Agro-Science Journal of Tropical Agriculture, Food, Environment and Extension
Volume 19 Number 3 (July 2020) pp. 32 - 39
ISSN 1119-7455

ATTRACTION OF *Callosobruchus maculatus* FABRICIUS (COLEOPTERA: CHRYSOMELIDAE: BRUCHINAE) TO PODS OF COWPEA PLANTS AT DIFFERENT DEVELOPMENTAL STAGES

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ABSTRACT

The cowpea beetle, Callosobruchus maculatus Fab. is an economically important pest of stored grain and causes serious damage to stored peas. The application of pesticides has been the generic control measure due to its effectiveness, affordability and ease of application. However, due to the apparent health and environmental consequences with pesticides, stakeholders are advocating for an alternative management approach that has less social and environmental impact and is more sustainable. Consequently, this study examined the attraction of mated female Callosobruchus maculatus to volatile blends collected from pods of cowpea plants (Borno-brown and black-eyed cultivars) at three developmental stages÷ developing podsie; 15-17 days after anthesis (daa), fully developed pods (18-20 daa) and mature pods (> 20 daa). The beetles' responses to the pods' odour were determined using a two-arm olfactometer, and gas chromatography-mass spectrometry (GC-MS) was used to identify and quantify the volatile compounds collected from the pods. The results showed that female C. maculatus attraction increased with the pod's age, and that the composition and abundance of volatile compounds varied between cowpea cultivars and the pod's developmental stage. These findings form an important bases towards developing alternative approach for the management of bruchids.

Key words: volatile compounds, cowpea plants, GC-MS, pod's developmental stage, days after anthesis

INTRODUCTION

The dry seeds of cowpea (*Vigna unguiculata* L. Walp) is an important food and cash crop to farmers (mainly, cowpea growers in tropical regions) and exporting nations. But its production is facing serious pest infestations, especially, from the cowpea bruchid, *Callosobruchus. maculatus*. The gravid females of this storage beetle lay eggs on pods of cowpea plant in the field (Taylor and Agbaje, 1974; Ouedraogo and Huignard, 1981; Djossou, 2006; Kebe and Sembene, 2011), and the egg-laden pods are harvested, kept in storage where cross infestations and re-infestations continue. Emerging adults leave a hole on the bean's surface and have mined out the internal tissue of the bean, thus damaging its economic value. As a result, farmers have to spray pesticides on their harvest to control the pest attack without understanding the consequences of their actions. The risks associated with pesticide usage have been well documented, and has triggered legislation banning its use, and the need for new, safer pest control methods.

Several studies have reported the attractiveness of plant volatile organic compounds (VOCs) to certain insect pests of agricultural importance (Agelopoulos *et al*., 1999; Dudareva *et al*., 2004; Webster *et al*., 2008). For example, the cosmopolitan granary pest *Acanthoscelides obtectus* (Say), the pea weevil, *Bruchus pisorum* L. and the legume pod borer (*Maruca vitrata* Fab.) are attractedby the volatile compounds from dry bean cultivars (Khelfane-Goucem *et al*., 2014), *Pisum sativum* L. (Ceballos *et al.*, 2015) and *Vigna unguiculata* (Bendera *et al.*, 2015; Zhou *et al.*, 2015), respectively. Also, the use of methyl eugenol and protein-bait from brewery waste on modified lynfield trap were effective in mass-trapping *Bactrocera invadens* D.T.W. (Ugwu *et al*., 2018). Most plant volatiles are sulphuric compounds, terpenoids, fatty acid derivatives and nitrogen-containing compounds (Pare and Tumlinson, 1999), and are mainly lipophilic products with molecular masses less than 300. The most extensively studied vegetative volatile is Isoprene (Sharkey and Yeh, 2001), a thermoregulatory which protects plants against heat-stress (Sharkey *et al*., 2008), whereas other substances are released to protect plants against natural enemies (Takabayashi and Dicke, 1996; Hammer *et al*., 2003) and attract pollinators (Reinhard *et al*., 2004). Studies have shown that the

Please cite as: Ahuchaogu C.E. and Ojiako F.O. (2020). Attraction of *Callosobruchus maculatus* Fabricius (Coleoptera: Chrysomelidae: Bruchinae) to pods of cowpea plants at different developmental stages. *Agro-Science*, **19 (3),** 32-39. DOI: https://doi.org/10.4314/as.v19i3.6

quantity and number of the volatile substances emitted are affected by low light conditions (Takabayashi *et al*., 1994) and high organic nitrogen fertilizers (Van Wassenhove *et al.*, 1990). These volatile substances are released from different parts of a plant, and induce behavioural interactions between organisms. They are used by pest insects to identify, home-in on and utilise a preferred host type (Ignacimuthu *et al*., 2000; Uechi *et al*., 2007; Bruce *et al*., 2005; Webster *et al.*, 2008).

Developing (Umar andTuraki, 2014; Zannou *et al*., 2003) and mature stages of cowpea plants (Caswell,1984) are susceptible to infestations by *C. maculatus* in the field. According to Messina (1984), female *C. maculatus* preferred fully developed pods to younger or mature pods, and were attracted to pods with exposed seeds compared to intact pods. In another study, a senesced banana leaf was preferred to other developmental stages (Abagale *et al.*, 2019). These preferences have been suggested to be triggered by the volatile compounds emitted at the developmental stages. For example, the senesced banana leaf was found to contain (2R, 5S)-the aspirane as an active component unlike other developmental stages (Abagale *et al.*, 2019) of the banana leaf. Also, soybean showed variances in the abundance and number of VOCs emitted at different developmental stages (Boué *et al.*, 2003). These findings suggest there may be important chemical cues correlated with the host's life-history stage. There is therefore considerable evidence that the beetle interacts with the host plant long before the seeds are stored by farmers (Ouedraogo and Huignard, 1981; Taylor and Agbaje, 1974; Djossou, 2006; Kebe and Sembene, 2011).

Analysing the chemical components of cowpea pods at various developmental stages and the examination of the pest's behavioural attraction to such chemicals is important to predict how the life cycle of the plant influences its vulnerability to infestations by the pest. With this in mind, this chapter examines the response of cowpea beetle to volatiles from the pods of two cowpea varieties at different growth stages, and predicts that odour cues from pods' categories would induce attraction of *C. maculatus.* As a field-to-store pest, this measure is to identify the most vulnerable pod's growth stage to infestation together with the volatile compounds moulding such action.

MATERIALS AND METHODS

Insect

A wild strain of *C. maculatus* collected from infested Borno-brown beans in a farmer's field in Taraba State, Nigeria was used in this study. The strain was cultured in breeding containers (17 × 11.5 cm), each having 200 g of uninfested whole Borno-brown bean. Lids of the containers were perforated to allow for ventilation. The cultures were kept in the lab. at a temperature of 28±2°C and relative humidity of 30±5%.

Growing of Cowpea

This was carried out in a greenhouse at Arthur Willis Environment Centre (AWEC), University of Sheffield, United Kingdom. The greenhouse day and night temperatures were maintained at 27±1°C and 22±1°C, respectively. Relative humidity was 30-60% used throughout the study. The photoperiod was set at 9 h light and 15 h dark. Nine pots (30 cm diameter) each, were used for the study. Three clean seeds of Borno-brown and California black-eyed cultivars were sown per pot which was later thinned to a plant stand per pot after one week of germination. The plants were tagged at the onset of flowering (anthesis) to accurately estimate the pods' age (Plate 1).

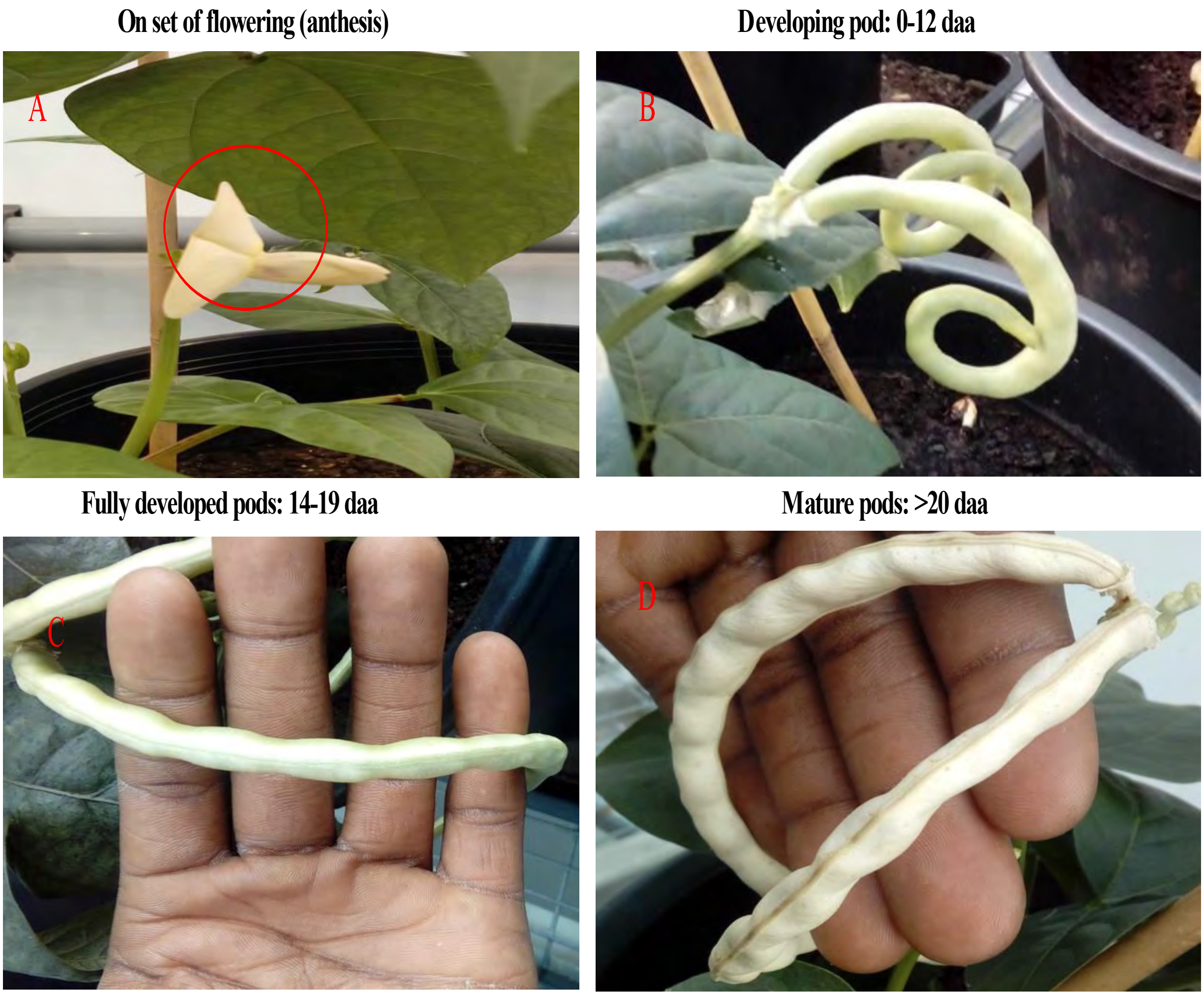
The age classes were based on the number of days after onset of flowering as categorized below; 15-17 days after onset of flowering (developing pods) 18-20 days after onset of flowering (fully developed pods) 20 days after onset of flowering (mature pods).

Headspace Samples Collection from Cowpea Pods

Headspace VOCs of the pods were trapped with air entrapment equipment from the cowpea plants at the defined developmental stages. All equipment was washed with detergent, rinsed with hexane and distilled water, and then dried in an oven at 120°C for 15 h. Transparent oven bags used for the study were also pre-conditioned by heating them in an oven at 120°C for 15 h. Each cowpea plant at various growth stage was enclosed with an oven bag, and charcoal-filtered air passed through a Porapak Q absorbent (Alltech Associates, Lancashire, UK) at a constant rate of 300 ml/ min (Plate 2). All the connections were made with Polytetrafluoroethylene (PTFE) tubing and tape (Supelco, Bellefonte, PA). The VOCs absorbed on Porapak Q were eluted with 1 ml of hexane. Extracted samples were further concentrated to 100 µl by a low stream of nitrogen, and stored in glass vials in a freezer at –80°C.

Beetles' Response to Headspace Volatile Samples

A two-arm olfactometer (Webster *et al.*, 2008) was used to examine the attractiveness of *C. maculatus* to volatile samples collected from the plant pods. The olfactometer consists of three layers; the base (floor), the observation layer and the cover clipped together to form an eight-sided shape with a two-arm exposure chamber. Each layer is made of a transparent Perpex with 6 mm thickness. The first layer (floor) was lined with a What man filter paper base (110 mm) to provide traction for the weevil. Another layer, the observation arena had a hole (3 mm diam.) drilled from both edges into the two arms to accommodate the odour chambers. Then, a third layer (cover), all of the same size and shape had a hole (4 mm diam.) drilled at the centre. Two 60-ml BD plastiak's (syringes) served as the odour chambers. A Teflon tube (1.5 mm ID × 3.2 mm OD) was used to connect each of the chambers to both arms of the olfactometer, and the connections were tightened with a PTFE tape. A 60-W light bulb was positioned 1m above the olfactometer to provide uniform illumination.



On set of flowering (anthesis)

Developing pod: 0-12 daa

Fully developed pods: 14-19 daa

Mature pods: >20 daa

Plate 1: Onset of an thesis (A); developing pods: 0-12 daa (B); Fully developed pods: 14-19 daa (C); Mature pods: > 20 daa (D)

Plate 2: Collection of volatile organic compounds from cowpea pods

Twenty microliters (20 µl) of volatiles samples from pods of the plants at the three different growth stages were applied on a piece of filter paper, and 1 min. was allowed for solvent evaporation. The treated filter paper was put into one of the odour chambers, while the second chamber was used as a control which contained a piece of filter paper treated with 20 µl of hexane. A mated normal female of the beetle was then introduced into the centre of the olfactometer (observation arena). Air was drawn through both arms using a vacuum, and regulated with a flow meter at a rate of 100 ml/min. After introduction, the beetle was given 3 min. to settle in the observation arena, and the movement towards both arms was observed for 15 min. Beetles that failed make any choice 5 min. after introduction were regarded as “non-responders” and discarded. All materials used were washed, rinsed with distilled water, and then cleaned with 70% ethanol. Between 8-10 replicates were used.

Coupled Gas Chromatography – Mass Spectrometry (GC – MS)

The candidate compounds associated with the volatile samples from the plants’ pods were identified using GC-MS. A 2 µL of the air headspace sample was injected onto a capillary GC column (30 m × 0.25

mm ID, 0.25 µm film thickness), which is directly coupled to a mass spectrometer (PerkinElmer, Clarus® SQ 8T). The carrier gas was helium with a flow rate of 1.02 ml min^{-1}. Ionization was achieved by electron impact at 70 eV, 230°C. The injection port was maintained on a splitless mode. The GC initial oven temperature was maintained at 30°C min^{-1}, then ramped at 5°C min^{-1} to 240°C, and held for 20 min. Mass spectrum acquisition was scanned using a *m/z* range from 35 to 450. Candidate compounds were identified by comparing the chromatograph retention index and mass spectra with library database spectra using the National Institute of Standards and Technology (NIST) mass spectra search programme (version 2.2, NIST 14, Gaithersburg, Maryland, USA). The retention index of each compound identified was calculated using a series of straight alkanes ($C_8 – C_{20}$). The abundance of each identified compound was calculated by integrating the peak areas of the total ion chromatograph and averaged (Webster *et al.*, 2008).

Data Analysis

The two-choice data on the beetles' responses to VOCs from each pod category were analysed using Chi-square (χ^2) test. Staked bars were used to present the proportion of time spent by the beetles in each arm of the olfactometer. To determine the similarities or differences among the compounds identified, the chemical analysis data on the abundance of volatile compounds from each pod category were subjected to permanova analysis and Tukey's HSD test was used for mean separation. The similarities of the compounds were interpreted using cluster analysis (Ward, 1963); whereas, principal component analysis (PCA) was used to indicate the ordination of the compounds and their relationships in the first two components. R statistical software (RStudio Version 1.1.456, 2009-2018) was used for all analyses.

RESULTS

Beetle Response to Headspace Volatile Compounds

Olfactometer bioassays with natural samples of pod volatiles from Borno-brown beans showed that samples from developing pods ($\chi^2 = 0.051$, df = 1, $p = 0.820$) and fully developed pods ($\chi^2 = 0.170$, df = 1, $p = 0.679$) did not elicit responses from mated females of *C. maculatus*. However, there was significant attraction ($\chi^2 = 10.397$, df = 1, $p = 0.001$) to volatiles from mature pods (Figure 1). When the odour sources from pods of black-eyed beans was tested, the results showed that beetles spent significantly more time on arms with fully developed ($\chi^2 = 7.255$, df = 1, $p = 0.007$) and mature pods ($\chi^2 = 5.215$, df = 1, $p = 0.022$) samples. But, when given a choice between volatiles from developing pods and the control, the female did not differentiate between the two treatments ($\chi^2 = 1.849$, df = 1, $p = 0.173$) (Figure 1).

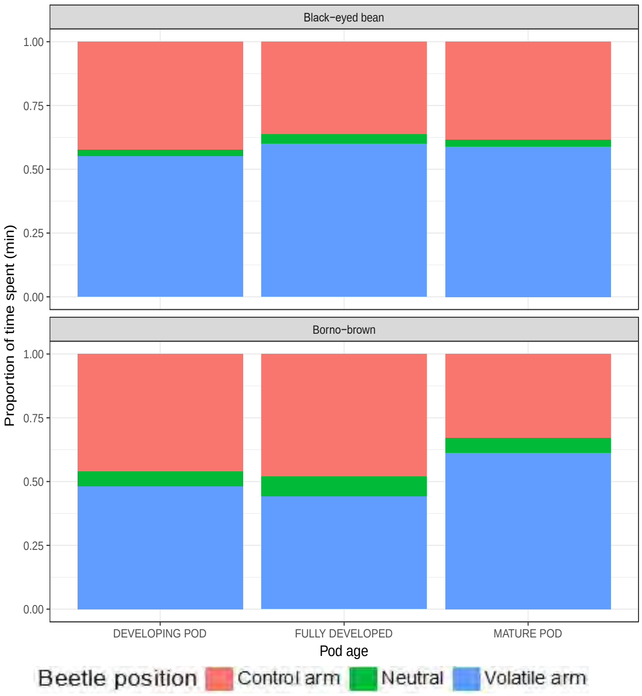


Figure 1: Proportion of time spent by mated female *C. aculatus* in response to volatile stimuli from cowpea pods in a two-arm olfactometer

Identification and Chemical Analysis of Volatile Compounds from Pods of Black-Eye Cowpea.

The analysis revealed a total of 12 volatile compounds in the cultivar; eight were present on the developing and the fully developed pods, whereas, 11 compounds were detected on the mature pods (Table 1). Each of Hexanal and 3-Hexen-1-ol-acetate was only present on the mature and developing pods of black-eye cowpea, respectively. The PCA showed that components, 1 and 2 explained more than 99 % of the variances in the abundance of VOCs examined on pods of black-eye cowpea (Figure 2), and the cluster analysis classified the compounds into three; benzaldehyde and ethanol, 2-(2-butoxyehoxy)-acetate representing cluster 2 have similar abundance profile (Figure 3). The PCA biplot and dendrogram fully describe how the other compounds are related. The chemical analysis of the compounds indicated that they varied significantly among pods of black-eye cowpea (F = 2351.6, df = 11, 35, $P < 0.01$; Tables 1 and 2). Benzaldehyde, was significantly more abundant, followed by p-xylene and m-xylene on developing pods of black-eye cowpea; whereas, in the fully developed pods, benzaldehyde was more abundant, followed by m-xylene and p-xylene, although, they do not differ significantly (Table1). Similarly, ethanol, 2-(2-butoxyehoxy)-acetate, followed by m-xylene and benzaldehyde were more abundant on mature pods of black-eye cowpea. However, 1-octane-3-ol was the least abundant compounds on developing pods of black-eye cowpea; whereas, Nonanal was the least abundant on the fully developed and mature pods of black-eye cowpea, respectively (Table 1).

Table 1: GC-MS analysis of volatile organic compounds emitted by 2µl of air entrainment sample of black-eye cowpea pods (mean ±SD)

Developing pods	Fully developed pods	Mature pods RI	
BENZALDEHYDE 16.790±0.241a	17.839±0.329a	18.818±0.071a	962
P-XYLENE 18.684±0	16.195±0.228b	17.267±0.191a	865
M-XYLENE 19.029±0.242a	15.072±0.103c	17.633±0.095a	866
LIMONENE 15.582±0.682cd	14.203±0.249d	15.592±0.011b	1030
NONANAL13.376±0.391f	13.375±0.065e	11.241±0.464c	1104
3-HEXEN-1-OL-ACETATE 1005	12.923±0.245ef	nd	nd
3-CARENE16.669±0.379b	12.875±0.296f	15.721±0.106b	1011
1-OCTEN-3- L14.811±0.025de	11.527±0.029g	nd	980
1-HEXANOL, 2-ETHYL1030	nd	14.996±0.704b	15.794±0.079c
a-PINENE18.585±0.000a	937	nd	17.083±0.213a
ETHANOL, 2-(2- BUTOXYETHOXY)- ACETATE nd	nd	19.167±0.000a	1366
HEXANAL	14.568±0.004e	nd	nd880

Within the row means with the same letter are not significantly different. Means separated using Turkey HSD test.
nd: not detected, RI: retention index

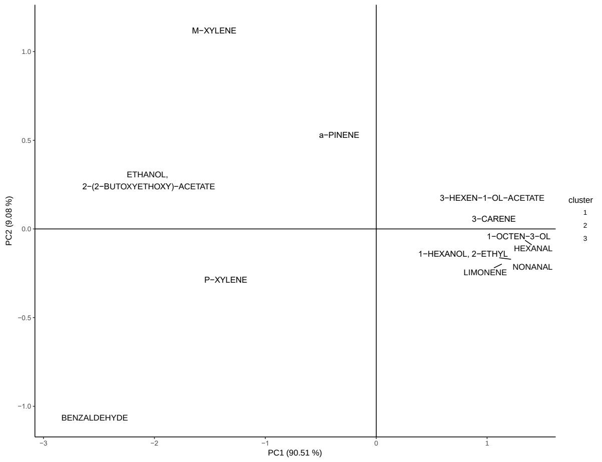


Figure 2: Biplot showing the ordination of the clustered volatile compounds

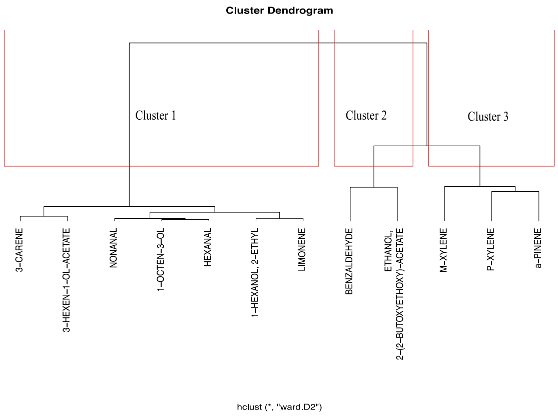


Figure 3: Dendrogram showing relationship among 12 volatile compounds from pods of black-eye cowpea based on their relative abundance

The rectangular boxes represent each cluster.

Table 2: PCA of the data set on 12 compounds from pods of black-eye cowpea showing the scores in the various components

Compounds	PC1	PC2	PC3
P-XYLENE	-1.2883	-0.2529	-0.2950
M-XYLENE	-1.3935	1.15360	0.1068
a-PINENE	-0.3953	0.4986	-0.0659
BENZALDEHYDE	-2.8638	-1.0622	0.1453
3-CARENE	1.1223	0.0251	0.0693
1-HEXANOL, 2-ETHYL	1.2135	-0.1707	0.0120
ETHANOL, 2-(2-BUTOXYETHOXY)-ACETATE	-2.6734	0.2737	-0.0121
LIMONENE	1.1304	-0.1986	0.0749
3-HEXEN-1-OL-ACETATE	0.9822	0.1416	0.0021
1-OCTEN-3-OL	1.4002	-0.0888	0.0058
NONANAL	1.4047	-0.1804	-0.0494
HEXANAL	1.3610	-0.1392	0.0062

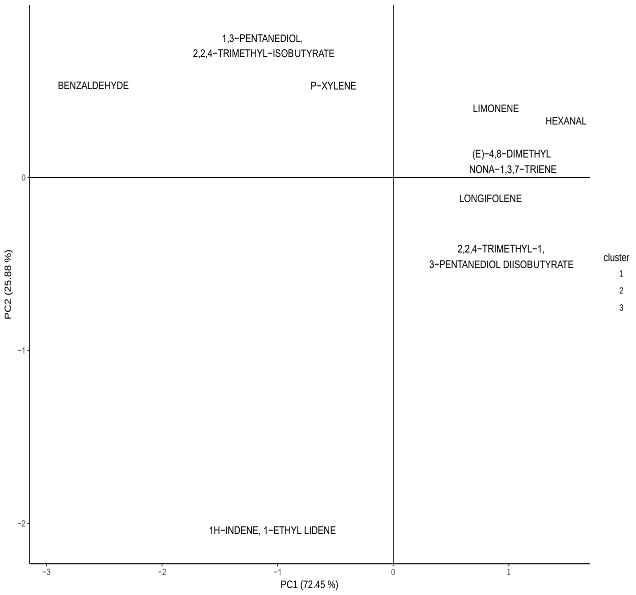


Figure 4: Biplot showing the ordination of the volatile compounds from pods of Borno-brown cultivar

Identification and Chemical Analysis of Volatile Compounds from Pods of Borno-Brown Cultivar

The analysis of the volatiles from the cowpea cultivars revealed that a total of 9 were emitted from the pods of Borno brown cultivar; all 9 compounds were detected on the developing pods, but longifolene was not present on the fully developed and mature pods (Table 3). The PCA showed that components 1 and 2 explained over 98% of the variances in the abundance of VOCs examined on pods of black-eye cowpea (Figure 4) and all the 9 compounds showed loadings in the first two components.

Table 3: GC-MS analysis of volatile organic compounds emitted by 2µl of air entrainment sample of Borno-brown pod (mean ± SD)

Developing pods	Fully developed pods	Mature pods	RI
BENZALDEHYDE 16.00±0.126a	17.234±0.08a	17.601±0.144abc	962
1H-INDENE, 1-ETHYL LIDENE 15.224±0.235ab	15.583±0.579c	18.218±0.261a	1335
P-XYLENE 15.155±0.721ab	16.463±0.112b	16.491±0.274cd	865
1,3-PENTANEDIOL-2,2,4-TRIMETHYL-ISOBUTYRATE 14.375±1.433abc	16.965±0.397ab	16.772±0.049bcd	1380
LIMONENE14.223±0.061abc	15.592±0.0134c	15.744±0.095d	1030
LONGIFOLENE 13.094±1.723bc	nd	nd	1405
2,2,4- TRIMETHYL-1, 3-PENTANEDIOL DIISOBUTYRATE12.965±0.813bc	15.076±0.014c	17.955±1.333ab	1580
HEXANAL 12.416±0.457c	13.706±0.0126d	13.873±0.257e	880
(E)-4,8-DIMETHYL NONA-1,3,7-TRIENE 12.097±0.080c	12.888±0.244e	15.599±0.017d	1116

Within the row means with the same letter are not significantly different. Means separated using TurkeyHSD test. nd: Not detected RI: retention index

The cluster analysis classified the compounds in three clusters; 1h-indene, 1-ethyl lindene, representing cluster 2 has no similarity with any other compound and other compounds in the same cluster have similar abundance profile (Figure 5). The PCA biplot and dendrogram fully describe how these compounds are related. The chemical analysis of the compounds indicated that they varied significantly on pods of Borno-brown cowpea (F = 100.59, df = 8, 26, $P < 0.01$; Tables 3 and 4). Benzaldehyde, was significantly more abundant, followed by 1h-indene, 1-ethyl lindene and p-xylene on developing pods of Borno-brown cowpea; whereas, in the fully developed pods, benzaldehyde was significantly dominant, followed by 2,2,4-trimethyl-isobutyrate and p-xylene (Table 3). On the mature pods, 1h-indene, 1-ethyl lindene was more abundant, followed by 3-pentanediol, diisobutyrate and benzaldehyde. However, (E)-4,8-dimethyl nona-1,3,7-triene was the least abundant compounds on developing and fully developed pods of Borno-brown cowpea; whereas, hexanal was the least abundant on the mature pods (Table 3).

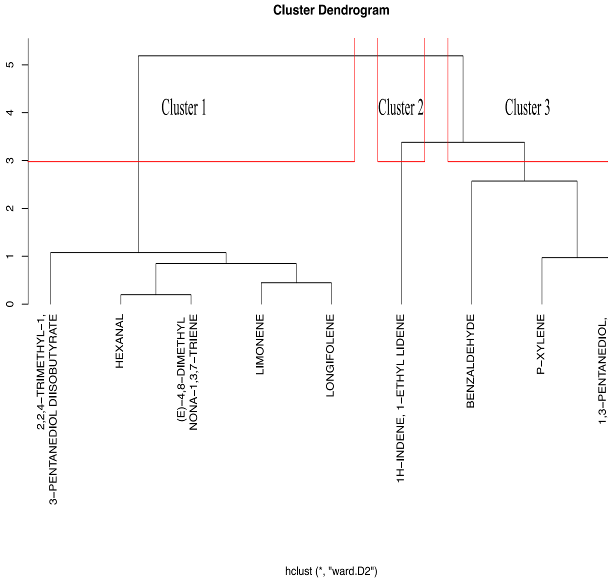


Figure 5: Dendrogram showing relationship among 9 volatile compounds from pods of Borno-brown cowpea based on their relative abundance

The rectangular boxes represent each cluster.

DISCUSSION

Our study identified 12 volatile compounds on the pods of black-eyed cowpea and 9 on the pods of Borno-brown cultivar. The compounds varied in abundance and numbers across the three developmental stages examined and majority of those identified in this work have been reported in other studies (Mushobozy, *et al*., 1993; Adhikary *et al*., 2015; Zhou *et al*., 2015) to trigger behavioural responses on insects. Benzaldehyde was present and abundant at all the developmental stages of both cowpea cultivars, thus suggesting it could be playing an important role in host location by the pest. Its role in mediating host-choice on *Acanthoscelides obtectus* (Khelfane-Goucem *et al.*, 2014), *Vicia faba* (Webster *et al*., 2008) and *Maruca vitrata* (Zhou *et al*., 2015) have been suggested.

The headspace volatile samples of the cowpea pods collected at different developmental stages elicited varying behavioural (olfactometer) attraction on mated females of *C. maculatus.* The results show that the beetles moved towards odour samples from the fully developed pods (of black-eyed cultivar) and mature pods (of black-eyed and Borno-brown cultivars), respectively. This suggests that the beetles' attraction to the host plant increases with the pod's age. The preference is likely driven by the fact that the host beans (the primary target) are developing as the pods mature. In the other hand, the beetle's preference for older pods could be due to the organic compounds associated with the developing seeds in the pods which may be difficult to detect at an early podding stage. Earlier work (Zannou *et al.*, 2003) revealed that cowpea plants with pods attracted more beetles compared to cowpea plants without pods. The findings in the present study also agree with Ouedraogo and Huignard, (1981) that dry and mature pods of cowpea are vulnerable to *C. maculatus* infestations. Similarly, Abagale *et al.* (2019) found that the banana weevil (*Cosmopolites sordidus*) was attracted to the odour of senesced banana leaf material. The variation in attraction to the pods are linked with the differences in the chemical composition of the plant part which affects the abundance and quality of VOCs (Li *et al*., 2016; Shiojiri and Karban, 2006).

Table 4: PCA of the dataset on 9 compounds from pods of Borno-brown cowpea showing the scores in the various components

Compounds	PC1	PC2	PC3
BENZALDEHYDE	–2.9247424	0.53395161	0.14308114
HEXANAL	1.4804515	0.28392084	0.10658366
P-XYLENE	–0.4480953	0.57735731	0.29599372
LIMONENE	0.8250549	0.35727251	0.08089173
1H-INDENE, 1-ETHYL LIDENE	–1.1065272	–2.08567898	0.02205382
2,2,4-TRIMETHYL-1,3-PENTANEDIOL DIISOBUTYRATE	0.8039782	–0.54510722	–0.2314637
1,3-PENTANEDIOL, 2,2,4-TRIMETHYL- ISOBUTYRATE	–0.9859765	0.85121692	–0.4637878
(E)-4,8-DIMETHYL NONA-1,3,7-TRIENE	1.4445936	0.09429446	0.06990006
LONGIFOLENE	0.9112631	–0.06722745	–0.0232527

The results of the GC-MS analyses of the headspace volatile samples from the pods showed that the VOCs profile differed with cowpea cultivar and pod's age. The variation in gene sequence has been suggested to be affecting the chemical composition of plant cultivars or ecotypes, thus, triggering the release of diverse blends of compounds (Köllner *et al.*, 2004). It has been shown that as a plant grows, the ratio of compounds present changes (Najar-Rodriguez *et al.*, 2010; Vallat and Dorn, 2005). Most of the compounds identified (Benzaldehyde, M-xylene, Hexanal, P-xylene, Limonene etc.), are among the common volatile compounds associated with most leguminous plant parts (Blight *et al.*, 1984; Webster *et al.*, 2008). Although they have been only identified as candidate compounds in this study, a probable role in eliciting behavioural attraction in the beetle still remains a strong possibility.

CONCLUSION

In summary, this study has (a) demonstrated that mated females of *C. maculatus* are attracted to fully developed and mature pods of cowpea, (b) identified the volatile compounds that could be inducing the beetles' behavioural attraction to older cowpea pods (c) shown that volatile compounds composition and abundance profile vary between cowpea cultivars at different pods' developmental stage. This is a step forward in confirming that volatile compounds in growing plants can drive host identification and selection by *C. maculatus.* The approach presents great potential for the management of the pest using semiochemical-based approach.

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