Chemical ecology of plant-to-plant communication and opportunities for maize stemborer management in Africa

TA Tolosa
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Thesis submitted for the degree *Philosophiae Doctor* in Environmental Sciences at the Potchefstroom Campus of the North-West University

Promoter: Prof J van den Berg

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Promoter:	**Prof J Van den Berg**
Co-promoters:	**Dr CAO Midega** **Prof ZR Khan**

May 2016

NORTH-WEST UNIVERSITY, SOUTH AFRICA

DECLARATION BY THE CANDIDATE

I, **TIGIST ASSEFA TOLOSA**, declare that this thesis which I submit to North-West University, Potchefstroom Campus, in compliance with the requirements set for the *Philosophiae Doctor* (Environmental Sciences) degree is my own original work and has not been submitted to any other university for a similar or any other degree award.

Signature: --- Date: ---

DECLARATION AND APPROVAL BY SUPERVISORS

We declare that the work reported in this thesis was carried out by the candidate under our supervision and approve its submission

Prof. Johnnie Van den Berg
School of Environmental Sciences and Development, North-West University, Private
Bag, X6001, Potchefstroom, 2520, South Africa.

Signature: --- Date: --

Prof. Zeyaur R. Khan
Habitat Management Programme, International Centre of Insect Physiology and
Ecology, P.O Box 30-40305, Mbita, Kenya.

Signature: --- Date: --

Dr. Charles A. O. Midega
Habitat Management Programme, International Centre of Insect Physiology and
Ecology, P.O Box 30-40305, Mbita, Kenya.

Signature: --- Date: --

DEDICATION

To my dearly loved parents Assefa Tolosa and Ayenalem Ayele for their constant sacrifices and supports.

To my beloved husband Kidanemariam Kassahun and our dear Son Nathan Kidanemariam for their encouragements and love.

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ACRONYMS AND ABBREVIATIONS

EAG - Electroantennography

EFN - Extra Floral Nectar

FAO - Food and Agricultural Organization

FARA - Forum for Agricultural Research in Africa

GC - Gas Chromatography

HIPV - Herbivore-Induced Plant Volatiles

ICIPE- International Centre of Insect Physiology and Ecology

IITA - International Institute of Tropical Agriculture

Ltd – Limited

MS - Mass Spectroscopy

VOC - Volatile Organic Compound

FID – Flame Ionization Detector

HP – Hewlett Packard

NIST – National Institute of Standards and Technology

GLV – Green Leaf Volatile

DMNT- (*E*)-4,8-dimethyl-1,3,7-nonatriene

SDDS- Stimulo-deterrent Diversionary Strategy

TMTT – (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene

Abstract

Maize is the most widely grown cereal crop worldwide, and is the most important staple crop in sub-Sahara Africa. The spotted stemborer, *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) is considered among the most important pests of maize and the damage it causes may result in yield losses of up to 88%. Previous studies showed that plants damaged by herbivores release huge amounts of volatile compounds, known as herbivore-induced plant volatiles (HIPVs) into the environment, which serve as attractant to natural enemies and repellent to herbivores. In addition, emitted HIPVs affect the defence responses of neighbouring plants. Previous studies reported that the non-host molasses grass, *Melinis minutiflora* P. Beauv. repels *C. partellus* moths and increases larval parasitism by *Cotesia sesamiae* Cameron (Hymenoptera: Braconidae) when intercropped with maize. However, the potential role of plant signalling between molasses grass and maize, and any subsequent effect on *C. partellus* and *C. sesamiae* behaviour remained unknown. Moreover, it was not known for how long the maize plant retained the information after removal from exposure to the emitter plant. Experiments were conducted by exposing plants of two maize landraces, "Nyamula" and "Jowi-red", and two hybrid maize varieties "WS505" and "PH4" to molasses grass volatiles for certain periods of time. In two-choice oviposition bioassays, gravid *C. partellus* moths preferred non-exposed maize landraces for oviposition compared to those exposed to molasses grass volatiles. Additionally, volatile samples collected from landrace maize plants were significantly more attractive to *C. sesamiae* compared to non-exposed maize plants in four-arm olfactometer bioassays. Similarly, maize plants previously exposed to molasses grass and removed for certain periods of time then infested by *C. partellus* larvae were not preferred for ovipostion by *C. partellus* moths, and headspace samples collected were more attractive to *C. sesamiae* compareared to non-exposed infested plants. Headspace samples were analysed using Gas Chromatography (GC), Coupled Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography-Electroantennography (GC-EAG). GC-EAG analysis with attractive headspace samples from exposed maize landraces revealed that *C. sesamiae* was responsive to certain compounds, namely, myrcene, (Z)-3-hexen-1-ol acetate, *(E)*-ocimene, (β)-ocimene, *(R)*-linanool, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), decanal, *(E)*-caryophyllene and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). Notably, with the commercial maize hybrids, there was no significant difference in the number of eggs laid by *C. partellus* moths on exposed and non-exposed plants. Similarly, there was no significant difference in *C. sesamiae* attraction towards volatiles obtained from exposed and non exposed hybrid maize plants. These findings suggest that volatile organic compounds released by molasses grass have the ability to induce defence responses in neighbouring maize landraces, a trait that the commercial hybrid varieties seem to lack, and demonstrate the potential of plant signalling as a component of management approaches for stemborer pests in subsistence farming in Africa.

Key words: *Chilo partellus*, *Cotesia sesamiae*, induced defence, *Melinis minutiflora,* neighbouring plants, oviposition.

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background

Agriculture is the most important enterprise in Africa, and is the backbone of the economy of most African countries (Abate *et al.,* 2000). About 60% of people in the continent earn their livelihood from the agricultural sector (FAO, 2011). In spite of the importance of agriculture in the economy of the continent, productivity remains low. This, combined with the high human population growth rates in the continent, result in high incidences of poverty and food insecurity (Sasson, 2012). Indeed Africa continually faces the challenge of feeding its population due to its failing agricultural sector. With reports indicating that income growth derived from agriculture having up to four times effectiveness in reducing poverty (World Bank, 2008), growth in agricultural productivity therefore remains the key to economic development in the continent (Midega *et al.,* 2015).

Maize (*Zea mays* L.) is the world's most abundantly grown cereal crop, with an annual production of over 870 million metric tons (Cairns *et al.,* 2013). It is rich in vitamins, carbohydrates, essential minerals and protein (IITA, 2014), and remains the most important cereal crop in developing countries (Morris, 2002). In developed countries maize is cultivated mainly as animal feed or sold for industries as raw materials for the production of corn oil, corn syrup, fuel (ethanol) and starch. However, in developing countries, it is the basic staple food for about 900 million consumers (FARA, 2009), with crop residues being basic elements of animal feed.

Lepidopteran pests such as *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) and the invasive *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) are considered among the most important pests of grain crops, causing up to 88% yield losses (Kfir *et al*., 2002). Approximately 21 economically important species of lepidopteran stemborers infest cultivated grasses in Africa (Seshu Reddy, 1983; Harris, 1990; Maes, 1998). Among these are the noctuids *B. fusca* and *Sesamia calamistis* Hampson, the pyralid *Eldana saccharina* (Walker) and 12 Crambidae species including *C. partellus* and *Chilo orichalcociliellus* (Strand) (Kfir *et al.*, 2002).

1.2 Statement of the problem and justification

The lepidopteran stemborers, *B. fusca* and *C. partellus,* are well known pests of maize and sorghum in Africa (Kfir *et al.*, 2002), causing significant yield losses depending on season, ecological zones and geographical location (Mailafiya and Degri, 2012). Their larvae damage crops by feeding on young leaf tissues and boring into the stems of the crop, causing reduction in photosynthetic area, damage to translocation vessels and foliar damage (Bosqu-Pérez and Schulthess, 1998; Maes, 1998). In addition to this, larvae attack and damage the maize ears (Mailafiya and Degri, 2012). The nature of damage depends on the stemborer species, crop growth stage, number of larvae feeding on the plant, plant reaction to the damage as well as agro-ecological conditions (Mailafiya and Degri, 2012).

In order to suppress stemborer infestation levels and damage, various management strategies have been developed. However, a number of these approaches have not been widely adopted due to various socio-economic and biological challenges. Additionally, use of chemical pesticides is largely ineffective, partly due to the cryptic and nocturnal habits of the adult moths, and the protection provided by the stem of the host crop for immature stages. Recently, the push-pull strategy has been developed by scientists at the International Centre of Insect Physiology and Ecology (*icipe*) and partners, including Rothamsted Research (United Kingdom) for stemborer management (Khan *et al.* 2001; Khan and Pickett, 2004).

The push-pull strategy uses selected plant species in the system, i.e. trap plants planted around the main crop together with repellent intercrops. The intercropped companion plants, molasses grass (*Melinis minutiflora* P. Beauv.) or desmodium (*Desmodium uncinatum* Jacq. and *Desmodium intortum* Urb.) repel gravid stemborer moths away from the main crop while trap plants, Napier grass (*Pennisetum purpureum* Schumach) or Sudan grass (*Sorghum vulgare sudanense* Pers.) attract the moths (Khan and Pickett, 2004), keeping them away from the target crop. In addition to stemborer management, the push-pull strategy plays an important role in the suppression of the parasitic *striga* weed (*Striga hermonthica* (Del.) Benth.) and provides added value in terms of increased soil fertility (Khan and Pickett, 2004). The push-pull system relies on stemborer repellent compounds such as *(E)*-β-ocimene and *(E)*-4,8,-dimethyl-1,3,7-nonatriene, from *Desmodium* species and molasses grass which lure parasitoids of the stemborers. In addition to this, perimeter trap plants such as Napier grass play an important role by emitting volatiles that are more attractive to the female stemborer than those emitted by maize plants (Khan *et al.,* 1997; Khan *et al.*, 2000; Birkett *et al.,* 2006; Khan *et al.,* 2010). However, when the eggs hatch the larvae are unable to survive or their development is constrained, thus reducing the number of pest individuals in the environment (Khan and Pickett, 2004; Pickett *et al.,* 2006).

Plants actively respond to attacks by releasing herbivore induced plant volatiles (HIPVs) that play a significant role in plant-to-plant communication. In addition to this, HIPVs could be used to repel pests and attract their natural enemies. It was recently observed that some plants are signalled by volatiles from neighbouring plants to produce HIPVs without being damaged by herbivores (Ramadan *et al.,* 2011; Ton *et al.*, 2006). Molasses grass has the unique characteristic of releasing constitutively "cry for help" volatile cues that repel stemborer moths and attract their natural enemies without being damaged. Therefore, the current study sought to investigate any induction and/or priming of defence on maize mediated by volatiles emitted by molasses grass. These findings will generate novel and useful information in this area of science and contribute to our understanding of plant-to-plant communication for subsequent exploitation in pest management in African farming systems and beyond.

1.3 Objectives

1.3.1 General objectives

To develop a maize stemborer management strategy based on understanding of plant-to-plant communication involving companion cropping.

1.3.2 Specific objectives

This study had four specific objectives, namely:

1. to assess any plant-to-plant communication between *Melinis minutiflora* and different maize varieties.
2. to examine the effects of any induced plant responses on female moths of *Chilo partellus.*
3. to examine any effects of induced plant responses on *Cotesia sesamiae,* a parasitiod of *C. partellus.*
4. to investigate any priming effects in the target plants arising from the effects of stimuli from neighbouring plants.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin and distribution of stemborers

Lepidopterous stemborers are among the most important insect pests of maize in Africa (Kfir *et al.,* 2002) where they cause significant yield losses. These include the maize stalk borer, *Busseola fusca* (Füller) (Lepidoptera: Noctuidae), the pink stalk borer, *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae), the African sugarcane borer, *Eldana saccharina* Walker (Lepidoptera: Pyralidae), Coastal stalk borer, *Chilo orichalcociliellus* Strand (Lepidoptera: Crambidae) and the spotted stalk borer, *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae). The first four are of African origin, and are present in most countries in sub-Saharan Africa (SSA), while *C. partellus* is native to Asia and is believed to have been accidentally introduced into Africa before 1930 (Tams, 1932). It is widespread throughout eastern and southern Africa (Figure 2.1) (Bosque-Pérez, 1995; Kfir *et al.,* 2002) whereas *B. fusca* is distributed throughout SSA (Kfir *et al.,* 2002).

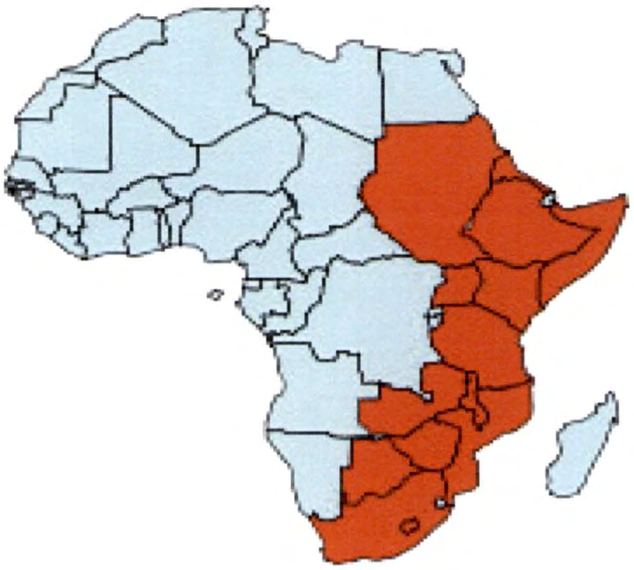

Figure 2.1: Geographical distribution of *Chilo partellus* in Africa (countries indicated in red are where the exotic *Chilo partellus* have been reported).

Source: http://www.infonet-biovision.org- Spotted stemborer

2.2 Biology and behaviour of *Chilo partellus*

Chilo partellus is a polyphagous species that has several alternative cultivated and non-cultivated gramineous host plants (Harris, 1990; Khan *et al.,* 1991; 2000; Kfir *et al.,* 2002). The behaviour and life cycle of *C. partellus* is similar to that of *B. fusca* but it does not undergo diapause in warm areas (Kfir *et al.,* 2002). The gravid female *C. partellus* mostly lays eggs on the underside of the leaf sheath of young host plants, often near the midrib (Hutchison *et al.,* 2007). Eggs hatch after an incubation period of 7-10 days. Newly hatched larvae feed on the funnel leaves of the plant, creating a characteristic damage pattern before they bore into the stems or migrate to neighbouring plants (Harris, 1962; Bosque-Pérez and Schulthess, 1998).

Stemborer larvae feed on leaf surfaces and tunnel inside stems of host plants. The larvae take 28-35 days to complete its developmental stages (Hutchison *et al.,* 2007), depending on the species. The larval developmental stage may last 28-58 days (Mailafiya *et al.,* 2011). Fully grown larvae or the last instar pupate inside the stem for 6-14 days after which adult moths emerge (Maes, 1998). Immediately after emergence the adults mate during the two to three subsequent nights (Berner *et al.,* 1993) and lay eggs on plants to continue their life cycle. Under favourable conditions the total life cycle take 30-60 days to complete (Figure 2.2). Depending on species, temperature and other related factors the length of the insect life cycle may vary. In general *C. partellus* may complete three or more generations per year, depending on the availability of resources and favourable temperatures.

Most stemborer species escape harsh environmental conditions by entering into diapause, or inactive stage, especially at the end of the cropping season. This diapause period may last for up to six months (Kfir *et al.,* 2002). *Chilo partellus* normally develops and continues its life cycle if there is an abundance of host plants and favourable environmental conditions (Kfir *et al.,* 2002).

Diapausing individuals pupate 10-12 days after the onset of rains before they emerge as adult moths (Bosque-Pérez and Schulthess, 1998). The adult moths are mostly sedentary during the day but become active at night and hence are classified as nocturnal (Kfir, 1998). It is also during the night time that they mate and lay eggs.

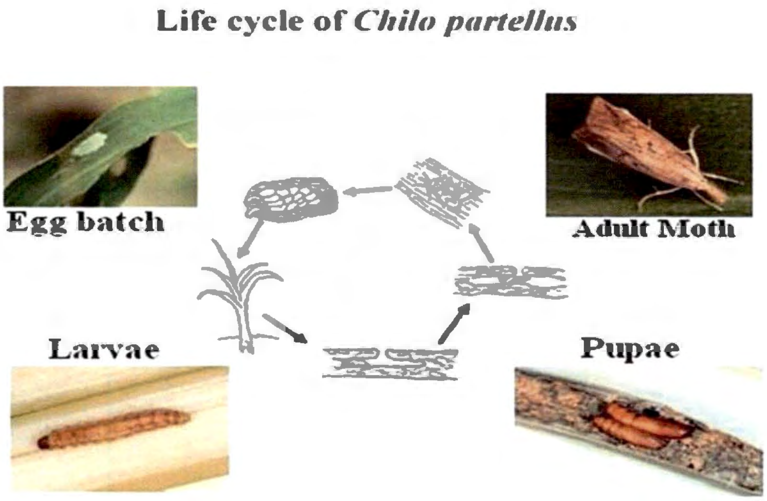

Figure 2.2: Life cycle of *Chilo partellus* (Source: *icipe*-push-pull.net)

2.3 Economic importance of *Chilo partellus*

Maize is the world's most widely grown cereal, the major staple crop in developing countries, and is mainly grown by small-scale farmers. Stemborers are serious and economically important pest of maize in sub-Saharan Africa, causing significant losses in the region (Kfir *et al.,* 2002). Stemborer larvae feed on the growing folded central leaves before penetrating into the stems where they then tunnel (Wisdom and Mary, 2012). Due to infestation by the pest, crop losses may result from death of the growing point (dead hearts), early leaf senescence, reduced translocation of nutrients, lodging and direct damage to the ears (Figure 2.3) (Kfir *et al.,* 2002).

Cereal yield losses due to stemborers greatly vary depending on season and ecological zones (Mailafiya and Degri, 2012). Examples include yield losses by *C. partellus* of 88% on sorghum reported in Kenya (Seshu-Reddy, 1988; Kfir *et al.,* 2002), and 56% recorded in

Uganda with infestation of the crop occurring early, at 20 days after seedling emergence (Starks, 1969). In Zimbabwe, yield losses due to *C. partellus* in sorghum were reported to range between 50 and 60% (Sithole, 1990). In Ethiopia yield losses due to stemborer pests ranged between 20 and 50% (Gebre-Amlak, 1985; Getu and Abate, 1999). In general, maize or sorghum yield losses caused by stem borers range between 40 and 88% (Kfir *et al.*, 2002, Van den Berg, 2009). Consequently, the damaged seed is easily exposed to fungal infection during storage and reduced food quality results (Kfir *et al.*, 2002).

Figure 2.3: Damage by stemborer larvae. © D. Cugala stemborer team, *icipe*.

Source: http://www.*infonet-biovision.org*-Spotted stemborer

2.4 Control strategies of stemborer

The key to an insect's success lies in its great reproductive potential, small size, dispersal mechanisms, and ability to survive harsh environments (Bosque-Pérez, 1995). Maize is damaged by more than 200 species of insects, of which the lepidopterous stemborer complex is probably the most serious (Wisdom and Mary, 2012). For those pests, control measures must be devised to minimize the economic impact of their damage.

2.4.1 Cultural control

Cultural control plays a significant role to make the environment less favourable for pest insects (Kfir *et al.,* 2002). The latter involves a number of strategies to disrupt their life cycles and to make the environment unfavourable for the pest to survive in. For example crop rotation can reduce pest populations and reduce the adaptive mechanism to different host plants. Adaptation in planting date causes differences in synchronisation between host plant and pest occurance, resulting in the plant escaping serious damage. Tilling and field sanitation, to keep fields clean of plants or materials that may harbour pests, are also commonly used strategies (Dent, 1991; Bosque-Perez, 1995). African smallholder farmers have been using different types of plant extracts to protect their crops from pest damage, including *Azadirachta indica* A. Juss (neem) (Marandu *et al*., 1987; Polazsek, 1998; Ogendo *et al*., 2013). However, the use of cultural control practices is limited due to shortage of labour, lack of finance and presence of alternative wild grasses that may host pest species.

2.4.2 **Biological control**

Biological control is an important strategy in stemborer management. It involves use of the natural enemies of stemborers such as parasitoids and predators, which contribute to the mortality of stemborers at different stages of their life cycle. *Cotesia flavipes* and *Cotesia sesamiae* (Cameron) (Hymenoptera: Braconidae), and *Trichogramma bournieri* Pintureau (Hymenoptera: Trichogrammatidae) are parasitic wasps that attack the larval and egg stages of *C. partellus* respectively, and have shown good results in biological control (Seshu Reddy, 1989). *Cotesia flavipes* alone has resulted in a 32-55% reduction of stemborer density in East and southern Africa (Kfir *et al.,* 2002). Parasitoids are insects whose larvae feed internally (endo-parasitoids) or externally (ecto-parasitoids) on other arthropods (Hassanali *et al.,* 2008; Wisdom and Mary, 2012). The parasitoids search for plants containing stemborer life stages by detecting volatile semiochemicals released by the plants as a result of the presence and/or feeding of these life stages on the plant (Hassanali *et al.,* 2008). *Cotesia* for example then lays about 40 eggs into a single stemborer larvae. Upon hatching the larvae of the parasitic wasp feed and move freely inside the host larvae (http://www.infonet-biovision.org).

Cotesia flavipes was first introduced into Kenya during 1991 by the International Centre of Insect Physiology and Ecology (*icipe*) for control of *C. partellus* (Overholt, 1993; Getu *et al.*, 2001). Following its introduction into Kenya, this parasitoid has been released in a number of African countries from where it has spread and established in countries where it was not released, for example Ethiopia and Tanzania (Getu *et al.,* 2001; Kfir *et al.,* 2002). Although the parasitoids were released in Ghana it failed to establish (Hordzi and Botchey, 2012). In addition to this, the distribution of *C. flavipes* in Ethiopia was affected by rainfall and temperature (Getu *et al.,* 2001). However, often there are insufficient numbers of the natural enemies to achieve economically significant control of the pests, posing a challenge to biological control of pests (Kfir, 1995).

2.4.3 Chemical control

Globally, chemical insecticides are effective in control of stemborers if used as a seed treatment before planting, or if applied before the larvae penetrate into the stems of the host plants. However, use of insecticides for pest control is not only expensive in the context of smallholder farmers, but may also have undesirable consequences such as resistance development, secondary pest outbreaks, environmental pollution and risk to spray operators (Van den Berg and Nur, 1998; Bruce *et al.,* 2010; Tamiru *et al.,* 2011). Additionally, use of chemical pesticides has been largely ineffective for stemborer control due to the cryptic and nocturnal habits of the adult moths, and the protection provided by the stem of the host crop for immature stages (Van den Berg and Viljoen, 2007; Khan *et al.*, 2008b).

2.4.4 Host plant resistance

Use of resistant host plant varieties is economically acceptable, compatible with other insect-control methods, has no adverse environmental effects and has been suggested as the most promising means of stemborer control for reducing yield losses (Bosque-Perez and Schulthess, 1998). This strategy enables the plant to avoid, minimize, tolerate or recover from the damage caused by the pests (Bosque-Perez, 1995). In general, it is target specific, increasing insect mortality and reducing reproduction rate of the pest by producing toxic substances (antibiosis), or through non-preference, a behavior of the insect pests towards certain varieties regarding its feeding, oviposition and shelter (antixenosis) (Polaszek, 1998).

In the last two decades efforts have been made to identify and incorporate stemborer resistance traits into cereal crops. Many resistant maize and sorghum lines/hybrids have been identified which show tolerance to stemborer damage in South Africa (van Rensburg and van den Berg, 1995; Kfir *et al*., 2002).

2.5 The push-pull strategy for stemborer control

Insect-plant communication is mediated by chemicals in their environment. Push-pull technology uses this channel of communication to manipulate agro-ecosystems in a manner that is unfavourable to pests, whilst simultaneously promoting crop yield through reduced pest damage (Khan and Pickett, 2004). This successful strategy for the control of cereal stemborers in smallholder systems in eastern Africa has been developed by *icipe*, Rothamsted research and partners (Khan *et al*., 2010; 2014). It involves repelling the pests away from the main crop using a repellent intercrop plant and attracting insect pests to trap plants (Pickett *et al.,* 2006). Selected companion plants are grown within the main crop as repellent or deterrent for insect pests from the main crop, whilst the perimeter plants act as the trap using attractive volatile blends which attract pests from the main crop (Figure 2.4) (Cook *et al.,* 2007; Khan *et al.,* 2010). In addition to attracting stemborer moths, perimeter plants provide space for laying their eggs but when the eggs hatch the larvae are unable to survive or enter the next developmental stage, thus reducing the number of pest individuals (Khan and Pickett, 2004; Pickett *et al.,* 2006). Furthermore, the intercropped plants also suppress weed growth and add value to the agro-ecosystems through nitrogen fixation (Pickett *et al.,* 2006). The push-pull components are generally nontoxic, improve the livelihoods of small-holder farmers, increase agricultural productivity and improve environmental sustainability (Khan and Pickett, 2004). The novel pest management strategy is currently being implemented by over 120,000 subsistence cereal producers in eastern Africa, including Kenya, Uganda, Tanzania and Ethiopia (Murage *et al*., 2015). Plants that have been identified as effective in the push-pull strategy include Napier grass (*Pennisetum purpureum* Schumach), Sudan grass (*Sorghum vulgare sudanense* Pers.), molasses grass (*Melinis minutiflora* P. Beauv.), and desmodium (*Desmodium uncinatum* Jacq. and *Desmodium intortum* Urb.) (Khan and Pickett, 2004).

Napier grass is one of the most attractive trap plants for stemborers in the push-pull strategy (Khan *et al.,* 2006), because of its ability to produce higher quantities of the attractive compounds than sorghum and maize (Birkett *et al.,* 2006; Khan *et al.,* 2010). The most common attractive green leaf volatiles released by the grass include (*E*)-2-hexanal, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexynyl acetate. The release rate of these compounds increases approximately 100-fold in the first hour of scotophase (Chamberlain *et al.,* 2006), the time during which stemborer moths are actively seeking host plants for oviposition (Päts, 1991). The gravid moths are therefore differentially attracted to Napier grass relative to the cereal host plants. However, larval survival and development on Napier grass is severely hampered (Khan *et al.,* 2006). This results from a sticky sap released by the grass upon injury by stemborer larvae in an attempt to enter into the stem which entangles the larvae causing mortality, both directly and through exposure to natural enemies. Additionally, Napier grass has insufficient nutrition to support growth and development of stemborer larvae, resulting in long developmental periods and smaller sized pupae and adults (Midega *et al.,* 2015). In addition to reducing larval development, Napier grass is also the main source of fodder for the smallholder dairy industry, and an important plant for soil conservation (Khan and Pickett, 2004).

Molasses grass is a non-host plant for stemborers and is used as an intercrop in push-pull systems, as it exhibits desirable direct and indirect defence traits (Khan *et al.*, 1997). This multi-functional grass also has well known anti-tick property (Kimani *et al.,* 2000). Volatile blends emitted by molasses grass are repellent to ovipositing moths and also result in increased parasitisim by parasitoids, *Cotesia sesamiae* Cameron (Hymenoptera: Braconidae) in intercropping systems (Khan *et al.,* 1997, 2000; Kimani *et al.,* 2000). The chemical compounds mediating these interactions have been identified as (*E*)-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, β-caryophyllene, humulene and α-terpinolene (Khan *et al.,* 1997;

2000; Pickett *et al.,* 2006). Some of these compounds are also known to be produced by maize in response to insect herbivore damage (Turlings *et al.,* 1990; 1995).

Desmodium plants emit semiochemicals that are repellent to stemborer moths and attractive to parasitoids (Khan *et al*., 1997; 2000; Pickett *et al*., 2006; Midega *et al.,* 2009). In addition to stemborer control, the desmodium intercrop controls the growth of a parasitic weed, *striga* (*Striga hermonthica* (Del.) Benth.). Plants in the genus *Desmodium* release allopathic root exudates that inhibit/ suppress the growth of *striga* (Khan *et al*., 2002; Hooper *et al*., 2015). The root exudates contain biologically active isoflavonones that stimulate germination of *striga* seeds while others inhibit radical growth (Tsanuo *et al*., 2003; Khan *et al*., 2008b; Hooper *et al*., 2010). This causes suicidal germination of *striga* seeds resulting in depletion of the seed bank in the soil even in the presence of host plants (Khan *et al*., 2008b).

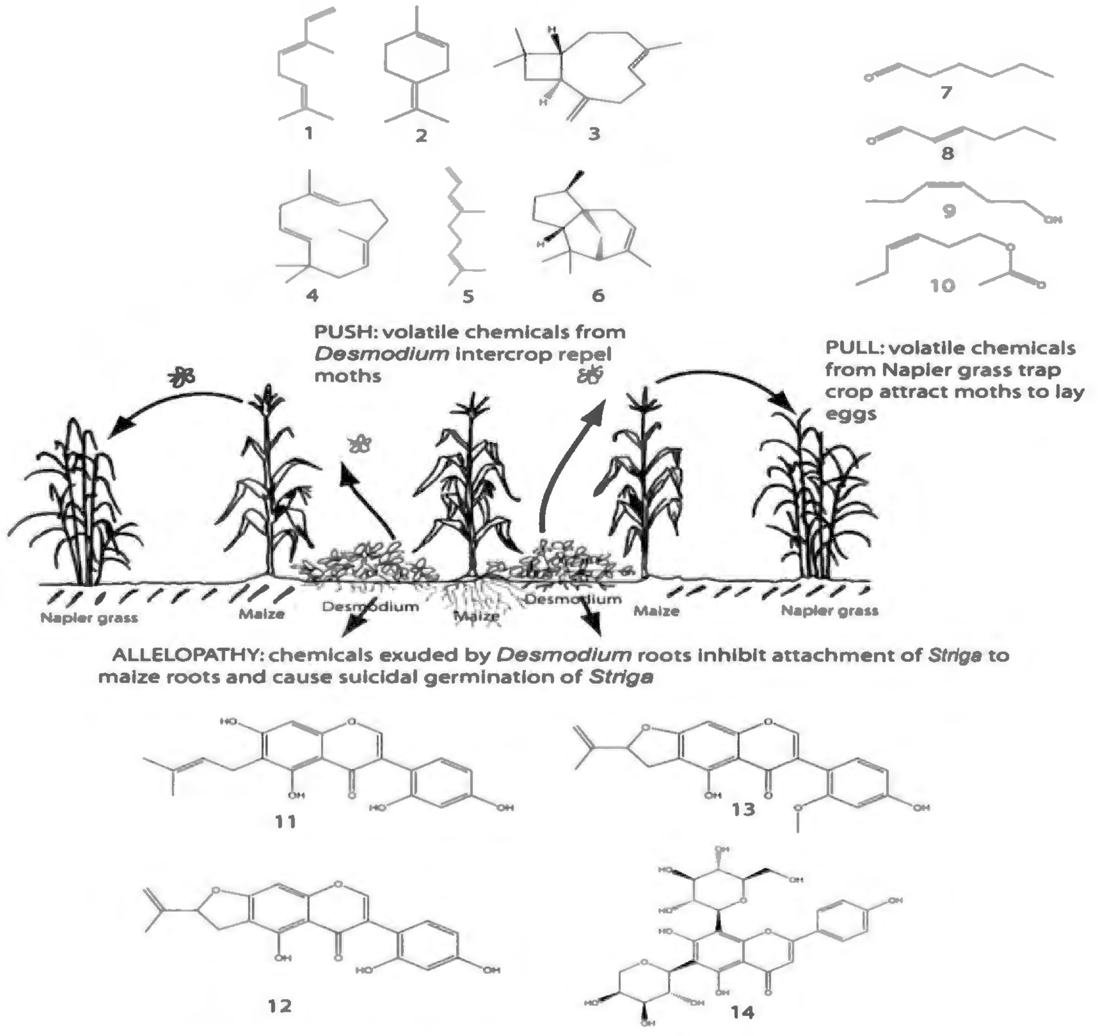

Figure 2.4: Chemical ecology of the push-pull system: stemborer moths are repelled by intercrop volatiles while attracted by trap crop volatiles. Root exudates from the *Desmodium* intercop cause suicidal germination of *Striga* and inhibit attachment to maize roots. 1, (*E*)-β ocimene; 2, α-terpinolene; 3, β-caryophyllene; 4, humulene; 5, (*E*)-4,8-dimethyl-1,3,7-nonatriene; 6, α-cedrene; 7, hexanal; 8, (*E*)-2-hexanal; 9, (*Z*)-3-hexen-1-ol; 10, (*Z*)-3-hexen-1-yl acetate; 11, 5,7,2',4'-tetrahydroxy-6-(3-methylbut-2-enyl)isoflavanone(uncinone A); 12, 4",5"-dihydro-5,2',4'-trihydroxy-5"-isopropenylfurano-(2",3";7,6)-isoflavanone (uncinone B); 13, 4",5"-dihydro-2'-methoxy-5,4'-dihydroxy-5"-isopropenylfurano-(2",3";7,6)-isoflavanone (uncinone C) and 14, di-*C*-glucosylflavone 6-*C*-α-L-arabinopyranosyl-8-*C*-β-D-glucopyranosylapigenin (Adapted from Khan *et al*., 2010).

2.6 Defence mechanisms of plants against herbivores

There are many more herbivorous insect species than plant species. To manage these herbivorous insects, plants have developed a number of defence mechanisms against the herbivores. Generally, plant defence mechanisms are broadly categorized as direct and indirect defences.

2.6.1 Direct defence mechanisms

Direct (pre-formed) defence mechanisms are considered as the first line of defence, and are present before insect attack takes place. This type of defence strategy relies on evolutionary antagonistic features which include morphological and chemical defence mechanisms that directly affect the herbivore (Kessler and Baldwin, 2001). Unlike indirect defence mechanisms, plants are always in defence mode even during the absence of the herbivorous organisms. Plant structural traits such as presence of waxy cuticles, bark, trichomes, thorns and spines are considered as the first physical barriers to feeding by the herbivores. In addition to this, secondary metabolites are not directly involved in the normal growth, development or reproduction of a plant but they directly defend the plant from attack by affecting herbivore growth and development by producing toxic or deterrent chemicals (Rhoades, 1983; Khan *et al.,* 2010).

2.6.2 Indirect defence mechanisms

Many plant species emit volatile organic compounds in varying quantities for a variety of reasons. Under normal conditions plants release small quantities of volatile chemical compounds compared to damaged plants. In response to attack by herbivores, plants produce a blend of volatile chemical compounds referred to as herbivore-induced plant volatiles (HIPVs) (Mumm and Dicke, 2010), which are important signals for herbivores, parasitoids, predators and neighbouring plants (Engelberth *et al.,* 2004; Khan *et al.,* 2008b; Penaflor *et al.,* 2011).

Indirect defences may attract or call natural enemies of the herbivores which kill or reduce the effectiveness of herbivores on the plant (Figure. 2.5). These defences either take the form of 'cry for help' and/or providing a service for predators (Khan *et al.*, 2010). In addition to indirect defence strategies, HIPVs also function as signalling cues between- and within-plants (Arimura *et al.*, 2010; Heil and Karban, 2010).

Figure 2.5: Female larval parasitoid *Campoletis sonorensis* Cameron (Hymenoptera: Ichneumonidae) ovipositing into larvae of *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) (De Lange, 2013).

2.7 Semiochemicals

The term semiochemical is derived from Greek "*semeon*" meaning "signal". Semiochemicals are a group of natural volatile chemical substances produced and used by organisms for intra- and interspecies communication (Petroski *et al.*, 2005). Based on the interaction between organisms, semiochemicals are classified as allelochemicals or pheromones, interacting between individuals of the same species (intraspecific) or interacting between different species (interspecific), respectively (Nordlund, 1981).

Allelochemicals (derived from *allelon*, in Greek=of each other) are comprised of allomones, kairomones, synomones and apneumones (Headrick and Gordh, 2001). Allomones are biofunctional molecules which evoke advantageous reactions such as defence compounds, in their producers (Furstenau, 2011). These compounds can act as repellents or feeding or oviposition deterrents which are consequently detrimental to the receiver organism (Norris *et al.*, 2003).

Kairomones are a class of compounds that are advantageous for the receiver. In the case of herbivorous damage, plants release volatile organic compounds which contribute to attract natural enemies. The term "kairomone" is derived from the Greek word "*kairos*," which means "opportunistic" (Nordlund, 1981).

The term apneumones was coined by Nordlund and Lewis (1976). Apneumones are chemicals derived from a non-living source that benefits the receiver (Kabeh, 2007). The other type of allelochemical is synomones (from the Greek "*syn*" for "with" or "together"), which are compounds that are beneficial to both the receiver and the sender.

Substances secreted to the outside by an individual and received by the same species of insect are termed pheromones. These are mostly mediated by olfactory cues in the surrounding environment. The term pheromone originates from "*Phereum*" in Greek, meaning to carry; hormone, to excite or to stimulate.

2.7.1 Use of semiochemicals in pest management

Globally, chemical insecticides have been applied since 1940 as an effective remedy for the control of insect pests. However, increasing numbers of resistant pest species, chemical residues in food and groundwater, health risks for humans and animals, side effects on beneficial organisms and high costs of pesticides (Pickett *et al.*, 2006) divert the attention of scientists to search for alternative control methods. In the past few decades, the use of semiochemicals has gained attention for the control of pest insects due to its non-toxic mode of action, high specificity, low risk of resistance evolution and affordability (Pickett *et al.*, 2006; Khan *et al.,* 2010). Currently semiochemicals are used in pest management strategies for pest monitoring, mating disruption, mass trapping, and to lure and kill (Norin, 2007).

Pheromones are one of the most widely utilized types of semiochemicals. It is produced by an insect to attract members of its own species or notify members of its own species that danger is present. Therefore, pheromones are practically applied in pest management strategies as anti-aggregation pheromones, oviposition deterring pheromones, and alarm pheromones (Ryan, 2002). In addition, semiochemicals are also used in the stimulo-deterrent diversionary strategy (push-pull) as described above.

2.8 Plant-to-plant communication

Plants synthesize and emit a large variety of volatile organic compounds (VOCs) from above ground (vegetative and floral parts) and below ground parts (roots) (Knudsen *et al.,* 1993; Steeghts *et al.,* 2004; Rodriguez-Saon *et al.,* 2013). Plants emit volatile organic compounds depending on the conditions where they occur (Holopainen and Gershenzon, 2010). Under normal conditions, undamaged plants release small amounts of VOCs into the atmosphere but when damaged by herbivores or pathogens, a blend of HPIVs are emitted (Bruinsma *et al.,* 2010; Hare, 2011; Das *et al*., 2013). However, emitted blend of HIPVs differs based on plants and herbivore species and developmental stage of the plant (Takabayashi *et al.,* 1995; De Moraes *et al.,* 1998; Turlings *et al.,* 1998). Plant VOCs create a communication channel between the emitter and receiver plants (between and within) as well as insects and pathogens (Arimura *et al.,* 2009; Rodriguez-Saon *et al.,* 2013), and in general it is considered to be a simple way of plant interaction with their environment. Damage to plants by herbivores influences the defence strategies of plants. However, HIPVs can deter pathogens and herbivores directly from the host plants or indirectly serve as foraging cues for natural enemies of the herbivores (De Moraes *et al.,* 2001; Kessler and Baldwin, 2001; Heil, 2004; Das *et al.,* 2013).

Volatile compounds are typically lipophilic chemicals with low molecular weights and high vapour pressure (Pichersky *et al.,* 2006). The non-conjugate volatile organic compounds are released freely through cell membranes into the atmosphere from their site of synthesis (Pichersky *et al.,* 2006; Das *et al.,* 2013). Upon damage by herbivorous or mechanical means many plants release green leaf volatiles (GLVs) immediately from the site of damage (Heil and Bueno, 2007a; Holopainen and Gershenzon, 2010) to attract natural enemies of the herbivores (McCormick *et al.*, 2012) and to repel or affect growth and development of the attacking herbivour (Howe and Jander, 2008). In addition, GLVs induce extra floral nectar (EFN) secretion in undamaged parts and neighbouring plants (Heil and Bueno, 2007a;b). HIPVs induce or prime the defensive responses in intact neighbouring plants or intact plant parts on the same (within-) plant (Engelberth *et al.*, 2004; Kessler *et al.*, 2006; Ton *et al.*, 2007; Rodriguez-Saona *et al.*, 2009; Muroi *et al.*, 2011), and allow them to prepare defence mechanisms for a future herbivore attack. Most plant volatiles are commonly released by all plant species, while others are specific to the plant species and the herbivore that damage the plant (Takabayashi *et al.,* 1991; Gouinguene *et al.,* 2001).

2.8.1 Intra-plant communication

Plants release a blend of volatile compounds into the environment that mediate communication between organisms. However, the composition of the released volatile profiles varies from species to species, with blends being more similar within than between plant species (Rodriguez-Saona *et al.,* 2013). The plant volatiles released from damaged parts of a plant induce intact (undamaged) parts of the same plant (Engelberth *et al.*, 2004; Choh and Takabayashi, 2006; Heil and Kost, 2006; Kessler *et al.*, 2006), before it communicates with neighbouring plants (Das *et al.,* 2013). GLVs and terpenoids are airborne signals responsible for rapid within-plant communication (Frost *et al.,* 2007; Heil and Bueno, 2007a). As Kost and Heil (2006) and Heil and Bueno (2007b) showed that EFN secretions are induced in undamaged parts of lima bean leaves immediately after volatiles are released from damaged leaves. The benefit arising from these EFN secretions is protection of the plant from subsequent herbivore damage through increased attraction of natural enemies (Kost and Heil, 2006).

2.8.2 Inter-plant communication

Plant-to-plant communication has been a highly debated topic since the idea was first conceived. However, Rhoades (1983) observed that Sitka willow plants (*Salix sitchensis* Sanson ex. Bong) grown near to herbivore-infested conspecific plants increased their level of resistance to herbivores. Similarly, when undamaged sugar maple trees (*Acer saccharum* Marshall) and poplar (*Populus x euroamericana* (Dole) Guinier) trees were exposed to damaged trees, the production of phenolic compounds increased drastically (Baldwin and Schultz, 1983). In addition, the level of defensive enzymes of wild tobacco plants increased after planted near to clipped sagebrush plants (Karban *et al.,* 2000). Similarly, resistance of Sagebrush plants and alder trees increased after exposure to clipped conspecific neighbouring plants (Dolch and Tscharntke, 2000; Karban *et al.,* 2004; 2006). In general, these are some of the evidence that illustrate how plants respond to cues produced by damaged neighbouring plants in spite of themselves not having been attacked by herbivores.

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CHAPTER THREE

3.0 VOLATILE CUES FROM NEIGHBOURING MOLASSES GRASSES ENHANCE THE EMISSION OF MAIZE VOLATILE ORGANIC COMPOUNDS

3.1 Abstract

Plant volatile organic compounds (VOCs) mediate interactions between plants, herbivores and parasitoids in the ecosystem. Previous observations in maize-molasses grass intercropping systems showed that molasses grass, *Melinis minutiflora* P. Beauv. emit volatile infochemicals, which can be perceived by the herbivores and their natural enemies. It was however not known whether these volatiles could signal defence responses in neighbouring maize seedlings. The aim of this study was to examine the effect of intercropping of molasses grass on maize plant volatile profiles. Three-week old maize seedlings were exposed to molasses grass volatiles in an intercropped system for 24 hr, 96 hr and 1 week, after which headspace samples were collected from maize exposed to molasses grass, non-exposed (control) maize plants, and molasses grass. Samples were analysed by means of Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS). Compounds were identified by calculating the retention time of individual GC peaks and compared with the Kovats indices and mass spectral library. The GC analysis revealed enhanced profile changes in the maize exposed to molasses grass compared to non-exposed maize seedlings. Identified compounds included (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT). Results suggest that changes in volatile emission in an intercropped system due to plant signaling may boost plant defence in the system since the identified compounds are known to repel stemborer moths and attract their natural enemies. Further investigation into the effect of these compounds on parasitoid and herbivore behavior may provide new strategies for crop protection against herbivores.

Key words: infochemicals, intercropping, *Melinis minutiflora,* retention time

3.2 Introduction

Plants synthesize and emit a range of volatile organic compounds (VOCs) from different plant parts (Knudsen *et al.,* 1993; Steeghts *et al.,* 2004; Rodriguez-Saon *et al.,* 2013). In response to damage by herbivores or any other mechanical means plants adjust their metabolic rate and respond by releasing a blend of VOCs, which can act as direct defence mechanisms to repel or interfere with growth and development of the attacking organisms (De Moraes *et al*., 2001; Kessler and Baldwin, 2001; Heil, 2004; Schoonhoven *et al*., 2005). Many of these direct defence mechanisms are the result of plant secondary metabolites. Indirectly, the emitted VOCs, also called herbivore induced plant volatiles (HIPVs), can serve as foraging cues for natural enemies (parasitoids and predators) of the herbivores (Dicke and Sabelis, 1988; Turlings *et al.,* 1990; De Moraes *et al.,* 1998; Dicke and Vet, 1999; Heil, 2004).

HIPVs are infochemicals received by different organisms in the environment and their functions in the ecosystem depend on the reaction of the receiver organism (Birkett *et al*., 2000; Arimura *et al.,* 2009). Emission of herbivore induced plant volatiles can play a great role in inter- and intra-plant communication, and could function to enhance direct and indirect defence systems of plants (Karban *et al*., 2000; 2001; 2006; Heil and Karban, 2010, Gols, 2014). Plants communicate systemically between damaged and undamaged parts of the same plant (Heil and Ton, 2008; Arimura *et al.,* 2009; Karban, 2011; Chamberlain, 2014) and emit more pronounced signals, which can be easily detected by parasitoids and herbivores (Dicke, 2009; Rodriguez-Saona *et al.,* 2009; War *et al.,* 2011).

Volatile-mediated defense strategies have complex ecological significance. Emission of plant volatiles in response to damage by herbivory can play a great role in plant-to-plant communication and initiate defence responses of undamaged neighbouring plants (Karban and Shiojiri, 2009; Heil and Karban, 2010; Bruce and Pickett, 2011; Lucas-Barbosa *et al*., 2011). However, emitted blends vary according to plant species, developmental stage of the plant and herbivore species (Arimura *et al.,* 2009). Receiver plants perceive and respond to reliable signals (Karban, 2008) by adjusting their physiology accordingly (Arimura *et al.*, 2000; Paschold *et al.*, 2006).

Constitutive emissions of VOCs are limited in most plant species, unless they perceive environmental stress stimuli (Paré *et al.,* 2005; Glinwood *et al.,* 2011). Surprisingly, molasses grass, *Melinis minutiflora* P. Beauv., which is currently used in the push-pull strategy, plays an important role by emitting a similar quality of VOCs as damaged maize plants do, without being damaged by herbivores (Khan *et al.,* 1997). The unique property of molasses plants in decreasing infestation by *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and increasing larval parasitism of stemborers by *Cotesia sesamiae* Cameron (Hymenoptera: Braconidae) have been reported in maize-molasses grass intercropping systems (Khan *et al.,* 1997; 2000; Midega *et al.,* 2015). Some of the compounds emitted from intact molasses grass have been identified as (*E*)-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, β-caryophyllene, humulene and α-terpinolene (Khan *et al.*, 1997; 2000; Pickett *et al.,* 2006). (*E*)-ocimene, (*E*)-*β*-caryophyllene and nonatriene are also known to be produced by maize in response to insect herbivory (Dicke and Sabilis, 1988, Turlings *et al.*, 1990; 1995). Moreover, the compound (*E*)-4,8-dimethyl-1,3,7-nonatriene has been demonstrated to be responsible for increased parasitoid activity within the main crop when intercropped with molasses grass (Khan *et al.*, 1997; 2000). Sharing a common environment allows plants to interact chemically with the surrounding organisms. However, plant-to-plant communication between undamaged plants has not been studied widely. Therefore this study intended to determine whether maize plants in a maize-molasses grass intercropping system can modify their volatile profiles in response to chemical cues received from undamaged neighbouring plants.

3.3 Materials and methods

3.3.1 Study site

This study was conducted at the Thomas Odhiambo Campus of the International Centre of Insect Physiology and Ecology (*icipe*), Mbita point (0°25'S, 34°12'E, 1 200 m a.s.l. and 900 mm annual rain fall). The site is situated on the eastern shores of Lake Victoria in the Suba district of Kenya, where the push-pull farming system has been adopted by many farmers.

3.3.2 Experimental plants and setup

Seed of the open pollinated maize variety (Jowi-red) used in this study were collected from farmers in western Kenya while those of molasses grass were collected from field plots maintained at *icipe* Mbita. The choice of smallholder farmers' maize variety was based on a previous study that indicated it had 'smart' defence traits inducible by *C. partellus* egg deposition (Tamiru *et al.*, 2012). The experimental seeds of the two plant species were planted individually in pots filled with fertilized soil in different insect-proof screen houses under natural conditions (25 ^{0}C, 65% RH; 12L: 12D). Twenty one days after planting, the pots with maize seedlings were transferred to stand in between the rows of pots of molasses grass for 24 hr, 96 hr and 1week (Figure 3.1a). Maize of the non-exposed Jowi-red seeds were also planted in a different screen house under similar environmental conditions without molasses grass (Figure 3.1b).

3.3.3 Volatile organic compound (VOC) collection

Volatile compounds were collected using headspace sampling (Agelopoulos *et al.*, 1999) (Figure 3.2) from molasses grass and maize seedlings exposed and non-exposed to molasses grass. The leaves of the experimental plants were placed gently inside different polyethyleneterephthalate (PET) bags (volume 3.2 L, ~12.5 mm thickness) through an open end of the bag. The PET bags were sterilized at 150 ^{0}C before use. Purified charcoal filtered air was pumped through the bottom of the bag at a rate of 600 ml/min. The adsorbent, Porapak Q (0.05 g, 60/80 mesh; Supelco) was placed at the outlet valve where air was drawn at 400 ml/min. A lower flow rate in the outlet valve allowed for enough time and pressure for the Porapak Q, to effectively adsorb the volatile organic compounds (VOCs). After 48 hr of entrainment, collected volatile samples from the Porapak Q were eluted with 0.5 ml of dichloromethane. The eluted samples were collected using 3.5 ml sample vials and kept in a freezer at -20 ^{0}C until use for chemical analysis. Each treatment (entrainment) from exposed and non-exposed Jowi-red seedlings were replicated four times.

3.3.4 Gas Chromatography (GC) Analysis

Plant VOCs samples were analyzed using a Hewlett-Packard 7890 GC machine (Agilent Technologies) equipped with a cool-on column injector, a non-polar HP-1 capillary column (50 m, 0.32 mm internal diameter, 0.52 µm film thickness) and a flame ionization detector (FID). Four µl of headspace sample were injected into the injector port of the GC instrument. The oven temperature was maintained at 30 ^{0}C for 2 min and then programmed at 5 ^{0}C/min to 250 ^{0}C. The carrier gas was hydrogen. Data were analyzed using HP Chemstation software.

3.3.5 Coupled GC-Mass Spectrometry (GC-MS) analysis

Aliquots of attractive headspace samples were analysed on a capillary GC column (HP-1, 50 m, 0.32 mm i.d., 0.52 µm) directly coupled to a mass spectrometer (VG Autospec; Fisons Instruments, Manchester, UK) equipped with a cool on-column injector. Ionisation was performed by electron impact (70 eV, 250 ^{0}C). The oven temperature was maintained at 30 ^{0}C for 5 min and then programmed at 5 ^{0}C/min to 250 ^{0}C. Tentative identifications of compounds were made by comparison of spectra with mass spectral databases (NIST, 2005) and confirmed through co-injection with the authentic standards.

3.3.6 Retention indices (RI)

To calculate the retention indices of individual FID peaks of the sample profile, an alkane series consisting of C7-C23 was injected on the same GC column with similar programme as used for sample analysis. Retention times of each alkane were recorded and used to work out retention indices of each sample FID peaks. The RI for a given compound indicates its elution time relative to the adjacent alkanes. FID peaks (X) eluted between two alkanes, with retention time z-1 and z+1 respectively, a retention index (RI) was calculated using the following equation (Bartle, 1993): The calculated value of each RI compared with the known value in the Kovats database (Kovats, 1958).

$$RI = \left(\frac{100(\log rt(x) - \log rt(z-1))}{(\log rt(z+1) - \log rt(z-1))} \right) + 100(z-1)$$

Where:
rt= retention time
x= compound of interest
z-1= alkane before the compound of interest
z+1= alkane after the compound of interest.

3.3.7 Statistical analysis

Retention indices (RI) of each GC-peak for the specific samples were calculated by using the retention time of the C7-C23 alkanes following the RI equation above (Bartle, 1993).

3.4 Results

3.4.1 GC analysis of maize volatiles

Gas Chromatography analysis (GC) of maize plants (Jowi-red) showed significant differences in volatile profile between plants exposed to molasses grass and non-exposed plants. Significant differences were also observed in the volatile blends produced after maize plants were exposed to molasses grass volatiles for different time intervals (Figures 3.4-3.6).

3.4.2 Comparison of calculated Retention indices (RI) of GC peaks with Kovats Retention Indices (RI) and with mass spectral database

Gas Chromatography profile peaks of exposed and non-exposed maize plants as well as molasses grass were tentatively identified by calculating the Retention Indices (RI) and compared with values in the database (Kovats Indices) to assign tentative identifications of the compounds from the collected samples. Further compounds identified by comparison of spectra with GC-MS spectral databases and confirmed by co-injection with authentic standared compounds included (*E*)-2-hexyn-1-yl acetate, hexyl acetate, decane, nonanal, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), decanal, *(E)-β*-caryophyllene, (*E*)-β-farnesene, and *(E,E)*-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) (Tables 3.1-3.4 and Figures 3.4-3.6).

3.5 Discussion

Current findings provide evidence that airborne communication is not only limited between plants of similar species, but also between plants of different species. In the experimental setup of a maize-molasses grass intercropping system, high chemical signalling interactions were observed as the exposure time to the source of the volatiles increased. Maize seedlings exposed to volatiles released from neighbouring molasses grass showed differences in volatile profiles compared to non-exposed seedlings, indicating possible induction of the plant defence system in the maize. Khan *et al.* (1997; 2000) and Pickett *et al.* (2006) identified the volatile compounds emitted from intact molasses grass as (*E*)-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, β-caryophyllene, humulene and α-terpinolene as being similar to those released by plants damaged by herbivores.

It is a well documented fact that plants exposed to herbivore-induced volatile organic compounds change their emissions in terms of abundance of defence related compounds (Arimura *et al.*, 2002; Engelberth *et al.*, 2004; Ruther and Kleier, 2005). Intact maize plants previously exposed to neighbouring damaged plant volatiles rapidly produced jasmonic acid (JA) and emitted sesquiterpenes (Engelberth *et al.*, 2004). Plants can also respond to volatiles released by undamaged plants (Runyon *et al.*, 2006). Results of the current study provide evidence suggesting that part of the *C. partellus* control in maize-molasses grass intercropping system is derived from the signalling on the maize defence system delivered by volatiles from molasses grass. Indeed, some of the compounds from maize exposed to molasses grass, including (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*Z*)-jasmone, have been reported as possible cues that can affect pest and natural enemy behaviour (Pickett *et al.,* 2006).

In the last few decades direct and indirect defence mechanisms of plants have been reported in different plant species in response to damage by arthropod herbivores and/or mechanical damage. However, the response of the plant varies. The blends of volatiles emitted from damaged plants vary according to the plant species, developmental stage of the plant, and species of the herbivore (Sabelis *et al.,* 2007; Turlings and Wackers, 2004; Arimura *et al.,* 2009). However, there is no specific evidence for intact molasses grass why and when it emits a high or low quantity of volatile compounds. Compounds that were identified from maize exposed to the volatiles of molasses grass in this study are known to be produced by maize in response to insect herbivore damage, which are a key for direct and indirect defence responses in plants (Pickett *et al.,* 2006).

Previous studies have reported beneficial consequences of plant signalling. For example development of resistance to herbivores was observed on sagebrush, *Artemisia tridentata* after it was exposed to experimentally clipped neighbouring plants (Karban *et al.,* 2006). In some species of plants, such as molasses grass, volatile organic compounds emitted without the plant being damaged by herbivores or mechanical means (Khan *et al.,* 2000) may warn neighbouring plants from impeding pest attack. However, the ecological relevance of emitting these compounds for the emitter is not known. In most plant species emission of volatile compounds are only produced after herbivory.This unique property of molasses grass was reported to contribute to the reduction of stemborer damage and increased parasitism in maize-molasses grass intercropping systems (Khan *et al.,* 1997).

In conclusion, this study demonstrated that in a maize-molasses grass intercropping setup, the volatile profiles of the intercropped maize seedlings changed as the exposure time to molasses grass increased. This can boost defence responses and limit pest build up in maize under field conditions. Understanding the ecological function of molasses grass in plant signalling is a key component for developing environmentally benign strategies for pest management.

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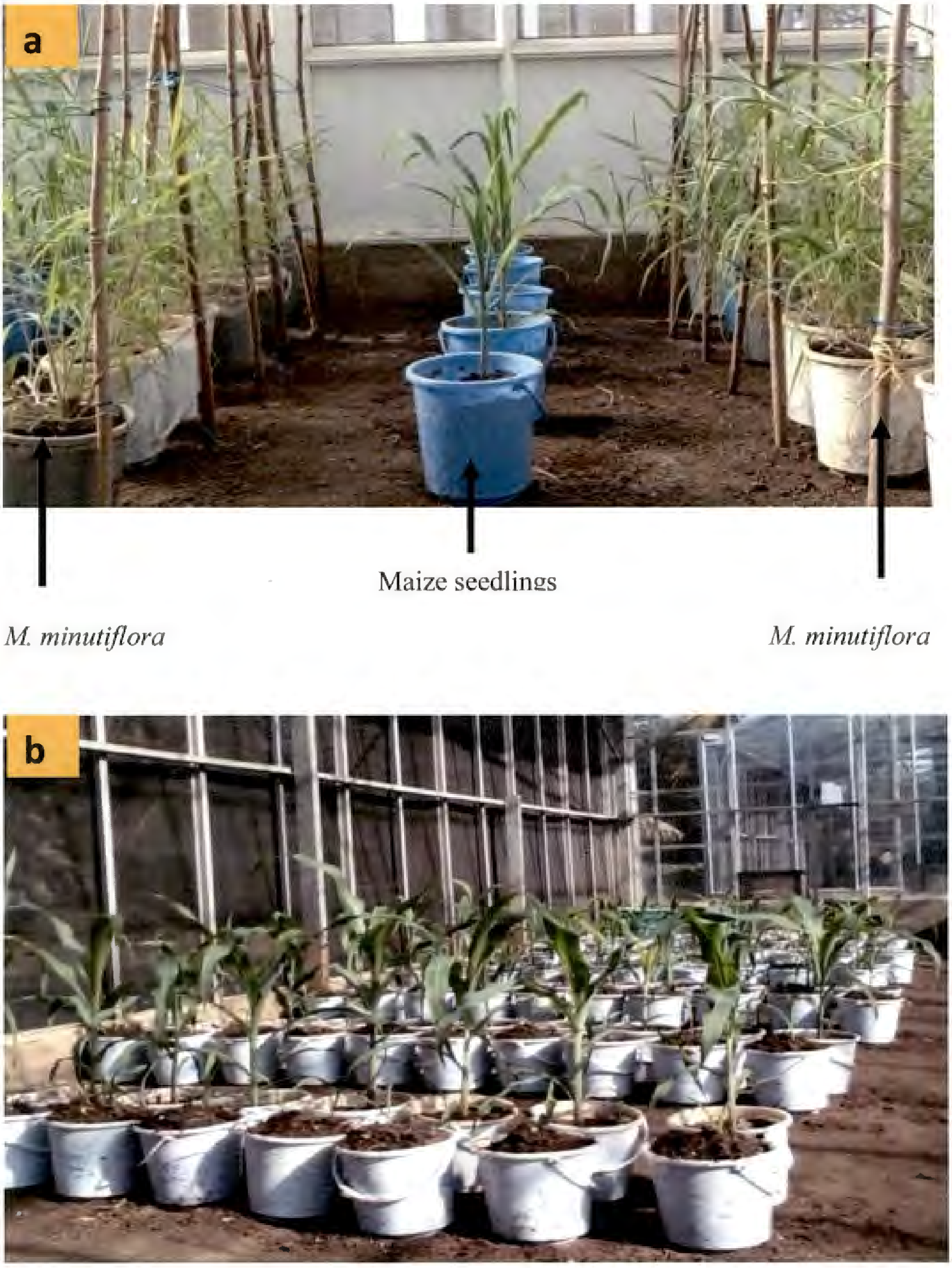

Figure 3.1: Experimental set up of maize seedlings exposed to molasses grass for 24 hr, 96 hr and 1 week (a) and non-exposed control maize seedlings (b).

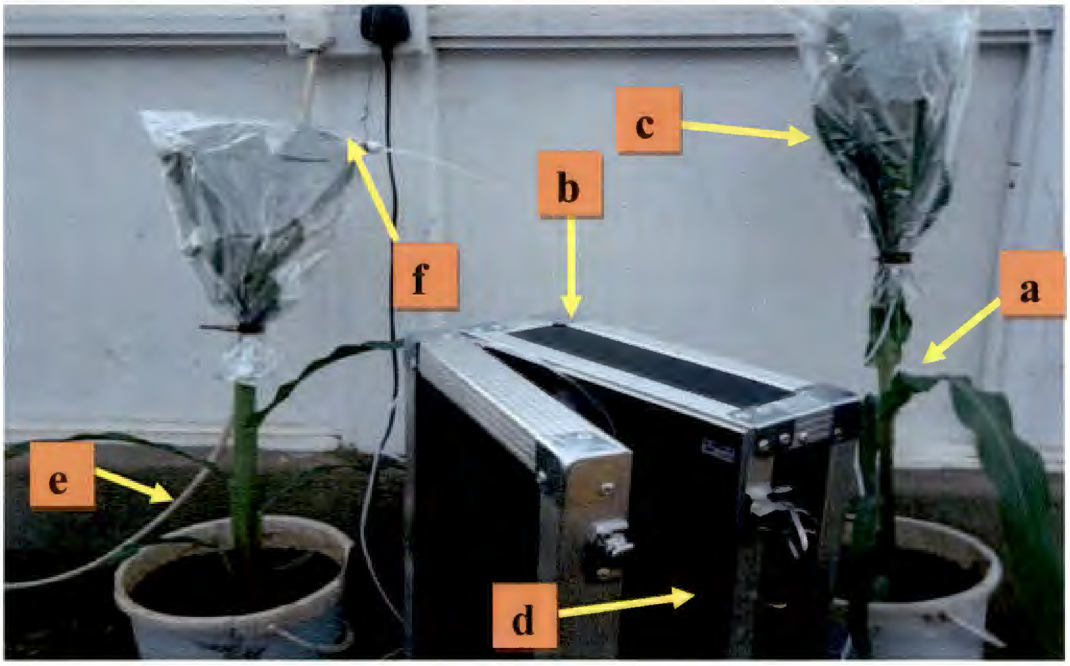

Figure 3.2: Headspace sampling set-up for volatile collection from maize seedlings exposed to molasses grass, non exposed control seedlings and molasses grass. The labels represent (a) Maize seedling, (b) Portable air entrainment kit, (c) Polyethyleneterephthalate bags, (d) Flow-metre controlling air flow rate, (e) Ethylene terephthalate tubes transporting air to/from the pump, (f) Porapak Q tubes.

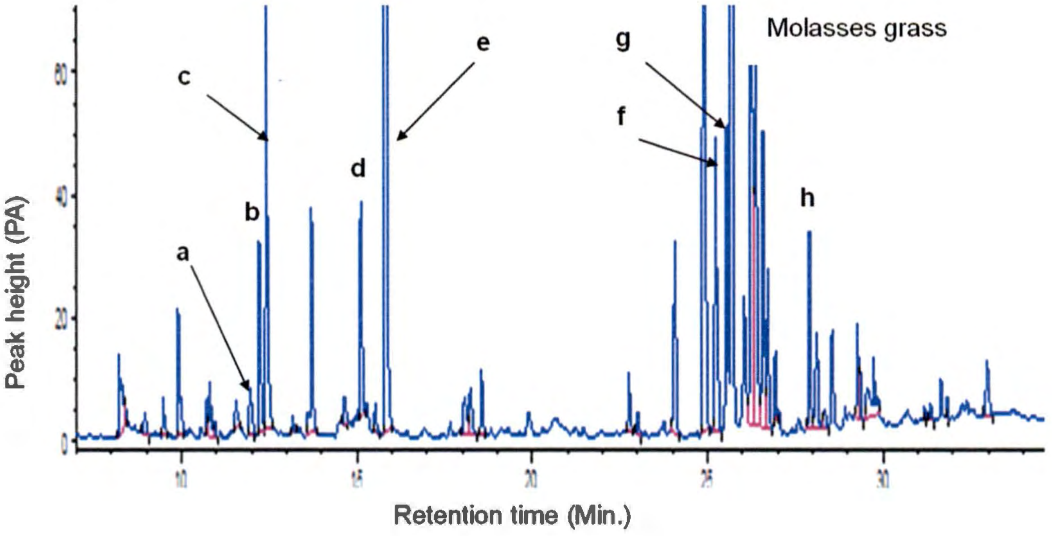

Figure 3.3: GC profiles of headspace volatiles from molasses grass used for intercropping. Compounds were tentatively identified by comparing the calculated value with known value in the Kovats database and compounds confirmed by co-injections with authentic standards were: (a) *(E)*-2-hexyn-1-yl acetate, (b) hexyl acetate, (c) decane, (d) nonanal, (e) (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (f) *(E)*-caryophyllene, (g) (*E*)-β-farnesene, (h) *(E,E)*-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT).

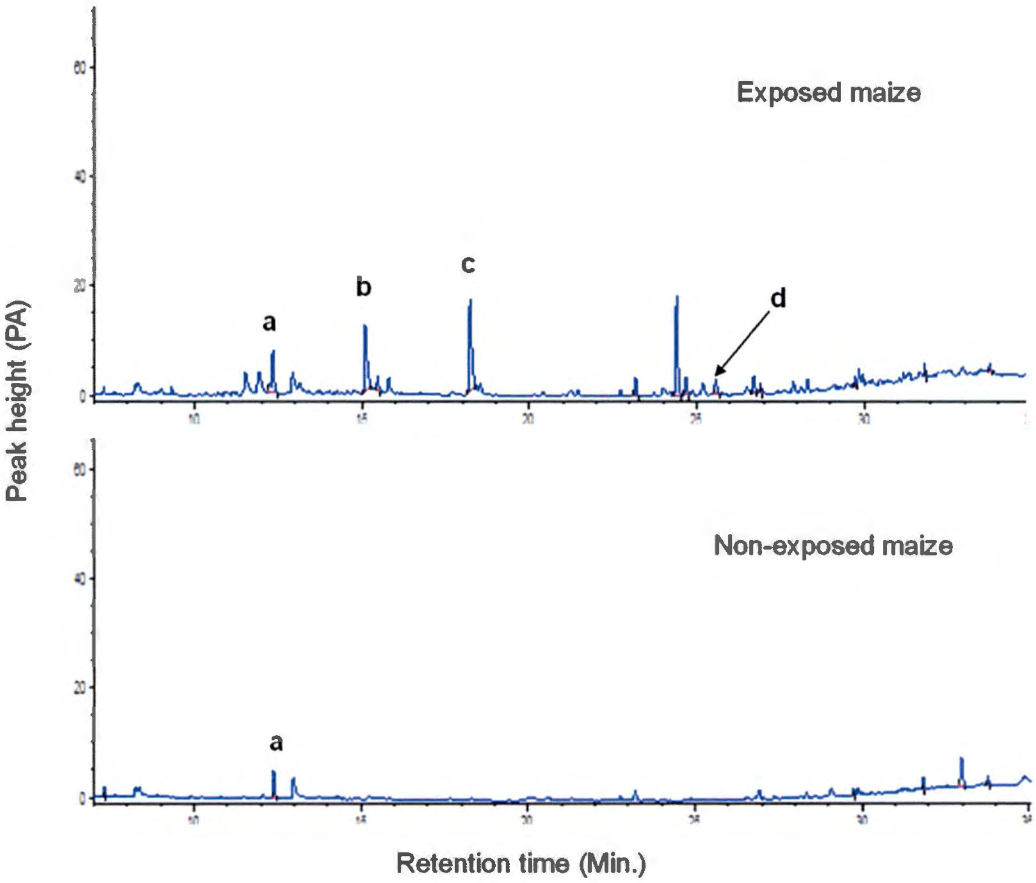

Figure 3.4: GC profiles of headspace volatiles from exposed maize plants to molasses grass volatile for 24 hr and non-exposed maize plants. Compounds were tentatively identified by comparing the calculated value with known value in the Kovats database and compounds that were confirmed by co-injections with authentic standards are: (a) hexyl acetate, (b) nonanal, (c) decanal, (d) (Z)-jasmone.

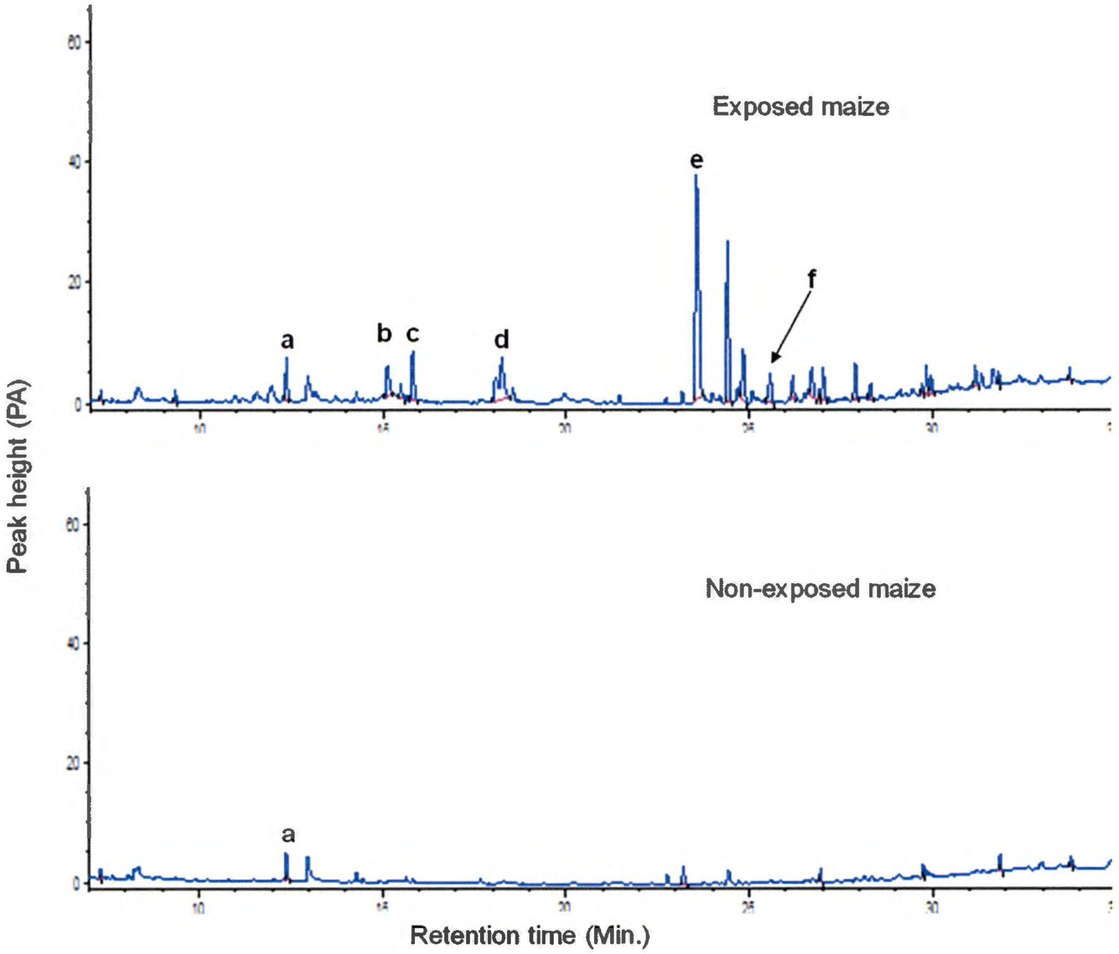

Figure 3.5: GC profiles of headspace volatiles from maize plants exposed to molasses grass volatile for 96 hrs and non-exposed maize plants. Compounds were tentatively identified by comparing the calculated value with known value in the Kovats database and compounds that were confirmed by co-injections with authentic standards are: (a) hexyl acetate, (b) nonanal, (c) (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (d) decanal, (e) (Z)-jasmone, (f) (*E*)-*β*-farnesene.

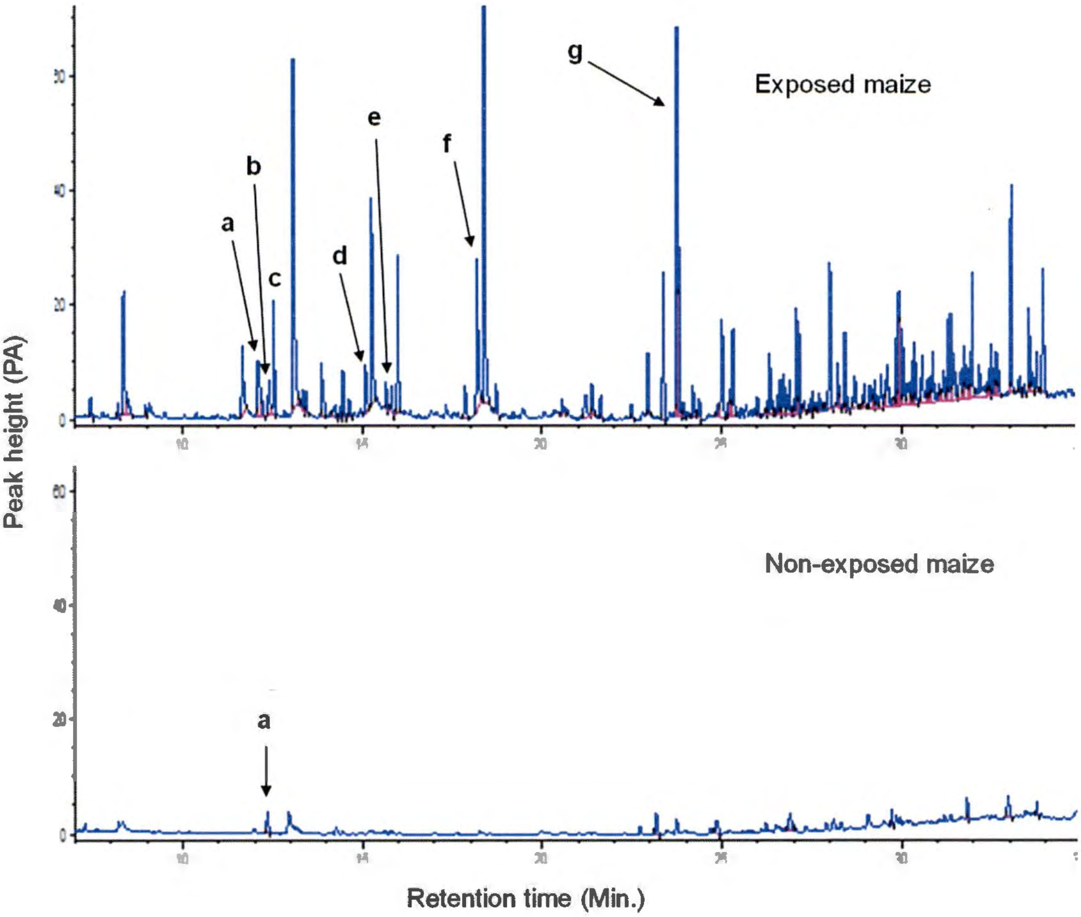

Figure 3.6: GC profiles of headspace volatiles from exposed maize plants to molasses grass for one week and non-exposed maize plants. Compounds were tentatively identified by comparing the calculated value with known value in the Kovats database and compounds that were confirmed by co-injections with authentic standards are: (a) *(E)*-2-hexyn-1-yl acetate, (b) hexyl acetate, (c) decane, (d) nonanal, (e) (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (f) decanal, (g) *(Z)*-jasmone.

Table 3.1: Kovats Retention Indices (RI) of GC peaks for the headspace samples collected from *Melinis minutiflora* plants.

Alkanes	**Retention time (min.)**	**Calculated RI**	**Tentative identification**	**Kovats RI**
9	**9.251**			
	12.112	986	*(E)*-2-hexen-1-yl acetate	993
10	**12.458**			
	12.414	994	Hexyl acetate	995
	12.553	999	Decane	1000
	15.096	1084	Nonanal	1084
11	**15.669**			
	15.833	1106	(*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT)	1106
12	**18.755**			
13	**21.681**			
14	**24.439**			
	25.232	1036	*(E)-β*-Caryophyllene	1032
	25.549	1450	*(E)-β*-farnesene	1450
15	**26.690**			
	27.914	1569	*(E,E)*-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT).	1570
16	**28.478**			

Retention time of alkanes with carbon numbers C7-C23 were used for calculating the value of the GC peaks. The Retention indices table was used to tentatively identify GC peaks of molasses grass samples by comparing the calculated retention indices with known values in the Kovats database (Kovats, 1958). Figures in "bold" under the second column are retention time of alkanes used to calculate respective RIs.

Table 3.2: Kovats Retention Indices (RI) of GC peaks for the headspace samples collected from Jowi-red plants exposed to molasses grass for 24 hrs and non-exposed plants.

Alkanes	**Retention time (min.)**		**Calculated RI**		**Tentative identification**	**Kovats RI**
	Exposed	**Non-exposed**	**Exposed**	**Non-exposed**		
9	**9.251**					
	12.351	12.395	997	998	Hexyl acetate	995
10	**12.458**					
	15.106		1084		Nonanal	1084
11	**15.669**					
	18.236	-	1184	-	Decanal	1186
12	**18.755**					
13	**21.681**					
14	**24.439**					
	25.579	-	1452	-	*(E)-β-*farnesene	1450
15	**26.69**					

Retention time of alkanes with carbon number C7-C23 were used for calculating the value of GC peaks. Retention indices table was used to tentatively identify GC peaks of 24 hr exposed and non-exposed maize plant samples by comparing the calculated retention indices with known value in the Kovats database (Kovats, 1958). Figures in "bold" under the second column are retention time of alkanes used to calculate respective RIs.

Table 3.3: Kovats Retention Indices (RI) of GC peaks for the headspace samples collected from Jowi-red plants exposed to molasses grass for 96 hrs and non-exposed plants.

Alkanes	**Retention time (min.)**		**Calculated RI**		**Tentative identification**	**Kovats RI**
	Exposed	**Non-exposed**	**Exposed**	**Non-exposed**		
9	**9.251**					
	12.359	12.394	997	998	Hexyl acetate	995
10	**12.458**					
	15.123	-	1085	-	Nonanal	1084
11	**15.669**					
	15.816	-	1105	-	(*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT)	1106
	18.242	-	1185	-	Decanal	1186
12	**18.755**					
13	**21.681**					
	23.573	-	1370	-	(Z)-jasmone	1373
14	**24.439**					
	25.579	-	1452	-	*(E)-β-*farnesene	1450
15	**26.69**					

Retention time of alkanes with carbon number C7-C23 were used for calculating the value of GC peaks. Retention indices table was used to tentatively identify GC peaks of 96 hr exposed and non-exposed maize plant samples by comparing the calculated retention indices with known value in the Kovats database (Kovats, 1958). Figures in "bold" under the second column are retention time of alkanes used to calculate respective RIs.

Table 3.4: Kovats Retention Indices (RI) of GC peaks for the headspace samples collected from Jowi-red plants exposed to molasses grass for one week and non-exposed plants.

Alkanes	**Retention time (min.)**		**Calculated RI**		**Tentative identification**	**Kovats RI**
	Exposed	**Non-exposed**	**Exposed**	**Non-exposed**		
9	**9.251**					
	12.112	-	991	-	*(E)*-2-hexen-1-yl acetate	993
10	**12.458**					
	12.414	12.360	999	997	Hexyl acetate	995
	12.553	-	1003		Decane	1000
	15.096	-	1084	-	Nonanal	1084
11	**15.669**					
	15.833	-	1106	-	(*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT)	1106
	18.197	-	1183	-	Decanal	1186
12	**18.755**					
13	**21.681**					
	23.762	-	1377	-	(Z)-jasmone	1373
14	**24.439**					

Retention time of alkanes with carbon number C7-C23 were used for calculating the value of GC peaks. Retention indices table was used to tentatively identify GC peaks of one week exposed and non-exposed maize plant samples by comparing the calculated retention indices with known value in the Kovats database (Kovats, 1958). Figures in "bold" under the second column are retention time of alkanes used to calculate respective RIs.

CHAPTER FOUR

4.0 EFFECTS OF MOLASSES GRASS ON HOST PREFERENCE OF *CHILO PARTELLUS*

4.1 Abstract

Chilo partellus (Swinhoe) (Lepidoptera: Crambidae) is one of the most serious pests of maize in sub-Saharan Africa. Previous studies reported that non-host molasses grass, *Melinis minutiflora* P. Beauv. repels *C. partellus* moths and increases larval parasitism by *Cotesia sesamiae* Cameron (Hymenoptera: Braconidae) when intercropped with maize. However, the potential role of plant signalling between molasses grass and maize, and any subsequent effect on *C. partellus* behaviour remain unknown. We examined the effect of intercropping with maize landraces and commercial hybrids on *C. partellus* oviposition. In two-choice oviposition bioassays, *C. partellus* preferred non-exposed maize landraces for oviposition compared to those exposed to molasses grass. In addition, volatile organic compounds (VOCs) collected from Nyamula and Jowi-red maize landraces exposed to molasses grass odour released higher amounts of electrophysiologically active compounds such as (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT). In contrast, there was no significant difference between the number of eggs oviposited by *C. partellus* on maize hybrids exposed to and not exposed to molasses grass odour. Our findings suggest that the VOCs released by molasses grass can induce direct defence responses in neighbouring landrace plants. However, the hybrids appear to lack the ability to detect or to respond to molasses grass VOCs. This study demonstrates the potential of plant signalling as a component of management approaches for stemborer pests in subsistence farming in sub-Saharan-Africa.

Key words: *Chilo partellus*, induce defence, maize landraces, *Melinis minutiflora*, oviposition

4.2 Introduction

Maize, *Zea mays* L. (Poaceae), is one of the most important staple and cash crops in eastern and southern Africa. In spite of the demand for the crop due to high rates of human population growth in these regions, productivity remains low, often <1t/ha, representing some of the lowest in the world (Cairns *et al.,* 2013). Lepidopteran stemborers, including *Chilo partellus* (Swinhoe) (Crambidae), are the most injurious pests of maize in many areas, causing yield losses ranging between 20 and 80% depending on planting date and infestation pressure (Kfir *et al.,* 2002). Management of cereal stemborers however remains a challenge as the use of insecticides is hampered by cryptic and nocturnal habits of the adult moths, and the protection provided by the stem of the host crop for immature stages (Khan *et al.,*1997a; Van den Berg and Nur, 1998; Slabbert and Van den Berg, 2009). Moreover, use of chemical pesticides is not economical for smallholder farmers in Africa and may result in undesirable consequences such as resistance development, secondary pest outbreaks, and environmental pollution (Kfir *et al.,* 2002; Obonyo *et al.*, 2008; Bruce *et al.*, 2010). Recently, for pest control, more emphasis has been given to habitat management strategies such as the "push-pull" or stimulo-deterrent diversionary strategy (SDDS), which effectively suppresses the pest population while maintaining environmental integrity (Midega *et al.,* 2015).

This strategy uses knowledge of plant chemistry and insect behaviour to manipulate agro-ecosystems in a manner that is unfavourable to pests, whilst simultaneously promoting crop yield through reduced pest damage (Pickett *et al.,* 2006; Khan *et al.,* 2010). It involves planting a repellent intercrop between the rows of the main crop that 'pushes' away ovipositing moths by releasing semiochemicals that indicate low quality of food or presence of competitors (Rasmann *et al.,* 2005; Pickett *et al.,* 2006; Dicke and Baldwin, 2010; Heil and Karban, 2010). Trap plants, such as Napier grass are planted around the main crops to attract the pest and provide a resource for laying their eggs. However, when the eggs hatch on trap crops such as Napier grass, the larvae are unable to survive or their development is constrained, thus reducing pest populations (Khan and Pickett, 2004; Pickett *et al.,* 2006).

Plants respond to insect attack by releasing a mixture of volatile organic compounds (VOCs), usually referred to as herbivore-induced plant volatiles (HIPVs), which directly repel the pest from the host plant or indirectly attract their natural enemies i.e. parasitoids and predators (Turlings and Tumlinson, 1992; Dicke and van Loon, 2000; Khan *et al.*, 2008). In addition, HIPV emissions can induce defence responses in neighbouring plants (Karban *et al.*, 2000; Kessler *et al.*, 2006; Rodriguez-Saona *et al.*, 2009). Previous studies reported significant reduction in stemborer infestation levels and increased larval parasitism by *Cotesia sesamiae* Cameron (Hymenoptera: Braconidae) on maize intercropped with non-host molasses grass, *Melinis minutiflora* P. Beauv. (Khan *et al.*, 1997a; b; 2000). The bioactive compounds responsible for repelling the pest and attracting the parasitoids are constitutively released from intact *M. minutiflora,* and include (*E*)-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*E*)-caryophyllene, humulene and α-terpinolene (Khan *et al.*, 1997a; 2000; Pickett *et al.*, 2006). (*E*)-ocimene, (*E*)-caryophyllene and DMNT are also known to be produced by maize in response to insect herbivory and/or egg deposition (Turlings *et al.*, 1990; Tamiru *et al.*, 2011). However, the role of intercropping on plant/plant communication between non-host and host plants and effects on direct defence against pests has not been established.

Here, we evaluated the oviposition behaviour of *C. partellus* on maize landrace and hybrid varieties, either previously exposed or non-exposed to *M. minutiflora*, and the induced production of VOCs from maize plants exposed to *M. minutiflora*.

4.3 Materials and methods

4.3.1 Study site

Studies were carried out at the Thomas Odhiambo Campus of the International Centre of Insect Physiology and Ecology (*icipe*), Mbita point (0°25'S, 34°12'E, 1 200 m a.s.l. and around 900 mm annual rainfall). The site is situated on the eastern shores of Lake Victoria in western Kenya, where *C. partellus* is the key insect pest of maize (Khan *et al.*, 2006).

4.3.2 Insects and plants

Chilo partellus were obtained from the mass rearing unit of *icipe*. This colony was established from field-collected *C. partellus* larvae that were reared on a semi-synthetic diet as described by Ochieng *et al.* (1985). The mass-reared culture was infused with a field-collected insect population every three months to avoid genetic decay and maintain the original behavioural characteristics of the species. Adults were sexed and females selected for oviposition experiments and electrophysiology. Maize seeds of local varieties (landraces) 'Jowi red' and 'Nyamula' were obtained from farmers in western Kenya, and hybrid varieties 'WS505' and 'Ph4' were obtained from commercial seed suppliers (Western Seed Company Ltd and Kenya Seed Company Ltd, respectively). Seeds of molasses grass were obtained from push-pull field plots at *icipe*. Seeds of the different maize varieties and molasses grass were planted individually in pots filled with fertilized soil inside insect proof screen houses under natural conditions (25 ^{0}C, 65% RH; 12L: 12D).

4.3.3 Plant/plant communication

The plant/plant communication experiment was conducted by placing pots of newly planted landrace or hybrid maize varieties in between rows of 5-6 weeks old potted molasses grass until the maize plants were three weeks old (Figure 4.1). After this time, all the plants exposed to molasses grass were removed and kept in a separate screen house until they were used for oviposition experiments and VOC collection. Each maize variety was subjected to six different treatments, based on the time interval between removal from the vicinity of molasses grass and use in oviposition preference and VOC collection: 0hr (immediately used), 24hr, 48hr, 72hr, 96hr and 1 week after removal. Control plants of the same maize varieties were planted in a different screen house under similar natural conditions but without the molasses grass.

4.3.4 Oviposition preference

Two-choice tests were conducted in oviposition cages (90x60x60 cm) covered by a fine wire mesh netting (Figure 4.2). To determine *C. partellus* oviposition preference, molasses grass-exposed and non-exposed maize plants were placed inside the oviposition cages adjacent to each other and five gravid female naïve *C. partellus* moths were introduced into each cage and allowed to oviposit overnight. The following morning, plants were removed from the cage and the number of eggs laid on each plant counted under a light microscope at 6.5X magnification. A total of 10 replicates were done for each treatment.

4.3.4 Volatile organic compound (VOC) collection

Volatile organic compounds (VOCs) were collected from molasses grass, as well as from molasses grass-exposed and non-exposed maize plants using dynamic headspace collection (Agelopoulos *et al.,* 1999). The leaves of the experimental plants were placed gently inside different polyethyleneterephthalate (PET) bags (volume 3.2 L, ~12.5 mm thickness) through an open end of the bag. The PET bags were sterilized at 150 ^{0}C before use. Purified charcoal filtered air was pumped through the bottom of the bag at a rate of 600 ml/min. The adsorbent, Porapak Q (0.05 g, 60 ⁄ 80 mesh; Supelco) was placed in a filter at the outlet valve where air was drawn at 400ml/min. A lower flow rate in the outlet provided positive pressure to prevent unfiltered air entering the system through any gaps. After 48hr of VOC collection, trapped VOCs were eluted from the Porapak Q filter with 0.5 ml of dichloromethane. The eluted samples were collected using 3.5 ml sample vials and kept in a freezer at -20 ^{0}C until they were required for chemical analysis.

4.3.5 Gas Chromatography (GC) analysis

Collected VOC samples were analysed using an Agilent 7890 GC instrument (Agilent Technologies) equipped with a cool-on column injector, a non-polar HP-1 capillary column

(50 m, 0.32 mm internal diameter, 0.52 μm film thickness) and a flame ionization detector (FID). Four μl of headspace sample were injected into the injector port of the GC instrument per analysis. The oven temperature was maintained at 30 ^{0}C for 2 min and then programmed at 5^{0}C min^{-1} to 250^{0}C. The carrier gas was hydrogen. Data were analysed using HP Chemstation software.

4.3.6 Coupled GC-Electroantennography (GC-EAG)

Coupled GC-electroantennography (GC-EAG) was carried out using antennae of gravid female *C. partellus* with VOC samples collected from molasses grass and maize plants. Five insects were used per selected plant samples. The glass Ag-AgCl electrodes were filled with saline solution (composition as in Maddrell (1969) but without glucose). Female moths were chilled for 1 min before the antennae were excised. The tips of the antennae were removed to ensure a good contact while suspended between the two saline solution-filled electrodes. Signals were passed through a high impedance amplifier (UN- 06; Syntech, Hilversum, The Netherlands) and analysed using a customised software package (Syntech). The GC-EAG system, in which the effluent from the GC column is simultaneously delivered to the antennal preparation and the GC detector, has been described previously (Wadhams, 1990). Separation of the VOCs was achieved on a GC (Agilent Technologies, 6890N) equipped with a cold on column injector and a FID using a HP-1 column (50 m, 0.32 mm ID, 0.52 μm film thickness). The oven temperature was maintained at 30^{0}C for 2 min and then programmed at 15^{0}C min^{-1} to 250^{0}C. The carrier gas was hydrogen. Outputs from the EAG amplifier and the FID were analysed using the Syntech software package.

4.3.7 Coupled GC-Mass Spectrometry (GC-MS) analysis

Aliquots of VOC samples were analysed on a capillary GC column (HP-1, 50 m, 0.32 mm i.d., 0.52 lm) directly coupled to a mass spectrometer (VG Autospec, Fisons Instruments, Manchester, UK) equipped with a cool on-column injector. Ionisation was performed by electron impact (70eV, 250^{0}C). The oven temperature was maintained at 30 ^{0}C for 5 min and then programmed at 5^{0}C min^{-1} to 250^{0}C. Tentative identification of compounds were made by comparison of spectra with mass spectral databases (NIST, 2005) and confirmed through co-injection with authentic standards.

4.3.8 Statistical analysis

Two-sample (unpaired) Student's t-tests were used to analyse data on the differences between treated and control maize plants of the different varieties with regard to the number of *C. partellus* eggs laid on plants using R-software (R core team, 2014).

4.4 Results

4.4.1 Oviposition preference (two-choice test)

The proportions of *Chilo partellus* eggs laid on 'Nyamula' and 'Jowi-red' maize landraces that were previously exposed to molasses grass, were significantly lower on all exposed plants (0hr, 24hr, 48hr, 72hr, 96hr and 1 week after removal from molasses grass) compared to non-exposed plants ($P<0.05$, Figure 4.3A and B). In contrast, except for the 0 hr treatments of WS505, the proportions of eggs laid on molasses grass-exposed and non-exposed commercial hybrid maize varieties (WS505 and Ph4) did not differ significantly ($P>0.05$, Figure 4.4A and B).

4.4.2 Identification of attractive volatile organic compounds

GC analysis of the VOCs collected from 'Jowi-red' maize landrace and hybrid maize WS505, exposed or non-exposed to molasses grass, revealed differences in VOC profiles (Figure 4.5). Increased VOC emission from landraces after exposure to molasses grass was observed, but this was not observed with the hybrid maize plants (Figure 4.5). Coupled GC-

EAG recordings from the antennae of female *C. partellus* using the VOCs collected from molasses-exposed 'Jowi-red' revealed responses to compounds which were identified by GC-MS and GC peak enhancement as (*R*)-linalool and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) (Figure 4.6).

4.5 Discussion

Our findings demonstrate that maize landrace varieties, previously exposed or non-exposed to the odour of molasses grass, are differentially preferred for oviposition by *C. partellus*. In the oviposition bioassay, a lower number of eggs was laid on exposed maize plants than for non-exposed plants, suggesting that molasses grass odour modifies the direct defence response of neighbouring maize plants leading to repulsion of gravid female *C. partellus*.

Like most other lepidopterous insects, host plant selection in *C. partellus* is made by the ovipositing female (Singer, 1986; Konstantopoulou *et al.*, 2002), and is influenced by a coevolutionary process (see review by West and Cunningham, 2002) and interaction with intrinsic properties of plants, such as plant chemistry (Nylin and Janz, 1996). However, adaptation is not always perfect and based on such plants' suitability for larval performance (Midega *et al.*, 2011). The current study demonstrated that *C. partellus* exhibited an oviposition preference for non-exposed 'Nyamula' and 'Jowi-red' plants suggesting that exposure may have induced emission of repellent VOCs. Relative preference for different plants is thought to arise from the balance between attractants and deterrents to which the insect responds (Renwick and Chew, 1994). This study provides evidence that the direct defence response of maize landrace varieties used in this experiment was higher even after one week of removal from molasses grass. This implies that the emission of VOCs from intact molasses grass triggers direct defence responses in heterospecific neighbouring landrace maize plants.

Host plant volatiles can either repel insects from ovipositing (De Moraes *et al.*, 2001; Bruce *et al.*, 2010; Dicke and Baldwin, 2010; Heil and Karban, 2010) or attract them for oviposition (Khan *et al.*, 2007; 2010). Plants with lower acceptance may have fewer positive or more negative stimuli (Renwick and Chew, 1994). Our results provide evidence that the presence of molasses grass modifies the direct defence response of the neighbouring maize landrace, making them less attractive to gravid moths. In addition, EAG studies showed that the main bioactive compound in the molasses grass VOCs was (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT). The hybrid maize plants appear to lack the defence response that was elicited by molasses grass VOCs in 'Nyamula' and 'Jowi-red'. In a related study, Mutyambai *et al.* (2014) reported that the same commercial hybrid maize varieties used in this study did not respond to insect egg signals, and that plants that were previously either oviposited by *C. partellus* or non-oviposited were equally preferred for subsequent oviposition by *C. partellus,* whereas moths preferred non-exposed maize landraces for subsequent oviposition. Taken together, these findings suggest that 'Nyamula' and 'Jowi-red' are more prepared to respond to insect attack through detection of plant stress VOCs associated with insect damage.

In some species of plants, large amounts of VOCs that are constitutively emitted without the plant being damaged or attacked by herbivores, can repel pest and attract parasitoids or alert neighbouring plants to switch on their defence mechanisms before they are exposed to attackers (Khan *et al.,* 2007). Interestingly, the identified compounds from intact molasses grass which are responsible for stimulating parasitism of stemborer larvae and repelling gravid stemborer moths were similar to the compounds released by damaged maize plants (Khan *et al.,* 1997a; 2000; Pickett *et al.,* 2006). However, the induced effect resulting from the interaction between intercropped maize and molasses grass plants has not been investigated previously. Here our findings provide evidence that VOCs released from intact molasses grass enhance the direct defence response of undamaged neighbouring maize landrace plants. This compound has been shown to be released following egg deposition on the plant surface (Tamiru *et al.,* 2011) and to reduce oviposition (Khan *et al.,* 1997a; b; 2000; Birkett *et al.,* 2006).

In conclusion, our study demonstrated that intercropping maize landraces with molasses grass enables these plants to deter *C. partellus* oviposition. It appears that the defence response gene of maize landrace varieties can be switched on by the VOCs released from the neighbouring molasses grass. However, the same effect did not occur with the commercial hybrid maize varieties. Moreover, the exposed maize landraces maintain the induced direct defence for up to one week after removal from the molasses grass. These findings provide a deeper understanding of the chemical ecology of intercropping and pave the way for the development of new ecologically sound plant protection strategies against stemborer pests. There is also scope for introgressing inducible defence traits into elite commercial hybrid maize varieties, especially for resource constrained smallholder farmers in sub-Saharan Africa who cannot afford pesticides for crop protection.

4.6 References

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Figure 4.1: Experimental set up of maize seedlings exposed to molasses grass from day of planting up to three weeks (a) and non-exposed control maize seedlings (b).

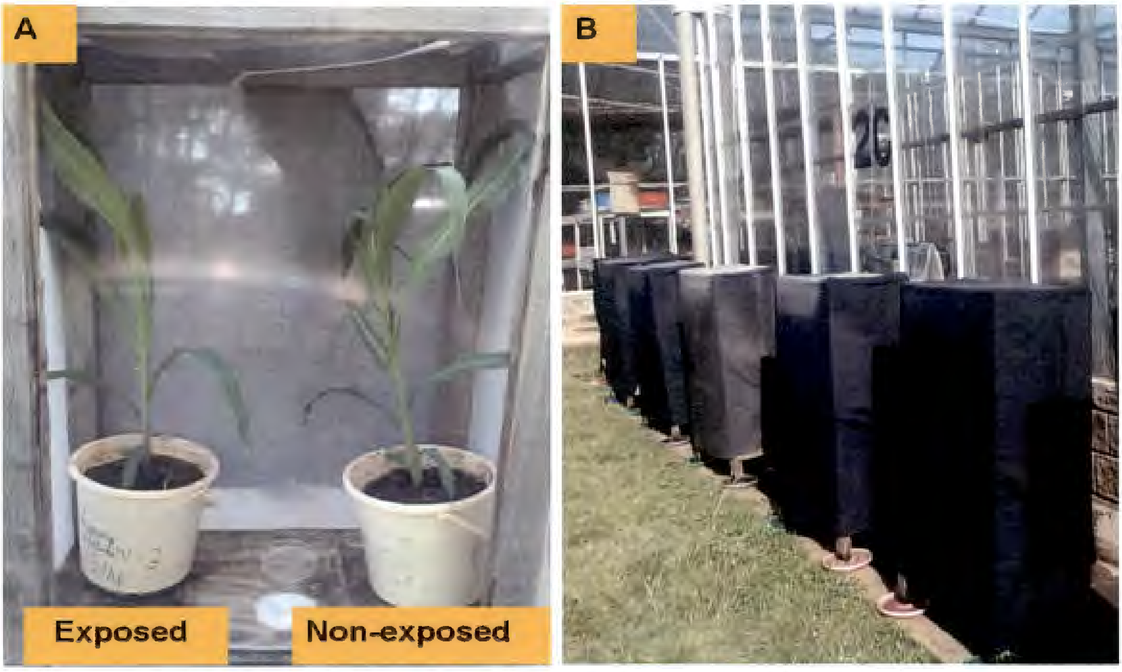

Figure 4.2: Two choice test set-up of cages: Exposed and non-exposed maize seedlings used for the choice test of gravid *Chilo partellus* bioassays (a), To prevent external light cages covered with black clothes after introducing the insects and plants (b).

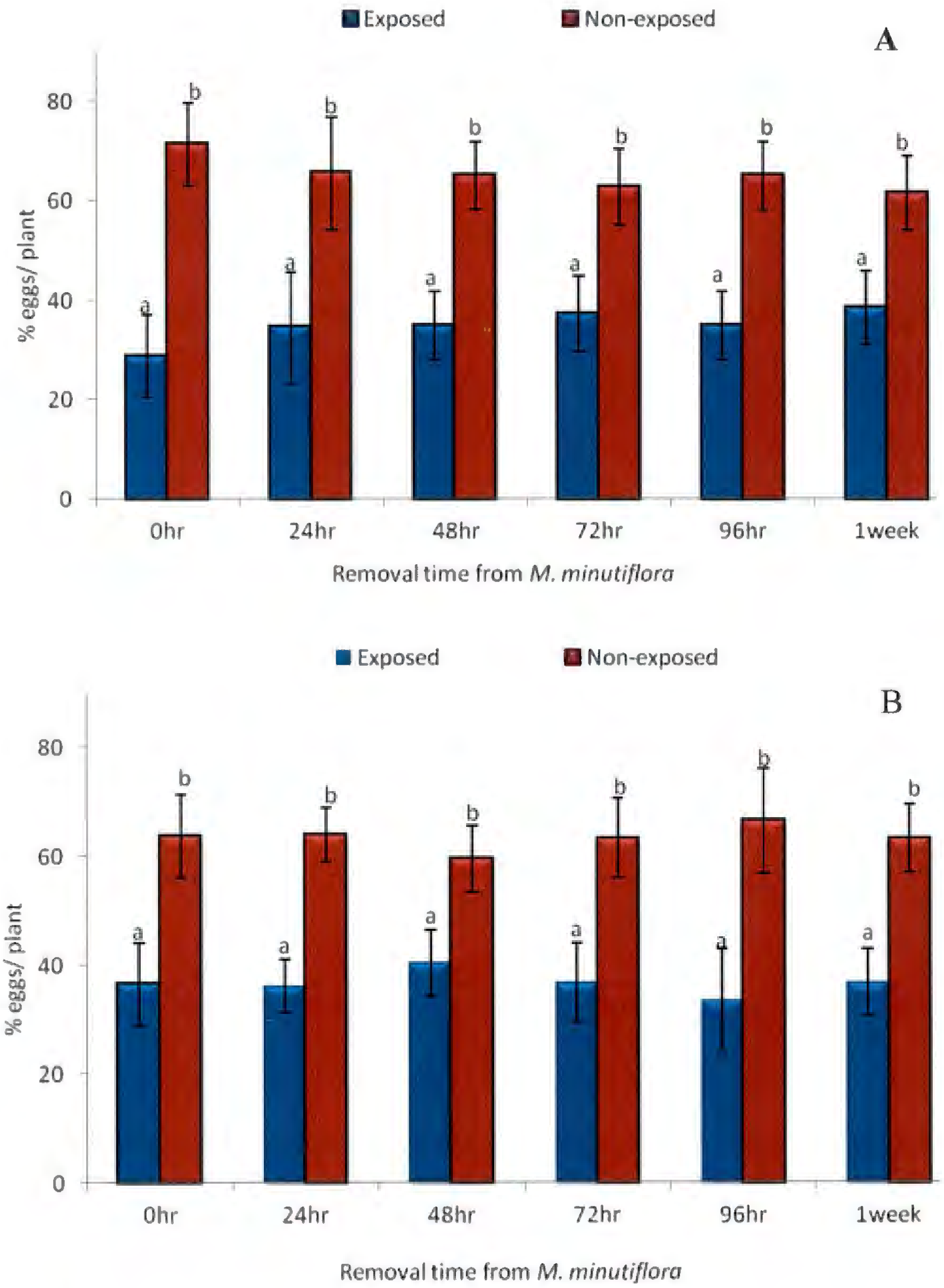

Figure 4.3: Mean (±SE) percentage of *Chilo partellus* eggs per plant laid on exposed and non-exposed landrace maize plants in two choice tests according to different removal time. Landrace maize varieties, Nyamula (A) and Jowi-red (B). Means with different letters above the bars differ significantly (Student's t-test: P<0.05).

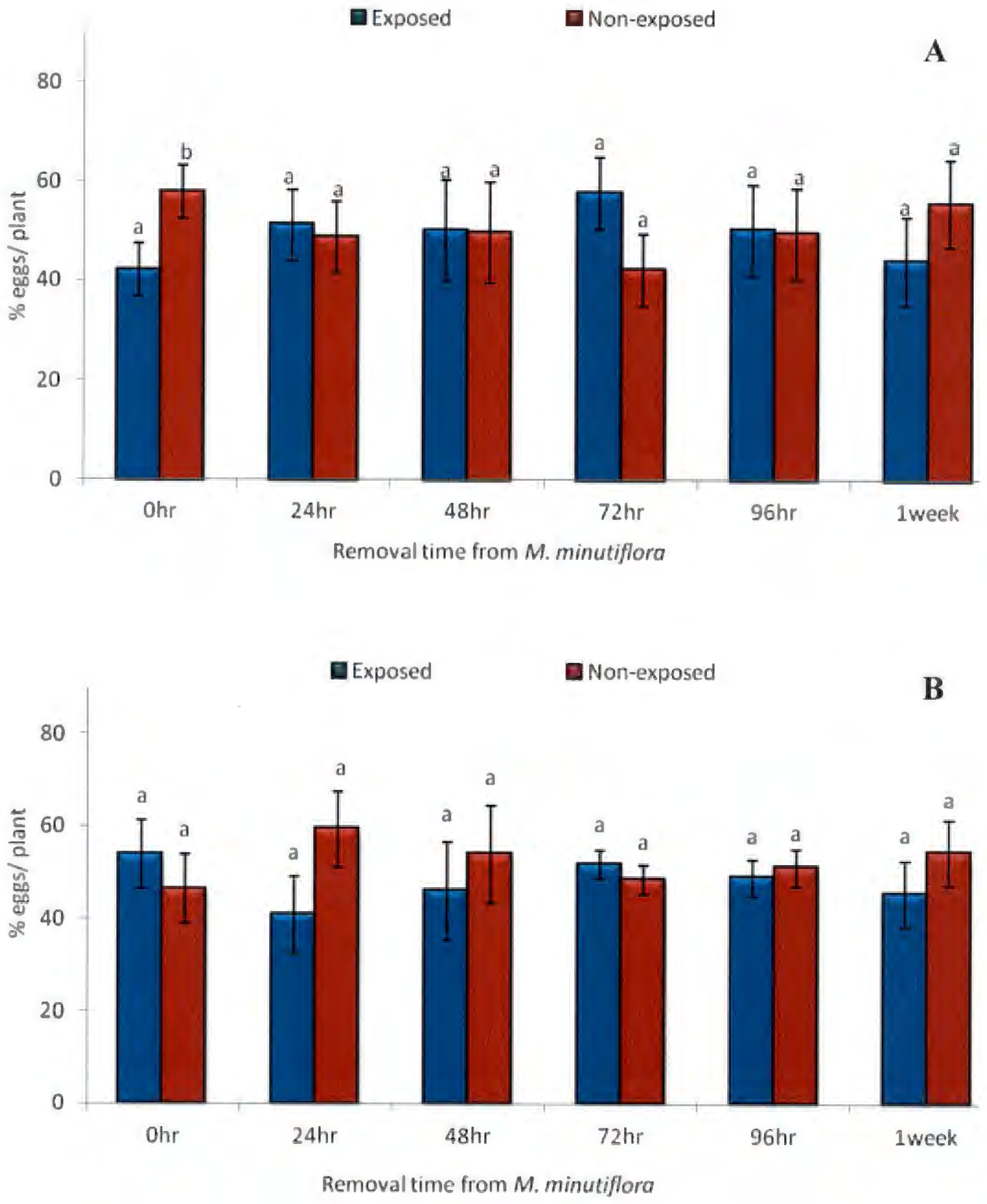

Figure 4.4: Mean (±SE) percentage of *Chilo partellus* eggs per plant laid on exposed and non-exposed hybrid maize plants in two choice tests according to different removal time. Hybrid maize varieties, WS505 (A) and Ph4 (B). Means with different letters above the bars differ significantly (Student's t-test: $P<0.05$).

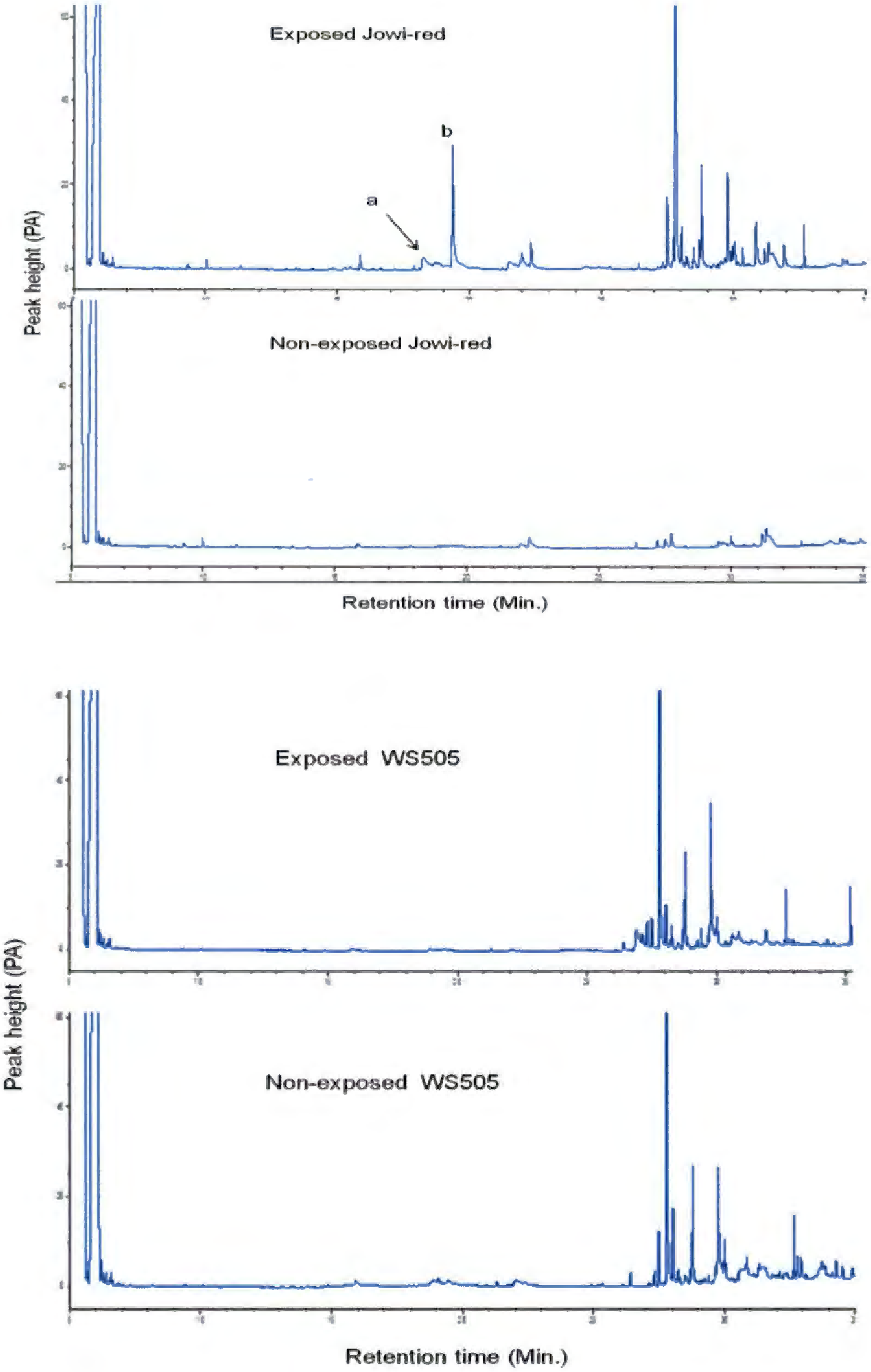

Figure 4.5: Representative GC profiles of VOCs collected from maize landrace variety, Jowi-red and the hybrid maize variety, WS505, exposed and non-exposed to molasses grass VOCs. EAG active compounds: (a) (*R*)-linalool, (b) (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT).

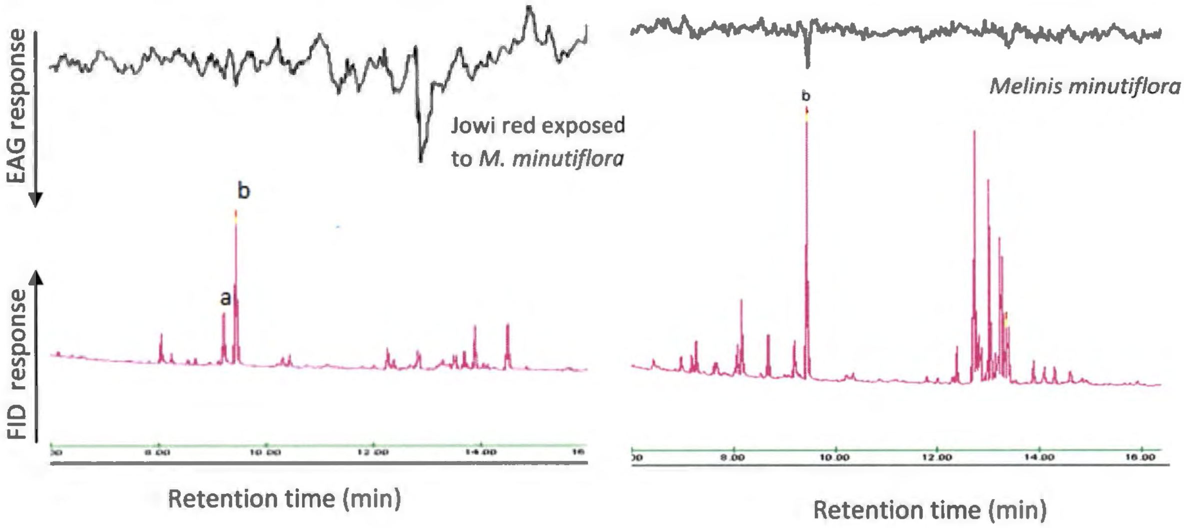

Figure 4.6: Representative GC-EAG responses of female *Chilo partellus* to volatiles collected from maize landrace "Jowi-red" (plants exposed to molasses grass) and molasses grass. The FID peaks marked are those which elicited antennal responses a= *(R)*-linalool and b= *(E)*-4,8-dimethyl-1,3,7-nonatriene (DMNT).

CHAPTER FIVE

5.0 VOLATILE EXCHANGE BETWEEN UNDAMAGED PLANTS IN AN INTERCROPPING SYSTEM BOOSTS THE INDIRECT DEFENCE OF NEIGHBOURING PLANTS

5.1 Abstract

Plants exposed to insect attack respond with increased volatile emissions that act as repellents to the attacking insect and/or attractants for their natural enemies. In addition, the emitted herbivore induced plant volatiles (HIPVs) warn neighbouring plants from impending attack. Previous studies reported that molasses grass, *Melinis minutiflora* P. Beauv. emit similar HIPVs as those emitted by damaged maize plants. However, the role of molasses grass volatiles in plant-to-plant communication in maize-molasses grass intercropping systems and the consequence on parasitoid recruitment had not been explored. This study investigated the induced indirect defence response of different maize varieties exposed to molasses grass volatiles. After three weeks of growing in close proximity to molasses grass, maize plants were removed and headspace volatile samples were collected immediately (0 hr), after 24 hr, 48 hr, 72 hr and 96 hr, as well as one week from time of removal. Behavioural bioassays were carried out in a four-arm olfactometer using the larval parasitoid, *Cotesia sesamiae* Cameron (Hymenoptera: Braconidae). The headspace volatile samples collected from exposed maize landraces were significantly more attractive to *C. sesamiae* than those from non-exposed landraces. Headspace samples were also analysed using Coupled Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography-Electroantennography (GC-EAG). GC-MS analysis revealed enhanced profile changes in the exposed maize landraces compared to non-exposed maize plants. Higher amounts of EAG-active volatile compounds such as (*E*)-4,8-dimethyl-1,3,7-nonatriene were emitted from exposed maize landraces compared to non-exposed plants. In contrast, there was no significant difference observed in the behavioural bioassay of *C. sesamiae* for headspace samples collected from exposed and non-exposed hybrid maize varieties. Results from chemical analyses and behavioural assays suggest that the indirect defence response of maize landraces is induced by the volatile organic compounds emitted by the neighbouring undamaged molasses grass. However, the hybrid maize varieties tested in this experiment lacked the ability to respond to neighbouring plant volatiles. This physiological trait in maize landraces may have significant impacts on tritrophic interactions through airborne signalling in the agro-ecosystem. Understanding the phenomenon of plant-to-plant communication between undamaged plants and introducing the responsible induced defence trait into future breeding maize lines may improve plant protection strategies by increasing predation pressure on herbivores.

Key words: exposed maize, landrace, olfactometer, parasitoid, tritrophic interaction

5.2 Introduction

Plants are sessile organisms which cannot escape or hide from attackers. However, they evolved with different defence mechanisms including volatile and non-volatile chemical compounds to deter herbivores from feeding or oviposition and attract natural enemies of the herbivores. Plants that are not under attack by herbivores usually emit small quantities of volatile chemicals into the environment, whereas the amount and quality of the compounds emitted by damaged plants may vary (Arimura *et al.,* 2009). In response to herbivore damage, large amounts of chemical compounds may be produced and emitted into the environment, serving as sources of information for parasitoids, herbivores and neighbouring plants (Engelberth *et al.,* 2004; Holopainen, 2004; Khan *et al.,* 2008; Arimura *et al.,* 2009; Heil and Karban, 2010; Karban, 2011; Penaflor *et al.,* 2011). However, the identity of emitted volatile organic compounds (VOCs) depends on the type of damage, species of the plant or herbivore (De Moraes *et al.,* 1998; Pare and Tumlinson, 1999; Schnee *et al.,* 2006) and environmntal factors (Gouinguene and Turlings, 2002)

Herbivore induced plant volatiles (HIPVs) have multiple functions that range from protecting the plant to mediating interaction between organisms. HIPVs emitted from damaged plants can warn undamaged neighbouring plants of the same or different species from impending attack (Baldwin and Schultz, 1983; Arimura *et al.*, 2000; Karban and Maron, 2002; Baldwin *et al.,* 2006; Heil and Silva Bueno, 2007). HIPVs may also affect the defense strategy of neighboring plants (Baldwin and Schultz, 1983; Rhoades, 1983). Plants therefore perceive and respond to reliable cues from their damaged neighboring plants in order to adjust their physiology by changing the abundance of phytohormones (Arimura *et al.*, 2002; Engelberth *et al.,* 2004), increased production of defense related metabolites such as terpenoids (Engelberth *et al.,* 2004; Ruther and Kleier, 2005), proteinase inhibitors (Tscharntke *et al.,* 2001) or phenolic compounds (Baldwin and Schultz 1983; Tscharntke *et al.,* 2001). It has been documented that maize plants exposed to wound induced green leaf volatiles, are able to trigger and produce much more defense related compounds including the terpenoid (E)-4,8-dimethyl-1,3,7-nonatriene (Yan and Wang, 2006). Similarly, parasitoids also perceive and use the chemical information emitted from damaged plants (Takabayashi and Dicke, 1996; Dicke and Vet, 1999; Fatouros *et al.*, 2012) or oviposited plants to locate their target prey (Tamiru *et al.*, 2011; 2012; Fatouros *et al.*, 2012; Mutyambai *et al.*, 2015).

Some plant species such as intact molasses grass, *M. minutiflora* emit similar VOCs as those emitted by damaged maize plants, and as such can repel pests and attract natural enemies without the plant being attacked by pests or being mechanically damaged (Khan *et al.*, 1997; 2000). In addition, the emitted volatiles from molasses grass are available as a cue for the intercropped maize plants to release specific volatiles (Chapter 3). Studies have shown that plants that are capable of "eavesdropping" on airborne signals emitted from neighbouring plants (Bruin *et al.*, 1992; Shonle and Bergelson, 1995; Chamberlain *et al.*, 2000; 2001; Karban *et al.*, 2000; Pickett and Poppy, 2001; Kost and Heil, 2006; Heil and Silva Bueno, 2007), change their volatile profile and respond to the signal accordingly or defend themselves before they are attacked (Ton *et al.*, 2007; Ninkovic *et al.*, 2009). Volatile chemical exchange between undamaged plants can affect insect-plant interactions (Glinwood *et al.*, 2011). In a maize-molasses grass intercropping system, volatile emissions of maize has been shown to increase after exposure to molasses grass volatiles (Chapter 3) and it was also shown that the signals cause neighbouring plants to be less attacked by herbivores (Chapter 4). However the effect of the induced response of maize plants on parasitoid recruitment has not been studied.

Therefore this study investigated whether the volatiles emitted from undamaged molasses grass induce indirect defense responses in neighboring maize plants and its effect on parasitoid recruitment.

5.3 Materials and methods

5.3.1 Study site

This study was carried out at the Thomas Odhiambo Campus of the International Centre of Insect Physiology and Ecology (*icipe*), Mbita point (0°25'S, 34°12'E, 1 200 m a.s.l.). The site is situated on the eastern shores of Lake Victoria in western Kenya.

5.3.2 Experimental insect

The larval parasitoid *C. sesamiae* was reared on stemborer larvae using methodologies described previously (Overholt *et al.,* 1994). The insects were maintained at the insect mass rearing unit of *icipe*-Thomas Odhiambo campus (24±3 ^{0}C, 70±5% RH, 12L: 12D). The mass-reared culture was infused with a field-collected insect population every three months to avoid genetic decay and maintain the original behavioural characteristics of the species. Naive mated female parasitoids were used in the experiments.

5.3.3 Experimental plants and setup

Seeds of the experimental plants of two landrace maize varieties 'Jowi-red' and 'Nyamula' were collected from farmers in western Kenya while the two hybrid maize varieties 'WS505' and 'PH4' were collected from commercial seed suppliers (Western Seed Company Ltd and Kenya Seed Company Ltd, respectively). Seeds of the "inducer" plant, molasses grass were collected from a push-pull field plot situated at *icipe's* Thomas Odhiambo Campus, Mbita Point. All plants were planted individually in pots filled with fertilized soil in insect-proof screen houses under natural conditions (25 ^{0}C, 65% RH; 12L:12D) at the same locality.

Maize plants were maintained between the molasses grass until the seedlings were three weeks old. Thereafter seedlings were transferred into a different screen house until they were used for the experiments. Each maize variety was subjected to six different treatments, based on the time interval they were used for the experiment. These treatments were: after 0hr (immediately used after removal from the neighbourhood of molasses grass), 24hr, 48hr, 72hr, 96hr and 1week. Control (non-exposed) plants of the same type of maize varieties were planted in a different screen house under similar natural conditions but without the molasses grass.

5.3.3 Volatile organic compound collection

Volatile compounds were collected using headspace sampling (Agelopoulos *et al.,* 1999) from hybrid and landrace maize plants exposed and non-exposed to molasses grass. Prior to volatile collection, exposed maize plants were removed from the screen house where it was grown together with molasses grass for the 3-week period. Volatile samples were then collected at different times after removal. The leaves of the experimental plants were placed gently inside different polyethyleneterephthalate (PET) bags (volume 3.2 L, ~12.5 mm thickness) through the open end of the bag. The PET bags were sterilized at 150 ^{0}C before use. Purified charcoal filtered air was pumped through the bottom of the bag at a rate of 600 ml/min. The adsorbent, Porapak Q (0.05 g, 60/80 mesh; Supelco) was placed at the outlet valve where air was drawn out at 400 ml/min. A lower flow rate in the outlet valve allowed for enough time and pressure for the Porapak Q, to effectively adsorb the VOCs. After 48hr of entrainment, collected volatile samples from the Porapak Q were eluted with 0.5 ml of dichloromethane. The eluted samples were collected using 3.5 ml sample vials and kept in a freezer at -20 ^{0}C until use for chemical analysis. Each treatment from exposed and non-exposed maize seedlings was replicated four times.

5.3.4 Behavioural bioassay

Effects of collected plant-derived volatile samples were tested on gravid larval parasitoids (*C. sesamiae*) in a Perspex four-arm olfactometer (Pettersson, 1970) (Figure 5.1). Aliquots of headspace samples (10 μl) from exposed and non-exposed maize plants were applied onto a piece of filter paper (4×25 mm) using a micropipette. The pieces of paper were then placed into the two arms at the opposite ends of the olfactometer, while the remaining two arms were used for the solvent control. Air was drawn through the four arms towards the centre of the olfactometer at a rate of 260 ml min^{-1}. Mated female *C. sesamiae* parasitoids without any previous exposure to plants or hosts were transferred individually into the central chamber of the olfactometer using a custom-made piece of glass tubing. Time spent and number of entries into each arm was recorded using 'Olfa' software (F. Nazzi, Udine, Italy) for 12 minutes. To avoid any directional effect of the response the olfactometer were rotated every 3 minutes. The experiments were replicated 10 times for each sample.

5.3.5 Gas Chromatography Analysis

Plant VOCs samples were analyzed using a Hewlett-Packard 7890 GC machine (Agilent Technologies) equipped with a cool-on column injector, a non-polar HP-1 capillary column (50 m, 0.32 mm internal diameter, 0.52 μm film thickness) and a flame ionization detector (FID). Four μl of headspace sample were injected into the injector port of the GC instrument. The oven temperature was maintained at 30 ^{0}C for 2 min and then programmed at 5 ^{0}C min^{-1} to 250 ^{0}C. The carrier gas was hydrogen. Data were analyzed using HP Chemstation software.

5.3.6 Coupled GC-Mass Spectrometry (GC-MS) analysis

Aliquots of attractive headspace samples were analyzed on a capillary GC column (HP-1, 50 m, 0.32 mm i.d., 0.52 lm) directly coupled to a mass spectrometer (VG Autospec; Fisons Instruments, Manchester, UK) equipped with a cool on-column injector. Ionisation was performed by electron impact (70 eV, 250 ^{0}C). The oven temperature was maintained at 30 ^{0}C for 5 min and then programmed at 5 ^{0}C min^{-1} to 250 ^{0}C. Tentative identifications of compounds were made by comparison of spectra with mass spectral databases (NIST, 2005) and confirmed through co-injection with the authentic standards.

5.3.7 Coupled GC-Electroantennography (GC-EAG)

Coupled GC-electroantennography (GC-EAG) was carried out using antennae of gravid female *C. sesamiae* with headspace samples collected from exposed and non-exposed landrace and hybrid maize plants (Figure 5.2). Five insects were used for the EAG recording for each of the selected plant samples based on the response of the parasitoids in the olfactometer bioassays. The glass electrodes Ag-AgCl were filled with saline solution composition as in Maddrell (1969) but without glucose. Female parasitoids were chilled for 1 min before the antennae were excised. The tip of the antennae was removed to ensure a good contact while suspended between the two saline solution-filled electrodes. Signals were passed through a high impedance amplifier (UN- 06; Syntech, Hilversum, The Netherlands) and analysed using a customised software package (Syntech). The GC-EAG system, in which the effluent from the GC column is simultaneously delivered to the antennal preparation and the GC detector, has been described previously (Wadhams, 1990). Separation of the volatiles was achieved on a GC (Agilent Technologies, 6890N) equipped with a cold on column injector and a FID using a HP-1 column (50 m,0.32 mm ID, 0.52 µm film thickness). The oven temperature was maintained at 30 ^{0}C for 2 min and then programmed at 15 ^{0}C min^{-1} to 250 ^{0}C. The carrier gas was helium. Outputs from the EAG amplifier and the FID were analysed using the Syntech software package.

5.3.8 Statistical analysis

Bioassay data from the four-arm olfactometer were analysed by use of analysis of variance (ANOVA) after the data were converted into proportions and log-ratio transformation. Means were separated using Tukey's test, with α set at 0.05. Statistical analyses were done using R software (R, 2014).

5.4 Results

5.4.1 Behavioural response of *C. sesamiae* to headspace samples collected from exposed and non-exposed maize varieties

Cotesia sesamiae females were significantly attracted ($P<0.05$) to the plant volatiles collected at any of the six collection times, from exposed plants of the maize landraces (Nyamula and Jowi-red) compared to the non-exposed and solvent controls (Figure 5.3A and B). No differences were however observed between exposed, non-exposed and solvent treatments for Jowi-red, one week after plant removal. In contrast, volatiles collected from exposed and non-exposed plants of the commercial hybrid varieties (WS505 and PH4) did not elicit different responses ($P>0.05$) from *C. sesamiae* individuals, and the times spent in arms containing volatiles from exposed, non-exposed and solvent control, irrespective of the time of entrainments of the volatiles (Figure 5.4A and B), were similar. The only exception to this was the first treatment of PH4 at 0 hr.

5.4.2 Comparison of volatiles emitted from maize varieties exposed and non-exposed to molasses grasses

While GC analyses showed that changes occurred in the volatile profiles released by maize landraces exposed to molasses grass, this was not the case with non-exposed plants of the same landraces (Figures 5.5-5.7). In contrast, no differences were observed in the volatile profiles of exposed and non-exposed hybrid varieties (Figures 5.8-5.10). In general, high response differences were observed between hybrid and landrace varieties to the volatiles released from neighboring molasses grass. Landrace varieties exposed to molasses grass volatiles emitted more EAG active compounds than non-exposed landraces (Figure 5.11).

5.4.3 Identification of attractive volatile organic compounds

GC-EAG recording with the attractive plant volatile samples from exposed Jowi-red plants revealed that *C. sesamiae* antennae were responsive to certain compounds (Figure 5.11). GC-MS identification of headspace samples from exposed maize landrace variety, Jowi-red showed that *C. sesamiae* antennae were responsive to myrcene, (*β*)-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), decanal, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) as well as two unknown compounds.

5.5 Discussion

Results from this study showed that volatile samples collected from maize landrace plants exposed to molasses grass volatiles were more attractive to females of the stemborer parasitoid *C. sesamiae* than volatiles of non-exposed plants. This demonstrated that volatile compounds emitted from molasses grass can induce indirect defense responses in undamaged plants of neighboring maize landraces through airborne signals. However, the effect was not observed with hybrid maize varieties. Previous studies have shown significant reductions in stemborer infestation levels and increased larval parasitism by *C. sesamiae* in the intercropped fields with molasses grass (Khan *et al.,* 1997). Certain maize varieties exposed to molasses grass volatiles for certain periods of time have shown volatile profile changes (Chapter 3), indicating that volatile cues released from neighboring plants change the physiology of receiver plants which can also affect the oviposition behavior of *C. partellus* (Chapter 4).

In response to damage by herbivores plants release much more volatile compounds than they usually do. Although this may benefit parasitoids and contribute to their efforts to locate their prey (Turlings and Wäckers, 2004), it may also make plants less attractive to herbivores (Tscharntke *et al.,* 2001). The VOCs released by plants during periods of biotic or abiotic stress are available as signals for neighboring plants. Many studies have shown that intact plants respond to the cues released from damaged plants in their close vicinity (Arimura *et al.,* 2002; Engelberth *et al.,* 2004; Kessler *et al.,* 2006). However, it has been shown that plants are able to sense and respond to volatiles released from undamaged neighboring plants (Chapter 3; Ninkovic *et al.,* 2003), to reduce their attractiveness to insect herbivores (Chapter 4; Ninkovic *et al.,* 2002; 2009; 2013; Glinwood *et al.,* 2011), or to be more attractive to natural enemies of herbivores.

The behavioral responses of *C. sesamiae* to the headspace samples collected from landrace varieties previously exposed to molasses grass volatiles showed that these volatiles elicited a positive (attraction) response compared to volatiles collected from non-exposed plants. However, a similar phenomenon was not observed on the commercial hybrid maize varieties used in this study. This study may be the first to demonstrate that the volatiles emitted from molasses grass induce indirect defence responses and alter the volatile profile of neighbouring maize landrace plants.

Volatile chemical compounds play an important role in interaction between organisms in the environment. Plants are able to detect volatile cues released in their environment and adjust their morphohology, physiology and phenotype according to the signal (Callaway *et al.,* 2003; Trewavas, 2005). In this experiment large quantities of VOCs were emitted from maize landraces exposed to molasses grass compared to non-exposed maize landraces. The GC analysis revealed differences in volatile profiles between exposed and non-exposed plants of maize landraces. However, the difference between exposed and non-exposed hybrid maize varieties was insignificant.

Insects rely on olfaction to locate their suitable hosts (Kegge and Pierik, 2010). VOCs are important cues for the foraging success of parasitoids, the results in this study showed that headspace samples collected from landrace varieties previously exposed to molasses grass volatiles were much more attractive to parasitoids in olfactometry bioassays than volatiles from non-exposed plants. Similarly, the EAG active compounds that were identified by GC-MS from exposed maize landraces included myrcene, (*β*)-ocimene, *(R)*-linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). DMNT is well known as a key to attractant of *C. sesamiae* (Khan *et al.,* 1997), and is known to be emitted during egg deposition on maize plants (Tamiru *et al.,* 2011; 2012; Mutyambai *et al.,* 2015). The EAG active volatile compounds produced by maize landrace varieties exposed to molasses grass volatiles were not observed with hybrid maize plants used in this experiment.

Identifying the genes responsible for induced indirect defenses and applying this knowledge in the future maize breeding programmes may increase the efficacy of plant protection strategies, especially for resource poor smallholder farmers in sub-Saharan Africa.

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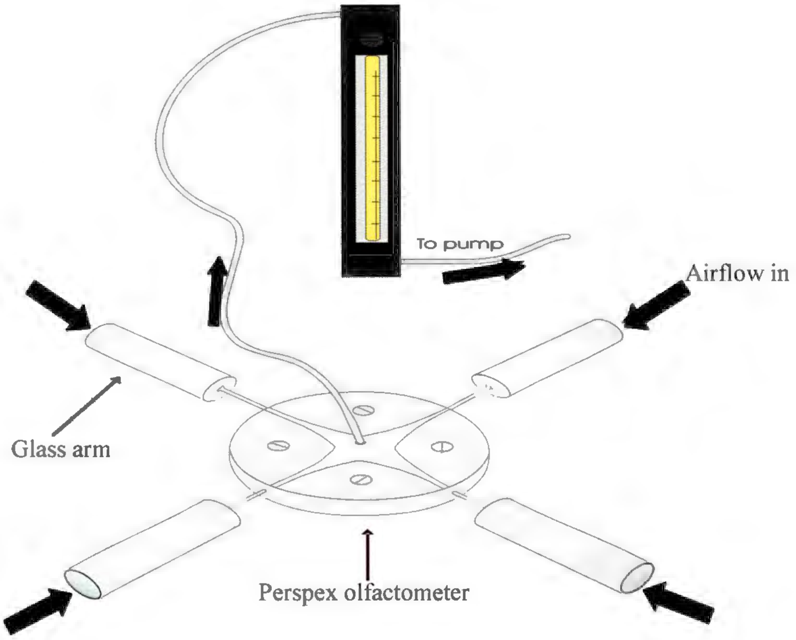

Figure 5.1: Schematic diagram of the four-arm olfactometer that was used to assay for behavioural responses in *Cotesia sesamiae*. Arrows indicate direction of airflow (Pettersson, 1970).

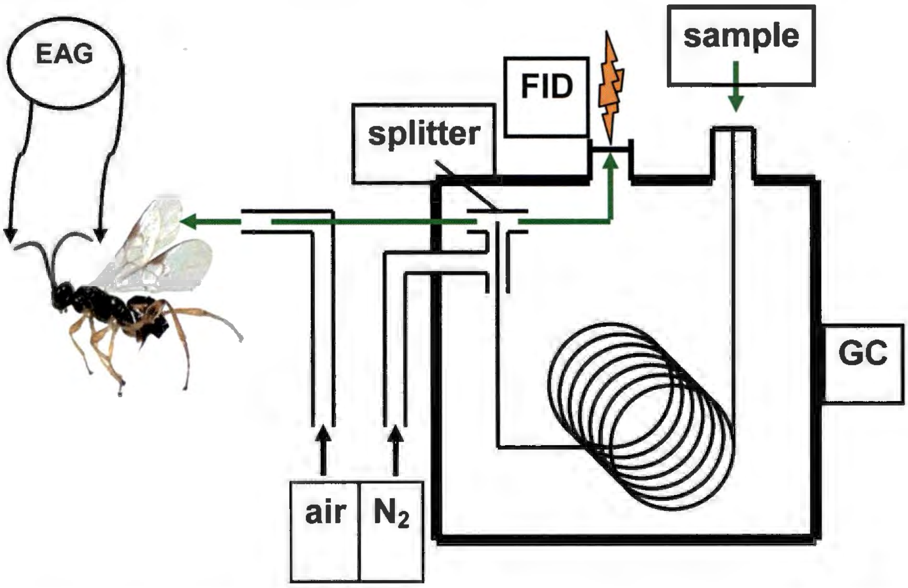

Figure 5.2: Schematic diagram of the coupled gas chromatography (GC)-electroantennogram (EAG) setup.

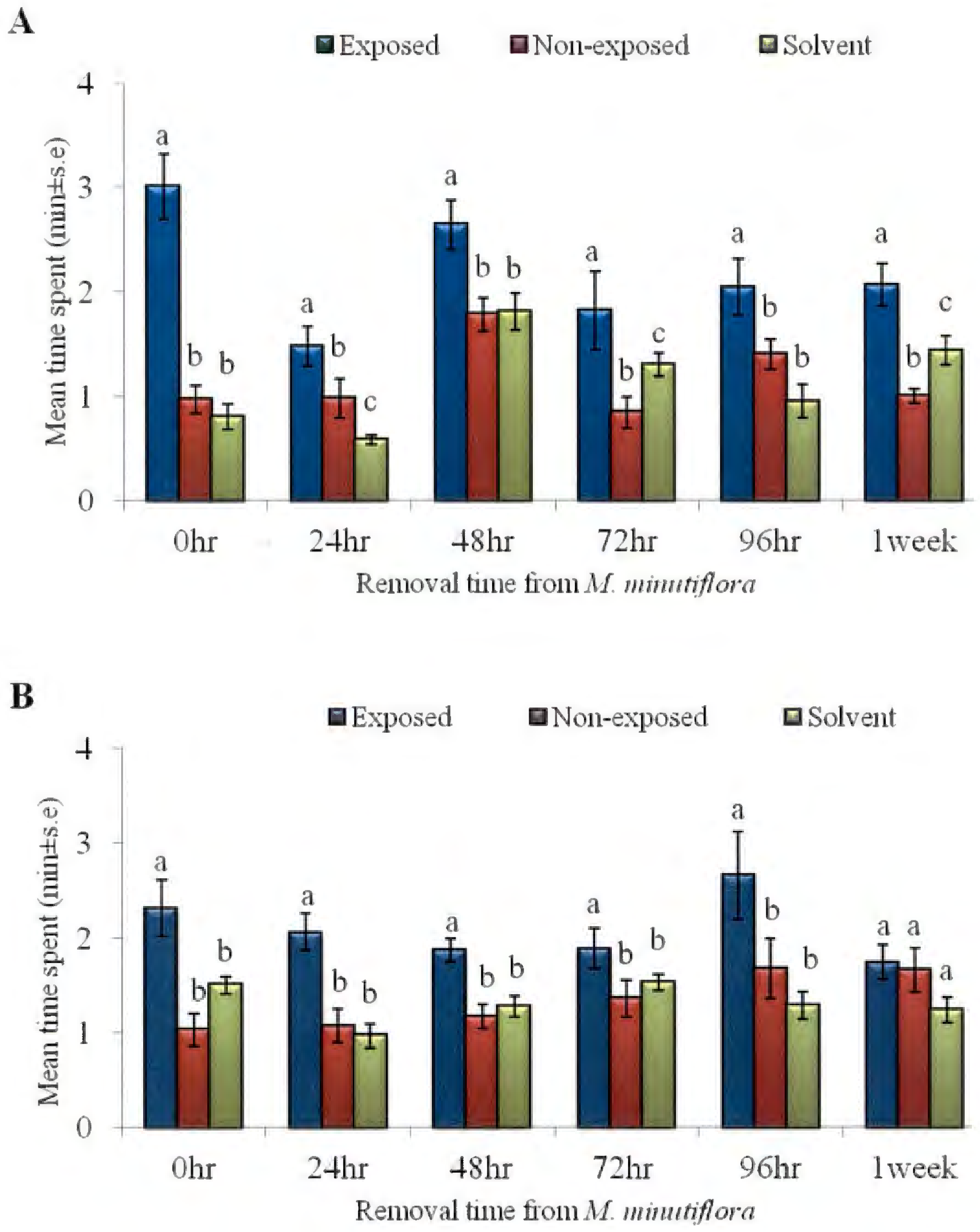

Figure 5.3: Behavioural responses of female larval parasitoids, *C. sesamiae*, to volatiles collected from exposed and non-exposed maize landraces to molasses grass and a solvent control in a four-arm olfactometer bioassay. Maize landrace varieties used in this experiment were, Nyamula (A) and Jowi-red (B). Each female parasitoid was observed for 12 minutes. Bars followed by different letters are significantly different (Tukey studentized range test: $P<0.05$).

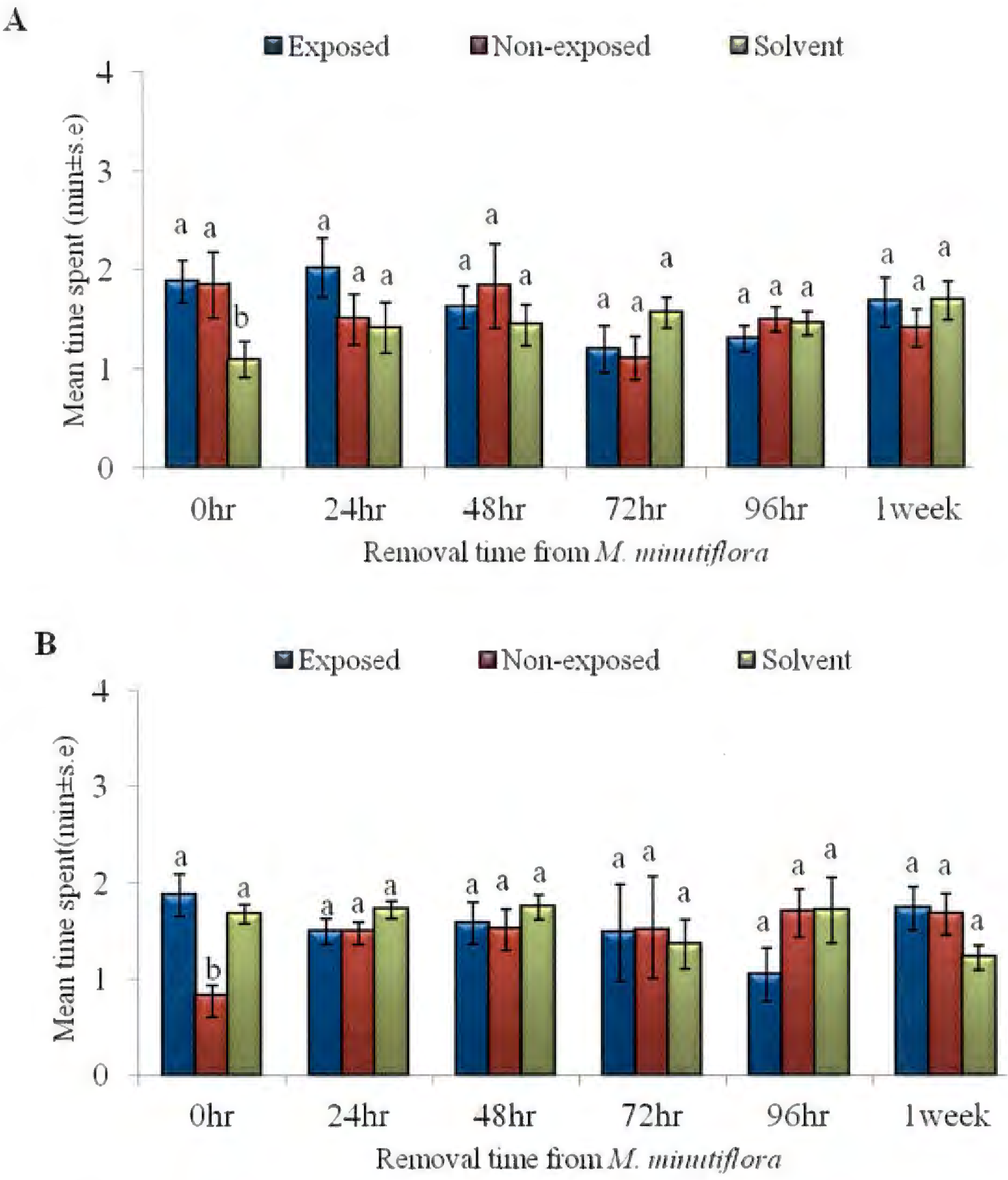

Figure 5.4: Behavioural responses of female larval parasitoids, *C. sesamiae*, to volatile collected from exposed and non-exposed hybrid maize varieties to molasses grass and solvent control in a four-arm olfactometer bioassay. Hybrid maize varieties used in this experiment are, WS505 (A) and PH4 (B). Each female parasitoid was observed for 12 minutes. Bars followed by different letters are significantly different (Tukey studentized range test: P<0.05).

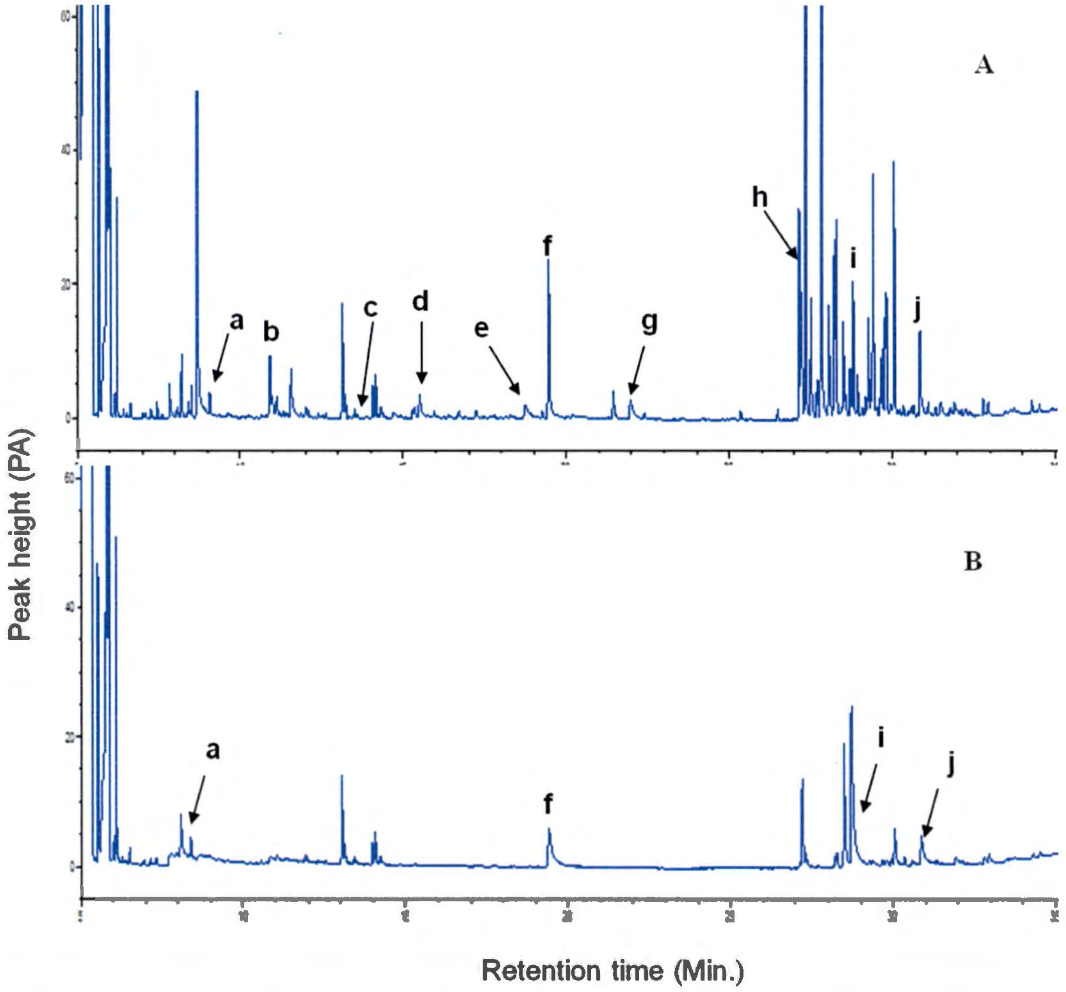

Figure 5.5: GC profiles of headspace volatiles taken immediately after removal (0 hr) of plants of landrace variety, Nyamula, from molasses grass (A) and a non-exposed Nyamula plant to molasses grass (B). Some of identified EAG active compounds are: (a) (Z)-3-hexenal, (b) hexanol, (c) decane, (d) β-ocimene, (e) nonanol, (f) (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (g) decanal, (h) (Z)-jasmone, (i) (E)-β-farnesene, (j) (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT).

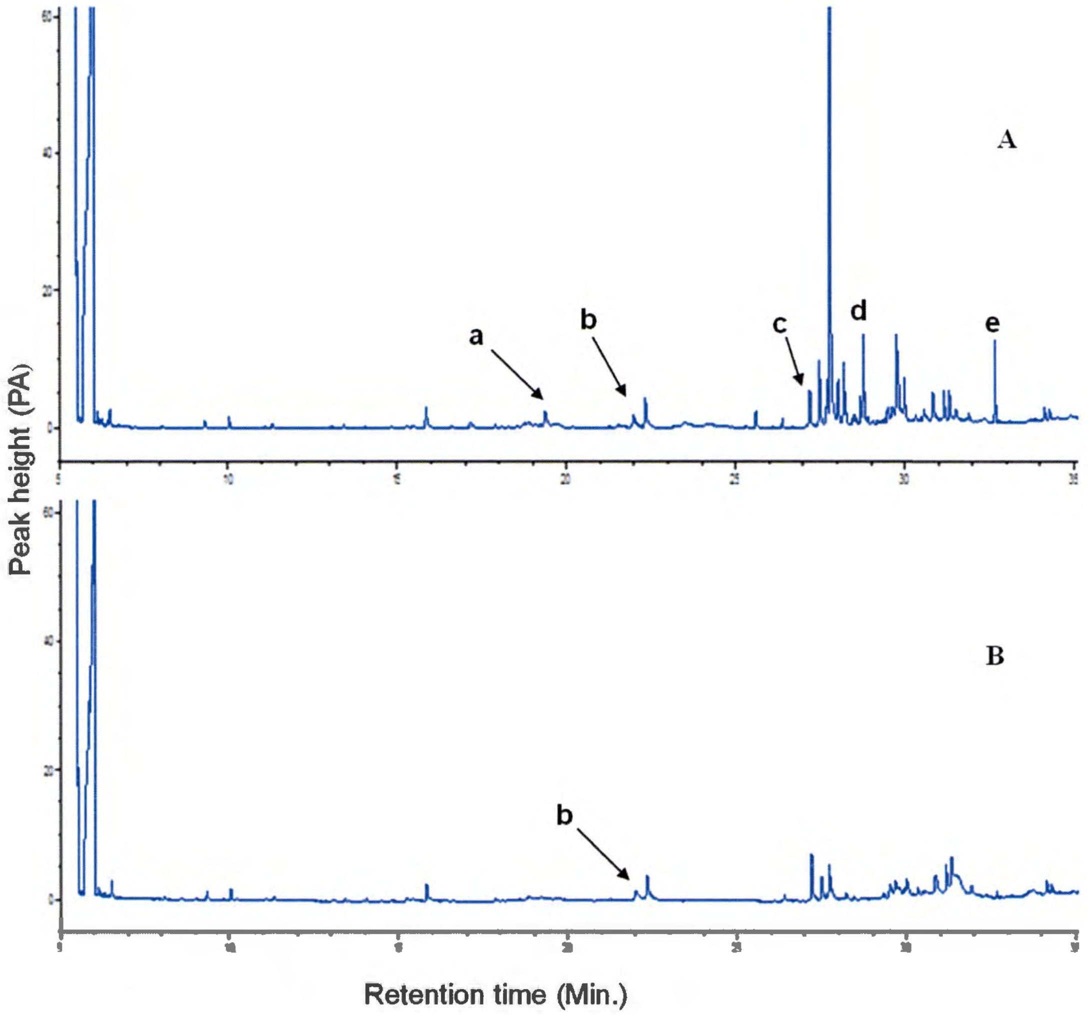

Figure 5.6: GC profiles of headspace volatiles from maize landrace variety, Nyamula, after 72 hr of removal from molasses grass (A) and non-exposed Nyamula plant to molasses grass (B). Some of identified EAG active compounds are: (a) *(E)*-4,8-dimethyl-1,3,7-nonatriene (DMNT), (b) decanal, (c) (Z)-jasmone, (d) *(E)*-*β*-farnesene, (e) *(E,E)*-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT).

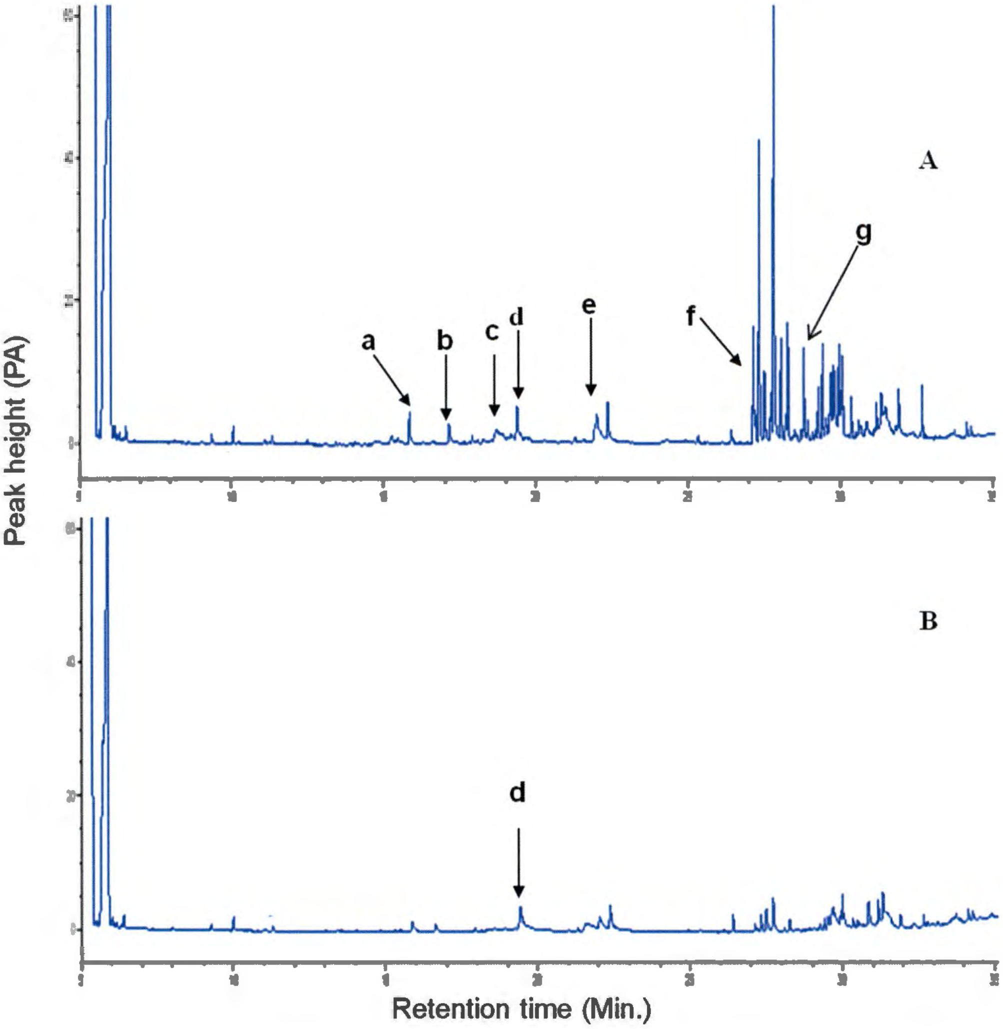

Figure 5.7: GC profiles of headspace volatiles from landrace variety, Nyamula, after one week of removal from molasses grass (A) and non-exposed Nyamula plant to molasses grass (B). Some identified EAG active compounds are: (a) α-pinene, (b) myrcene, (c) nonanal, (d) *(E)*-4,8-dimethyl-1,3,7-nonatriene (DMNT), (e) decanal, (f) *(Z)*-jasmone, (g) *(E)*-*β*-farnesene.

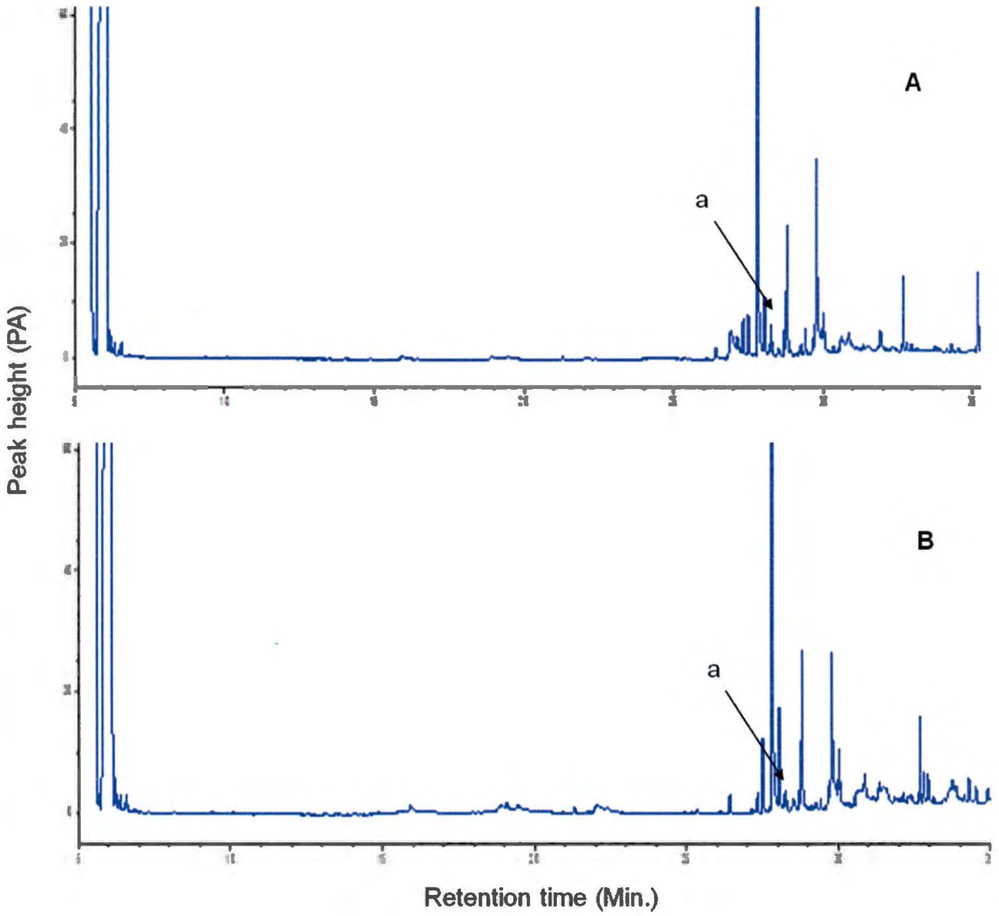

Figure 5.8: GC profiles of headspace volatiles taken immediately after removal (0 hr) of plants of hybrid maize variety, WS505, from molasses grass (A) and non-exposed WS505 plant to molasses grass (B). The identified EAG active compound, indicated by (a) is *(E)-β-*farnesene.

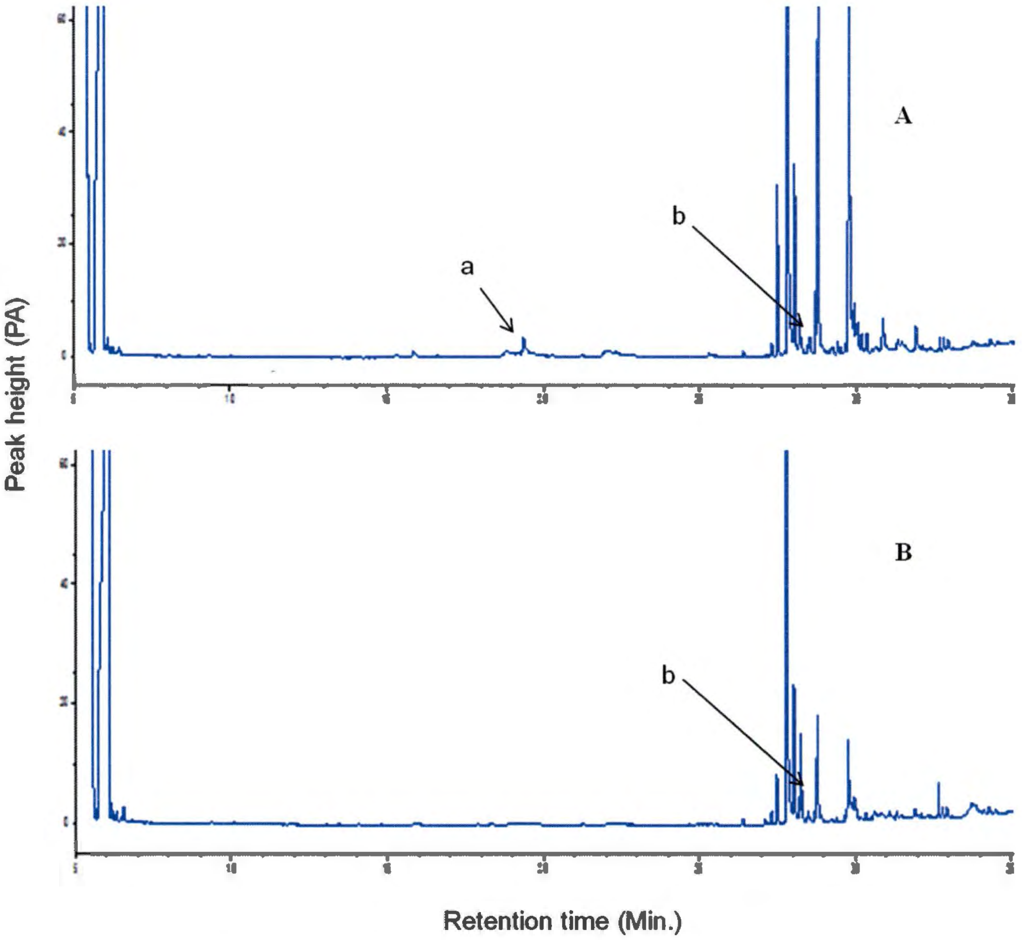

Figure 5.9: GC profiles of headspace volatiles from hybrid maize variety, WS505, 72 hr after removal from molasses grass (A) and non-exposed WS505 plant to molasses grass (B). Some of identified EAG active compounds are: (a) *(E)*-4,8-dimethyl-1,3,7-nonatriene (DMNT), (b) *(E)*-*β*-farnesene.

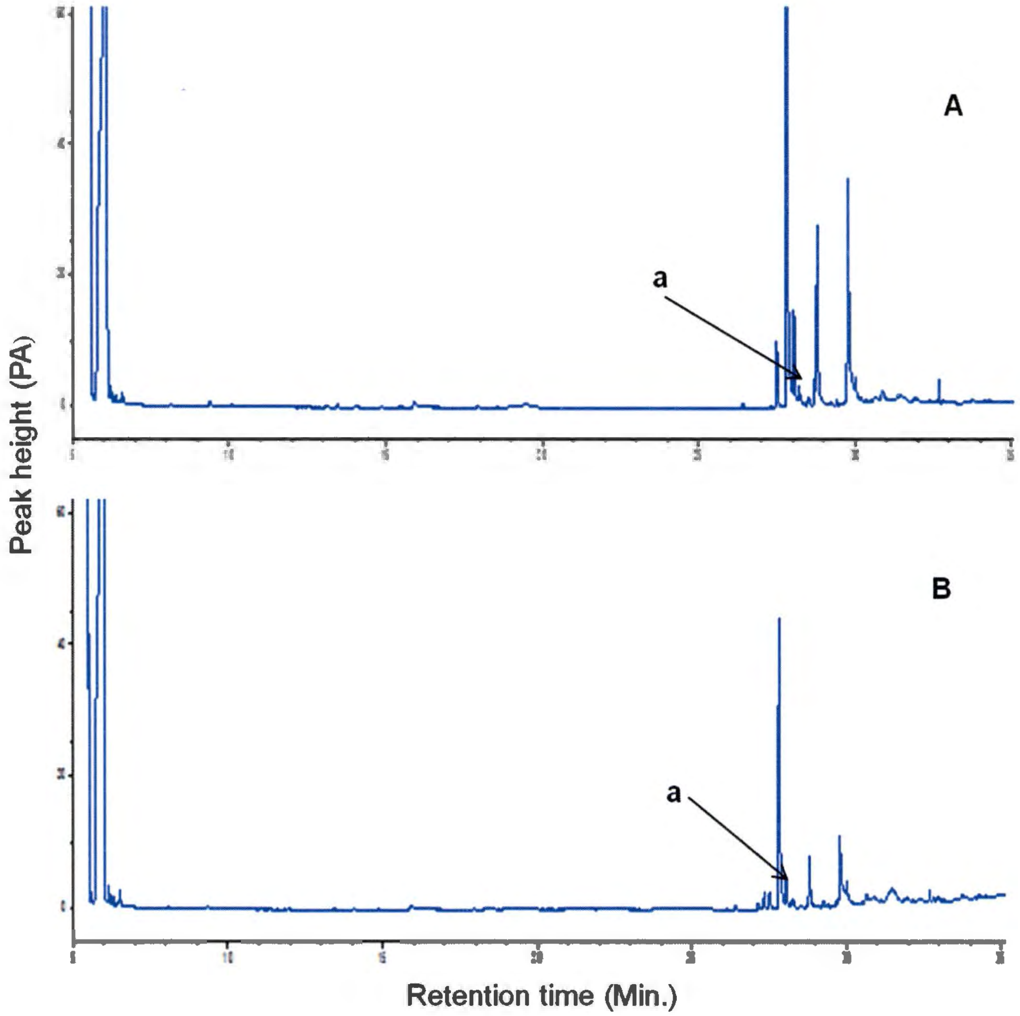

Figure 5.10: GC profiles of headspace volatiles from WS505 plants, one week after removal from molasses grass (A) and non-exposed WS505 plant to molasses grass (B). The EAG-active compound indicated by (a) is *(E)-β*-farnesene.

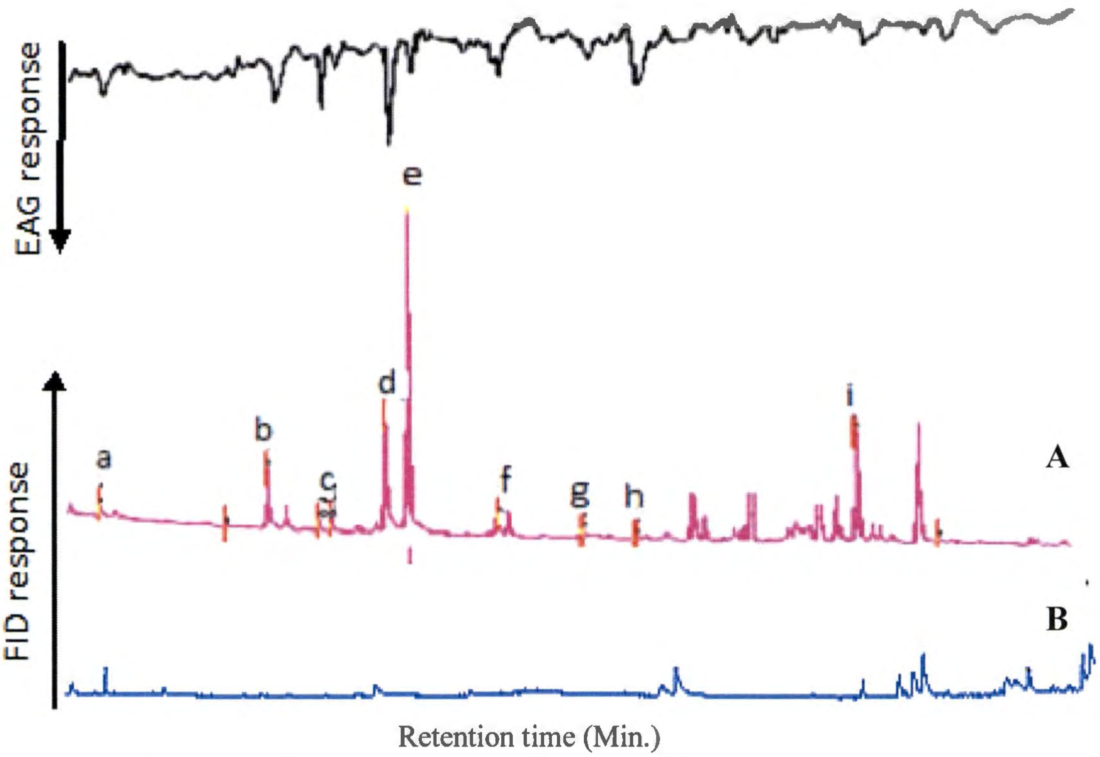

Figure 5.11: Representative GC-EAG responses of *Cotesia sesamiae* female to volatiles collected from landrace maize of Jowi-red plants (A) exposed to molasses grass and (B) non-exposed Jowi-red volatiles, GC traces underneath shown for comparison. The marked FID peaks are those which elicited antennal response in coupled runs: a= unknown, b= myrcene, c= *(β)*-ocimene, d= *(R)*-linalool, e= *(E)*-4,8-dimethyl-1,3,7-nonatriene (DMNT), f= decanal, g= unknown, h= unknown, i= (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT).

CHAPTER SIX

6.0 MAIZE PLANTS PRIME DIRECT AND INDIRECT DEFENSE RESPONSES BY RETAINING INFORMATION RECEIVED FROM AIRBORNE SIGNALS

6.1 Abstract

When plant tissue is disrupted by herbivores, herbivore-induced plant volatiles (HIPVs) are released. These HIPVs may function as a direct defence mechanism repelling herbivores or negatively affect their growth and development and/or indirectly attract their natural enemies. In addition, the emitted HIPVs can warn neighbouring plants from impending attack. A previous study reported that volatile compounds emitted from intact molasses grass, *Melinis minutiflora* P. Beauv. can induce the direct and indirect defence responses of neighbouring maize plants. However the priming effect of maize plants when challenged by *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) larvae had not been studied. Here, maize plants previously exposed to molasses grass volatiles for three weeks were removed from the vicinity of the emitter plants for 0 hr, 24 hr, 48 hr,72 hr, 96 hr and 1week and damage induced through feeding by five 3rd instar larvae of *C. partellus*. Results showed that maize landraces previously exposed to molasses grass volatiles then infested by *C. partellus* larvae attracted more larval parasitoids, *Cotesia sesamiae* Cameron (Hymenoptera: Braconidae) in olfactometry bioassays than volatiles emitted by non-exposed infested plants. In contrast, there were no significant differences in behavioural responses of *C. sesamiae* with regard to headspace samples collected from exposed infested and non-exposed infested plants of maize hybrids tested. In addition, no significant differences were observed in the number of eggs oviposited by *C. partellus* moths on exposed infested and non-exposed infested hybrid plants. However, *C. partellus* preferred to lay their eggs on non-exposed infested landrace maize plants compared to previously exposed and infested landraces. Headspace samples were analysed using Coupled Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography-Electroantennography (GC-EAG). GC-MS analysis revealed enhanced profile changes in the exposed infested maize landraces compared to non-exposed infested plants. Higher amounts of EAG-active volatile compounds including (*E*)-4,8-dimethyl-1,3,7-nonatriene were emitted from exposed infested landraces compared to non-exposed infested plants. These results demonstrate plant-to-plant signalling that occurs in maize-molasses grass intercropping systems and that may play an important ecological role by priming indirect and direct defence responses of neighbouring maize plants. The effect was not only observed immediately after removal of maize plants from molasses grass but also after 1 week of removal and infestation by *C. partellus* larvae. Restoring such traits into commercial hybrid maize varieties could be an effective way to increase plant volatile-based pest control strategies.

Key words: *Cotesia sesamiae*, headspace samples, plant-to-plant signalling, priming effects

6.2 Introduction

Plants are sessile organisms that evolved with diverse arrays of defence strategies to overcome herbivore attack. The presence of waxy cuticles, bark, trichomes, thorns and spines in some plant species are considered as physical defence mechanisms (Schoonhoven *et al.* 2005) which are always present even in the absence of herbivores. However, in response to attack or tissue disruption by herbivorous insects, pathogens or mechanical means, most plant species react immediately by releasing defence related compounds into the environment (Heil and Bueno, 2007; Holopainen and Gershenzon, 2010). Some of these compounds have toxic properties or antifeedant effects on the feeding insects and negatively affect their growth and development (Howe and Jander, 2008; Mithofer and Boland, 2012). Volatile chemical compounds (VOCs) such as (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate which are called green leaf volatiles (GLVs) are rapidly emitted after plants are damaged or tissues ruptured and attracts natural enemies of the herbivores (Mc Cormick *et al.*, 2012). Whereas terpenoids, aromatic compounds, sesquiterpenes and other compounds commence synthesis and release only after several hours of damage (Turlings *et al.,* 1998). Many of these defence compounds are dependent on plant secondary metabolites which are not involved in the growth and development of plants.

Secondary metabolites may contain toxic or deterrent compounds which directly affect the herbivore growth and development after feeding (Rhoades, 1983; Khan *et al.,* 2010) or repel pests away from the plant and/or indirectly attract natural enemies of the herbivores (Pare and Tumlinson 1999; Mc Cormick *et al.*, 2012). Compounds released due to herbivore damage are however considerably different from those released because of mechanical damage (Turlings *et al.,*1990). This might be due to the elicitors from insect saliva which may trigger specific VOCs that provide specific information to nearby organisms (Schmelz *et al.,* 2006). Following the release of these compounds, undamaged parts of the plant as well as neighbouring plants also can respond to these signals.

The influence of volatiles in tritrophic interactions have been illustrated in many studies showing that neighbouring plants also have the ability to produce quick and strong induced reactions upon following abiotic and biotic stress (Ton *et al.,* 2007; Frost *et al.,* 2008; Kant *et al.,* 2009; Kim and Felton, 2013; Oluwafemi *et al.,* 2013). This process is referred to as "priming", and it functions through reducing vulnerability to insect attack by increasing direct and indirect defence (Engelberth *et al.,* 2004; Ton *et al.*, 2007; Conrath, 2009; Karban, 2011; Kim *et al.*, 2011). After damage by herbivores, primed plants adjust their physiology to produce and release more volatile compounds that are attractive to parasitoids and predators (Turlings and Ton, 2006). Several studies showed in the past few years (review by Conrath *et al.,* 2006) that non-damaged plants exposed to neighbouring damaged plants or to an individual or blend of synthetic volatiles, respond immediately following herbivore damage or regurgitant application on artificially wounded plant parts. Therefore, natural or synthetic volatile organic compounds can prime plant defence mechanisms in neighbouring undamaged plants by inducing resistance to attack in the latter. Some commercially available synthetic compounds that can induce the defence response of undamaged plants during abiotic and biotic stresses are Brotomax, BABA, Oryzemate and other priming-inducing compounds to enhance resistance of the plants (review by Conrath *et al.,* 2006).

Engelberth *et al.* (2004) showed that maize seedlings previously exposed to GLVs exhibited strong and rapid responses after application of larval regurgitant on mechanically damaged plant parts. Similarly, lima bean plant exposure to synthetic volatile blends (Heil and Kost, 2006) and maize seedlings pre-treated with cis-Jasmone (Oluwafemi *et al.*, 2013) showed enhanced levels of volatile production after infestation by herbivores. Ton *et al.* (2007) also showed that undamaged maize plants exposed to volatiles from neighbouring plants infested with larvae of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) showed high levels of priming after being infested by herbivores. Similarly, native plants of tobacco (*Nicotiana attenuata*) transplanted adjacent to damaged sagebrush plants (*Artemesia tridentata tridentata*) showed accelerated production of trypsin proteinase inhibitors when damaged by *Manduca sexta* (L.) (Lepidoptera: Sphingidae) larvae (Kessler *et al.,* 2006). In addition to immediate responses to volatile released from neighbouring plants, maize plants have also shown the capability of recalling the information after some time (Ali *et al*., 2013; Sugimoto *et al*., 2013).

Previous studies reported that the molasses grass, *M. minutiflora*,, which is not a host plant of *C. partellus*, repels moths and increases larval parasitism by *C. sesamiae* when intercropped with maize (Khan *et al.,* 1997a; b; 2000; 2010). Chemical compounds that are constitutively released from molasses grass and that play a role in these interactions have been identified as (*E*)-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-caryophyllene, humulene and α-terpinolene (Khan *et al*., 1997a; 2000; Picket *et al.,* 2006). Recently, it has been observed that maize plants exposed to molasses grass volatiles are able to induce direct and indirect defence responses (Chapter 4 and 5). However, the priming effect of maize plants following the interaction with molasses grass volatiles has not been studied.

This study addresses the following questions: (1) can molasses grass volatiles prime neighbouring maize plants to activate direct and indirect defence mechanisms against future herbivore attack? (2) do different maize varieties differ in terms of their ability to memorize priming information and the duration of the priming period? (3) do primed plants change their volatile profiles?

6.3 Materials and methods

6.3.1 Insect rearing

Chilo partellus moths were collected from field and reared in the mass insect rearing units at International Centre of Insect Physiology and Ecology (*icipe*), Thomas Odhiambo campus on a semi-synthetic diet as described by Ochieng *et al.* (1985). Field collected larval parasitoid, *C. sesamiae* was reared on stemborer larvae according to methodology developed by Overholt *et al.* (1994). The rearing colonies were maintained at 24 ± 3 ^{O}C, 70 ± 5% RH, 12L: 12D.

6.3.2 Experimental plants

The study was conducted at *icipe* in western Kenya. Two maize landraces 'Nyamula' and 'Jowi-red' were collected from farmers in western Kenya and two hybrid maize varieties 'WS505' and 'PH4' were obtained from commercial seed suppliers (Western Seed Company Ltd and Kenya Seed Company Ltd, respectively). Seeds of molasses grass were collected from the push-pull field plots maintained at *icipe*. Seeds were planted individually in pots filled with fertilized soil in insect-proof screen houses under natural conditions (25 ^{0}C, 65% RH; 12L: 12D). Molasses grass seeds were planted 5-6 weeks before the maize was planted or until the grass seedlings reached a height of approximately 20 cm. Pots containing maize seedlings were exposed to molasses grass volatiles by placing them in between the rows of molasses grass based on the recommended spacing (Khan *et al.,* 1997b) until the plants were three weeks old. Maize plants were then transferred into a different screen house and kept for 0 hr (immediate following removal), 24 hr, 48 hr, 72 hr, 96 hr and 1 week before infesting them with *C. partellus* larvae. Each maize variety was subjected to six different treatments, based on the time interval after removal from exposure to molasses grass. Control (non-exposed) plants of the same varieties were also planted in pots in a different screen house under similar environmental conditions but without any molasses grass plants being present. Sufficient numbers of plants were grown to allow for use of plants in bioassays and to have plants to use for entrainment of headspace volatiles.

6.3 3 Oviposition bioassay

Plants that had been previously exposed to molasses grass volatiles and non-exposed (control) maize plants were transferred into different oviposition cages (80 X 40 X 40 cm). Five 3rd instar *C. partellus* larvae were inoculated onto each plant and allowed to feed inside the whorls of plants for 24 hr before choice tests were conducted. One exposed infested plant was put into an oviposition cage of (90 x 60 x 60 cm) together with a non-exposed infested plant and oviposition preference of *C. partellus* determined by releasing five gravid naïve female *C. partellus* moths to lay their eggs overnight. In each oviposition cage a moistened wad of cotton wool (10 cm diam.) was introduced for the moths to have access to water. The number of eggs laid on each plant was determined after removal of egg batches the following morning. Ten replicates were done for each variety and time interval.

6.3.4 Collection of headspace samples

To determine differences in volatile emissions and its effect on *C. sesamiae* behaviour, headspace samples were collected from exposed and non-exposed maize plants following 24 hrs of larval infestation. Headspace samples were collected from plants of all treatments for a period of 48 hr using methods described by Agelopoulos *et al.* (1999). The leaves of infested exposed and non-exposed maize plants were placed gently inside different polyethyleneterephthalate (PET) bags (volume 3.2 L, ~12.5 mm thickness) through an open end of the bag. The PET bags were sterilized at 150 ^{0}C before use. Purified charcoal filtered air was pumped through the bottom of the bag at a rate of 600 ml/min. The adsorbent, Porapak Q (0.05 g, 60 / 80 mesh, Supelco), was placed at the outlet valve where air was drawn at 400 ml/min. A lower flow rate in the outlet valve allow for enough time and pressure for the Porapak Q, to effectively adsorb the VOCs. After 48 hr of entrainment, collected volatile samples were eluted from the Porapak Q adsorbent with 0.5 ml of dichloromethane. The eluted samples were collected using 3.5 ml sample vials and kept in a freezer at -20 ^{0}C until they were used for bioassays and chemical analysis.

6.3.5 Perspex four-arm olfactometer bioassay

The responses of the larval parasitoid to collected headspace samples were evaluated in a Perspex four-arm olfactometer (Pettersson, 1970). Air was drawn through the four arms towards the centre of the olfactometer at a rate of 260 ml/min. Aliquots of headspace sample (10 µl) were applied onto a piece of filter paper (4×25 mm) using a micropipette, after which the filter papers were placed at the end of each olfactometer arm. The two opposite arms held the test stimuli collected from exposed infested and non-exposed infested plants while the remaining two opposite arms were used for the solvent control. Mated females of *C. sesamiae*, without previous exposure to plants or hosts were transferred individually into the central chamber of the olfactometer using a custom-made piece of glass tubing. Time spent and number of entries into each arm over a 12 min period was recorded using 'Olfa' software (F. Nazzi, Udine, Italy). To avoid any directional effect of the response the olfactometer was rotated every 3 minutes. The experiment was replicated 10 times for each plant sample.

6.3.6 Gas Chromatography (GC) Analysis

Headspace samples were analyzed using a Hewlett-Packard 7890 GC machine (Agilent Technologies) equipped with a cool-on column injector, a non-polar HP-1 capillary column (50 m, 0.32 mm internal diameter, 0.52 µm film thickness) and a flame ionization detector (FID). Four µl of headspace sample were injected into the injector port of the GC instrument. After injection the oven temperature was maintained at 30 ^{0}C for 2 min and then programmed at 5 ^{0}C min^{-1} to 250 ^{0}C. The carrier gas was hydrogen. Data were analyzed using HP Chemstation software.

6.3.7 Coupled GC-Electroantennography (GC-EAG)

Coupled GC-electroantennography (GC-EAG) was carried out using antennae of mated female individuals of *C. sesamiae* with headspace samples of maize plants that had elicited positive responses during olfactometer bioassays. Five insects were used for EAG recording for each of the selected plant samples. The glass Ag-AgCl electrodes were filled with saline solution (composition as in Maddrell (1969) but without glucose). Female parasitoids were chilled for 1 min before the antennae were excised. The tips of the antennae were removed to ensure a good contact while suspended between the two saline solution-filled electrodes. Signals were passed through a high impedance amplifier (UN- 06; Syntech, Hilversum, The Netherlands) and analysed using a customised software package (Syntech). The GC-EAG system, in which the effluent from the GC column is simultaneously delivered to the antennal preparation and the GC detector, has been described previously (Wadhams, 1990). Separation of the volatiles was achieved on a GC (Agilent Technologies, 6890N) equipped with a cold on column injector and a FID using a HP-1 column (50 m,0.32 mm ID, 0.52 lm film thickness). The oven temperature was maintained at 30 ^{0}C for 2 min and then programmed at 15 ^{0}C min^{-1} to 250 ^{0}C. The carrier gas was helium. Outputs from the EAG amplifier and the FID were analysed using the Syntech software package.

6.3.8 Coupled GC-Mass Spectrometry (GC-MS) analysis

Aliquots of headspace samples found to be attractive to *C. sesamiae* were analysed on a capillary GC column (HP-1, 50 m, 0.32 mm i.d., 0.52 µm) directly coupled to a mass spectrometer (VG Autospec; Fisons Instruments, Manchester, UK) equipped with a cool on-column injector. Ionisation was performed by electron impact (70 eV, 250 ^{0}C). The oven temperature was maintained at 30 ^{0}C for 5 min and then programmed at 5 ^{0}C min^{-1} to 250 ^{0}C. Tentative identification of compounds were made by comparison of spectra with mass spectral databases (NIST, 2005) and confirmed through co-injection with the authentic standards.

6.3.9 Statistical analysis

Number of eggs laid on exposed infested and non-exposed infested plants of the different maize varieties were analysed using two-sample (unpaired) Student's t-tests (within-variety tests). Bioassay data from the four-arm olfactometer assays were analysed using analysis of variance (ANOVA), after the data were converted into proportions and log-ratio transformed. Means were separated using Tukey's test, with α set at 0.05. Statistical analyses were done using R software (R, 2014).

6.4 Results

6.4.1 Oviposition preference

In two-choice tests, fewer eggs were laid on landrace maize plants that had been exposed to molasses grass volatiles and which were infested by *C. partellus* larvae than similarly infested non-exposed maize landrace plants. This was the case for each of the treatments with different time intervals after removal from exposure to molasses grass volatiles ($P<0.05$; Figure 6.1A and B). However, there was no significant difference in the number of eggs laid on exposed and non-exposed plants of the maize hybrids ($P>0.05$; Figure 6.2A and B), except for the 48 hr treatment of WS505.

6.4.2 Behavioral responses of *C. sesamiae* to headspace samples

Headspace samples collected from landrace plants that were exposed to molasses grass volatiles and infested by stemborer larvae were significantly more attractive to *C. sesamiae* in the four-arm olfactometer bioassays than non-exposed infested maize plants of the same variety and solvent control ($P<0.05$; Figure 6.3), except for Jowi-red treatment at 96 hr (Figure 6.3E). However, no differences were observed in parasitoid responses to headspace samples collected from hybrid maize plants of the different treatments ($P>0.05$; Figure 6.3), except for hybrid PH4 after the 48 hr of removal (Figure 6.3C).

6.4.3 Comparison of volatiles emitted from exposed infested and non-exposed infested maize plants.

GC analyses of the headspace samples revealed changes in the volatile profiles emitted by maize landrace plants exposed to molasses grass volatiles and infested by *C. partellus* larvae in comparison to the non-exposed infested landrace maize plants (Figures 6.4 and 6.5). However, a few differences were observed in the volatile profiles of exposed and non-exposed infested hybrid varieties (Figures 6.6 and 6.7). Generally volatile emissions varied markedly among hybrid and landrace varieties used in this experiment.

6.4.4 Identification of attractive volatile organic compounds

GC-EAG recordings with the attractive sample from landrace plant showed that *C. sesamiae* antennae were responsive to certain compounds. Subsequent GC-MS identification showed that (*Z*)-3-hexen-1-ol acetate, (*E*)-ocimene, (*R*)-linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*E*)-caryophyllene and two other unknown compounds were attractive to *C. sesamiae* (Figure 6.8).

6.5 Discussion

Results from this study demonstrate the potential of volatile compounds emitted from molasses grass plants to prime defence responses in neighbouring maize plants. Maize plants seemed to be able to recall airborne information from previous exposure during subsequent pest attack. In this study, data from behavioural assays and chemical analysis used to determine the effectiveness of volatiles from neighbouring plants to induce priming of indirect and direct defences of maize plants. Oviposition bioassays revealed that maize landraces previously exposed to molasses grass volatiles and then infested by *C. partellus* larvae were less preferred for oviposition by *C. partellus* compared to non-exposed infested maize landraces. In contrast, with hybrid varieties no differences in oviposition were observed between exposed and non-exposed infested plants. A previous study reported that hybrid maize plants exposed to molasses grass volatiles were equally preferred to non-exposed plants for oviposition by *C. partellus* (Chapter 4).

Plants respond to biotic and abiotic stress by emitting a high quantity of volatile compounds (Baldwin and Preston, 1999; Walling, 2000; Laothawornkitkul *et al.*, 2009; Holopainen and Gershenzon, 2010) which are available as cues to mediate interactions in the environment (Hare, 2011). HIPV signals can be received at the time of damage or after a few hours of damage (Turlings *et al.*, 1998) and can induce direct and indirect defence responses of neighbouring undamaged plants (Tscharntke *et al.,* 2001; Dicke and Baldwin, 2010). However, receiver plants do not show immediate changes in their level of defence, but respond stronger and faster when damaged by herbivores (Engelberth *et al.,* 2004; Kessler *et al.,* 2006; Ton *et al.,* 2006; Frost *et al.,* 2008; Arimura *et al.,* 2010; Sugimoto and Arimura, 2013). Undamaged plants can also prime their defence responses upon receiving volatiles from damaged neighbouring plants (Arimura *et al.* 2000; Baldwin *et al.,* 2002; Karban *et al.,* 2003; Heil and Kost 2006; Kessler *et al.,* 2006; Frost *et al.,* 2007). Volatile interactions between undamaged plants can also occur in the plant ecosystem and boost the direct and indirect defence response of the undamaged receiver plants (Glinwood *et al.,* 2011). A previous study reported that maize plants exposed to volatile compounds emitted from intact molasses grass were able to induce direct and indirect defence responses of neighbouring undamaged maize plants (Chapter 4 and 5).

Plants previously exposed to HIPVs respond more strongly and more rapidly than non-receiver plants upon damage by herbivores (van Hulten *et al.,* 2006; Arimura *et al.,* 2010). Here, the olfactory responses of *C. sesamiae* to headspace samples revealed higher attractiveness to the volatiles collected from exposed infested maize landrace plants than to non-exposed infested plants.

A recent study by Ali *et al.* (2013) showed that maize plants exposed to HIPVs, do not show direct gene expression until plants are damaged by herbivores. Here, in response to infestation by *C. partellus* larvae previously exposed maize landraces emitted high levels of volatile compounds including green leaf volatiles ((*E*)-2-hexenal and (Z)-3-hexen-1-ol acetate) and terpenoids *(E)*-ocimene, (*R*)-linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*E*)-caryophyllene and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT)), which are well known defence compounds. The compound DMNT, which is known to be electrophysiologically active and play a key role in parasitoid attraction (Khan *et al.,* 1997a; Tamiru *et al.,* 2011; Mutyambai *et al.,* 2015), was found in higher quantities in exposed infested landrace maize varieties than non-exposed infested plants and hybrid maize varieties. Primed plants were therefore able to develop the resistance induced from their previous experience when they are attacked by herbivores.

Selective breeding of crop plants with the aim of yield and quality improvement only may negatively affect other important defence related traits (Rodriguez-Saona *et al.*, 2011) and reduce genetic diversity (Doebley *et al.*, 2006). In this study hybrid maize varieties previously exposed to molasses grass volatiles and non-exposed emitted similar types of compounds after infestation by *C. partellus* larvae. A recent study confirmed that certain commercial hybrid maize plants have lost the ability of calling natural enemies in response to egg deposition by *C. partellus* (Tamiru *et al.*, 2011; Mutyambai *et al.*, 2015). However the difference in volatile production were more pronounced between exposed and non-exposed maize landrace plants, 'Nyamula' and 'Jowi red' after the plants were infested by *C. partellus* larvae.

Studies on plant-insect interactions have shown that previously damaged plants can develop resistance for a more robust and rapid defence response upon subsequent attack (van Hulten *et al.*, 2006). In addition, maize plants also retain information for up to 5 days after plant exposure to HIPVs (Ali *et al.*, 2013; Sugimoto *et al.*, 2013). The present findings provide evidence that plants of certain maize landraces are able to retain defence related compounds even after one week of removal from molasses grass volatiles, and are able to repel *C. partellus* from oviposition and attract more *C. sesamiae*. Primed plants increase their resistance against biotic stress (Tsai *et al.*, 2011) at low cost (van Hulten *et al.*, 2006). Selection of appropriate varieties which have appropriate defence traits could add ecological value for pest control. For instance, herbivores challenged *Arabidopsis* and tomato plants to be more resistant to subsequent attack in the next generation (Rasman *et al.*, 2012). Similarly, Jasmonic acid treated seeds primed plant resistance for a period of approximately 8 weeks (Worrall *et al.*, 2012).

In summary, maize landraces previously exposed to molasses grass volatiles are able to retain the information even after a week of removal from molasses grass. In addition, the volatiles emitted from molasses grass can prime direct and indirect defence responses of neighbouring maize landraces when infested by *C. partellus* larvae. However, the effect was not observed with the hybrid maize plants used in this experiment. These findings pave the way for development of new ecologically sound plant protection strategies by using plant volatile-based pest management methods.

6.5 References

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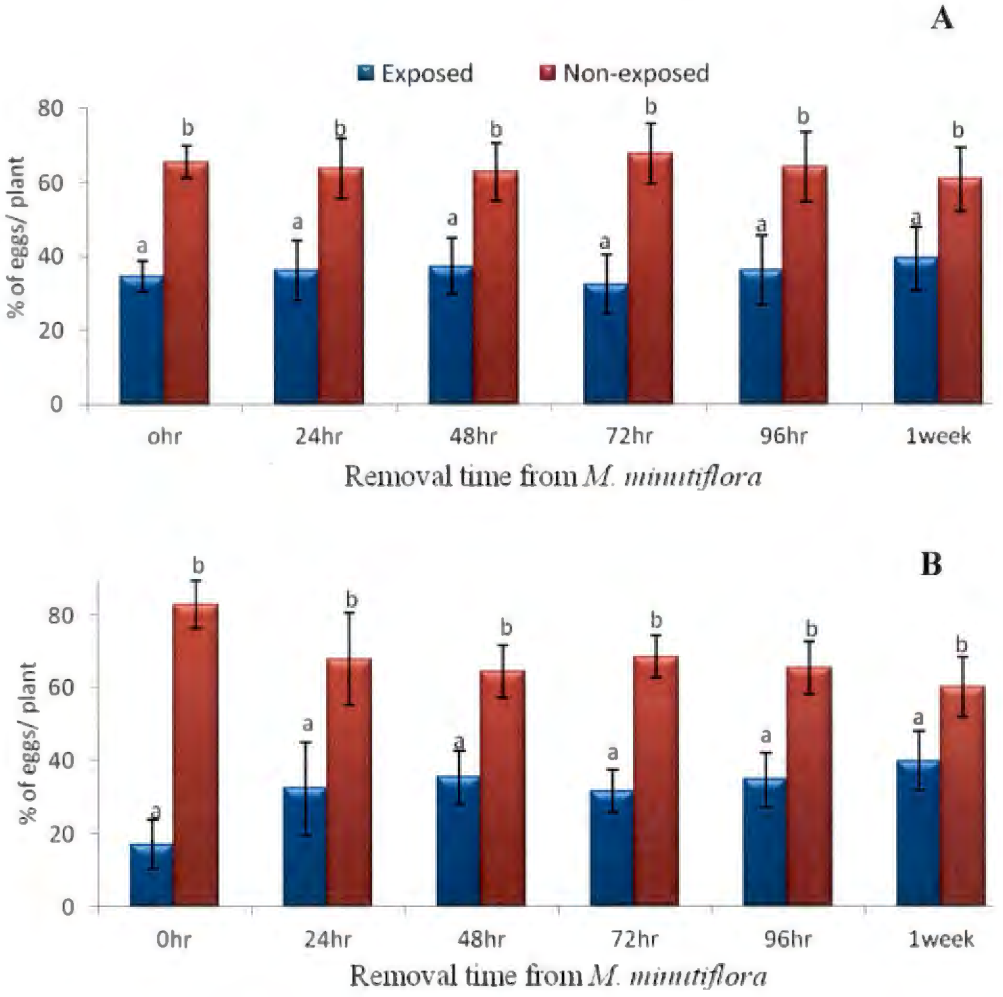

Figure 6.1. Mean (± SE) percentage of *C. partellus* eggs laid on exposed and non-exposed infested landrace maize plants, Nyamula (A) and Jowi-red (B). Two-choice tests were done for each maize variety, using six different treatments, based on the different removal time intervals from molasses grass. Means with different letters above the bars differ significantly (Student's t-test: P<0.05).

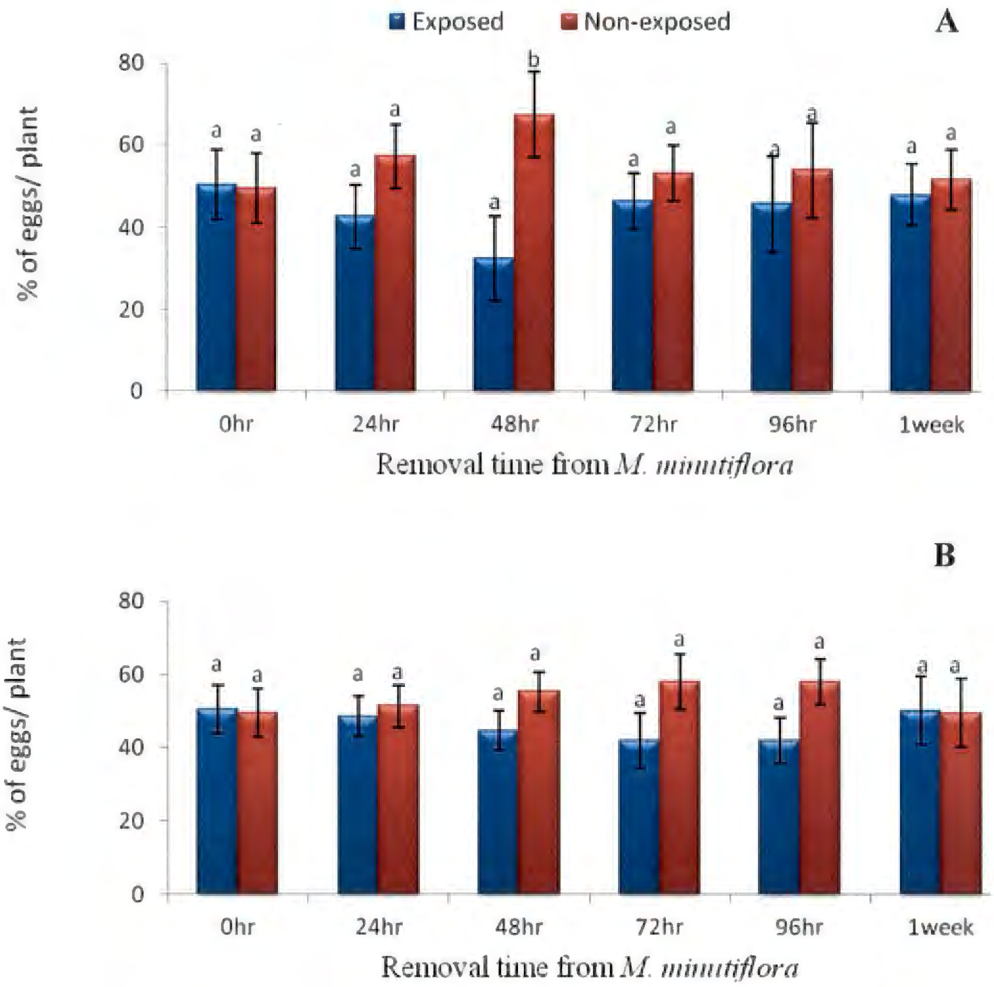

Figure 6.2: Mean (± SE) percentage of *C. partellus* eggs laid on exposed and non-exposed infested hybrid maize plants, WS505 (A) and PH4 (B). Two-choice tests were done for each maize variety, using six different treatments, based on the different removal time intervals from molasses grass. Means with different letters above the bars differ significantly (Student's t-test: $P<0.05$).

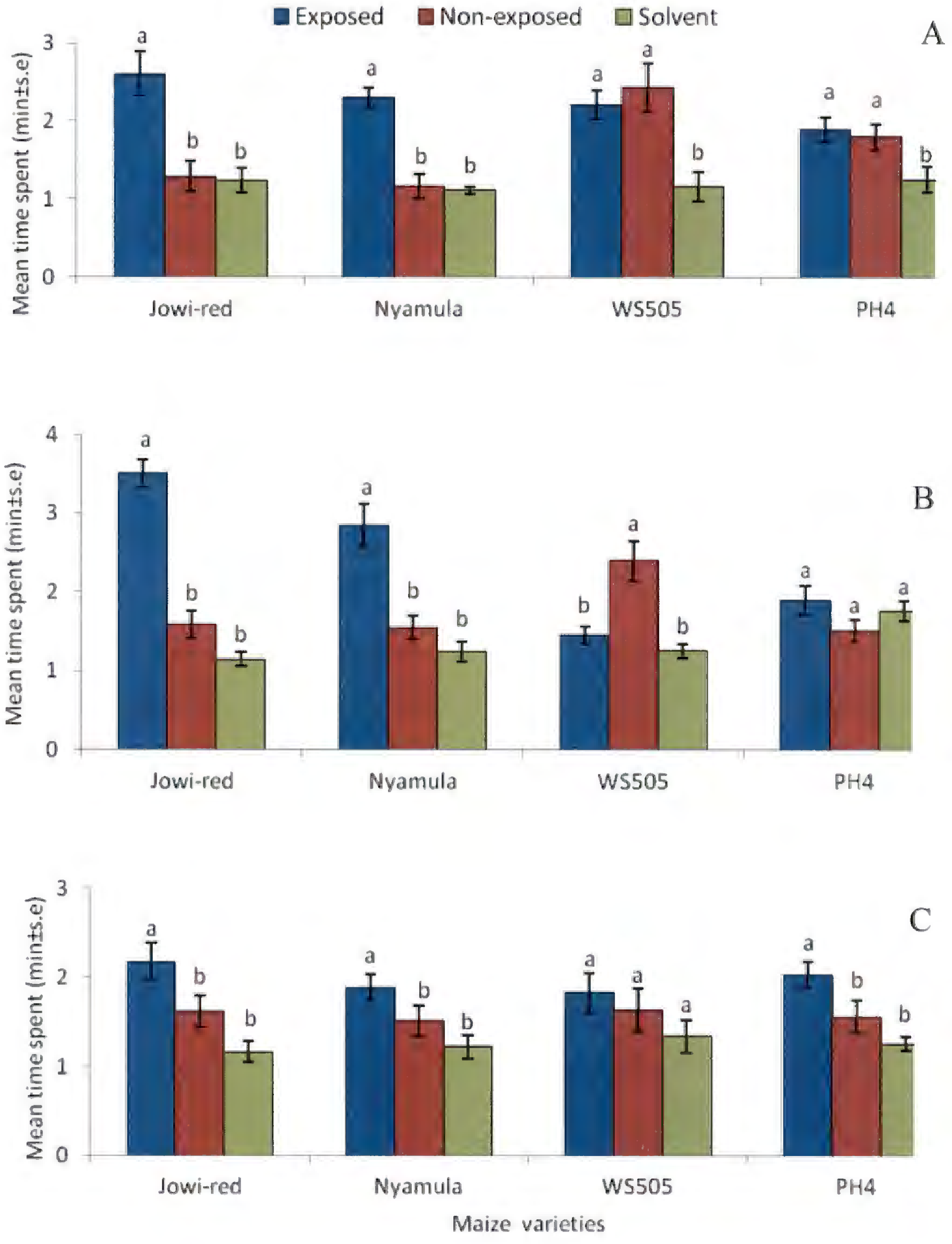

Figure 6.3: Behavioural responses of female *C. sesamiae* individuals to volatiles collected at different time intervals from plants of four maize varieties, evaluated in a four-arm olfactometer. The treatments were: Exposed = exposed to molasses grass and infested with *C. partellus* larvae, Non-exposed = non-exposed to molasses grass and infested with *C. partellus* larvae and Solvent = solvent control. Headspace samples were collected from exposed plants based on the different removal time intervals. (A) 0 hr, immediately after removal from exposure, (B) 24 hr after removal from exposure and (C) 48hr after removal from exposure. Bars followed by different letters are significantly different at P<0.05.

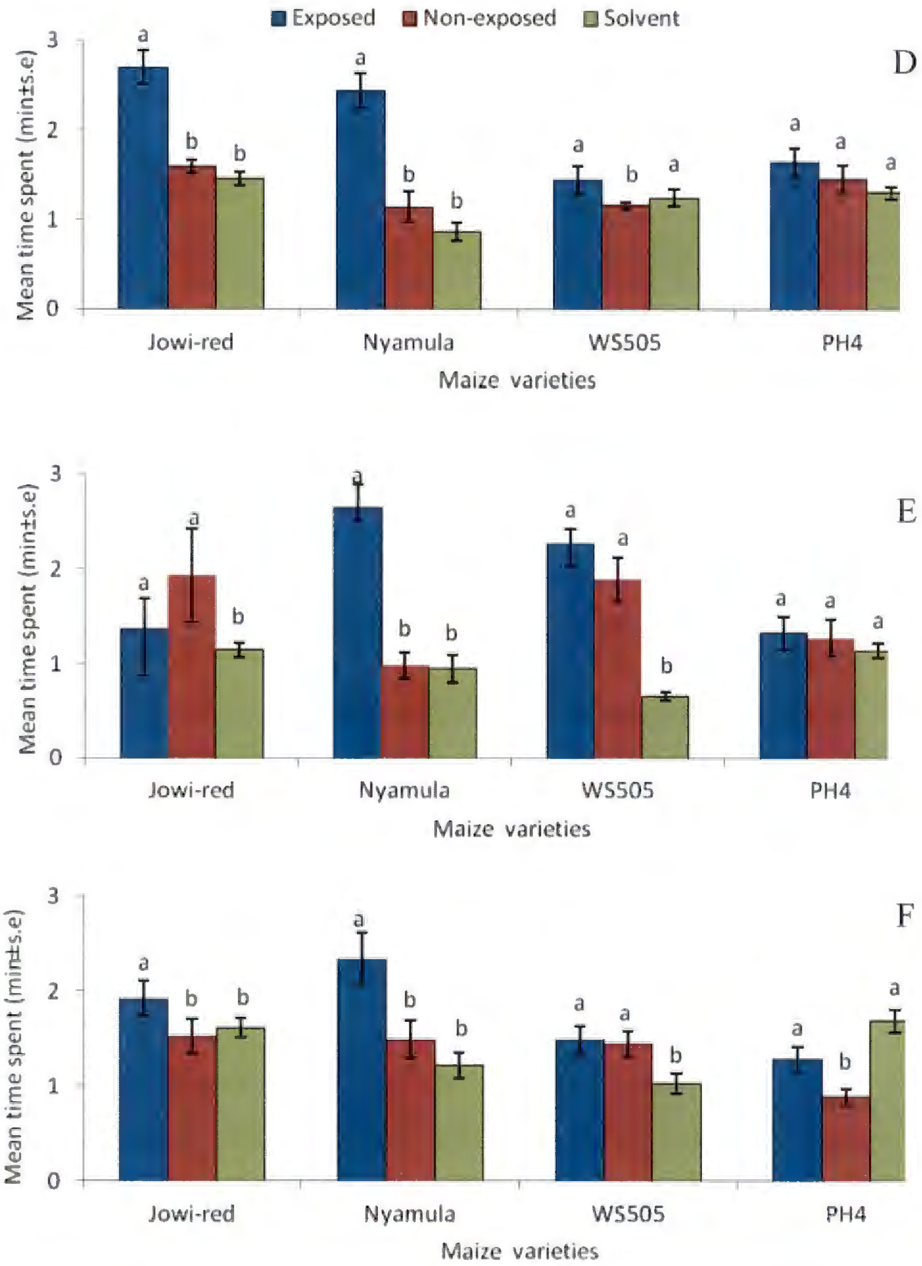

Figure 6.3: Continued. Behavioural responses of female *C. sesamiae* individuals to volatiles collected at different time intervals from plants of four maize varieties, evaluated in a four-arm olfactometer. The treatments were: Exposed = exposed to molasses grass and infested with *C. partellus* larvae, Non-exposed = non-exposed to molasses grass and infested with *C. partellus* larvae and Solvent = solvent control. Headspace samples were collected from exposed plants based on the different removal time intervals. (D) 72 hr after removal from exposure, (E) 96 hr after removal from exposure and (F) 1 week after removal from exposure. Bars followed by different letters are significantly different at $P<0.05$.

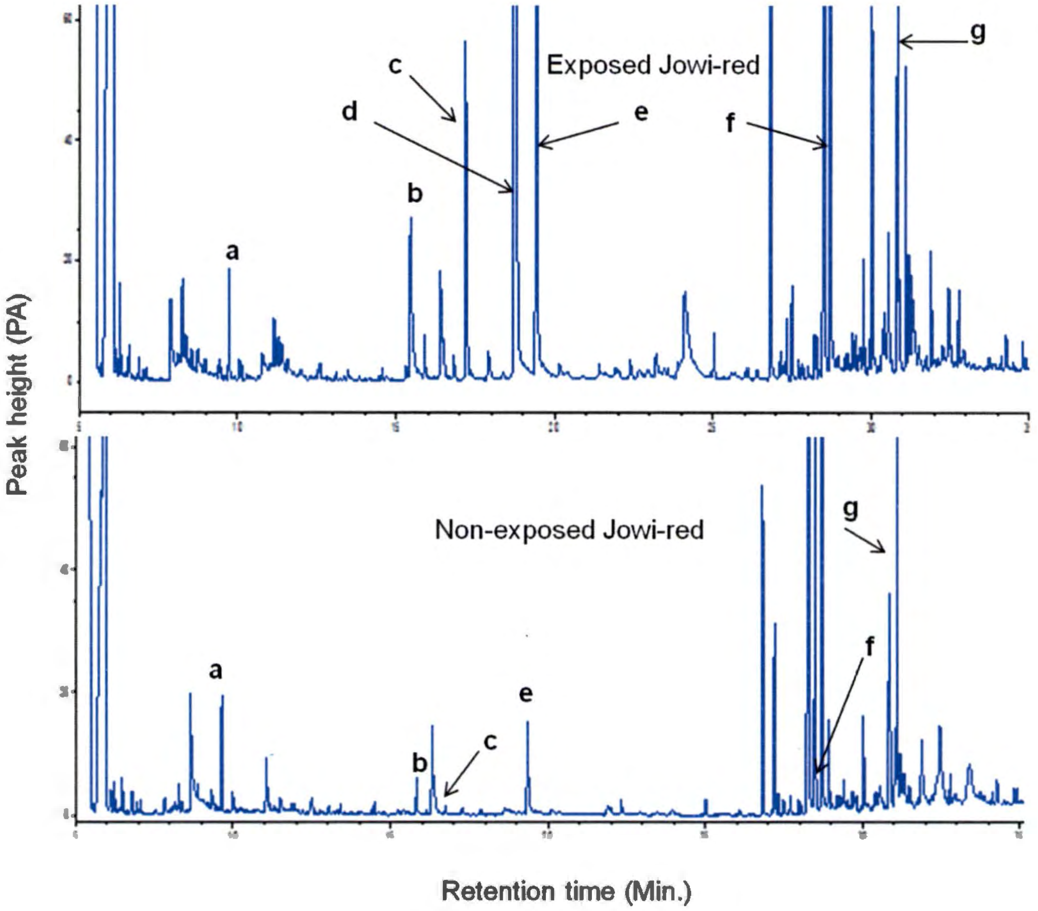

Figure 6.4: A representative GC profile of headspace volatiles from 'Jowi-red' maize plant exposed and non-exposed to *M. minutiflora* plants volatile. Maize plants were infested with 3rd instar *C. partellus* larvae for 24 hr, before entrainment of volatiles. Some of the identified compounds elevated due to larval damage are: a = *(E)*-2-hexenal, b = (Z)-3-hexen-1-ol acetate, c = (Z)-ocimene, d = *(R)*- linalool, e = *(E)*-4,8-dimethyl-1,3,7-nonatriene (DMNT), f = *(E)*-caryophyllene, g = (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT).

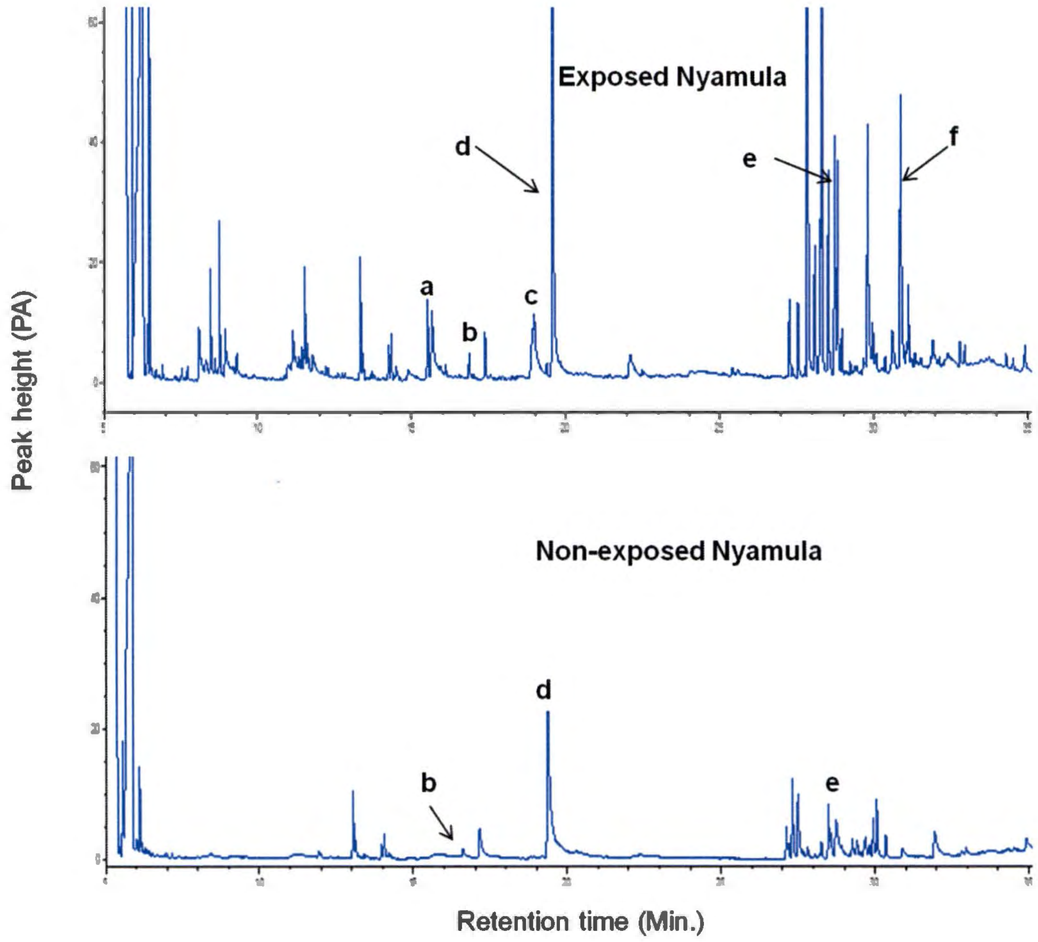

Figure 6.5: A representative GC profile of headspace volatiles from 'Nyamula' maize plant exposed and non-exposed to *M. minutiflora* plants volatile. Maize plants were infested with 3^{rd} instar *C. partellus* larvae for 24 hr, before entrainment of volatiles. Some of the identified compounds elevated due to larval damage are: a= (Z)-3-hexen-1-ol acetate, b= *(E)*-ocimene, c = *(R)*-linalool, d = *(E)*-4,8-dimethyl-1,3,7-nonatriene (DMNT), e= *(E)*-caryophyllene, f= (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT).

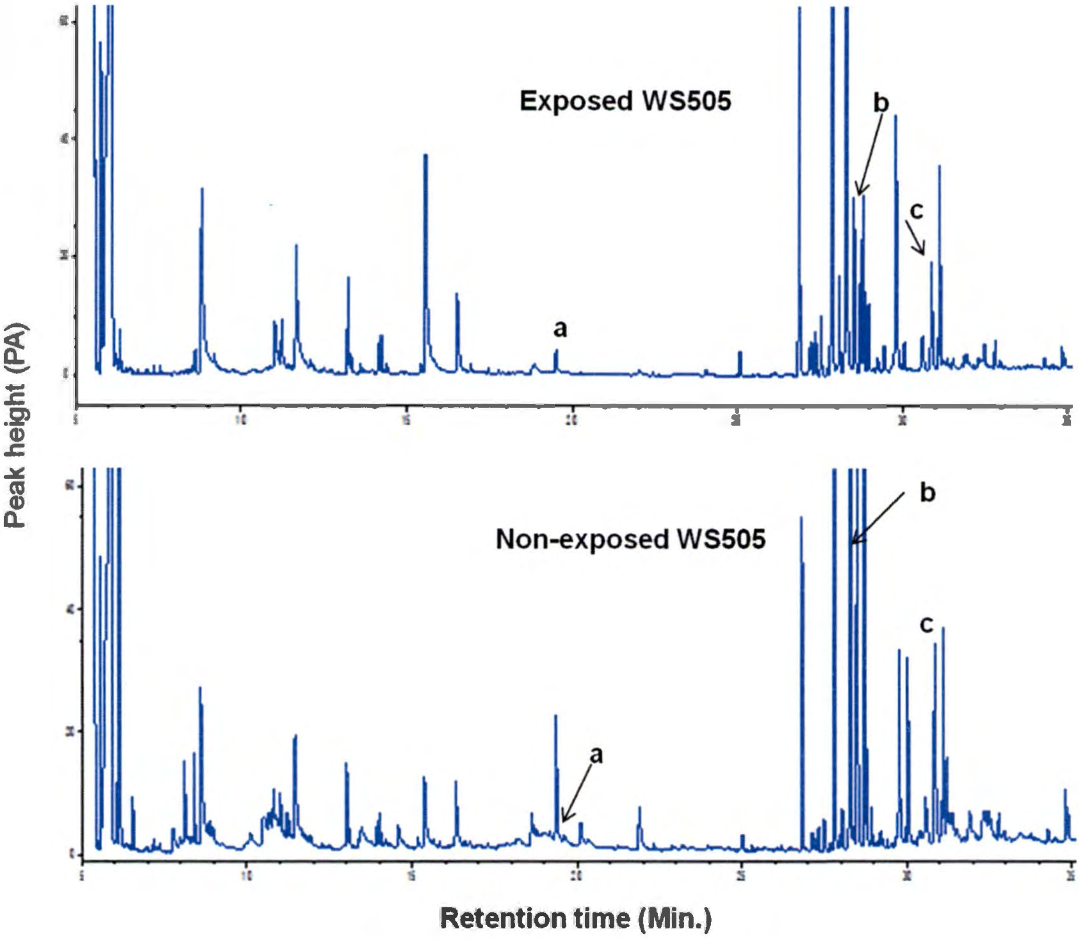

Figure 6.6: A representative GC profile of headspace volatiles from 'WS505' maize plant exposed and non-exposed to *M. minutiflora* plants volatile. Maize plants were infested with 3rd instar *C. partellus* larvae for 24 hr, before entrainment of volatiles. Some of the identified compounds elevated due to larval damage are a = *(E)*-4,8-dimethyl-1,3,7-nonatriene (DMNT), b = *(E)-β*-caryophyllene, c = (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT).

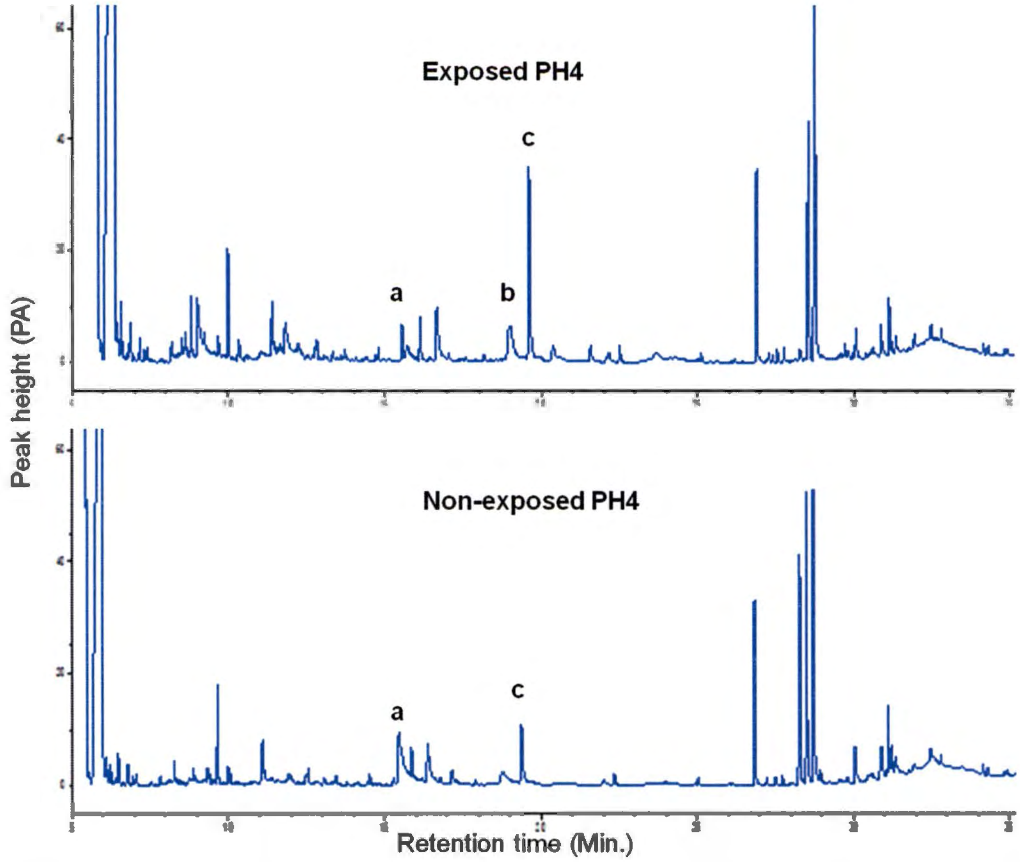

Figure 6.7: A representative GC profile of headspace volatiles from 'PH4' maize plant exposed and non-exposed to *M. minutiflora* plants volatile. Maize plants were infested with 3rd instar *C. partellus* larvae for 24 hr, before entrainment of volatiles. Some of the identified compounds elevated due to larval damage are a =*(Z)*-3-hexen-1-ol acetate, b = *(R)*-linalool, c = *(E)*-4,8-dimethyl-1,3,7-nonatriene (DMNT).

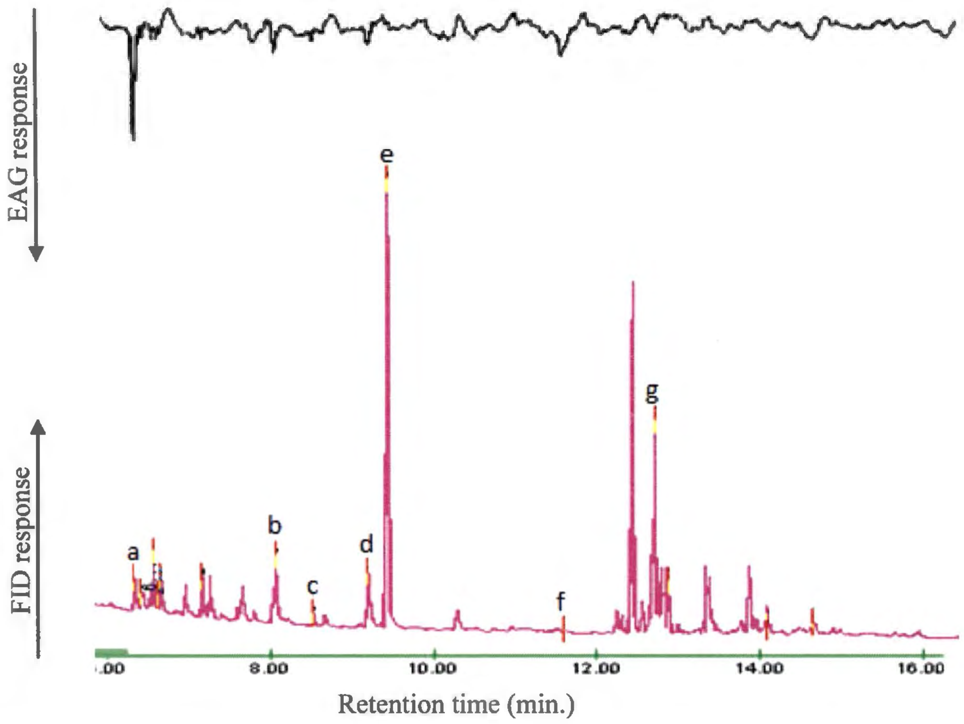

Figure 6.8: A representative GC-EAG response of female *C. sesamiae* to volatiles collected from landrace 'Nyamula' plants exposed to *M. minutiflora* plants volatile and infested with 3rd instar *C. partellus* larvae for 24hr, before entrainment of volatiles. FID peaks marked are those which elicited antennal response in coupled runs: a = unknown, b = (Z)-3-hexen-1-ol acetate, c = *(E)*-ocimene, d = *(R)*-linalool, e = *(E)*-4,8-dimethyl-1,3,7-nonatriene (DMNT), f = unknown, g = *(E)*-caryophyllene.

CHAPTER SEVEN

7.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 General discussion, conclusions

The invasive stemborer *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) is considered one of the most important pests of maize and may cause yield loss of up to 88% (Seshu-Reddy, 1988; Kfir *et al*., 2002). In order to suppress stemborer infestation levels and damage, various management strategies have been developed in the past but these have not been widely adopted due to various socio-economic and biological challenges. Additionally, use of chemical insecticides to control *C. partellus* is often considered unsatisfactory due to the cryptic and nocturnal habits of the adult moths, and the protection provided for borer larvae by the stem of the host crop.

To address constraints arising from the above, emphasis has been given to ecological management approaches such as the "push-pull" or stimulo deterrent diversionary strategy, which has been shown to effectively suppress stemborer populations while maintaining environmental integrity (Cook *et al.,* 2007; Khan *et al.,* 2010). This strategy uses knowledge of plant chemistry and insect behaviour to manipulate agro-ecosystems in a manner that is unfavourable to pests, whilst simultaneously promoting crop yield through reduced pest damage (Pickett *et al.,* 2006; Khan *et al.,* 2010).

The push-pull strategy was developed by scientists at the International Centre of Insect Physiology and Ecology (*icipe*) and partners (Khan *et al.* 2001; Khan and Pickett, 2004) and involves the simultaneous use of repellent plants and attractant trap plants in the cropping system (Khan *et al.,* 1997a; b; 2000; 2001). Repellent crops are used in the strategy to repel the ovipositing moths away from the main crop plants. Among the popular repellent plants are molasses grass (*Melinis minutiflora* P. Beauv) and two legumenious desmodium species (*Desmodium uncinatum* Jacq. and *Desmodium intortum* Urb.). The trap plants used in the push-pull system are Napier *(Pennisetum purpureum)* and Sudan grass (*Sorghum vulgare sudanense* Pers.). Trap plants play an important role by emitting volatiles that are more attractive to the female stemborer than those emitted by maize and sorghum plants (Khan *et al.,* 1997b; Khan *et al.*, 2000; Birkett *et al.,* 2006; Khan *et al.,* 2010). However, when the eggs hatch on the plant larvae are unable to survive or their development is constrained (Khan and Pickett, 2004; Pickett *et al.,* 2006).

The unique property of companion crops such as molasses grass is that it has the characteristic of releasing constitutively "cry for help" volatile cues that repel stemborer moths and attract their natural enemies into the system. Previous studies of the maize-molasses grass intercropping system revealed significant reductions in *C. partellus* infestation levels and increased larval parasitism by *Cotesia sesamiae* Cameron (Hymenoptera: Braconidae) (Khan *et al.,* 1997a). The identified bioactive compounds responsible for repelling the pests and attracting the parasitoids that are constitutively released from molasses grass include (*E*)-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*E*)-caryophyllene, humulene and α-terpinolene (Khan *et al.*, 1997a; 2000; Picket *et al.,* 2006). (*E*)-ocimene, (*E*)-caryophyllene and DMNT are also known to be produced by maize in response to insect herbivory and/or egg deposition (Turlings *et al.*, 1990a; Tamiru *et al.*, 2011). However, plant-to-plant communication in a maize-molasses grass intercropping system had not been investigated. This study investigated the possibility of plant-to-plant communication between maize after exposure to molasses grass volatiles, and its possible influence on *C. partellus* and its larval parasitoid, *C. sesamiae.* In addition, we also identified different maize varieties that exhibit inducible defence responses when primed by volatiles from molasses grass.

Plants actively react to biotic and abiotic stresses by emitting volatile organic compounds (VOCs) into the environment. In response to attack by herbivorous insects, plants produce blends of volatile chemical compounds referred to as herbivore-induced plant volatiles (HIPVs) (Mumm and Dicke, 2010). HIPVs are important signals used to communicate with herbivores (De Moraes *et al.,* 2001; Kessler and Baldwin, 2001), parasitoids, predators (Turlings *et al.,* 1990b; Tamò *et al.,* 2006; Penaflor *et al.,* 2011) and neighbouring plants (Engelberth *et al.,* 2004). Volatile-mediated defence strategies are complex and can have ecological significance for both the emitter and receiver plants in the environment. HIPVs play an important role in plant-to-plant communication between conspecific and heterospecific plant species as well as systemically within damaged and undamaged plant parts (Heil and Ton, 2008; Arimura *et al.,* 2009; Karban, 2011; Chamberlain, 2014). HIPV emission can also induce defence responses in the neighbouring plants (Karban *et al.,* 2000; Kessler *et al.,* 2006; Rodriguez-Saona *et al*., 2009), which are then used to repel pests and attract their natural enemies (Turlings and Tumlison, 1992; Dicke and van Loon, 2000; Khan *et al*., 2008). In addition it provides information that facilitates host finding by parasitoids and predators (Turlings *et al.,* 1990b; Turlings and Wäckers, 2004). In the present study the behavioural response of the larval parasitoid, *C. sesamiae* and the maize stemborer, *C. partellus* were elucidated after maize plants were exposed to molasses grass volatiles for certain periods of time.

It was recently observed that some plants are signalled by volatile compounds from their neighbouring plants to produce HIPVs despite not being damaged by herbivores (Chapters 3; 4; 5; Ton *et al.,* 2006; Ramadan *et al.,* 2011). In a maize-molasses grass intercropping system higher rates of stemborer parasitism by *C. sesamiae* have been reported (Khan *et al.,* 1997a). Volatile compounds emitted from plants provide reliable cues for parasitoids, herbivores, as well as for undamaged neighbouring plants. Plants exposed to VOCs can change and boost the abundance of defence related compounds (Arimura *et al*., 2002; Engelberth *et al*., 2004; Ruther and Kleier, 2005) that can possibly affect pests, predators and parasitoid behaviour. These initiated defence responses may be due to volatile adsorption to the receiver plants' epidermis and re-emitted, or they may directly induce physiological responses in plants. In the present study, changes in volatile profiles were observed in landraces exposed to molasses grass volatiles for certain periods of time (Chapter 3). Some plant species produce and emit defence related compounds to the environment which can influence pest and natural enemies of the pest without being damaged by herbivores. The volatiles emitted constitutively from molasses grass have a multi-functional role, from defending the plant itself to initiating the direct and indirect defence response of neighbouring plants. Identified volatile compounds from molasses grass that have positive impact on neighbouring plants are known to be produced by maize in response to egg deposition (Tamiru *et al.,* 2011;

Mutyambia *et al.,* 2015) and insect herbivore damage (Chapter 3; Khan *et al.*, 1997a; 2000; Pickett *et al.,* 2006).

Oviposition preference of moths is also influenced by the volatiles emitted from host and non-host plants. In addition, the biological performance of herbivores is determined by host plant quality (Awmack and Leather, 2002). In the present study, non-exposed plants were highly preferable for oviposition by gravid *C. partellus* moths compared to maize plants exposed to molasses grass (Chapter 4 and 5). Similarly, the GC-EAG study showed that *C. partellus* antennae were responsive to certain volatile compounds collected from exposed maize plants and molasses grass. The identified bioactive compound was (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) (Chapter 4), which is a compound known to repel *C. partellus* moths (Khan *et al.,* 1997a).

Insects rely on olfaction to locate their suitable hosts (Kegge and Pierik, 2010). In olfactometer bioassays *C. sesamiae* was attracted to headspace samples collected from landraces exposed to molasses grass volatiles (Chapter 5). Plants that are able to respond to neighbouring plant volatiles and emit defence related volatile compounds into the environment have an advantage of minimizing pest build-up in the field through increased foraging efficacy of parasitoids. However, in a similar experiment with hybrid maize varieties *C. sesamiae* was not attracted to samples collected from exposed and non-exposed hybrid maize in olfactometer bioassays (Chapter 5). This study may be the first to report that the volatiles emitted from molasses grass can induce direct and indirect defence responses of neighbouring maize landraces. However a similar phenomenon was not recorded for WS505 and PH4 hybrid maize varieties. Selective breeding of crop plants with the aim of yield and quality improvement only may negatively affect other important defence related traits (Rodriguez-Saona *et al.*, 2011) and reduce genetic diversity (Doebley *et al.,* 2006). Introgression of desirable defence traits such as those described above should be considered in future breeding programmes.

Plants can perceive VOCs from damaged as well as undamaged neighbouring plants and contain the information to respond faster and stronger to attack that may happen in future.

This phenomenon is known as priming. Upon attack by herbivores, primed plants respond immediately by emitting defence related compounds (Frost *et al.,* 2008). Priming can be accelerated by natural or synthetic compounds. Previous studies reported that plants exposed to volatile compounds showed enhanced levels of VOC production after infestation and damage by herbivores (Englberth *et al*., 2004; Heil and Kost, 2006; Ton *et al.*, 2006; Oluwafemi *et al*., 2013). Priming can occur within species (Engelberth *et al.,* 2004; Ton *et al.,* 2006) as well as between species (Kessler *et al*., 2006). In the present study maize landraces previously exposed to molasses grass volatiles showed higher levels of VOC production than non-exposed maize plants while infested by *C. partellus* larvae. *C. sesamiae* was attracted to the headspace samples collected from exposed infested landrace maize plants (Chapter 6). This research demonstated that volatile compounds emitted from molasses grass to defend itself from herbivores pests played a role in the switching on of the defence responses of neighbouring maize plants. The observed attraction of parasitoids towards headspace samples collected from exposed maize plants and reduced attraction of *C. partellus* moths to the exposed landrace maize plants indicated that the direct and indirect defence traits were more prevalent in maize landraces than in the hybrid maize used in this study (Chapters 4; 5 and 6). In addition, these two landraces showed higher volatile profile changes following exposure to the emitter plant volatile.

Maize plants exposed to molasses grass volatiles for certain periods of time were able to retain the information and emit mixtures of volatile compounds while infested by *C. partellus* larvae, even after one week of removal from exposure to volatiles of molasses grass. These mixtures of HIPVs resulted in plants being more attractive to parasitoids, while deterrent to *C. partellus* moths. Electrophysiologically active compounds were detected by the antennae of *C. sesamiae* and *C. partellus*. The EAG-active peaks were identified by GC and GC-MS analysis. The identified electrophysiologically active compounds found in exposed landraces headspace samples included myrcene, (*Z*)-3-hexen-1-ol acetate, *(E)*-ocimene, (β)-ocimene, (*R*)-linanool, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), decanal, (*E*)-caryophyllene and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). The compound DMNT is known to have dual effects in that it attracts *C. sesamiae* and repels *C. partellus* (Khan *et al*., 1997a).

The levels of this compound were elevated in plants previously exposed to molasses grass volatiles and as well as on primed infested landraces.

In general, this study has demonstrated that maize landraces are able to detect and respond to neighbouring plant volatiles while hybrid maize plants appear to lack the signalling trait.

7.2 Recommendations

This study identified research gaps which need further investigation. The following areas are recommended for future study:

1. the role of volatiles emitted by molasses grass on other crop plants.
2. investigate the induced effect of maize plants on other stemborer species.
3. investigate the induced effect on gain yeild under field condition.
4. the biochemical pathways involved in induced plant defence.
5. evaluation of "smart" cereals with inducible traits during early stages of pest attack for incorporation into adaptive push-pull strategies.
6. introgression of inducible defence traits into hybrid maize lines to increase the efficacy of defence against herbivores.

7.3 References

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