ROLE OF PLANT METABOLITES IN MEDIATING HOST SELECTION AND OVIPOSITION OF *Busseola fusca* (FULLER) (LEPIDOPTERA: NOCTUIDAE)

A research thesis submitted for the fulfilment of the requirements for the award of the degree of Master of Science in Biochemistry of Jomo Kenyatta University of Agriculture and Technology (JKUAT).

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May 2005

DECLARATION

I hereby declare that this is my own original work and that it has not been presented for a degree award in any other university.

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Dedication

Dedicated to mum, Kalasina, daughter, Faith and my late brother Kenneth.

To mum and Ken:

Your love, endurance, care and

prayers made me reach this far.

To Faith

You're the source of my inspiration.

Acknowledgements

I would like to acknowledge with gratitude my sponsors, Institut de Recherche pour le Développement (IRD) and International Centre of Insect Physiology and Ecology (ICIPE) for the financial support they provided during the research. I wish to specially thank Dr. Paul-André Calatayud for his immense contribution to this work, from the time of conceptualizing the project, constructive advice during research work and critically reviewing the manuscript.

I wish to sincerely thank my supervisors Dr. Calatayud, Dr. Magoma and Prof. Hassanali for the deep interest they took in this work and for the immense guidance and critical discussion rendered during various stages of the study period. I also extend my sincere thanks to Dr. Wamunyokoli and Prof. Imbuga for their guidance at the initial periods of the research, Dr. Le Rü and his team for their continued supply of wild insects and Dr. Njagi for carrying out the EAG experiments.

.I am grateful indeed to Dr Sabine for molecular identication of insects, Dr. Derridj and Dr. Frerot who provided GC and HPLC-MS facilities and Dr. Kerhoas and Mr. Gaertner for carying HPLC-MS analysis of my samples. A lot of appreciation goes to Mr. Wanyama for carrying out the GC-MS analyses of my samples, Mr. Nyandat for the technical support and Mr. Angira for the technical support and his continued supply of solvents. Special thanks goes to all colleagues and staff of Chemical Ecology and Biodiversity departments for their assistance, support, cooperation and encouragement during my work.

My deepest gratitude goes to my wife Carolyne and our daughter Faith, who endured periods of prolonged absence from home and for their patience, love and unfailing encouragement. I remain indebted to my mother and siblings who have been there for me all this trying time.
To to all who I have not mentioned but in one way or another contributed towards the completion of this work, I say a big thank you.

Finally, I am grateful to the almighty God for giving me the courage, wisdom, strength and motivation and enabling me to complete this work successfully.
Good bless you all.

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ABSTRACT

In a previous study using three plant species (maize, sorghum and Napier grass), naïve gravid *B fusca* females significantly preferred sorghum and maize over Napier grass for oviposition a phenomenon, which was partly related to plant physical characteristics such as leaf surface texture and sheath toughness. The purpose of this study was therefore to determine the importance of plant chemical characteristics (volatiles as well as surface compounds) in host selection for oviposition using the same plants species as used in previous works.

Differences in the reproductive behaviour (oviposition) between the artificially reared and wild naive *B. fusca* females in relation to their host plant specificity were compared in laboratory bioassays. Unlike wild insects, artificially-reared insects tends to lose their host plant specificity for oviposition after several generations. These insects accepted oviposition supports totally outside their original host plants such as paper surrogate stem and showed no preference for oviposition on artificial stems baited with stimulatory plant extracts in contrast to the wild population. In addition few laboratory-reared insects exhibited an oriented flight behaviour towards maize plants in wind tunnel conditions in contrast to the wild population. However, the females after being reared artificially conserved the same antennal sensitivity towards host plant volatiles as the wild ones. All these results indicate that laboratory-reared *B. fusca* insects differ from natural population in the host plant specificity and this limits their representativeness of the species in the wild. Therefore it is important to use wild insects in future related studies involving the influence of plant chemistry in host plant selection for oviposition.

The volatiles emitted by maize, sorghum and Napier grass were analysed by GC and identified by GC-MS. The blend of volatiles emitted by these plants were in trace quantities though comparable and comprised mainly the green leaf volatile, *(Z)*-3-hexenyl acetate and the isoprenoids *(Z)*-4,8-dimethyl-1,3,7-nonatriene and caryophyllene. Comparatively, larger odor quantities were emitted by Napier grass than sorghum or maize. No correlation was evident between the amount of volatiles emitted and insect orientation to the host plants according to the wind tunnel results obtained in another study.

Water-soluble compounds from the surface of maize, sorghum and Napier grass did not stimulate *B. fusca* oviposition. Gas chromatographic analyses of the water extracts and comparison with retention times of authentic standards indicated the presence of simple sugars, amino acids and organic acids in the extract in similar composition among the plants species analysed, confirming the non-involvement of such compounds in oviposition

stimulation of *B. fusca*. In contrast, chloroform-soluble compounds from the surface of maize and particularly sorghum were stimulatory to oviposition but no stimulation was observed when using Napier grass extracts. Preliminary analyses of these extracts by HPLC-MS revealed significant difference in the number of compounds present in these chloroform extracts. Apart from the common compounds present in all the three extracts, sorghum had three more compounds with two eluting at low and another at a higher acetonitrile concentration. The compound in sorghum extract that eluted at high acetonitrile concentration was tentatively identified as a high molecular weight of intermediate polarity. These results suggest that oviposition stimulation in *B. fusca* is mostly influenced by chloroform-soluble compounds from the surface with low or medium polarity.

Chapter 1
General introduction

Cereals crops particularly maize, sorghum and millet are the most important staple food crops grown in wet and cool climates of the middle and high altitudes of Africa (Harris & Nwanze, 1992; FAO, 1995). Approximately sixty million hectares of land are under cultivation of these crops yearly (FAO & ICRISAT, 1996). These crops are generally cultivated by resource-poor, small-scale farmers (Kfir, 1998; Seshu Reddy, 1998), primarily for human consumption and surpluses may be used for feeding livestock (Sibanda, 1985). It is estimated that three quarter of the total production of these crops is consumed (FAO & ICRISAT, 1996). Though there has been a significant advancement in technology towards production of cereals in Africa, the productivity is hampered by several abiotic and biotic constraints, which may cause losses up to 80**%** (Pinstrup-Andersen *et al.*, 1999; Pingali & Pandey, 2001). Abiotic constraints in this region are mostly due to seasonal unreliability of rain-fed agriculture, poverty and limited access to remedial inputs. Biotic stress, which include diseases and pests, are ever present, and require effective management processes to support productivity and environmental protection.

In tropical Africa, several insect species are known to damage these crops (Ingram, 1958; Seshu Reddy, 1991). Lepidopteran stem borers are far the most important insect pests for these crops because they are ubiquitous and far the most injurious (Ingram, 1958; Nye, 1960; Youdeowi, 1989). These insects infest many graminaceous crops including maize, millet rice, sorghum and sugarcane, from seedling stage until maturity. With the exception of sugarcane, these are often the subsistence food crops in the developing countries. It is reported that the yield losses of maize and sorghum caused by stem borers vary widely in different regions depending on the pest population density and phenological stage of the crop at infestation. Recent estimates of yield losses due to stem borers alone in sub-Saharan Africa are in the neighbourhood of 20-40 percentage of the potential yield (Warui & Kuria, 1983; Youdeowi, 1989, Seshu Reddy & Walker, 1990).

These losses indicate the importance of stem borers as a limiting factor affecting crop productivity in this region.

Twenty-one species of lepidopteran stem borers that infest cultivated grasses have been identified and includes 7 noctuids, 2 pyralids and 12 crambids (Maes, 1998). Among them, two crambids *Chilo partellus* Swinhoe, *Chilo orichalcociliellus* Strand, one pyralid *Eldana saccharina* Walker, and two noctuids *Sesamia calamistis* Hampson and *Busseola fusca* (Fuller), are by far economically the most important pests of cereals in Eastern and Southern Africa (Seshu Reddy, 1998; Kfir, 1998). These species infest a large number of economically important plants such as maize, sorghum, sugarcane, rice and millet.

Most cereal stem-borers of maize and sorghum are generally polyphagous and have several cultivated and non-cultivated graminaceous host plants (Seshu Reddy 1983; Khan *et al*., 1991).

During off season when there are no cultivated plants in the field, the stem-borers remain present on wild host plants, or hibernate in crop residues and subsequently infest the cultivated hosts after planting (Van Emden, 1971). These wild habitats, often harbour food sources for these insect species and may encourage their outbreak in the neighbouring agro-ecosystems (Seshu Reddy, 1991). On the contrary, the adjacent wild habitat can be used to suppress insect outbreak by keeping the pest on the so-called wild traps (Khan *et al*., 1997; 2000). In addition, intercropping host and non-host plants may also support lower insect pest levels than the corresponding monocultures (Ampong-Nyarko *et al.,* 1994). Hence a more complete understanding of the role of such wild hosts and non-hosts in determining outbreak of stem-borers can generate suitable management strategies of the stem borers.

Control against stem-borers, where this is carried out, has been mainly by the use of insecticides. However the social and environmental considerations like resistance or tolerance to insecticides increased costs, health hazards and ecological imbalances have encouraged scientists to search for alternative methods of crop protection (Saxena, 1989). The use of resistant cultivars has been one such approach (CIMMYT, 2002). Developing a new strategy to protect crops against insect pests by the use of non-polluting and specific method has been highly recommended but requires a basic knowledge of insect-plant relationships.

It is well documented that different behavioural responses shown by an insect that lead to the selection of its host plant for feeding and / or oviposition are influenced in part by plant semiochemicals. For example, the cyclic hydroxamic acid (2,4-dihydroxy-7-1,4-benzoxazin-3-one or DIMBOA), a secondary compound produced by young maize leaves, has been recognised as the major feeding deterrent to the European corn borer *Ostrinia nubilalis* (Hubner). DIMBOA inhibits the digestive proteases of the insect larvae thereby interfering with its growth and development (Klun & Robinson, 1969; Robinson *et al*., 1978). In relation to oviposition, it has been reported that while pentane maize leaf extracts stimulate oviposition the corresponding methanolic extracts deter oviposition in both *S. nonagrioides* and *O. nubilalis* (Udayagiri & Mason, 1997; Konstantopoulou *et al*., 2002). In addition, volatiles semiochemicals emitted from plants also mediate interactions between Lepidopteran insects and their host plants particularly in oviposition. For example, a volatile semiochemical, α-terpilone emitted by *Melinis minutiflora* P. Beauv. (Poaceae) a non-host plant of maize stemborers is reported to deter oviposition in *B. fusca* and *C. partellus* while eugenol emitted by maize is attractive to the ovipositing moths (Khan *et al*., 2000). These findings imply that semiochemicals can be exploited to manipulate the oviposition behaviour of maize stem borers in the field and hence can be used in the overall pest management programmes. Stimulatory plant volatiles and surface extracts may be used to disrupt oviposition behaviour or to attract moth females away from valuable crops. In this context, habitat-management strategies involving the use of "push-pull" or stimulo-deterrent diversionary tactics have been tried in effort to control maize stemborers in the field (Khan *et al*., 2000). Using this strategy, the stem borers are attracted and retained on trap plants (pull) planted as border rows, while at the same time repellent intercrops (push) prevent the insects from infesting the maize crop. It has been suggested that the efficiency of this strategy partly relies on the interactions between the insect pest and the plant semiochemicals. Hence, adoption and efficient utilisation of the strategy needs in part, a good understanding of the host plant semiochemicals involved in the host selection and oviposition of the insect pest.

Hence this study sought to determine and evaluate the role of host plant metabolites (volatiles and contact –*i.e.* non-volatiles-) in the host selection for oviposition of *B. fusca.*

1.1-Hypothesis

Our null hypothesis was that undamaged graminaceous plants produce taxon-related metabolites with only slight variations. These variations are exploited by gravid moths during their preferential selection of host for oviposition.

1.2-Rationale

The review of the oviposition allelochemicals of the maize stalk borer *B. fusca* shows that no detailed and methodical study has been carried out to identify the chemicals from host graminaceous plants, that stimulate oviposition of the gravid female insects. Furthermore, the available data on this subject suggests that no work has been undertaken to demonstrate the relationships between host plant semiochemicals and differential oviposition preferences as shown by this species to different host plants.

Hence the present study entailed to characterize host chemicals involved in eliciting oviposition and also to develop an oviposition bioassay that allowed the effects of these semiochemicals to be investigated independently from physical stimuli associated with the common host plants of *B. fusca.* Data on the chemical compounds involved in host selection for the oviposition could be used to select putative trap or intercrops or used to disrupt the oviposition of *B. fusca* in the field and hence contribute to the development of an efficient 'push-pull' strategy for the effective management of the species populations in the field. In addition, the physiological and biochemical process involved in insect orientation to these semiochemicals may open up the possibility of enhancing crop yields by development of insect resistant cultivars through, selective breeding or even molecular manipulation.

1.3-Aim

The purpose of this study was to determine the relative roles played by both host plant volatile and surface metabolites in the overall host selection process for oviposition by *B. fusca*.

1.4-Objectives

This study was undertaken with the following objectives:

1 To compare differences in the host selection process for oviposition between artificially mass-reared and wild insect populations;

2 To assess the role of volatiles and surface plant metabolites in host selection process for the oviposition of *B. fusca.*

Chapter 2
Literature review

2.1-*Busseola fusca* (Fuller)(Lepidoptera: Noctuidae)

The African maize stalk borer*, B. fusca,* is a noctuid moth closely related to the genus *Sesamia*. Its larvae feeds inside the stems of grasses and cereal crops, especially maize and sorghum. It was first classified and named by Fuller in 1901 (see Harris & Nwanze [1992] for review). It is known to be a species indigenous to Africa and is not known to occur anywhere outside the Africa continent, although there must be some danger that it could be accidentally introduced elsewhere. It occurs widely in mainland Africa, south of the Sahara, but not on the Islands of Indian Ocean.

Recently, 307 individuals of this insect species were collected during growing season of maize and cultivated sorghum from 13 West African localities, 3 localities from Central Africa, and 13 localities from East Africa. A fragment of the gene coding for cytochrome *b* was sequenced from these individuals. Complementary methodological approaches were used to develop and test hypotheses about the history and demographic structure of this species. According to phylogenetic and Nested Clade Phylogeographical (NCPA) analyses, the species populations can be separated into three mitochondrial clades: one from Western Africa, and two designated Kenya I and Kenya II, from Eastern and Central Africa. The similarity of intraclades and interclades distances observed in these three large population units, suggests that they were isolated at the same period in three different refuges from subsaharan Africa and have had comparable demographic history. Notably, sorghum domestication, maize introduction in Africa and therefore the switch from wild to cultivated plants do not seem to have influenced the mitochondrial geographic patterns observed in the species (Sezonlin *et al*., unpublished data).

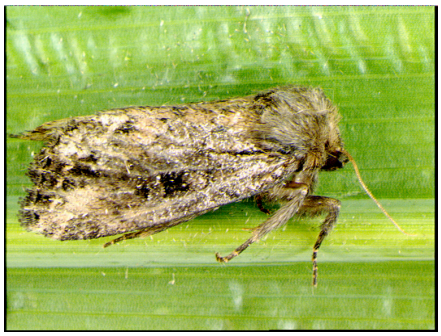
2.2-Pest status

B. fusca larvae cause major yield losses in subsistence cereal production throughout sub-Saharan Africa. Feeding and stem tunnelling by larvae on plants results in crops losses as a consequence of destruction of the growing point, early leaf senescence, interference with translocation of metabolites and nutrients that results in malformation of the grain, stem breakage, plant stunting, lodging and direct damage to ears (Appert, 1970; Bosque-Perez & Mareck, 1991; Kfir, 1998). In addition, infestations by borers increase the incidence and severity of stalk rots (Bosque-Perez & Mareck, 1991). Maize cobs may be directly damaged by tunnelling larvae, while tunnelling and breakage of peduncles may indirectly affect grain development of sorghum and pearl millet. The insect posses a great threat to cultivated grasses like sorghum, maize and pearl millet, and some wild grasses like Napier grass. It is estimated that the percentage loss accruing from the pest damage ranges in the region of 10-39% in southern Africa (van den Berg & Ebenebe, 2001).

2.3-Biology of *B. fusca*

The females oviposit round flattened eggs (see eggs in Figure 1) under the inner surfaces of leaf sheaths in batches of 30 – 100 eggs (see Harris & Nwanze [1992] for review). On average, a gravid female lays up to 200 eggs. The larvae hatch about a week later, and initially disperse over the host plants before they enter the leaf whorls and start to feed on the leaves. After establishment on the plant surface, these larvae bore into stem tissues for feeding and produce extensive tunnels in stems and in maize cobs. The larval period varies between 24 and 45 days depending on temperature, presenting six to eight larval instars (see larva stage in Figure 1). Adults emerge 9 to 14 days after pupation (see male and female adults in Figure 1). It is reported that generally two generations occur before the maturation of maize crop within 6 months cycle (Harris, 1962). The first generation attacks young maize or sorghum causing severe damages while the second generation attacks the crop later causing stem and ear damages. Some larvae at the second generation do not complete their development before the end of the cropping season. They undergo diapause in the stem tunnels until the onset of rains when they pupate.

Okuda (1990) demonstrated that water contact is a significant factor terminating larval diapause of *B. fusca*. The complete life cycle of *B. fusca* is around 66 days in the rainy season and increases up to 200 days during the dry season (Ingram, 1958).



Female

Male

Eggs

Larva

Figure 1. Photographs showing *B. fusca* at adult stage (both female and male), the eggs after oviposition on maize and also *B. fusca* at larval stage (Photos P. Zagatti).

2.4-*B. fusca* distribution in Africa

B. fusca is primarily a pest in the dry savannah zone of Africa. It occurs throughout mainland Africa, south of the Sahara and has been formally recorded in West, East and in Southern Africa (see Harris & Nwanze [1992] for review). The distribution status of *B. fusca* varies by region. In West Africa it occurs from sea level to an altitude of in excess of 2000 m, and is mostly abundant in the wetter parts of the tree savannah in Ghana

(Tams & Bowden, 1953) and Burkina Faso (Nwanze, 1988), and in the drier regions of the tree savannah and thorn-scrub savannah of Nigeria (Harris, 1962) where sorghum is extensively cultivated. In Eastern and southern Africa, *B. fusca* is generally a pest of higher altitudes, occurring at altitudes between 600 and 2700 m (Sithole, 1989). *B. fusca* is the dominant stemborer at altitudes above 900 m in Southern Africa but it also occurs at lower altitudes, indicating the ability of this insect to adapt to low-lands and warmer areas (Sithole, 1989). Though some studies have pointed out the absence of the species in the coastal areas of Kenya and Tanzania (see Harris & Nwanze [1992] for review), some *B. fusca* populations have been recently found in the coastal area of Kenya, suggesting that this insect can spread out in more areas than those earlier reported (B. Le Rü, personal communication).

2.5-Host plants for *B. fusca*

Most cereal stemborers are generally polyphagous and have several host plants (Ingram, 1958; Seshu Reddy, 1983). The major host plants for stemborers in Africa are the Gramineae (Poaceae) family. Other lesser preferred host plant families are the Cyperaceae and Typhaceae (see Polaszek & Khan [1998] for review).

Comparison of number of borers according to species found on some common host plants and across all wild host plant species in Kenya showed some discrepancies between findings by Randriamananoro (1996), Khan *et al.* (1997) and collection by Le Rü *et al*. (2004). From the latter, *B. fusca* appeared to be exceedingly rare on wild hosts (Table 1). On the other hand, *Chilo* spp. and *S. calamistis* were either found to be absent on some of the hosts described by Randriamananoro (1996), and Khan *et al.* (1997) and on some hosts they were much more common than found by these authors. These results indicate that most stemborer species are localized and specialized attacking only a narrow range of host plant species (Table 1). In particular they indicate that *B. fusca* is nearly absent on wild grasses, that more than 95% of *C. partellus* were found on wild sorghum and 71.3% of *C. orichalcocillielus* were found on *Panicum sp* and the host range appeared to be wider than for *C. partellus*.

Some cultivated graminaceous host plants for stemborers include sorghum, maize, millet, rice and sugarcane (Table 2). Sorghum and millet are native to Africa while maize, rice and sugarcane are exotic to Africa. Maize originated from Central America, probably within the area equivalent to modern Mexico while sugarcane and rice are originated from Asia. Although maize has existed in Africa for over 300-400 years, this crop is still considered a relatively recent introduction in comparison to the more traditional African cereals, such as millet and sorghum in terms of both agro-ecology and insect plant co-evolution (Polaszek & Khan, 1998). Due to its recent introduction into Africa, maize is highly damaged by native stem borers such as *B. fusca* as compared to the native cereals like sorghum that share a much longer common history with the local phytophagous insects.

Table 1. Comparison of number of borers found according to species on several common host plants between Randriamananoro (1996) and Khan *et al.* (1997) (plain), and Le Rü *et al*. (2004) (bold).

					Other borer species		
	C sp	Bf	Sc	Es	Pyr	Noct	# species
Sorghum sp 1	512 **1815**	432 **13**	28 **25**	1 **0**	**65**	**48**	**8**
Panicum sp 1	36 **274**	11 **0**	11 **5**	0 **0**	**505**	**1481**	**5**
Panicum sp 2	0 **3**	40 **0**	0 **0**	0 **0**	**1**	**251**	**6**
Pennisetum sp 1	63 **8**	181 **2**	79 **6**	0 **0**	**27**	**520**	**5**
Pennisetum sp 2	8 **70**	1 **0**	3 **67**	0 **0**	**0**	**95**	**3**
Echinochloa sp 1	23 **0**	86 **0**	43 **2**	0 **2**	**33**	**153**	**8**
Rottboellia sp 1	80 **41**	1 **0**	8 **1**	0 **0**	**52**	**0**	**2**
Phragmites sp 1	0 **0**	0 **0**	5 **0**	0 **0**	**0**	**10**	**2**
All wild host plants together	1077 **2183**	893 **15**	591 **170**	482 **25**	**1138**	**5083**	**75**

C sp. *Chilo* sp, Bf *B. fusca*, Sc *S. calamistis*, Es *E. saccharina*, Pyr Pyralidae, Noct Noctuidae, # species number of species

Table 2. Relative importance (number in plain and percentages in bold) of the 4 main lepidopteran stemborers species in cultivated and wild plants in Kenya (Le Rü *et al*., 2004)

	Bf	Sc	Cp	Co
Maize, sorghum, finger and candle millet, sugar cane	22695	2383	34835	408
	37.6	**4.0**	**57.7**	**0.7**
All wild grasses	15	170	1853	330
	0.6	**7.2**	**78.3**	**13.9**
Sorghum sp1	13	55	1812	13
	0.7	**2.9**	**95.7**	**0.7**

Cp *C. partellus,* Co *C. orichalcocillielus*, Bf *B. fusca*, Sc *S. calamistis*

2.6-Management of *B. fusca*

Various pest management strategies have been adopted in attempt to reduce the losses due to the insect pests. These includes chemical control, biological control, use of host plant resistance, cultural practices and the recently ‘’push-pull” strategy.

2.6.1-Chemical control

The use of insecticides in pest control provides curative control, as their effect on the pest is instant (Warui & Kuria, 1983). These authors demonstrated that selected chemicals treatment timed appropriately reduced losses caused by all stem borer species in maize by about 20%. However, their use is difficult to justify in low input agricultural systems because of high costs (see Kfir *et al*. [2002] for review). In addition, effective chemical control is hampered by the biology of the borers. Timing of insecticide sprays is crucial as their insecticidal activity is only effective against young borers. Older borer larvae penetrate the stalks and are not reached by conventional insecticides (Kfir, 1998). Therefore regular application may be required which the resource poor farmers cannot

afford (van den Berg & Nur, 1998). The use of insecticides is also not generally favoured in a durable pest management system due to eco-toxicity and insect resistance reasons.

2.6.2-Biological control

Classical biological control involving use of exotic parasitoids has been used as a strategy for controlling populations of moth pests in the tropics. The success of classical biological agents is generally attributed in part to the high searching efficiency of the natural enemy for its hosts (Waage, 1990). An important exotic parasitoid of stemborers, *Cotesia flavipes* (Cameron), introduced in Kenya in 1993 for the control of *Chilo partellus* (swinhoe) has shown varied degree of success in the control of insects (Overholt, 1998). However, *B. fusca* seems to be unsuitable host for the parasitoid. Laboratory studies indicates that although *C. flavipes* can search for, and attack *B. fusca* (Ngi-Song *et al*., 1996; Ngi-Song & Overholt, 1997), it is not able to complete its development in this host since all the parasitoid eggs become encapsulated (Ngi-Song *et al.,* 1995, 1998). *Cotesia sesamiae* (Cameron) has been reported as the predominant larval parasitoid of *B. fusca* in most parts of Africa (Polaszek & Walker, 1991; Kfir, 1992; Kfir & Bell, 1993*;* Omwega *et al.,* 1995; Kfir 1995; Kfir, 1997). However, other studies (Ngi-Song *et al.,* 1995; 1998; 2000) reported that *C. sesamiae* can not successfully develop in *B. fusca* populations originating from the coastal area of Kenya. Although there appears synergistic parasitism of *B. fusca* by *C. sesamiae* and *C. flavipes* (Ngi-Song *et al.,* 2001), these parasitoid species have not been shown to be efficient in the field.

Figure 2. Photograph showing a parasitoid, *Cotesia flavipes*, stinging a stem borer larva (Photo A. J. Ngi-Song).

2.6.3-Host plant resistance

Host plant resistance is another management strategy that has been tested to control lepidopteran moths (see Kfir *et al*. [2002] for review). Host plant resistance as an approach to pest management in graminaceous crops confers many advantages. Resistant crop varieties provide an inherent control that involves no environmental problems, and they are generally compatible with other insect control methods. Resistant varieties control even a low pest density, whereas insecticides use is justifiable only when the density reaches the economic injury level. Unfortunately host resistance is fraught with resistance erosion (Schumann, 1991) and besides no cultivars with sufficient levels of host plant resistance to insect have been identified. However efforts are underway in Africa to identify sources of *B. fusca* resistance in cereal crops (CIMMYT, 2002).

2.6.4-Cultural practices

Cultural control is the most relevant and economic method of stem borers management available for resource poor farmers in Africa. It includes many tactics among others, crop rotation, intercropping, management of crop residues, manipulation of planting dates, use of trap crops and the management of wild host plants. Though many cultural practices are labour intensive, they have less adverse effects on the environment and are readily available without extra investment on equipment. However, none among the cultural

methods has been shown to be efficient and therefore reliable in the control *B. fusca* populations in the field (Nwanze & Mueller, 1989; Skovgård & Päts, 1996; Grisley, 1997). In addition, most African farmers lack management capabilities to adopt the cultural control methods (Harris, 1989).

2.6.5-The "push-pull" strategy

This strategy involves the use of semiochemicals and other methods that manipulate insect behaviour in the management of stemborers. Semiochemicals are chemical compounds that act as signals to modify insect behaviour or development. Semiochemicals are reported to be widely used in the control of insect pests (Smart *et al*., 1994; Pickett *et al.,* 1997; Khan *et al*., 2000). However, semiochemicals often give ineffective or insufficiently robust pest control when employed alone. Use of semiochemicals should therefore be combined with other approaches in integrated management strategies that involve population reducing agents such as pesticides or biological control agents. The main components of such strategies are pest monitoring (to allow accurate timing of treatments), combined use of semiochemicals, host plant resistance and trap crops (to manipulate pest behaviour) and selective insecticides or biological control agents (to reduce pest populations).

Based on the understanding of the volatile semiochemicals employed by he stemborers in locating suitable hosts and avoiding non-host plants, a novel and highly promising integrated pest management strategy has been developed to control these pests in East Africa (Pickett *et al.*, 1997; Khan *et al*., 2000).

This habitat management system often referred to as the "push-pull" or stimulo-deterrent diversionary tactics involves 'pushing' the insects away from the harvestable, agronomic crops, and pulling them onto trap crops where their populations are reduced by biological control agents or a highly specific but slow acting pesticide. Hence the strategy involves combining a harvestable agronomic crop, trap crop, and intercrop. Host masking agents, repellents, anti-feedants, oviposition deterrents or compounds associated with plant defence protect the harvestable crop from attack by the insect (push). At the same time, aggregative semiochemicals, including host plant attractants and sex pheromones,

stimulate colonisation of the 'pushed' pests on the nearby trap crops or entry into traps where pathogens can be deployed (pull). The individual components of the 'push-pull' strategy are not in themselves highly efficient, and therefore, do not select for resistance as strongly as conventional toxicant pesticides, making the approach intrinsically more sustainable (Pickett, 1998).

Therefore by combining this approach with other approaches in the integrated management strategies, a robust and efficient control method for cereal stemborers can be developed.

Using three graminaceous plant species, maize (*Zea mays)*, sorghum (*Sorghum bicolor)* and Napier grass (*Pennisetum purpureum*), it was shown that under laboratory conditions gravid females of *B. fusca* preferred to oviposit on maize and sorghum over napier grass (Le Rü *et al.*, 2004). In this study it was suspected that both host plant semiochemicals and plant physical characteristics were involved in the preferential choice of hosts and hence could be key factors involved in the "push–pull" strategy.

2.7-Host selection for oviposition in Lepidoptera

Various sensory cues are generally involved in host plant location and assessment by an insect (Bernays & Chapman, 1994). Before landing on the host, visual and olfactory cues play essential roles (Prokopy & Owens, 1983; Rojas & Wyatt, 1999), whereas after landing, olfactory and tactile cues seem most crucial (Harris & Rose, 1990; Foster *et al*., 1997; Hora & Roessingh, 1999).

Among polyphagous insects, host selection (host-finding and host acceptance) is limited to a few potential host plants. Host preferences show a strong heritable component trait and are thought to represent the suitability of hosts for larval survival (Singer, 1983; Courtney *et al*., 1989; Singer *et al*., 1989; Thompson, 1998). Suitability can depend on a number of factors such as nutritional quality, host plant defence chemicals, prevalence of natural enemies or micro-environment (Thompson & Pellmyr, 1991).

In most lepidopteran insects, host selection is a function of the adult female (Renwick, 1989). Oviposition in such species is crucial as progeny survival is dependent on

recognition by the adult females of host plants that are suitable for larval development (Städler, 1992; Hamilton & Zalucki, 1993).

Lepidopteran cereal stemborers are known to prefer certain plant species, growth stage and parts of the plant for oviposition (Phiri, 1995; Schulthess *et al*., 1997). For example, it has been observed that the ovipositional response of *B. fusca* is selective and is related to the plant age and the host plant species, probably reflecting variation in phytochemical composition and concentration among these plant species. Among the graminaceous plants, maize plants are most attractive to the ovipositing moth 3-6 weeks after germination of the crop (van Rensburg *et al*., 1987). Additionally, the insect prefer to oviposit on the young, fully, unfolded leaf sheath of the healthy and vigorously growing plants (van Rensburg *et al*., 1989). Among several crop species *B. fusca* shows a strong oviposition preference to *Sorghum sudanense* and *Zea mays* but is repelled by *Melinis minutiflora* and legumes (Adesiyun, 1983; Khan *et al*., 2000). Differential oviposition phenomenon displayed by many Lepidoptera towards host selection may hence partly arise from the presence of phytochemical cues in host plants (Udayagiri & Mason, 1995; 1997; Jallow *et al*., 1999). Oviposition preference studies conducted in East Africa using maize (*Zea mays)*, sorghum (*Sorghum bicolor)* and napier grass (*Pennisetum purpureum* demonstrated that plant phenology (stem diameter, plant surface texture and leaf sheath toughness) (Le Rü *et al*., 2004) and phytochemicals (Pickett *et al*., 1997; Khan *et al.,* 2000) are both important for *B. fusa* oviposition.

Therefore, identification of such host plant chemicals that act as oviposition attractants and stimulants for insect species is of economic importance due to their potential for use in the manipulation of pest behaviour in the field (Udayagiri & Mason, 1997).

2.8-Phytochemicals mediating host plant selection for oviposition in Lepidoptera

Studies on phytochemicals mediating host plant location in phytophagous moths are scarce in comparison to those involved in mate-finding behaviour (Honda, 1995). Orientation to and recognition of hosts by ovipositing females are guided and governed largely by secondary plant metabolites, though nutritional profiles and water content of individual plants also exert some effects on the final decision by the females.

It has been demonstrated that both plant surface chemicals and volatiles are involved in the host selection and oviposition behaviour of Lepidopteran insects (Hilderbrand, 1996; Udayagiri & Mason, 1997; Jallow *et al*., 1999; Khan *et al.,* 2000; Konstantopoulou *et al*., 2002). The chemical information is received during the sequence of oviposition behaviour by chemoreceptors located on the antennae, tarsi, ovipositor or proboscis and is transmitted together with other sensory inputs to the central nervous system for further processing (Ramaswamy 1990; Marrion-Poll & Tobin, 1992). Both plant volatile and surface metabolites are thought to be involved in oviposition response of various Lepidoptera.

2.8.1-Plant volatiles

Plants usually release a variety of hydrocarbons during periods of high temperatures (Sharkey & Singsaas, 1995). The volatile chemical profile released is time dependent and is related to the physiological state of the plant. A large number of compounds have been identified in the collection of headspace volatiles from different plant species. Included in the list are fatty acid derived aldehydes and alcohols (Croft *et al*., 1993), terpenes derived from mevalonic acid pathway, and aromatic metabolites such as indole and methyl salicylate derived from shikimic acid pathway (Mann, 1987). Although intact plants release volatiles, their release rate is much lower (see Schoonhoven *et al*. [1998] for review). Great amounts of plant volatiles are often released upon plant damage. Upon herbivore damage however, the blend of volatiles radically changes from those released by intact plants with the surge of individual constituents (Takabayashi *et al*., 1995) and quantities (Agelopoulos & Keller, 1994). For example, several compounds, particularly green leaf volatiles, (3*E*)-4,8-dimethyl-1,3,7-nonatriene and (*3E,7E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene and to a lesser degree linalool, ocimenes and farnesenes are induced in many plant species as a result of herbivore damage (Ngi-Song *et al.,* 2000). The types and relative amounts of volatiles emitted by plants are usually unique for a given plant species (Bernays and Chapman, 1994; Schoonhoven *et al.,* 1998).

2.8.1.1-Insect olfaction

An insect olfaction appears to play an important role in host finding and selection behaviour in Lepidoptera (Visser, 1986). A variety of phytophagous insects use plant odors as cues for the orientation to food sources either for their own nutrition, oviposition or for avoidance of unsuitable hosts (Schoonhoven *et al.,* 1998). The characteristic of insect olfactory hairs or sensilla is generally multiporous. The entire sensillum wall or plate is perforated by up to thousand of pores for trapping volatile compounds. Olfactory sensilla are predominantly present on antennae but also on palpi and ovipositor. The role of plant odors in host selection can be clearly traced from the orientation manoeuvres of the insect towards particular plants, and in the ultimate recognition of host plants for feeding and or oviposition. Though, plant odors are often composed of tens of hundreds of compounds, studies of insect olfaction reveal that only a minority of the components in the complex odor blend are detected by the insect antennae (Jonsson & Anderson, 1999; Zhang *et al.,* 1999; Cossé & Baker, 2000). The olfactory receptors systems of the insects enables the insect to perceive some of the plant odors, which then compile an odor that acts as a chemical message. This chemical signal among other factors guides and orients the insect to its particular host for feeding or oviposition. Several studies have reported the importance of plant volatile semiochemicals in orienting many lepidopteran insects to their prospective hosts either for feeding or oviposition. Headspace volatile collections of some common graminaceous host plants including maize, sorghum and napier grass are reported to elicit some behavioural response of *B. fusca*.. A total of 45 compounds have been isolated and identified from these plants and among them, (*Z*)-3-hexen-1-ol, 3-methylbutyl acetate, alpha-pinene, 6-methyl-5-hepten-2-one, octanal, (*Z*)-3-hexenyl acetate, alpha-terpinolene, nonanal, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, methyl salicylate and decanal were identified to be electrophysiologically active on *B. fusca* antennae (Khan & Hassanali, 2004).

2.8.1.2-Isolation and analysis of plant volatiles

In studies of insect-plant interactions, it is essential to identify and quantify the chemicals mediating those interactions to elucidate the factors affecting their release, and to obtain information on the time course of their emission. The techniques used to collect plant volatiles should provide all the above information without disturbing the plant. Handling of plant material before and during the collection of volatiles is crucial. Techniques requiring maceration or chopping of the plant material followed by extraction and distillation, allow enzymatic and chemical reaction to occur that causes changes in volatile composition of the sample (Blight, 1990). Although such procedures are useful for the isolation of large amounts of specific chemicals, they do not provide information on what is emitted by intact plants. Furthermore practices that involve cut stems, detached leaves or uprooted plants placed in water (Dicke *et al.,* 1990; Whitman & Eller, 1990; Croft *et al.,* 1993; Loughrin *et al.,* 1995; Takabayashi *et al*., 1995; Geervliet *et al*., 1997) may also alter the chemistry of the plant. Mechanical injury of plant tissue also induces changes in the chemistry of intact plants (Pare & Tumlinson, 1997). To overcome these factors researchers have used potted plants enclosed in large entrainment chambers, thus avoiding injury to the plant (Blaakmeer *et al.,* 1994; Bolter *et al.,* 1997). In such systems plants suffer no physical injury, but contaminants released from pot soil, or micro-organisms inhabiting the soil may mask part of, or contribute to the volatile profile recorded. This has necessitated the design of specially entrainment chambers (Heath & Manukian, 1992; Agelopoulos & Keller, 1994; Heath & Manukian, 1994; Jacobsen & Olsen, 1994; Tollesten & Müller, 1996; Agelopoulos *et al*., 1999).

The techniques employed in chemical ecology for the isolation, identification and quantification of volatile semiochemicals are the common pre-concentration techniques used for headspace analysis in gas chromatography, modified specifically to serve the needs of the living organisms under study (Charmas *et al.,* 1994; Thomas, 1991). Routinely, dynamic headspace analysis is employed, although static headspace analysis is sometimes used (Jacobsen, 1997). In dynamic headspace analysis, the sample is confined in an entrainment chamber and a carrier gas (usually purified air) is passed over the sample. The volatile chemicals released by the sample are carried by the gas to a solid

trap, usually a porous organic polymer such as porapak Q, 'Tenax TA'' or activated charcoal, where the analyte are adsorbed and concentrated. The desorption of the analyte from the solid trap for gas chromatography can be achieved either by elution with a solvent (solvent desorption) or rapid heat treatment (thermal desorption). In static headspace analysis, the sample is tightly closed into a vessel, where it comes into equilibrium with its vapour at a pre-determined temperature. The headspace can be sampled using a syringe or a similar device and injected directly into the gas chromatograph (Agelopoulos & Pickett*,* 1998).

Advantages associated with solvent desorption are that it results in a liquid sample that can be stored in the freezer, sealed under nitrogen in glass ampoules, and used a number of times when replication is required such as coupled runs of gas chromatography coupled with electro-antenno-graphy detection (GC-EAD) (Wadhams, 1990), peak enhancements co-injections (Pickett, 1990) and bioassays. Quantification is also facilitated by having a liquid sample in which an internal standard can be readily added. However, only a small volume of the sample can be injected at a time into the gas chromatography (GC), and the solvent peak can mask compounds with short retention times. On the other hand, thermal desorption, although more sensitive since all sample goes through GC column, results in no sample at the end of the run. Other problems associated with thermal desorption are the difficulty of introducing an internal standard for quantification, a problem associated not only with thermal desorption of polymers but also when using a syringe or a solid phase micro-extraction (SPME) fibre, and the temperature of desorption may affect the thermal stability of some analytes.

Solid phase micro-extraction (SPME) is a new isolation method that has been used to extract and concentrate a wide range of volatile and semi-volatile organic compounds from various matrices such as air, water and soil (Berlardi & Pawliszyn, 1989; Arthur & Pawliszyn, 1990; and Zhang & Pawliszyn, 1993). The technique was developed for sampling organic contaminants in water by directly immersing the fibre into the sample, but it has also been applied with success to sampling headspace of liquid and solid samples. SPME has also found applications in environmental and flavour analysis (Steffen & Pawliszyn, 1996) and has also been used for recording the release of insect pheromones (Molasse *et al.,* 1995; Frérot *et al*., 1997). The technique uses a polymer-

coated fused silica fibre that has the ability to absorb chemicals relying on a three-phase equilibrium between the sample, its vapours, and the fibre. While the fibre is in contact with air-borne volatiles, the volatiles partition from the sample matrix into the stationary phase until equilibrium is reached. The fibre is then inserted into the injector port of a gas chromatograph where it is heated, and the volatiles are rapidly thermally desorbed into a capillary GC column for analysis.

Gas chromatography is the technique of choice for the separation and analysis of thermally unstable and volatile organic plant compounds. Separation is accomplished by partitioning the components of a chemical mixture between a moving (mobile) gas phase and stationary phase of a high boiling point liquid material such as silicone grease held on an inert granular solid. Since the partition coefficients are different, the individual components are carried along the column at different rates and emerge from the opposite end of the column in distinct 'zones'. A detector such as flame ionisation detector (FID), thermal conductivity detector (TCD), Electron capture (ECD) is present at the exit of the separation column of the GC from which the electronic signal is fed into a recorder which draws out a set of peaks corresponding to the quantitative percentage of each component present in the test mixture. The retention time of the test chemical is compared with that of the known compound for identification (Hoshika, 1981).

The combination of gas chromatography with mass spectrometry gives another powerful method for analysis and characterisation of complex volatile mixtures. Mass spectra obtained rapidly on compounds emerging from a gas chromatographic column afford informative correlation of mass spectrometric and chromatographic data. Full use is made of the separating power of GC together with structural information derivable from mass-spectrometer (MS). This gives exceptional power of discrimination between closely related structures. Molecules are identified by comparing the spectra of the unknown with those of the reference collection (NIST, NBS or Willey libraries) to find out ions of similar M/e value and relative abundance.

2.8.2-Plant surface chemicals

As outlined by Schoonhoven *et al*. (1998), the first contact between an insect and a plant occurs when the insect lands or otherwise touches the plant surface. Numerous insects species are known to explore the leaf surface before feeding (Woodhead & Chapman, 1986; Chapman & Bernays,1989; Bernays & Chapman, 1994) or ovipositing (Städler, 1986; Städler & Roessingh, 1991; Renwick & Chew, 1994) on suitable host plant. Combinations of both physical and chemical stimuli influence the evaluation process of the leaf surface by the insect. Plant glandular structures and secretions, leaf epicuticular waxes (fatty acids, alkanes, primary alcohols esters of the alcohols, aldehydes, ketones) (Eigenbrode & Espelie 1995), non-polar substances (generally secondary compounds extractable in organic solvents from the waxes) (Städler, 1992), water soluble secondary metabolites embedded in epicuticular waxes such as flavonoid glycosides and primary metabolites such as sugars, amino acids leaking out of waxes (Derridj *et al* 1989; Fiala *et al*., 1990) are perceived by the insects through the leaf surface. Some of these compounds are individually or synergistically involved in stimulating or inhibiting feeding and or oviposition in various insect species (Derridj *et al.,* 1989). The role of contact phytochemicals in eliciting oviposition of phytophagous insects has been reported in many studies (Kumar, 1986; Udayagiri *et al.,* 1995, 1997; Derridj *et al.,* 1996; Jallow *et al*., 1999). Primary metabolites-simple sugars (mostly sucrose, fructose and glucose) and free amino acids present on maize leaf phylloplane stimulate oviposition behaviour of the polyphagous European corn borer *Ostrinia nubilalis* (Derridj *et al*., 1989; 1996). It is also reported that non-polar, low volatile, *n*-alkane plant surface compounds elicit oviposition in *Ostrinia nubilalis* (Renwick *et al*., 1992; Udayagiri & Mason, 1995; 1997). In contrast corn methanol extract were deterrent to ovipositing *O. nubilalis* (Udayagiri & Mason, 1995) and *Sesamia nonagrioides* (Konstantopoulou *et al*., 2002). These *n*-alkane compounds are widely distributed in plants at various concentrations and combinations depending on plant species, plant tissue or developmental stage. Epicuticular lipids also strongly elicit ovipositional response in Lepidoptera (Städler & Schöni, 1990; Grant & Langevin, 1994), although the mechanism by which insects detect them is still largely unknown (Chapman & Bernays, 1989). Chemicals emanating from surfaces of various

plant tissues are likely to be different and this could also affect oviposition behaviour. Selection of a specific site for oviposition on a plant that is more suitable for establishment by the emerging larva could be based on specific chemicals associated with the particular tissue.

2.8.2.1-Insect tasting

Insects generally rely upon contact chemoreception when searching for food, oviposition sites, mating partners as well as social communication [see Schoonhoven *et al*. (1998) for review].

The behavioral responses of an insect to contact plant substances is based on the perception of gustatory (tasting) sensilla predominantly located in the preoral cavity, mouth parts, tarsi, ovipositor and the antennae. Extremities equipped with gustatory sensilla often move in such a way that the sensilla make brief intermittent contacts with the plant surface or the interior during contact evaluation behaviour [see Schoonhoven *et al*. (1998) for review]. The sensilla involved in contact chemoreception are uniporous with a single pore located on the tip of the sensillum for being into contact of non-volatile compounds present on the plant or insect surface.

2.8.2.2-Isolation and analysis of plant surface compounds

Techniques employed for the collection of plant surface compounds must ensure that the collection time for plant surface metabolites are short to prevent biochemical modification of compounds on the plant surface as well as to avoid contamination with internal constituents and damage to epidermal cells. Currently, the techniques used for collection and analysis of plant surface compounds are limited and often do not give reproducible results (Fiala *et al.*, 1990). Traditional techniques such as solvent extraction, chopping, maceration or homogenisation in a blender often lead to destruction of the plant tissue and lead to a mix-up of plant surface and internal compounds. These procedures are also amenable to enzyme catalysed oxidation products that are normally not present in the intact plant and hence may mask the original compounds. In addition

such techniques lead to leaching of soluble substances from inner tissues to result in overestimates of substances on the plant surface (Derridj *et al.,* 1996). Brief extractions (10-60 s) of fresh foliage at room temperatures in appropriate organic solvents are usually recommended for the isolation of plant surface chemicals (Eigenbrode & Espelie, 1995).

Two extraction techniques that simulates plant natural conditions to which leaves are usually exposed (rain, dew) have been described for collecting water soluble compounds especially carbohydrates present on the leaf surface of the plants:

(i) Dipping technique: plant leaves are dipped in water for different durations,

(ii) Spraying technique: that involves spraying the leaf with pure water (Fiala *et al*., 1990).

Although these techniques were initially developed to quantitatively determine the amount of water-soluble carbohydrate present on corn-leaf phylloplane, they have been also used to collect other organic compounds such as phenolic acids and amino acids (Derridj *et al*., 1996). Epicuticular lipids are reported to influence insect oviposition in a number of insect species (Udayagiri & Mason*,* 1997).

Epicuticular lipids can be extracted from a plant surface by briefly dipping intact plants into organic solvents such as chloroform or a mixture of solvents with a sequential gradient of increasing polarities (Torto *et al*., 1991; Eigenbrode & Espelie, 1995). Although dried plant tissues are best suited for the quantitative removal of surface lipids, their use increases the possibility of extracting internal lipid components. Brief extractions (10-60 s) of fresh foliage at room temperatures is hence recommended for the isolation of epicuticular chemicals so that only surface compounds are extracted (Eigenbrode & Espelie, 1995)

The identification methods used for the extracted chemicals need to be adapted to the properties of the chemicals and to their quantities. Simple sugars in extracts may be determined by coupled enzyme assays (Bergmeyer *et al.,* 1974) or by use of high performance liquid chromatography (HPLC) (Pecina *et al.,* 1984). Column chromatography is usually used when the extract is suspected to have many fractions. Thin layer chromatography has been used to reveal the distribution of the major lipid classes of an epicuticular lipid extract where they migrate from the origin in a relatively non-polar organic solvent system. Further fractionation of the TLC fractions is usually

achieved by column chromatography (Udayagiri & Mason, 1997; Anupam *et al.,* 2003). Coupled GC-MS is used to analyse surface compounds upon appropriate derivatisation (Shepherd *et al.,* 1995). However, many of the naturally occurring epicuticular lipids are not commercially available and spectra of most these lipid components are not in the National Bureau of standards mass spectral library, making difficult their identification.

2.9-Electrophysiological Techniques

Electrophysiology is the study of electrical properties of living cells and how information instigated by chemical and electrical changes in these cells is conveyed within the nervous system. Signals initiated in the peripheral receptor neurones and response characteristics transmitted to the central nervous system can be recorded by electrophysiological methods. Electrohysiological experiments are usually performed on olfactory or gustatory on the sensilla of isolated heads, legs or intact insects [see Schoonhoven *et al.,* (1998) for review]. For olfactory and gustatory sensilla two types of electrophysiological techniques can be used: electroantennogram (EAG) (Arn *et al*., 1975) and standard tip-recording technique (Hodgson *et al.*, 1955).

The EAG technique measures the total response of all antennal receptor cells to particular odorous stimuli (only volatile compounds). When classifying plant volatiles for their capacity to evoke olfactory activity, the EAG appears to be a useful technique as it provides a screening of the entire antennal receptor population. In this case, an antennae is excised and placed between two glass capillaries filled with saline solution. One end of the antenna is placed in the recording electrode and the other end is grounded with indifferent electrode. Whole animal preparations can be used when the antennae is very small or a long lasting preparations is desired, in this case the indifferent electrode is positioned in contact with the haemolymph near the base of the antennae, while the recording electrode is put in contact with the excited antennal tie. The recording electrode is connected to a high impedance amplifier and detects the electrical responses when a biologically active compound is blown over the antennae. An electroatennographic response is registered as a direct current deflection over the antennae. The amplified electrical signal can be viewed on an oscilloscope and processed in a computer (Guerin &

Visser, 1980). The EAG method has also been used in combination with GC technique in the identification of pheromone gland extract from female moths (Arn *et al*., 1975) or in the identification of a plant volatile complex mixtures and simultaneous determination of biological activity of individual order components (Thiéry *et al* ., 1991; Marion-Poll & Thiéry, 1996). In this case the outlet of the GC is split with 50% of the effluent allowed to pass on the flame ionisation detector while the rest is released into an air stream flushed over the GC detector where it simultaneously elicits a response from the electro-antennographic detector (EAD). This makes it possible to very quickly pin-point a biologically active component.

For gustatory sensilla, a standard tip-recording technique is used (Hodgson *et al*., 1955). The technique registers the action potentials given by a single sensillum. An indifferent electrode is inserted into the insect body and the recording electrode, a micropipette filled with a tested solution (e.g. salt solutions), comes into contact with a single gustatory sensillum tip located on the tarsi, ovipositor or even on the antennae. When a good tip contact of the sensillum with the recording electrode is made, the electrical conductivity changes and the global nervous activity of the sensillum in response to a tested solution can be registered.

Electrophysiological studies provide useful information on the sensitivity and selectivity of sensilla receptors. However they do not allow any conclusion about behavioural attractiveness or repellence of a stimulus. Moreover a compound that stimulates chemosensory receptors may not elicit behavioural activity in an organism until the component is blended with other compounds (Tichenor & Seigler, 1980).

Chapter 3

Differences in ovipositional response between laboratory-reared and wild insects of *Busseola fusca*

3.1 – Introduction

The ovipositional responses of female insects are usually influenced by visual, physical, volatile and contact stimuli. Visual and volatile stimuli are distance perceivable, eliciting orientational responses of the insects. Variations in ovipositional response of female insects may be attributed to factors such as age, generation, strain, physiology or the rearing conditions of the insects.

The stem borer, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), is an important pest of maize and sorghum in East Africa (Kfir *et al*., 2002). It is possible to rear this insect species under laboratory conditions using a meridic diet and surrogate paper stems for female oviposition (Onyango & Ochieng'-Odero, 1994; Khan & Saxena, 1997). Since 1998, for studies purposes this insect has been continuously artificially reared at ICIPE Mass Rearing Unit, adding three times in a year new individuals from the wild (Onyango F.O., personal communication).

It has been reported in the literature that laboratory-reared insects generally tend to differ from natural (wild) population in genetic, behavioural and physiological characteristics and this limits their representativeness of the species in the wild (Tingey, 1986). Moreover, insects, reared under laboratory conditions, have been found to lose the ability to succesfully grow on their original host (Guthrie & Carter, 1972) or to accept plant totally outside their natural host range (Schoonhoven, 1967). The rearing conditions can therefore strongly influence the insect behaviour and physiology.

For future related studies, it was necessary to verify first if the laboratory-reared *B. fusca* differ from the natural population in their reproductive biology especially in oviposition of the females with a special attention to the original host plant recognition.

3.2 - Materials and methods

3.2.1-Insects

For laboratory-reared insects, *Busseola fusca* pupae were provided by ICIPE's mass rearing unit (Nairobi, Kenya). These insects were continuously reared on artificial diet under laboratory conditions for more than 60 generations following the method of Onyango and Ochieng'-Odero (1994). Wax papers rolled helicoidally to form a cylindrical surrogate stems were used as oviposition supports.

For wild insects, larvae were collected from field infested maize plants from several cultivated areas of Kenya. They were then reared up to pupae on the aforementioned artificial diet.

The pupae were sexed and, males and females maintained separately until emergence in a plastic box (21 cm length, 15 cm width, 8 cm high). A piece of moistened cotton wool maintained the relative humidity at 80 % inside the box. The insects were kept in a controlled room maintained at 25.9 ± 0.05 °C, 58.5 ± 0.4 % r.h. (means ± SE) and L12:D12 reversed photoperiod (scotophase from 7.00 to 19.00 h).

3.2.2-Plants

Maize (*Zea mays* L., cv. 511) seeds were provided by Simlaw, Kenya Seeds Company (Nairobi, Kenya). The maize seeds were allowed to germinate for three days between two layers of moisted cotton wool. For oviposition experiments, some plants were grown in plastic pots (13 cm diameter, 12 cm high) containing peat in a greenhouse at ICIPE (Kenya). The other plants were grown in the ICIPE field for plant surface chemical extraction. The environmental conditions were approximately 31/17°C (day/night) with L12:D12 photoperiod. The plants were used for experiments after three weeks of growth.

3.2.3-Maize extracts

Maize extracts were obtained following the method of Derridj *et al*. (1996) for collecting cuticular waxes and sugars from the maize cuticle. Briefly, maize plants grown in the field were excised at the base towards the end of the day (around 18 h). Forty entire plants were collected and individually dipped into 500 ml of chloroform for 2s. After filtration, the extract was concentrated in rota-vapor up to 40 ml (= 1 equivalent plant per ml) and stored at –20°C before use.

3.2.4-Ovipositional response experiments

For both populations, adult males and females were taken out on the night of emergence and placed into a mosquito-net cage (40 cm length, 40 cm width, 63 cm high). A moistened cotton wool pad induced a moisture of about 61 ± 1 % r.h. into the cage.

To obtain a maximum proportion of mated insects (96%) the following steps were developed. The females (24 h aged) were placed first at the beginning of the night in the mosquito-net cage. At the sixth hour of the night, females generally started to exhibit calling behaviour. Several males were introduced at eighth hour of the night to induce a choice situation of the sexual partner successful for the mating of wild insect (Calatayud P.-A., personal observations). During the subsequent 1-hour period, mating occurred and the mating pairs were removed from the cage. Once the sexes had separated, naive females were used in the succeeding night for the experiments outlined below.

Both laboratory-reared and wild mated females were placed individually in transparent plastic jars (16 cm high, 9 cm diameter) (with over 80 % r.h. maintained by moist cotton wool pad) with one rectangular piece of nylon (15 length, 5 cm width) rolled helicoidally from top to bottom to form a cylindrical surrogate stem. Such stem is known to be acceptable to *B. fusca* for oviposition and to elicit a good ovipositional response of the insect (personal observations). Since no eggs were recorded on stems during photophase (personal observations), indicating that no oviposition activity occurred during this phase, the experiments were started at the onset of scotophase and ended with onset of photophase. The oviposition response was estimated by recording the number of eggs laid per night over a period of 3 successive nights, with old surrogate stem being replaced

by newer ones after 24 hours. The results were recorded as percentage of the total number of eggs laid during the 3 nights as a replicate.

The same experiment was repeated with other mated females placed individually in a mosquito-net cage with one maize plant and one rectangular piece of wax paper (60 cm length, 7 cm width) rolled helicoidally (Khan & Saxena, 1997). After 3 nights, the number of eggs laid on the plant or on artificial support was counted, and the results were recorded as percentage of the total number of eggs laid per replicate.

To determine the role of maize surface extracts chemicals on oviposition, other mated females from both laboratory-reared and wild insects, were placed individually in aforementioned transparent plastic jars with two aforementioned nylon surrogate stems in a dual choice situation. One control stem was imbibed with 1 ml of chloroform alone and another with 1 ml of maize chloroform extracts. After about 8 hours of night, the number of eggs laid on each support was counted, and results recorded as percentage of the total number of eggs laid per replicate.

For all experiments, the number of eggs was estimated by weighing (with a 1-mg sensitive Sartorius balance) and the weight converted into numbers using a calibration curve (number = 11.896 x weight in mg).

All experiments were done in the same aforementioned room conditions as those used to maintain the insects.

3.2.5-Female attraction towards the plants

Female attraction toward maize plants was studied by comparing laboratory-reared with wild *B. fusca* populations in a plexiglass wind tunnel (184 cm long, 60cm wide and 40cm high). A speed-controlled fan was used to push air through the tunnel. Illumination was provided by a 40-W red incandescent light bulb mounted 70 cm above the wind tunnel. Individual mated females from both populations were allowed to acclimatize to the experimental room for at least 1 h before experiments. Two pots each containing 5 maize plants were placed together in the up-wind end of the tunnel. Each observation began by placing the female on a 15 cm high platform (release platform), 20 cm away from the wind tunnel exhaust end. To eliminate putative interaction among females, a single insect

was released and observed for 10 min. Forty females were tested for each experiment. The total number of individuals showing an orientated flight toward the plants was recorded.

3.2.6-Electrophysiology

Antennal receptivity of *B. fusca* females of both laboratory-reared and wild populations to selected plant volatile compounds was determined by electroantennography (EAG). An antenna was excised at its base and mounted between two glass micropipettes containing Beadle-Ephrussi saline solution (Ephrussi & Beadle, 1936). The micropipettes were sheathed over Ag wire electrodes. Synthetic (*Z*)-3-hexenyl acetate and (*Z*)-3-hexen-1-ol [green leaf volatiles of maize and many other plant species (Schoonhoven *et al*., 1998) and known to stimulate *B. fusca* antennae (Khan & Hassanali, 2004)] were tested individually at five doses (0.01 – 100 μg in decadic steps) prepared in light paraffin oil (Alison Products). To stimulate the olfactory receptors, a 200 msec puff of activated charcoal-filtered air was blown into a humidified air-stream at 4 ml/sec and over the antenna through a Pasteur pipette containing a given dose of the test compound impregnated onto a filter paper strip (Whatman no 1: 2.5 cm length, 1.0 cm width). The electroantennograms (EAGs) generated in the olfactory receptors were acquired through a high-impedance amplifier (UN 05; Syntech) and displayed on a monitor PC that had an EAD card (Syntech) for processing EAG data. Fresh antennae were used for repeated sample analysis. Antennae of five to eight individuals of each insect population were used for each tested compound.

3.2.7-Data analysis

Statistical tests were performed with Statview software (Abacus Concept, version 5.0, USA). Data were subjected to *F*-test and Kolmogorov-Smirnov method for homogeneity of variance and data normality respectively. For figure 1, they were analysed using one-way analysis of variance (ANOVA). Means were separated by Fisher's PLSD (Protected Least Significant Difference) test or Student's *t*-test. Chi-square test was used to compare proportions of individual showing orientated flight of females toward the plants in wind

tunnel experiment between laboratory-reared and wild populations. Mann-Whitney U-tests were used to compare EAG amplitudes between laboratory-reared and wild insects at each volatile dose.

3.3-Results

The oviposition response of the females in the succeeding night intervals varied significantly depending on the population origin of *B. fusca* (Figure 3). For wild females, the average number of eggs laid by one insect per night was 230, 113 and 67 respectively for the 1st, 2nd and 3rd nights, resulting in a relative proportion of about 45, 33 and 21% respectively. For laboratory-reared females, the average number of eggs laid per insect per night was 105, 116 and 42 respectively for the1st, 2nd and 3rd nights, to give a relative proportion of about 29, 45 and 25% respectively. The wild females showed the tendency to oviposite mostly during the first night, after one night of mating while the laboratory-reared females oviposited mostly during the second night. The influence of the support on ovipositional response in relation to the population origin of naive gravid *B. fusca* females was also investigated in a dual choice situation using maize (common host plant of *B. fusca* in the field) and paper surrogate stems. With wild insects, the average number of eggs laid on maize was 199 and 18 on paper, resulting in a relative proportion of about 91% and 9% respectively (Figure 4). A significant higher proportion of eggs were laid on maize than on paper. Hence wild insects preferred to oviposite on maize plant than on paper stem. For laboratory-reared insects, the average number of eggs laid was 154 on maize and 66 on paper, resulting in a relative proportion of about 56 and 44% respectively. Hence the laboratory-reared insects did not show strong preference for oviposition as compared to wild insects: almost equal proportion of eggs was laid on each support for these insects.

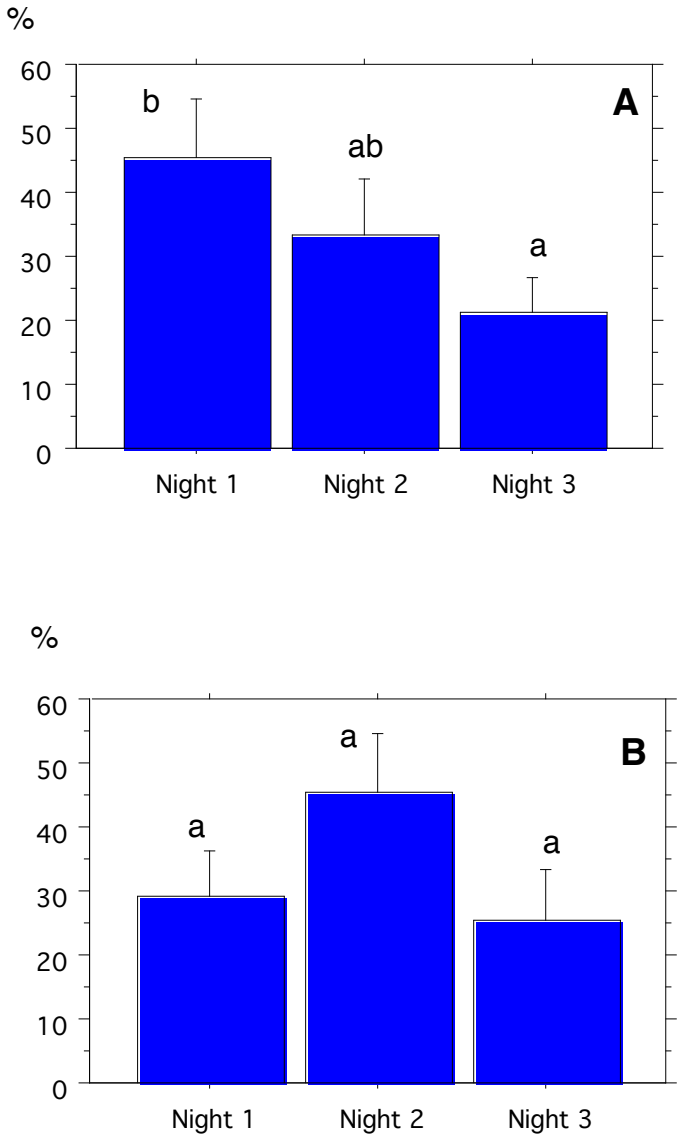


Figure 3. Relative proportions (in %, means ± SE) of eggs laid on nylon surrogate stem over 3 successive nights by females from wild population (in A, n=12) and from laboratory-reared population (in B, n=14). Bars with different letters are significantly different using an experiment-wise error rate of a=0.05 (PLSD's Fisher test following ANOVA).

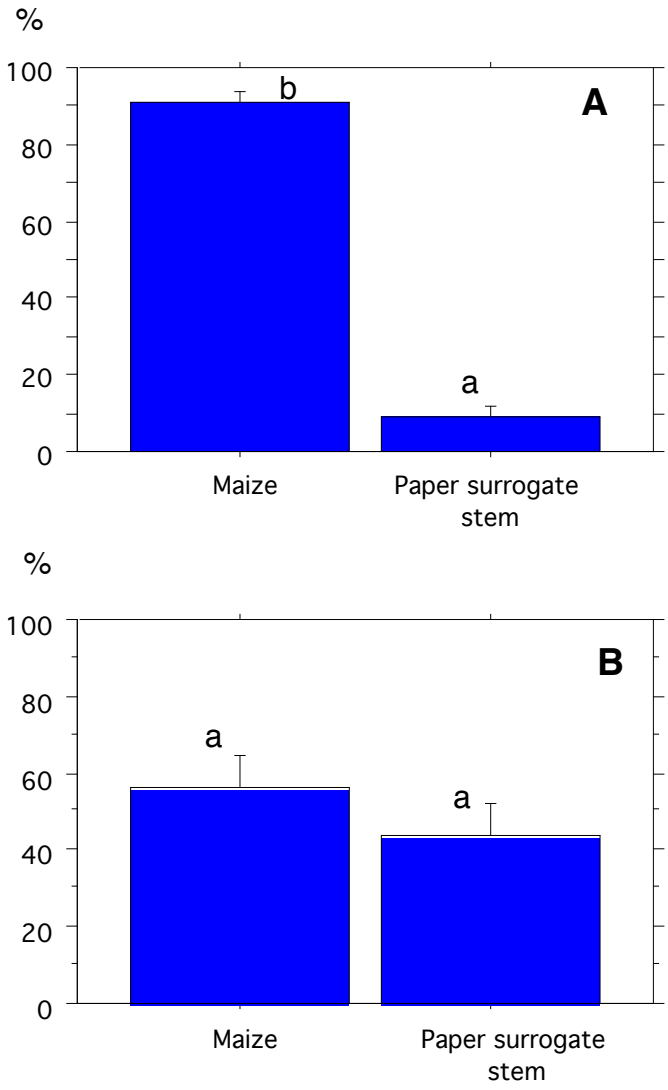


Figure 4. Relative proportions (in %, means ± SE, n=20) of eggs laid by females from wild population (in A) and from laboratory-reared population (in B) on maize plant or paper surrogate stem in dual choice situation after 3 nights. Bars with different letters are significantly different using an experiment-wise error rate of a=0.05 (Student's *t*-test).

A bioassay was used to study the role of contact host chemicals (*i.e.* plant surface chemicals) on oviposition response according the population origin of *B. fusca* by using two nylon surrogate stems (one imbibed with chloroform maize extract and another with

chloroform alone) in a dual choice situation. Using naive gravid wild females, the average number of eggs laid per insect were 57 on surrogate stem imbibed with chloroform alone and 104 with maize extract, resulting in a relative proportion of about 38 and 62 % respectively (Figure 5). A significant higher proportion of eggs were laid on surrogate stems imbibed with maize extract, indicating that wild insects preferred to oviposite on maize extract than on control (chloroform alone). However, for laboratory-reared insects, the average number of eggs laid was 123 and 113 on surrogate stem imbibed with chloroform alone and with maize extract respectively resulting in a similar relative proportion of about 57 and 42% respectively. These insects did not show any preference in oviposition relative to these supports.

The rearing conditions of *B. fusca* also significantly influenced the insect orientation towards the plants (Table 3).

Table 3. Proportion of *B. fusca* females showing orientated flight towards the maize plants in wind tunnel according to the population origin

Population origin	Orientated flight proportion (%)* [n]
Wild insects	52 [42] b
Laboratory-reared insects	31 [45] a

* Proportions followed by different letters are significantly different at $P < 0.05$ (Chi-square test).

The proportion of females showing an orientated flight toward maize plants was significantly lower with laboratory-reared insects than wild ones. However, the females regardless of the population origin reacted similarly towards the host plant odours. This was illustrated by the EAG amplitude responses recorded for two synthetic plant volatiles (Figure 6). Although significant differences were found at one dose for each compound tested, the EAG dose-response elicited by (*Z*)-3-hexenyl acetate and (*Z*)-3-hexen-1-ol was similar among female antennae of the laboratory-reared and wild populations.

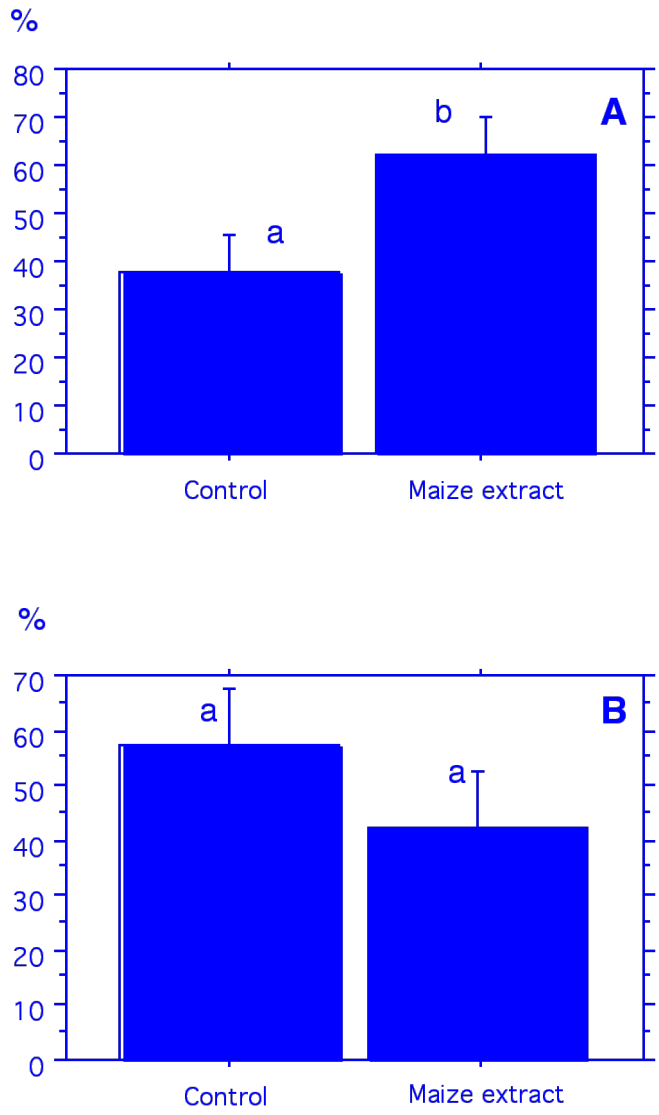


Figure 5. Relative proportions (in %, means ± SE) of eggs laid by females from wild population (in A, n=20) and from laboratory-reared population (in B, n=17) on nylon surrogate stem imbibed with chloroform alone (control) or with chloroform maize extracts (maize extract) in dual choice situation after 8 hours of night. Bars with different letters are significantly different using an experiment-wise error rate of a=0.05 (Student's *t*-test).

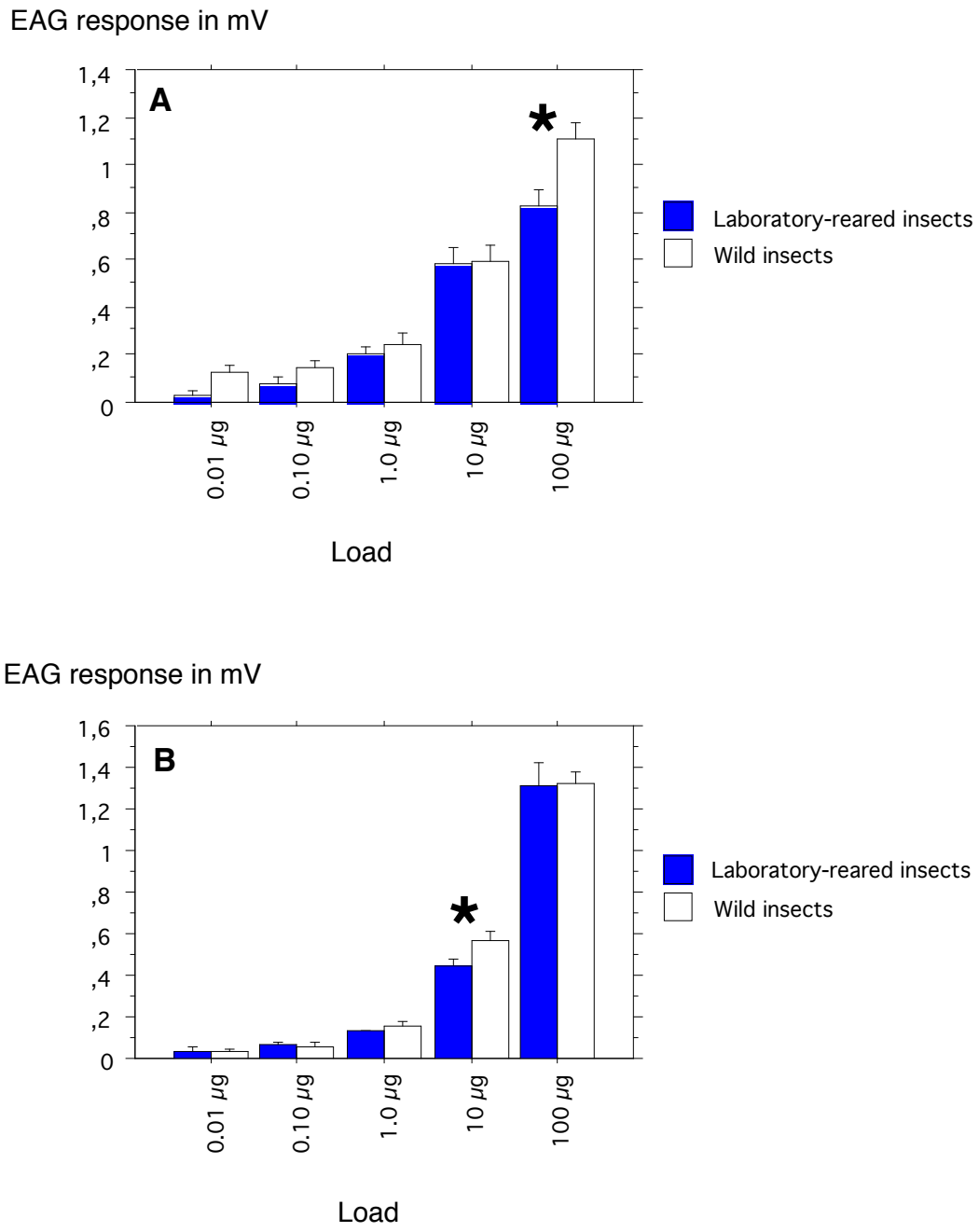


Figure 6 Mean (± SE) EAG amplitude responses of *B. fusca* females from laboratory-reared and wild population to different doses of two synthetic plant volatiles [A for (*Z*)-3-hexenyl acetate and B for (*Z*)-3-hexen-1-ol]. Bars with stars are significantly different using an experiment-wise error rate of a=0.05 (Mann-Whitney U test, laboratory-reared *vs* wild insects comparisons).

3.4-Discussion

The rearing conditions of *B. fusca* (laboratory or field conditions) strongly influence the oviposition of the insect.

Under laboratory conditions after several generations, the insects seem to completely adapt to artificial conditions. This is clearly demonstrated by the duration at which the insects take to oviposite as well as their ability to perceive the host chemicals. The females oviposite generally during the three successive nights after the mating night whereas the wild ones oviposite mostly during the first night (Figure 3). This difference could be due to the readily available suitable oviposition supports for laboratory-reared insects to insert their ovipositors any night after mating as compared to the wild ones. However, the later must undergo all the process of searching for appropriate oviposition sites and on finding one takes advantage of this as soon as possible.

Laboratory-reared insects seem to have lost the host plant specificity for oviposition. This is shown by the ease at which they accept oviposition support totally outside their original host plant such as paper or nylon surrogate stems. In addition, these insects also, show no preference for oviposition between paper surrogate stem or maize plant, in contrast to wild insects (Figure 4).

The rearing conditions of *B fusca* also seem to significantly influence insect orientation to the host plant odours. The proportion of gravid females that showed an oriented flight towards maize plants was significantly lower with laboratory-reared insects than wild population (see Table 3). However they reacted similarly regardless of the population origin to the host plant odours as exemplified by similar EAG amplitude responses recorded for both populations using two synthetic green leaf plant volatiles, (*Z*)-3-hexenyl acetate and (*Z*)-hexen-1-ol (see Figure 6). Such discrepancy between antennal and behavioural responses has also been observed in *Helicoverpa armigera* (Hull *et al*., 2000) and indicates that there is no relationship between antennal receptivity and the resulting behaviour. As hypothesized by Schoonhoven (1967) for *Manduca sexta* (Johan.) (Lepidoptera: Sphingidae), the lower proportion of laboratory-reared insects to orient to

the plants can be due to a change for these individuals in behaviour with regard to host plant specificity. This may be due to a change in interpretation of the incoming chemosensory information by the central nervous systems (C.N.S.) or to a change in the information sent by the chemoreceptors to the C.N.S. or a combination of both processes but not to a change in antennal receptivity. However, having lost host plant specificity, few artificially reared insects tended to orient to host volatiles as compared to the wild populations. Hence, although using artificially reared insects is more often convenient to use in behavioural studies, these results suggest that there is a risk that such insects may differ so radically from natural populations as to limit their representativeness of the species in the wild and therefore it is important to use wild insects in future studies.

Besides, this study indicates that prior food and environmental conditioning of *B fusca* allows the insect to lose the original host specificity and simultaneously adapt to new environment in terms of oviposition supports or altogether change the host plant. Although it is reported that host plant selection (recognition and avoidance) is determined genetically thus cannot be changed basically by experience (Jermy, 1987), experience may play a considerable role in insect host plant finding, acceptance and preference (Jermy, 1987). For example, insect learning can change the egg laying preferences of the adult females towards different host plant species (Cunningham & West, 2001). Repeated exposure of an insect to a host plant can lead to an increased preference for that particular host species. This is consistent with Hopkins (1917) host selection principle, which implies that experience during the larval period conditions the choice of the host plant for oviposition by the adult females. It has also been reported that the insect's feeding history might influence its behavioural and physiological responses to normal food plants through preference induction (Jermy, 1987).

Maize was recently introduced in Africa (Ristanovic, 2001). *B. fusca*, a native African species, must have been feeding on one or several other native graminaceous resources before maize introduction. *B. fusca* that is now feeding mostly on cultivated maize and sorghum (Le Rü, unpublished data) during the last century has experienced a host-shift from wild host plant(s) towards cultivated plants. The reasons why *B. fusca* has switched to cultivated plants are still unclear. Therefore, there is a need to understand how such a

host-shift has occurred and what factors (insect life traits and plant life traits) have helped in such a major ecological change.

Chapter 4

Role of plant volatiles and surface metabolites in host selection for oviposition of *Busseola fusca*

4.1-Introduction

The stem borer *Busseola fusca* (Fuller)(Lepidoptera: Noctuidae) is an important pest of maize and sorghum in Africa (Kfir *et al*., 2002). To control this pest, novel habitat-management strategies, the "push-pull" or stimulo-deterrent diversionary tactics (Khan *et al*., 2000), have recently been developed by ICIPE. The strategy uses a repellent maize intercrop to drive the insects from the agronomic fields (push) and at the same time attract and retain these insects on highly susceptible trap plants planted as border rows (pull) around the agronomic crops. However, the efficient use of such strategies requires a good understanding of the host selection process for oviposition of the insect pest.

It is reported that gravid female insects are usually influenced by plant related stimuli such as visual, tactile, olfactory and contact stimuli for selection of appropriate oviposition sites (Renwick & Chew, 1994). Visual and olfactory stimuli are distance perceivable, and usually elicit orientational responses of the insects whereas, contact stimuli influence host assessment (Renwick, 1989). The role of tactile stimuli in the choice of a suitable host for oviposition by insect has been extensively studied (Harris & Miller, 1988; Hattori, 1988 ; Ramaswamy, 1988 ; Harris & Rose, 1990 ; Roessingh & Städler, 1990 ; Foster *et al.,* 1997). Le Rü *et al*. (2004) investigated the role of host plant physical characters in *B. fusca* oviposition response, and concluded that there existed a strong influence of plant physical characters with oviposition. Gravid females significantly preferred maize and sorghum over Napier grass for oviposition a phenomenon, which was partly related to plant surface texture and leaf sheath toughness.

In addition to the importance of visual and tactile cues in selection of suitable oviposition sites gravid female insects also strongly respond to semiochemical cues associated with their host. The primary sensory modality in host plant finding of female lepidopteran insects is partly influenced by phytochemical stimuli (Honda 1995, Hern & Dorn, 2002). The balance of opposing positive and negative cues evoked by these

phytochemicals determines whether a plant is accepted or rejected by a herbivore (Huang & Renwick, 1993; Renwick & Chew, 1994). Host plant derived semiochemicals have been shown to attract insects and ultimately stimulate their feeding and oviposition (Visser, 1986; Udayagiri & Mason, 1995; 1997; Konstantopoulou *et al.,* 2002). Wind tunnel tests with *B. fusca* showed that the insect could select at long range the host plant for oviposition. Olfactory and visual stimuli seemed to guide the insect to the plant. Using maize, sorghum and Napier grass, both maize and sorghum were more frequently selected for oviposition than Napier grass (Le Rü *et al*., 2004). Electrophysiological studies showed that the insect antennae respond strongly to some of the volatiles emitted by some the host plants of *B. fusca* (Khan *et al.,* 2000; Khan & Hassanali, 2004).

Although volatile cues are almost certain to stimulate insect orientation to their specific hosts, comparative studies on post alighting insect behaviour, such as insect positioning, surface prospecting, surface evaluation, ovipositor sweep, on host plants clearly suggests that tactile and contact-chemoreception stimuli from the plants seems also to play a major role in oviposition decision of *B. fusca* (Le Rü *et al*., 2004). Identification of host plant chemicals that act as oviposition stimulants for economically important insect species is gaining importance due to their potential for use in the manipulation of pest behaviour in the field. For *B. fusca,* the chemical basis of oviposition site selection is still unknown.

In this context, the purpose of this study was to analyse the volatiles emitted by maize, sorghum and Napier grass at the insect preferred stage of growth and to determine the influence of polar and less polar plant surface compounds involved host selection for oviposition of *B. fusca*.

4.2-Materials and Methods

4.2.1-Insects

Wild insects larvae were collected from field infested maize plants from several cultivated areas of Kenya. These insects were reared on artificial diet under laboratory conditions following the method of Onyango and Ochieng'-Odero (1994) until pupation. The pupae were sexed and, males and females maintained separately until emergence in a

plastic box (21 cm long, 15 cm wide, 8 cm high). A piece of moist cotton wool maintained the relative humidity at about 80 % inside the box. The insects were kept in a controlled room maintained at 25.9 ± 0.05 °C, 58.5 ± 0.4 % r.h. (means ± SE) and L12:D12 reversed photoperiod (scotophase from 7.00 to 19.00 h).

4.2.2-Plants

Three plant species, maize (*Zea mays* L., cv. 511, a non-pubescent cultivar), sorghum *(Sorghum bicolor* [L.] Moench, cv. serena), commonly cultivated in Kenya, and Napier grass (*Pennisetum purpureum* Schumach), used for animal forage, were tested for *B. fusca* oviposition choice. Maize and sorghum seeds were provided by Simlaw, Kenya Seeds Company (Nairobi, Kenya). They were first placed between two moist cotton layers in a plastic box (30 cm long, 12 cm wide, 10 cm high) under dark conditions at 30°C for 3 days to allow germination. Napier grass was provided by ICIPE (Nairobi, Kenya) and obtained from cuttings.

All plants were grown directly in the ICIPE field. The environmental conditions were approximately 31/17°C (day/night) with L12:D12 photoperiod. The plants were used for volatile and surface compound extraction after 3-4 weeks of growth.

4.2.3-Collection of headspace plant volatiles

Headspace plant volatile samples were collected by solid phase micro-extraction technique (SPME) (Berlardi & Pawliszyn, 1989; Arthur & Pawliszyn 1990). The SPME manual holder device (Supelco, Bellefonte, PA, USA) equipped with a fused-silica fibre, coated with a 65 µm layer of polydimethylsiloxane/divinylbenzene (PDMS/DVB), was used to absorb the volatile components from the plant headspace. For each plant species, five plants growing in the field were excised at the base at the end of the day (around 18.00 h) and the excised stem immediately wrapped in a moist cotton wool in an aluminium foil. The plants were put in a 2 litres measuring cylinder and the cylinder tightly secured with aluminium foil made to resemble a cylinder cap using parafilm. Sandwiched between the aluminium foil cap was a self sealing gas tight septum through

which the SPME septum-piercing needle was allowed to pierce through into the plant headspace. The fibre was first thermally desorbed in the GC injector for 15 minutes at 250°C before being extended into the plant headspace and left to adsorb for two hours. After adsorption, the fibre was extruded from the plant headspace and immediately introduced into the split less GC injector pot (250°C) where it was allowed to desorb for five minutes. Four plant headspace replicates were carried out for each plant species.

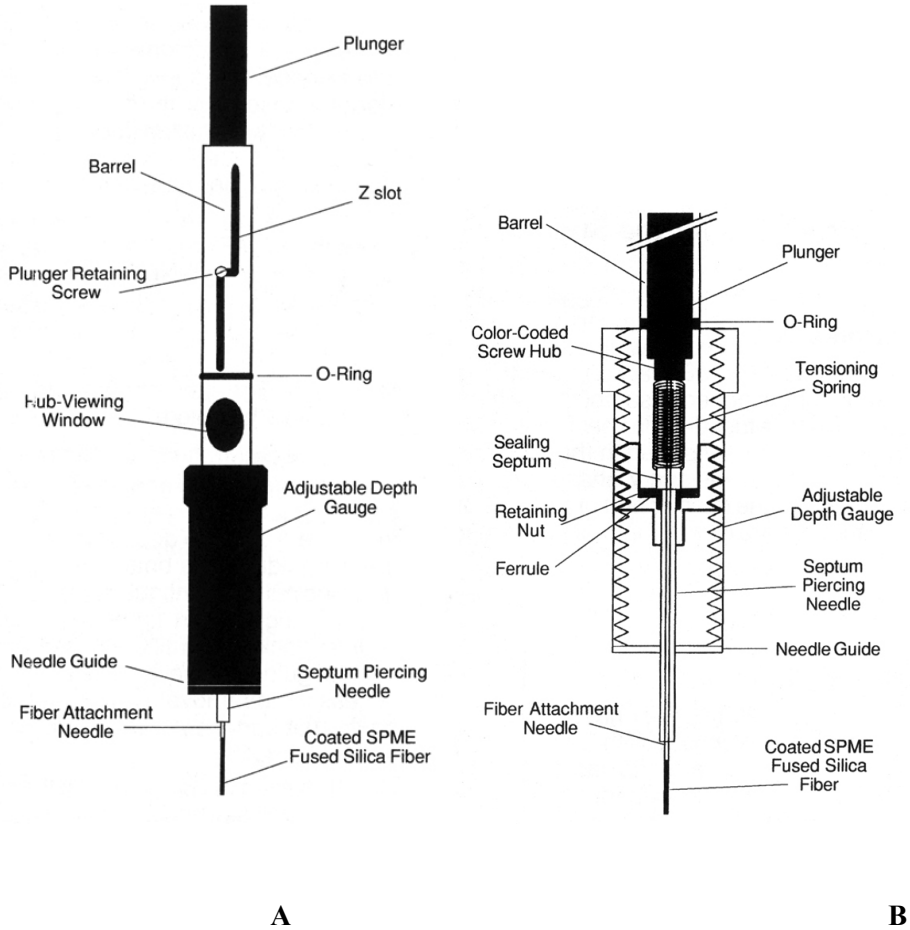


Figure 7. SPME fibre holder and fibre assembly (A) with its section view (B).

4.2.4-Analysis of plant volatiles

The headspace volatile components were separated using a HP 5890 series II gas chromatograph equipped with a HP capillary column (ultra-1-crosslinked methyl silicone, 50 m x 0.2 mm I.D. x 0.33 μL film thickness) using nitrogen as the carrier gas at a flow rate of 0.35 ml/min with flame ionisation detector (FID) heated at 270°C. The GC oven temperature was initially at 60°C then programmed at 5°C/min to 280°C where it was held for 20 minutes. GC analysis was operated in a split less mode with nitrogen as the carrier gas. The injection port septum purge flow was programmed to turn of and on, respectively, at the beginning and end of the desorption period. The fibre was subjected to thermal desorption in the injector at 250°C for 5 minutes which corresponded to the split less time. The identity of the headspace compounds was tentatively assigned by comparison of their retention times with those of authentic samples.

The compounds present in the volatile profile of plant headspace were identified using coupled gas chromatography-mass spectrometry. The volatile adsorbed fibre was thermally desorbed for five minutes in a split less injector (250°C) of Hewlett Packard 5790 gas chromatograph coupled to a VG MassLab 12-250 mass spectrometer operating in an electron impact mode (70eV). The analytes were separated using similar column and conditions as used in GC analysis. The ratios of compounds were represented by GC peak areas, normalized so that the total peaks of interest equalled 100%.

The identification of chromatographically separated compounds was carried out by comparing GC retention times to those of authentic samples, comparison of spectral data with those of the library database installed in the MS-computer (NIST Registry of mass spectral data, 1995), comparison of order of elution and by comparing their spectra to those already published (Kjaer *et al*., 1963; Spencer & Daxenbichler, 1980). The test samples were compared to the control samples from the apparatus of collection to ascertain chemicals specific to each treatment.

The relative proportion (peak area / total peak areas of all compounds * 100) was calculated for the repetitively detected compounds for each plant species and for the major compounds emitted among the plant species.

4.2.5-Extraction of host-plant polar surface metabolites

Host plant polar metabolites were collected according to the method of Fiala *et al.,* (1990). Briefly, plants were carefully cut at the base near the soil towards the end of the day and with the cut end held over aluminium foil, each plant was splashed with distilled water for one minute. Forty plants were used for each plant species. Samples were filtered using Whatman qualitative filter paper, divided into small volumes (5 ml) and then freeze dried in a stoppering tray drier (Labconco Missouri- Kansas City). The resulting solid was then recombined and re-suspended in an equivalent amount of distilled water to give an extract containing 1 plant equivalent per 1 ml of water. The extracts were refrigerated at –20°C until used for bioassay.

4.2.6-Extraction of host-plant less polar surface metabolites

Host plant surface extracts were obtained using the dip extraction method as used to collect epicuticular compounds from plants (Derridj *et al*., 1996). Briefly, plants growing in the field were in turn excised at the base towards the end of the day (around 18 h) and quickly and singly dipped in 500 ml cold chloroform (Analytical grade-Merck Euro lab) for two seconds. Forty plants of each species were used in each case. After filtration, the extract was concentrated in a rota-vapor up to 40 ml (= 1 equivalent plant per ml) and stored at –20°C before use.

4.2.7-Ovipositional response experiments

Adult males and females were allowed to mate in a mosquito-net cage (40cm long, 40cm wide, 63cm high) one night after emergence. A moistened cotton wool pad induced humidity of about 61 ± 1 % r.h. into the cage.

To determine the role of plant surface extracts chemicals on oviposition, naïve mated females were placed individually in cylindrical transparent plastic jars (16cm high, 9cm

diameter with over 80 % r.h.) with nylon surrogate stems in a dual choice situation. The surrogate stems were made from a rectangular piece of nylon cloth rolled helicoidally. One surrogate stem was imbibed with 1ml of plant extract while the control with extraction solvent (water or chloroform) and the solvent allowed to evaporate before introduction into the jar. The number of eggs laid on each support was counted after the eighth hour of the night and results recorded as percentage of the total number of eggs laid per replicate. All experiments were done in the same aforementioned room conditions as those used to maintain the insects.

4.2.8-Analysis of water extracts compounds

For sugars and organic acids analysis, the samples were first derivatised by acylation with N, O-*bis*-trimethylsilyl trifluoroacetamide (BSTFA). Aliquots of 2 µL of the derivatised samples were analysed on a Sur GC-FID (DN200, auto sampler, ALS 104) gas chromatograph equipped with 5m long and 0.25 I.D deactivated guard column connected to a 5% capillary column (ultra-1-crosslinked methyl silicone, 30 m x 0.25 mm I.D x 0.25 µm film thickness). Helium was the carrier gas at a flow rate of 1ml/min. The oven temperature was initially isothermal at 80°C for 1.5 min, then programmed at 14°C/min to 190°C/min for 12 min then at 12°C/min to 320°C for 8 min. The GC analysis was performed in split less mode. Compounds were identified by comparing their order of elution and GC retention times relative to those of authentic standards.

For free amino acids analysis, the samples were first acylated with N-methyl N-tert-buthyldimethylsilyl trifluoroacetamide (MTBSTFA). Acylation was done by adding 50 µl MTBSTFA to 100 µl of acetonitrile and then heating at 70°C for a half an hour. Aliquots of 2 µl of the derivatised samples were analysed on a Sur GC-FID (DN200, auto sampler ALS 104) gas chromatograph equipped with a 5 m long and 0.25 I.D. deactivated guard column connected to 5% capillary column (ultra-1-crosslinked methyl silicone, 30m x 0.25mm I.D. x 0.25um film thickness) using helium as the carrier gas at a flow rate of 1ml/min with column head pressure of 1.2 bar. The oven temperature was initially isothermal at 70 °C for 2 min, then programmed at 6°C/min to 220°C/min then at 8°C/min to 310°C for 2 min. The GC analysis was performed in a split/split less mode.

Compounds were identified by comparing their order of elution and relative retention times and GC retention times to those of authentic standards.

4.2.9- Analysis of chloroform extract compounds

The 1-plant equivalent chloroform samples were dried completely and re-suspended in 1ml acetonitrile. 20μL of the sample was injected into a Water Alliance 2695 HPLC and compounds separated on a C-8 reverse phase (RP) column. The compounds were separated under isocratic conditions with a water acetonitrile 40:60 mobile phase and detected by UV absorption at 250nM. Compounds were identified by Water Alliance 2695 HPLC coupled to MassLynx 4.0 SP2 RC001. 20 μl were separated on C8 RP-HPLC column (Nucleodur) with a mobile phase of aqueous acetonitrile and water at flow rate of 1.0 ml/min under gradient conditions. The conditions were programmed as follows, A: 40% acetonitrile, 0.2% acetic acid; B: 60% H2O, 0.2% acetic acid; 40-80% A in 25 min, 80% A during 5 min, 80-100% A in 10 min, 100% A during 5 min, and finally 100-30% A in 2 min. Each separated compound was detected by MS detector (SE+/SE- system).

4.2.10-Data Analysis

Data were subjected to F-test and Kolmogorov-Smirnov method for homogeneity of variance and data normality respectively. Means were separated by Fisher's PLSD (Protected Least Significant Difference) and for non-normal distributed data by Mann-Whitney U test.

In all oviposition tests, the number of eggs laid on the control (C) and treatment (T) were recorded. The mean number of eggs laid on each treatment with extract was compared with the mean number of eggs laid on the corresponding controls by a non-parametric Mann-Whitney U test. To compare the mean number of eggs laid among the three plant extracts in the dual choice tests, the oviposition stimulation index (OSI) was calculated according to the formula of Udayagiri and Mason (1997), where

$$OSI = \frac{\text{No. eggs on extract (T) - No. eggs on control (C)}}{\text{No. eggs on extract (T) + No. eggs on control (T)}}$$

The null hypothesis that equal number of eggs were laid on control and treatment (OSI = 0) was tested and the results examined using the students t test at α =0.05.
All statistical tests were performed with Stat view software (Abacus Concept, version 5.0, USA).

4.3-Results

4.3.1-Analysis of plant volatiles emitted by uninfested maize, sorghum and Napier grass

Small quantities of volatiles were detected for each plant species used, with (*Z*)-3-hexenyl acetate being the major green leaf volatile emitted (Figure 8). By magnifying the detector response by a factor of 186, three, seven and nine more other compounds were visible in the headspace of excised plants of maize, sorghum and Napier grass respectively (Figure 9). Compounds in the volatile profile ranged from n-C6 to n-C16 carbon atoms. GC-MS analysis identified seven of the total thirteen compounds detected as (*Z*)-3-hexen-1-ol (3), (*Z*)-3-hexenyl acetate (4), nonatriene derivative (5), (*E*)-4,8-dimethyl-1,3,7-nonatriene (6), nerylacetate (9), alpha-(*E*)-bergmotene (12), caryophyllene (13) and (*E*)-cyclodecene (14). Compounds 1, 2, 7, 8, 10 and 11 were detected in very trace amounts hence making it difficult for their definitive identification. The major compound that seemed to be widely spread in appreciable amounts among the three plant species was (*Z*)-3-hexenyl acetate representing more than 90% of all the compounds emitted (Figures 8 and 10). Among the plant species, Napier grass emitted many volatiles and in larger quantities than sorghum and maize (Figures 9 and 11). This plant species also emitted significantly high amounts of (*E*)-4,8-dimethyl-1,3,7-nonatriene (Figure 11) than the two other plants.

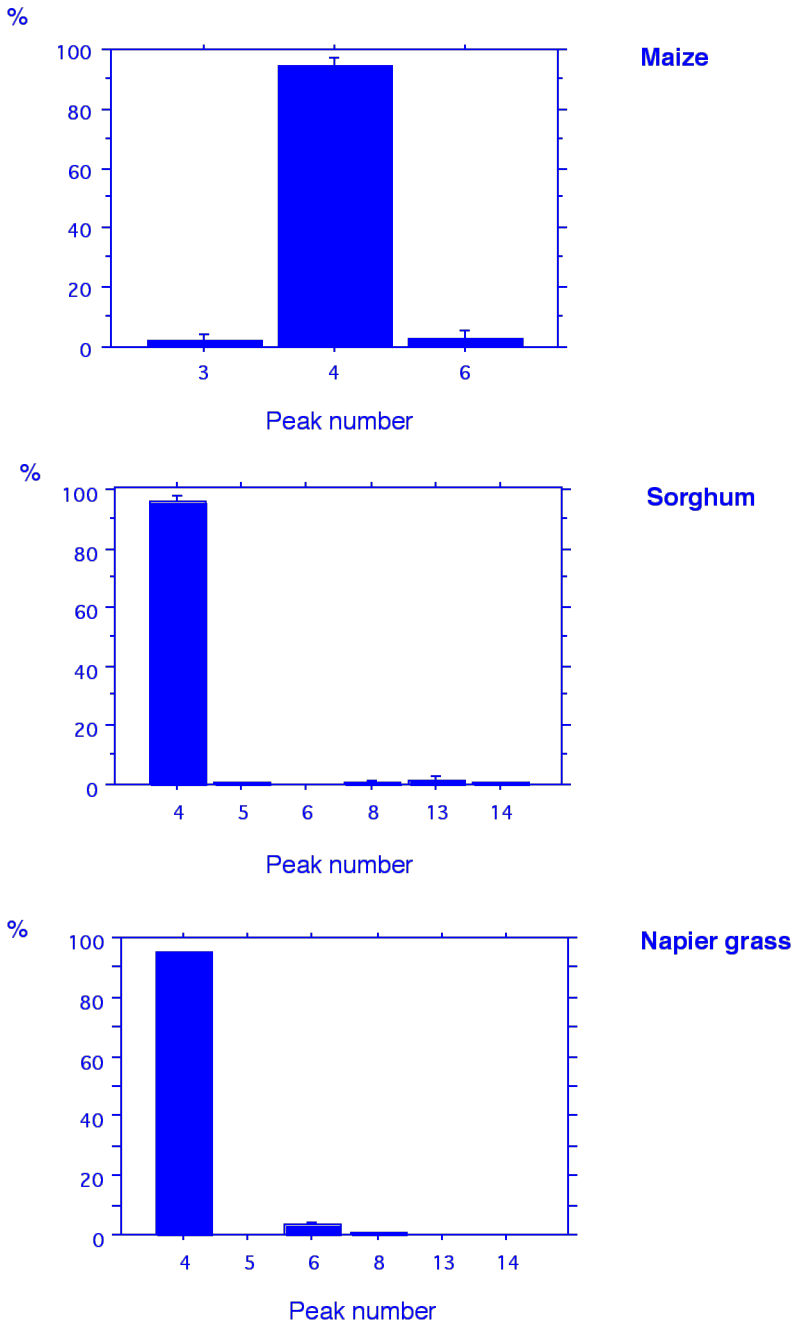


Figure 10. Relative proportion (%, means ± SE, n=3) of volatiles represented by peak number (see figure 9 for identification) detected in maize, sorghum and Napier grass [3 (*Z*)-3-hexen-1-ol; 4, (*Z*)-3-hexenyl-acetate; 5, nonatriene derivative; 6, (*E*)-4,8-dimethyl-1,3,7-nonatriene; 8, unknown; 13, caryophyllene and 14, (*E*)-cyclodecene].

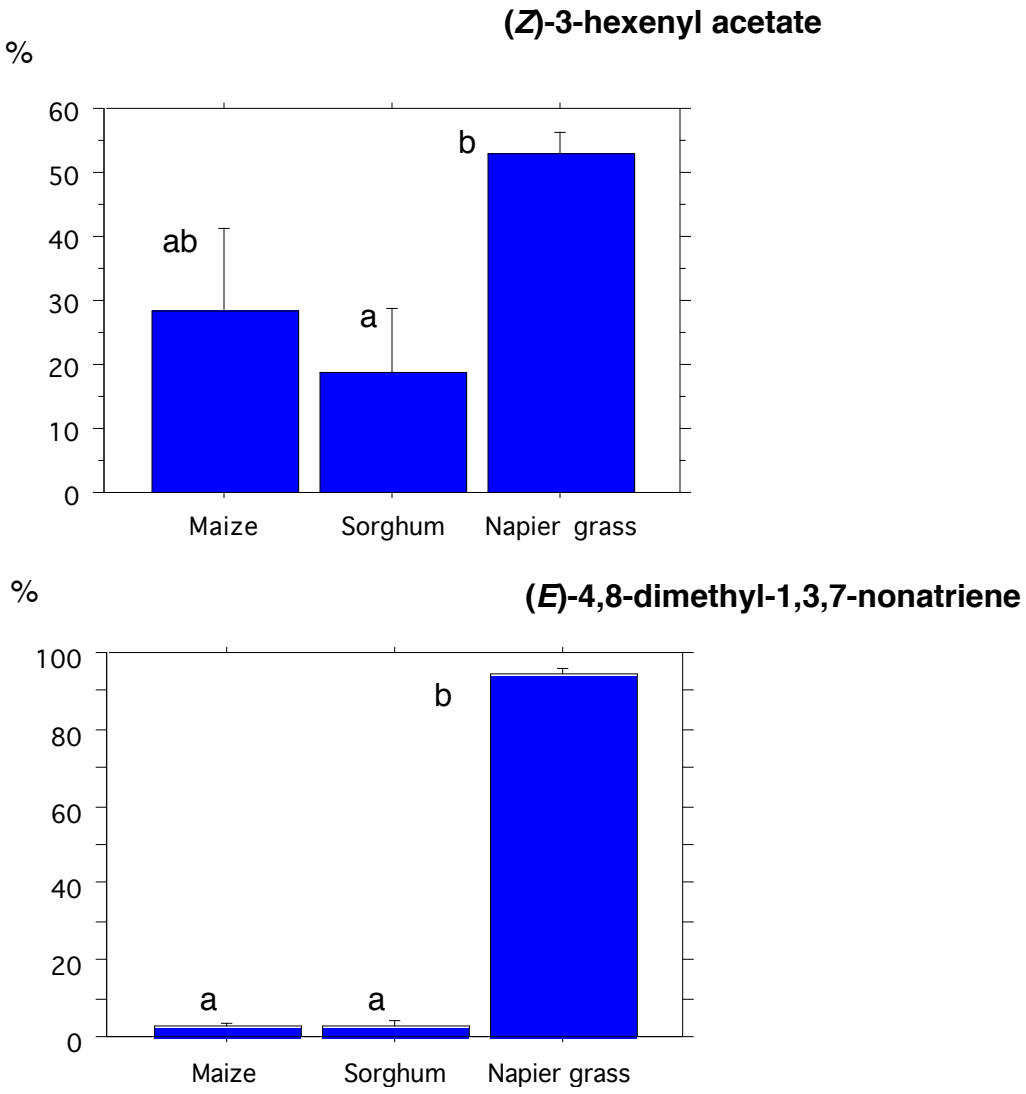


Figure 11. Relative proportion (%, means ± SE, n=3) of (*Z*)-3-hexenyl acetate and (*E*)-4,8-dimethyl-1,3,7-nonatriene major compounds emitted by uninfested maize, sorghum and Napier grass. Bars followed by different letters are significantly different at 5% level according to the PLSD's Fisher test following the ANOVA.

4.3.2-Influence of water and chloroform extracts on oviposition response of *B. fusca*

To validate whether the artificial nylon surrogate stems used in dual choice experiments did not contain putative insect stimulatory factors, two neutral stems were tested in dual choice situation. Both stems received similar proportion of eggs, 49 ± 6.7 and 51 ± 6.7 % respectively (U=196, p=0.9138 according to Mann-Whitney U test) after the eighth hour of oviposition. This indicated that no oviposition preference occurred between the two stems validating the use of such set up in subsequent oviposition preference tests.

Using plant water extracts of maize, sorghum and Napier grass in a dual choice situation (extract *vs* control(water)), no differences were observed in the number of eggs laid on surrogate stems with water plant extracts when compared to their corresponding controls (Table 4). In all tests, almost equal proportion of eggs was laid on each plant extract.

Table 4. Relative proportions (in %, means* ± SE, n=20) of eggs laid by wild females after 8 hours of night on nylon rolled helicoidally to surrogate stems imbibed with plant water extract (extract) or with water alone (control) (A = maize, B = sorghum and C = Napier grass extracts)

	A	B	C
Extract	55.5 ± 6.5 a	46.9 ± 6.7 a	56.8 ± 8.0 a
Control	44.5 ± 6.5 a	53.1 ± 6.7 a	43.2 ± 8.0 a

*Means followed by same letter are not significantly different at 5% level following the Mann-Whitney U test (column comparisons: extract *vs* control).

The proportion of eggs laid on each of the surrogate stem with the chloroform extracts differed among the plants used. Extracts of maize and sorghum received significantly more eggs than their corresponding controls (Table 5). There was no statistical difference in the proportion of eggs laid on Napier grass extract as compared to the control.

Table 5. Relative proportions (in %, means ± SE, n=20) of eggs laid by wild females after 8 hours of night on nylon rolled helicoidally to surrogate stems imbibed with plant chloroform extract (extract) or with chloroform alone (control) (A = maize, B = sorghum and C = Napier grass extracts)

	A	B	C
Extract	62.1 ± 7.8 b	70.5 ± 7.8 b	56.8 ± 8.0 a
Control	37.9 ± 7.8 a	29.5 ± 7.8 a	43.2 ± 8.0 a

*Means followed by different letters are significantly different at 5% level following the Mann-Whitney U test (column comparisons: extract *vs* control).

To further validate the role of both water and chloroform extractable chemicals on oviposition response the result was presented as oviposition stimulation index (OSI) (see Materials and Methods). The oviposition index can have a positive or negative values, which identifies extracts that are preferred by females for oviposition (positive values) and those that are not preferred (negative values). Positive indices were obtained for all chloroform and water maize plant extracts (Figure 12). In contrast, negative stimulation indices were observed for sorghum and Napier-grass water extracts. Moreover, the stimulation index was significantly higher and different with respect to the dual blank test (N *vs* N) only for sorghum extract, further validating that this extract was more stimulatory for oviposition, and thus probably contained compound(s) involved in oviposition stimulation of *B. fusca*.

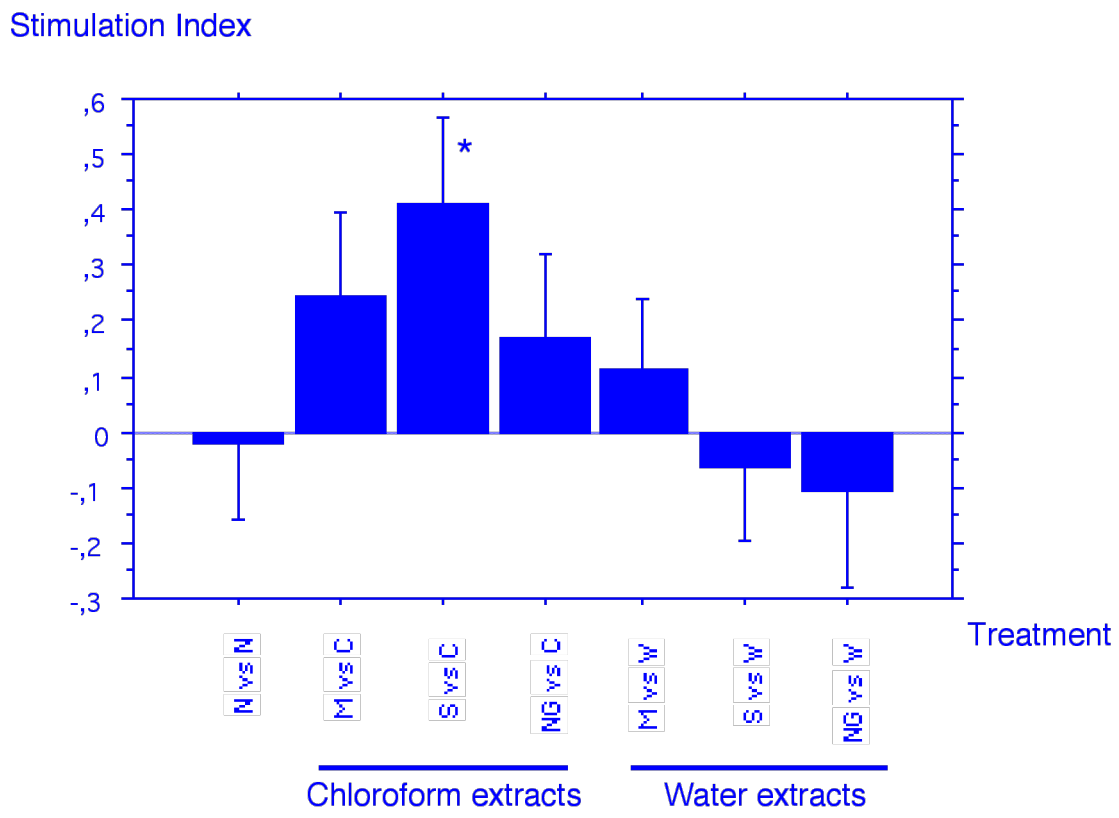


Figure 12. Oviposition stimulation index (OSI) calculated for the following dual tests: N *vs* N (nothing *vs* nothing = blank test), M *vs* C (maize chloroform extract *vs* chloroform), S *vs* C (sorghum chloroform extract *vs* chloroform), NG *vs* C (Napier grass chloroform extract *vs* chloroform), M *vs* W (maize water extract *vs* water), S *vs* W (sorghum water extract *vs* water) and NG *vs* W (Napier grass water extract *vs* water). Bar with star was significantly different at 5% level to the blank test N *vs* N according to Student's t-test.

4.3.3-Analysis of water and chloroform extracts of maize, sorghum and Napier grass

Gas chromatographic analysis to ascertain the nature of compounds present in the water extracts was performed. All the extracts contained simple sugars, organic acids and free amino acids. No difference in quantity and composition was observed among the three plant species used. Based on peak areas ratios of the plant species, sucrose, fructose and glucose were the major sugars, while alanine, leucine and arginine were the major free

amino acids detected (Figure 13). The similarity in compositions of these primary metabolites analysed among the plant species used confirmed the non-involvement of such compounds in host selection for oviposition of *B. fusca*.

HPLC analysis of the chloroform dip extracts indicated that the retention times of the maize peaks closely matched those of Napier grass (Figure 14),that is, the chromatogram profile of maize and Napier grass were similar in composition. Sorghum chromatogram however, had at least three additional major peaks (Rt 7.17, 8.00 and 33.52 min) that were absent in maize and Napier grass extract profiles. Subsequent preliminary analysis of the peak at 33.52 min by mass spectrometer showed the molecular ion of about 814 m/z and tentatively assigned the compound as a high molecular weight compound (Figure 15). These analysis confirmed the presence of putative compound involved in host selection of oviposition of *B. fusca in the* sorghum extract.

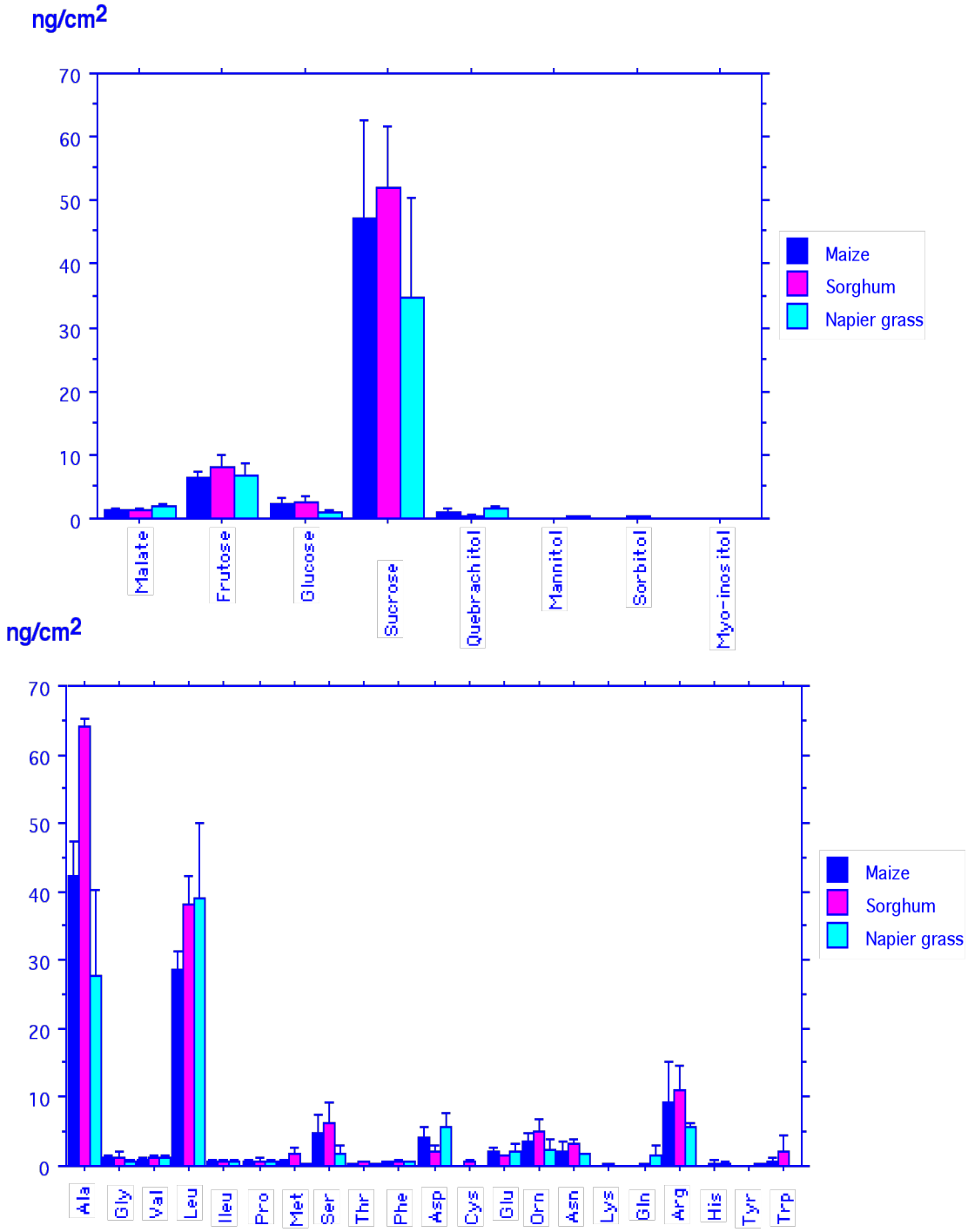


Figure 13. Concentrations (in ng/cm2, mean ± SE, n=4) of sugars and organic acids (A) and free amino acids (B) detected in the water extracts. For each compound, no difference was found among the plant species according to the Kruskal-Wallis test.

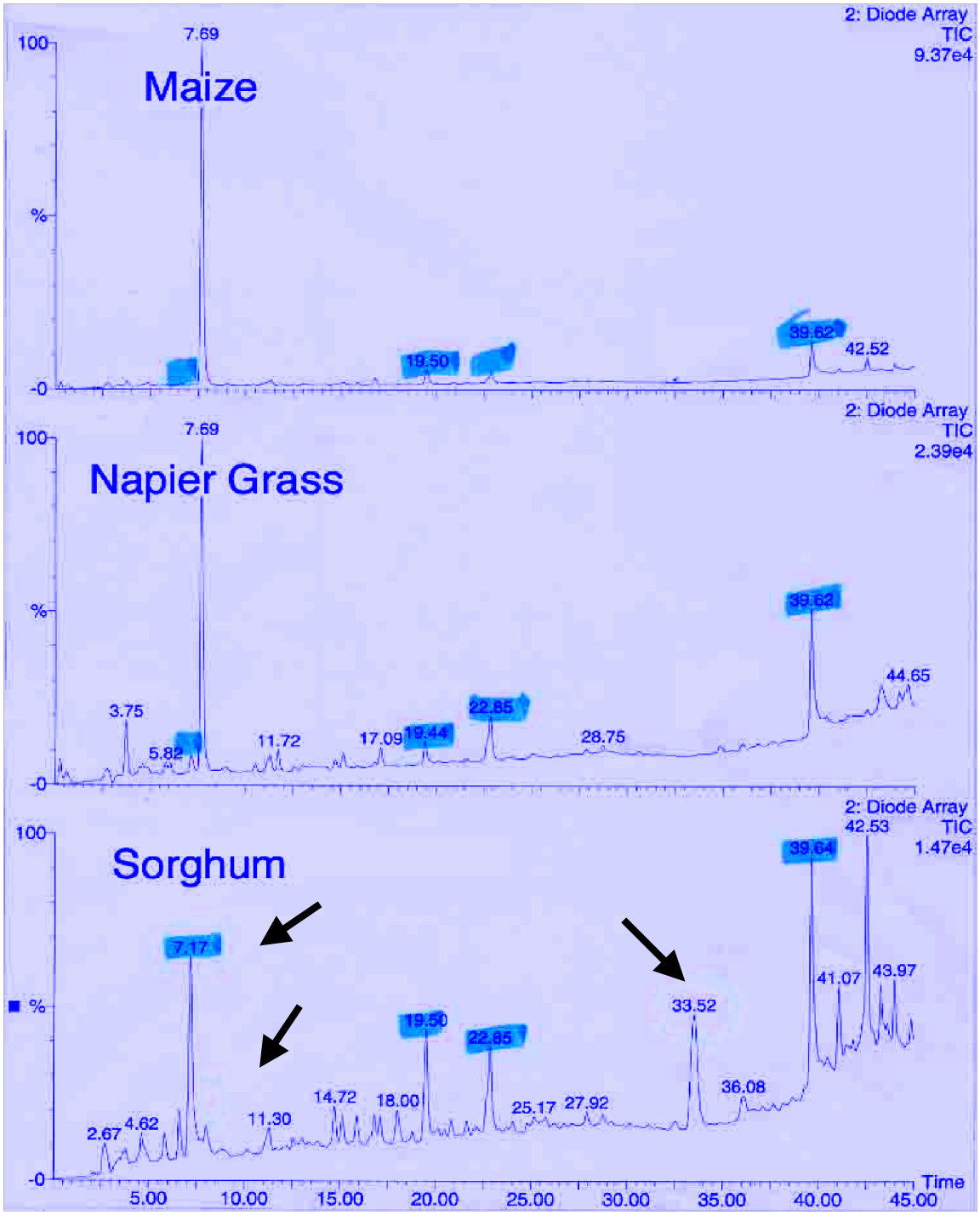


Figure 14. Chromatograms of chloroform extracts of maize, Napier grass and sorghum after HPLC analysis. The blue spots represent the common peaks found among the three plant extracts and the peaks shown by the arrows were only found in the sorghum extract.

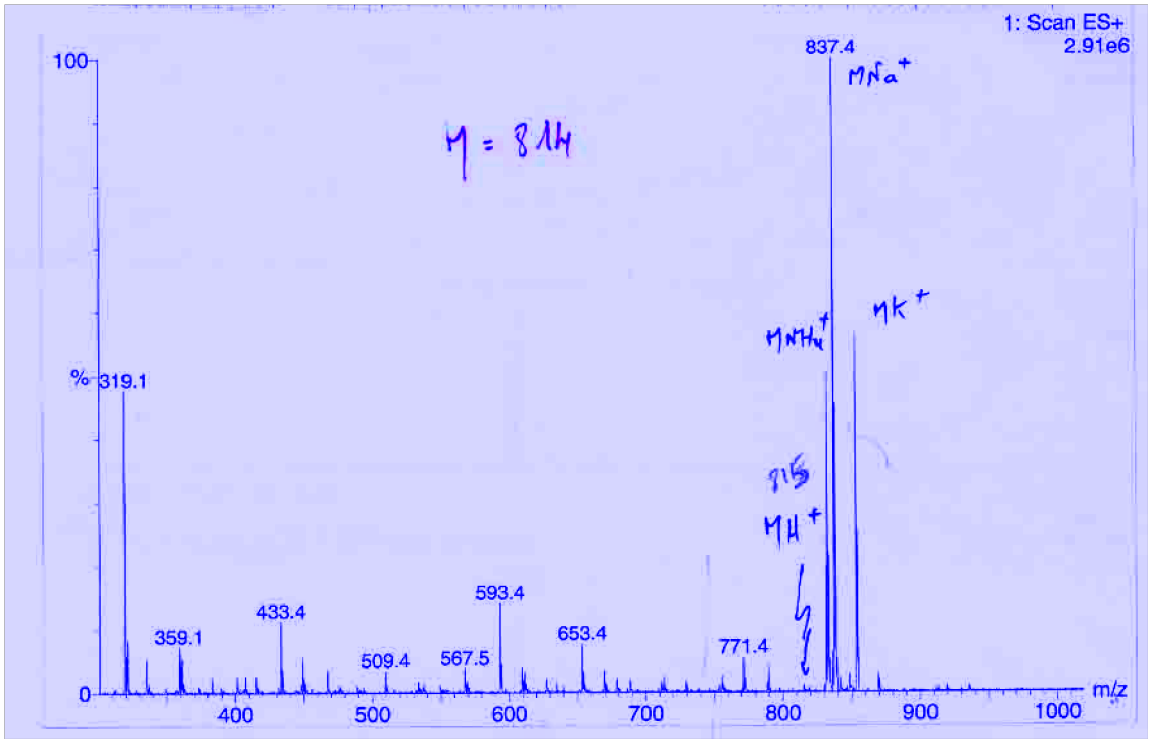


Figure 15. MS spectra of the unknown peak, Rt 33.52 min. of sorghum extract.

4.4-Discussion

Similar to previous reports (Turlings *et al*., 1994; Ngi -Song *et al*., 2000), uninfested plants emit small quantities of volatiles (Figure 8). The major constituent of the three uninfested plants in this study was the green leaf volatile, (*Z*)-3-hexenyl acetate accounting for over 90% of the total quantity of volatiles based on the peak area ratio. The other major volatiles identified were the isoprenoid, (*E*)-4,8-dimethyl-1,3,7-nonatriene and caryophyllene. (*Z*)-3-hexen-1-ol was detected only in maize while (*E*)-cyclodecene was detected only in sorghum and Napier grass (Figure 9). The green leaf volatiles consist generally of a number of compounds of saturated or monosaturated aldehydes, alcohols and acetates which occur in all plants, but in very varying proportions depending on the plant species (Hansson *et al*., 1999). In our study, Napier grass emitted more volatiles than sorghum and maize and in larger quantities (Figures 9 and 11), particularly more (*E*)-4,8-dimethyl-1,3,7-nonatriene. Most of the compounds identified in Figure 9 have been shown to be electrophysiologically active to *B*. *fusca* antennae (Khan & Hassanali, 2004). Antennal receptors of different lepidopteran species

were stimulated in response to green leaf volatiles, which included (*Z*)-3- hexenyl acetate (Visser, 1986; Bengtsson *et al.,* 2001). This class of compounds, possibly in combination with other constituents of plant odours, is assumed to be involved in herbivore orientation to their host plants (van Tol & Visser, 2002). The high level of the volatile released by Napier grass as compared to maize and sorghum could possibly be involved in the attraction of stem borers and this could explain why the plant is used as a trap crop in the "push–pull" strategy of insect control (Khan *et al.,* 2000). However, it was observed that *B. fusca* females alighted significantly more on maize and sorghum than on Napier grass for oviposition when the plants were put together in wind tunnel experiments, indicating that Napier grass is relatively less attractive from distance to *B. fusca* (Le Rü *et al*., 2004). Therefore, it seems that there is no correlation between insect attraction for oviposition and levels of major constituents of released volatiles. It could be possible that green leaf volatiles are indicative of insect orientation by distance to graminaceous plants and then afterwards, a close contact is necessary for *B. fusca* to make a decision to select the host plant for oviposition. Alternatively, very minor constituents of the plant volatiles may be involved in insect orientation. Additional experiments are needed to explore those possibilities.

In addition, while olfactory cues may be involved in plant orientation, chemo-tactile stimuli may play a decisive role in insect host plant selection for oviposition (Le Rü *et al*., 2004). Insects probably detect chemicals on the leaf surface as behavioural cues important for ovipositing. Though plant surfaces contain a large number of chemicals, several or only a few of which may be responsible for stimulating oviposition in phytophagous insects. In this study, based on gas chromatography analysis, comparable quantities of sugars, organic acids and amino acids were present in the water-soluble extracts of all the plant species studied. Although such compounds have been shown to elicit some ovipositional response in other Lepidotera species (Derridj *et al.,* 1989), the similarity in concentration and composition among the three plant species used does not reflect the preferential ovipositional response displayed by *B. fusca* to these plants as confirmed by behavioural tests. This indicates that such compounds have no crucial role in stimulating oviposition in this insect species.

Although water-soluble extracts may have little role in stimulating oviposition in *B. fusca*, it was observed that chloroform-soluble compounds from sorghum applied on nylon surrogate stems play a significant role in *B. fusca* oviposition stimulation. Stimulatory chemicals may be present on the plant surface. Brief extractions of plant foliage at room temperature is recommended for the extractions of surface compounds (Eigenbrode & Espelie, 1995; Derridj *et al*., 1996). Hence in this study, a 2 seconds extract provided evidence that epicuticular chemicals of sorghum are stimulatory. Preliminary analyses of these chemicals revealed that one of the potential compound involved in oviposition stimulation is a molecule of medium polarity that eluted at high acetonitrile concentration (Rt 33.52 min) and possessed a high molecular weight of about 814 m/z (Figures 14 and 15). However, two more polar compounds eluted with lower acetonitrile concentration (Rt 7.17 and 8.00 min) and were also characteristic of sorghum extract. This could also explain the enhanced oviposition stimulations of this extract as compared to that of maize and Napier grass. However, additional research is needed to identify, quantify and confirm the involvement of these compounds in host selection of *B. fusca* for oviposition. Less polar compounds such as lipids have been reported to elicit a positive ovipositional response in other Lepidoptera species (Udayagiri & Mason 1997, Rivet & Albert 1989).

Chapter 5

Conclusions and perspectives

Sorghum, maize and millet are important staple food crops cultivated in most parts of Africa. Despite of the technological advancement in food production, the productivity of these crops is very low due to the damage caused by various insect pests. Among the insect-pests, the stem borer *Busseola fusca* (Fuller)(Lepidoptera: Noctuidae) has become one of the dominant and most economically important, especially in East Africa.

Diverse control methods including among others, chemical, cultural and biological have been adopted to control this pest but with little success. Recently, a novel habitat-management strategy, the "push-pull" or stimulo-deterrent diversionary tactics has been developed to control the insect pest. The strategy involves the attraction and retention of stem borers on a highly susceptible trap plants (pull) and preventing them to reach the harvestable (maize) crops by a repellent and unsuitable intercrops (push). Adoption and utilisation of such strategy needs in part a good understanding of the host selection process for oviposition of the insect pest.

It has been reported in the literature that laboratory-reared insects generally tend to differ from natural (wild) population in genetic, behavioural and physiological characteristics and this limits their representativeness of the species in the wild. The rearing conditions can therefore strongly influence the insect behaviour and physiology.

For future studies on a good understanding of the host selection for oviposition of *B. fusca*, it was first necessary to verify if the laboratory-reared *B. fusca* differ from the natural population in their reproductive biology especially in oviposition of the females with a special attention to the original host plant recognition.

After having been reared under laboratory conditions for several generations, the insects have lost the host plant specificity for oviposition. They accept support totally outside their original host plants such as paper surrogate stem and show no preference for oviposition on artificial stems imbibed with stimulatory plant extracts and fewer exhibit an oriented flight behaviour toward maize plants in wind tunnel conditions. However, the females after being reared artificially conserved the same antennal sensitivity towards

host plant volatiles as the wild ones. These results indicate that laboratory-reared *B. fusca* insects differ from natural population in the host plant specificity and this limits their representativeness of the species in the wild. Therefore it is important to use wild insects in future studies such as studies on the influence of plant chemistry in host plant selection for oviposition. Moreover these preliminary findings underline the ability of *B. fusca* to change oviposition supports and even maybe to change the original host plants.

Previous work showed that host plant physical characters play a greater role in host plant selection for oviposition of *B. fusca*. Using three plant species (maize, sorghum and Napier grass), naïve gravid females significantly preferred maize and sorghum over Napier grass for oviposition a phenomenon, which was partly related to plant surface texture and leaf sheath toughness. In addition to the importance of physical cues in selection of suitable oviposition sites, gravid female insects also strongly responded to semiochemical cues associated with their hosts.

In this context, the volatiles emitted by maize, sorghum and Napier grass at the insect most preferred stage of growth were analysed and the influence of polar and less polar plant surface compounds involved host selection for oviposition of *B. fusca* determined.

Uninfested maize, sorghum and Napier grass emit small quantities of volatile metabolites. The major constituent of the three uninfested plants was the green leaf volatile, (*Z*)-3-hexenyl acetate accounting for over 90% of the total quantity of volatiles based on the peak area ratio. The other major volatiles identified were the (*E*)-4,8-dimethyl-1,3,7-nonatriene and caryophyllene. (*Z*)-3-hexen-1-ol detected only in maize and (*E*)-cyclodecene detected only in sorghum and Napier grass. Napier grass emitted more volatiles than sorghum and maize and in a larger quantities particularly (*E*)-4,8-dimethyl-1,3,7-nonatriene. However, in previous wind tunnel experiment, it had been observed by using these three plants species together, that *B. fusca* females alighted significantly more on maize and sorghum than on Napier grass for oviposition, indicating that Napier grass is relatively less attractive from distance to *B. fusca*. Therefore, there is no correlation between insect attraction for oviposition and levels of major constituents of released volatiles. It could be possible that these green leaf volatiles are mostly indicative of insect orientation by distance to graminaceous plants and then after, a close contact is necessary to make *B. fusca* decision to select the host plant for oviposition. Alternatively,

very minor constituents of the plant volatiles may be involved in insect orientation. Additional experiments are needed to explore these possibilities.

Moreover, while olfactory cues may be involved in plant orientation, chemo-tactile stimuli may play a decisive role in insect host plant selection for oviposition. Although water-soluble extracts may have little role in stimulating oviposition in *B. fusca*, chloroform-soluble compounds from sorghum play a significant role in *B. fusca* oviposition stimulation. Stimulatory chemicals may be present on the host plant surface. Preliminary analyses of such extract revealed that one of the compound potentially involved is a low polar molecule eluted at high acetonitrile concentration (Rt 33.52 min) and possess a high molecular weight of about 814 m/z. However, two more polar compounds were also eluted but at a lower acetonitrile concentration, (Rt 7.17 and 8.00 min) and were also characteristic of sorghum extract. This could also explain the enhanced oviposition stimulations of this extract compared to those of maize and Napier grass. Additional research is needed to identify and confirm the involvement of these compounds in host selection of *B. fusca* for oviposition.

Recently, it has been shown by molecular analyses that *B. fusca* populations can be separated into three mitochondrial clades: one from Western Africa, and two designated Kenya I and Kenya II, from Eastern and Central Africa. The similarity of intraclades and interclades distances observed in these three large population units, suggests that they were isolated at the same period in three different refuges from sub-Saharan Africa and have had comparable demographic history. However, Kenya II appears more largely distributed into Central, East and South Africa while Kenya I is more localised, mostly present in Eritrea and fewer in Ethiopia and Kenya. Recently, it has been observed that the females of Kenya I possess smaller antennae than females of Kenya II. It has been shown a positive relationship between antennal length and EAG amplitude response for *B. fusca.* It could be therefore probable that females of Kenya I possess a less antennal sensitivity towards plant volatiles than females of Kenya II explaining in part the larger distribution of Kenya II in Africa.

Chapter 6

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