounts of 3-methylbutyraldehyde, ethyl butyrate and acetophenone in beans of the traditional cocoa type compared to the amounts of these flavour volatiles in the new hybrid varieties. Manipulation of flavour volatile levels during breeding programmes should be adopted as a control measure against insect pest infestation in storage. Reducing the amount of acetophenone and ethyl butyrate but increasing the amount of 3-methylbutyraldehyde should deter *Tribolium* adults from feeding on cocoa beans in storage and provide an environmentally friendly control option.

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