

**CHEMICAL ECOLOGY OF THE COFFEE BERRY
BORER, *HYPOTHENEMUS HAMPEI* Ferrari
(COLEOPTERA: SCOLYTIDAE): THE ROLE OF
TWO SPIROACETALS IN THE INSECT-HOST
COMMUNICATION SYSTEM**

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Chemical Ecology of the Coffee Berry Borer, *Hypothenemus Hampei* Ferrari (Coleoptera: Scolytidae): The Role of Two Spiroacetals in the Insect-Host Communication System

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

I dedicate this work to my son Brian Njihia and my dear parents, Samuel Njihia & Esther Wambui. Their unwavering love and support has been my motivation to work hard and face life with a lot of optimism.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
CBB	Coffee Berry Borer
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
GC-MS	Gas Chromatography-Mass Spectrometry
<i>icipe</i>	International Centre of Insect Physiology and Ecology
JKUAT	Jomo Kenyatta University of Agriculture and Technology
SAS	Statistical Analysis Software
SNK	Student-Neuman-Keuls
SPME	Solid phase micro- extraction
χ^2	Chi-Square

ABSTRACT

The Coffee Berry Borer, *Hypothenemus hampei* Ferrari is the most important pest of coffee worldwide. The predominant method for the pest control is application of broad spectrum pesticides, but they are often toxic to humans and pollute the environment, hence the need to search for safer alternatives. Spiroacetals represent a group of semiochemicals often used to manage various pests through manipulation of their behaviour. This study which was carried out between January 2013 and December 2013 sought to investigate the effect of two synthetic spiroacetals viz. brocain (1,6-dioxaspirol[4.5] decane) and frontalín (1,5-dimethyl-6,8-dioxabicyclo[3.2.1] octane) obtained from volatiles of coffee berries on the behaviour of Coffee Berry Borer. To achieve the objectives, the behavioural response of *H. hampei* females to brocain and frontalín was evaluated in three experiments, comprising a Y- tube olfactometer and petridish arena, both in laboratory assays and a field experiment in a coffee plantation.

In olfactometer assays, a Y- tube arena (1 cm i.d; stem 8.5 cm; arms 7.5 cm at a 60° angle to the stem) was used to study *H. hampei* response to various concentrations of authentic standards of brocain, frontalín, and blends of the two compounds. During the assays, Coffee Berry Borer females that walked towards the arm with the test compound were considered to have made a positive response (attraction) while those that chose the control arm (solvent) were considered to make a negative response (repellence). In a subsequent experiment, *H. hampei* infestation levels in coffee berries whose exocarp was treated with brocain and frontalín was compared with solvent treated berries (control). The choice tests were done for *Coffee Arabica* var. Ruiru 11 in the most attractive stage (150 days old, yellow exocarp stage) and conducted in a petri dish arena (14cm diameter). In addition, volatility of brocain and frontalín was analysed and quantified (ng/µl/min) by gas-chromatography-mass spectrometry (GC-MS). Field experiments were conducted between 18th November 2013 and 9th December 2013

in three coffee plots located in Kiambu County, Kenya to investigate responses of *H. hampei* to Brocap® traps baited with frontalin and brocain.

Olfactory tests revealed that *H. hampei* are repelled by frontalin while brocain has a dual function, being significantly attractive at low concentration and repellent at high concentrations. In petridish assays, *H. hampei* infestation levels in coffee berries treated with brocain, was twice higher than the control (berries with solvent) while frontalin treated berries were significantly avoided. Tests investigating release rates of the spiroacetals showed that brocain amounts significantly reduced over time unlike frontalin. In the field trials, frontalin baited traps failed to catch any *H. hampei* and when mixed with the pest's commercial attractant, (1:1 methanol : ethanol mixture) the captures were reduced to 23%. Traps captures with brocain were not statistically different from the solvent baited traps (95%water+5%DMSO). Moreover, when brocain was mixed with the commercial attractant, it neither improved nor inhibited the attractants' performance.

This study established that frontalin is a repellent of *H. hampei* and masks the pest's attractants. The findings suggest that frontalin may be produced by coffee berries as a defensive compound (allomone) to avoid overcrowding of Coffee Berry Borers in an already attacked host. Brocain was observed to be highly attractive at a single concentration ($40 \text{ ng } \mu\text{l}^{-1}$) and repellent at high concentrations in olfactory assays. These results suggest that brocain is perhaps used by *H. hampei* as a host kairomone (attractant) at low concentration and as a repellent at high concentrations. Frontalin may therefore be incorporated in an integrated pest management program for *H. hampei*.

CHAPTER ONE

1.0. INTRODUCTION

Coffee is the most important agricultural commodity in the globe with annual revenues exceeding US \$ 70 billion (Vega, 2008). Small stakeholders with less than 5 ha of farming land supply 70% of the total global output which is about 9 million metric tonnes annually. In Kenya, the crop ranks third foreign exchange earner after horticulture and tea, with approx. 6% share (Monroy *et al.*, 2013). Almost 100% of the Kenyan coffee is Arabica species which is very marketable and fetch premium prices due to high quality (EPZ, 2005; Medina *et al.*, 2006). However, coffee production in the country has been on a steady decline especially among small holder farmers who on average produce 2.8 kg/tree/year whereas a few privately owned large estates produce about 6 kg/tree/year (Mugo *et al.*, 2011). Low production is a result of global fluctuating coffee prices (Karanja & Nyoro, 2002) as well as low investment on farm inputs such as fertilizers, pesticides and fungicides by resource poor farmers (Monroy *et al.*, 2013). Coffee Berry Borer, *Hypothenemus hampei* Ferrari, which is a key coffee pest remains a major threat to coffee production reported to cause up to 80% yield losses (Nyambo *et al.*, 1996; USAID, 2010; Mugo *et al.*, 2013).

Coffee Berry Borer is similarly regarded as the most serious pest of coffee worldwide (Vega *et al.*, 2009). It is native to Africa but has spread to almost all the 80 tropical coffee producing countries (Jaramillo *et al.*, 2006; Vega *et al.*, 2009) in Africa, Asia and America. The primary hosts of *H. hampei* are two coffee cultivars, *Coffea arabica* (arabica) and *Coffea canephora* (robusta), which are the only commercially cultivated coffee species (Vega *et al.*, 2009). Annual global losses due to the pest are estimated at US \$ 500 million (Vega *et al.*, 2009), and due to diminished yields and quality (Damon, 2000).

The borer spends most of its lifetime inside coffee berries complicating its control (Vega *et al.*, 2009), hence up to 100% infestation levels occurs (Periera *et al.*, 2012). In Kenya, various broad- spectrum pesticides and cultural methods are common for managing the pest (Nyambo *et al.*, 1996). Globally, pesticides application is the predominant method of control although there are

reports of *H. hampei* developing resistance to some of commonly used pesticides (Brun *et al.*, 1994). In addition, endosulfan which is a prevalent pesticide sprayed against the pest (Barbosa *et al.*, 2010) has lately been banned in many countries including Kenya in 2011 due to its high toxicity levels (PAN, 2008; PCPB, 2011).

Scolytid species often rely on bioactive plant volatiles and insect pheromones (semiochemicals) for host selection and locating mates (Atkin, 1966; Zhang and Schlyter, 2004; Wermelinger, 2004; Byers & Zhang, 2012). Such behaviourally active semiochemicals are often synthesized for commercial use in pests management for surveillance, mass trapping, disrupting mating patterns and repellence (El-Sayed *et al.*, 2006; Cook *et al.*, 2007). They are preferred to pesticides because they are often non-toxic, effective in small amounts and target specific (El-Sayed *et al.*, 2006; Witzgall *et al.*, 2010). Consequently, there have been calls to investigate semiochemicals utility for sustainable management of *H. hampei* (Mendesil *et al.*, 2009; Jaramillo *et al.*, 2013a).

Mass trapping of *H. hampei* with 1:1 methanol and ethanol semiochemical mixture is the only existing commercial semiochemical-based management strategy, but there are concerns about its relatively low capture rates (Vega *et al.*, 2009). The mixture does not work effectively because alcohols are common fermentation products that are not host -specific (Njihia *et al.*, 2014). Hence, it is important to investigate other potential semiochemicals from coffee with narrow host range as they stand a better chance to perform better than the existing product. This study expanded on a recent study by Jaramillo *et al.* (2013a), which identified various semiochemicals from coffee berries amongst them typical spiroacetals that are popular pheromones among scolytid species.

Spiroacetals represent a special group of volatile compounds that act as insect pheromones but may also be by-products of secondary metabolites of micro-organisms (Aho *et al.*, 2005; Barluenga *et al.*, 2009). Spiroacetals that were reported by Jaramillo *et al.* (2013a), included conophthorin, brocain and frontalin although behavioural studies with frontalin and brocain were

inconclusive. While Jaramillo *et al.*, 2013a detected the spiroacetals in healthy *C. arabica* var. Ruiru 11 berries, Njihia *et al.*, 2014 established that frontalinal amounts rapidly increase in *H. hampei* infested coffee berries in the initial stages of infestation. According to Jaramillo *et al.*, 2013a, an association between coffee berries and micro-organisms could result to production of the spiroacetals in coffee.

Previous studies depict frontalinal as a common multifunctional pheromone particularly amongst *Dendroctonus spp* (Scolytinae) acting as a host marker compound, sex pheromone, both an aggregation and an anti-aggregation pheromone (Ryker & Libbey, 1982; Pureswaran *et al.*, 2000; Blomquist *et al.*, 2010; Strom *et al.*, 2013; Liu *et al.*, 2013). Brocain is a new natural compound that is structurally similar to conophthorin which is a *H. hampei* kairomone (attractant) (Jaramillo *et al.*, 2013a).

The aim of the current study was to investigate the influence of spiroacetals, frontalinal and brocain in Coffee Berry Borer behaviour and explore their potential in the pest management in coffee farming systems.

1.1 Justification of the study

Coffee Berry Borer is the most serious pest of coffee in the world with infestation levels reaching up to 100% (Pereira, *et al.*, 2012). Despite this, the existing knowledge of the chemical ecology of *H. hampei* is limited (Vega *et al.*, 2009). For instance, it is still unclear, why each berry is attacked by a single initial ‘pioneer’ Coffee Berry Borer female (Jaramillo *et al.*, 2006), although there have been speculations about a host marking pheromone or resistant host compounds being responsible (Vega *et al.*, 2011).

The cryptic nature of *H. hampei* inside berries almost its entire lifetime make the pest difficult to control (Vega *et al.*, 2009). A major control strategy of the pest is application of non-selective pesticides (Mejia & Lopez, 2002) which has led to development of insecticide resistance in *H. hampei* and growing environmental concerns (Brun *et al.*, 1994). In addition, organic coffee is a scarce commodity contributing barely 1% of the total global output (Van der Vossen, 2005). Although, certified organic coffee fetches premium prices that can contribute to poverty eradication amongst small holder farmers, adherence to high production standards including limited use of pesticides is mandatory (Van der Vossen, 2005). Development of benign control strategies for Coffee Berry Borer is therefore vital in order to empower small holder farmers and protect the environment.

Chemical signalling in *H. hampei* has been studied with the intention of identifying and utilizing behaviourally active semiochemicals in the pest management (Mendesil *et al.*, 2009; Jaramillo *et al.*, 2013a). Semiochemicals have a wide range of applications such as mass trapping and repelling pests from a host. In addition, they can be used for surveillance, and hence avoid unnecessary application of pesticides. Currently, 1:1 mixture of methanol and ethanol is the only existing commercialised semiochemical-based management strategy for *H. hampei*. The mixture is widely accepted as a mass-trapping device but its capture rates are relatively low compared to *H. hampei* population in the field (Vega *et al.*, 2009). This study sought to investigate the behavioural response of Coffee Berry Borer females to spiroacetals, frontalin

and brocain in laboratory and field trials in order to understand their function in the pest ecology as well as evaluate their potential in the pest management.

1.2. Research hypotheses

The null hypotheses were:

1. Spiroacetals, frontalinalin and brocain do not elicit behavioural responses in Coffee Berry Borer
2. Frontalin and brocain cannot be used for monitoring Coffee Berry Borer populations

1.3. Objectives

1.3.1 General Objective

To investigate the effect of spiroacetals, brocain and frontalinalin in the chemical communication of the Coffee Berry Borer for the development of environmentally benign tools for its management in coffee

1.3.2 Specific Objectives

1. To investigate the behavioural response of the Coffee Berry Borer to frontalinalin (1,5-dimethyl-6,8-dioxabicyclo[3.2.1] octane) and brocain (1,6-dioxaspirol[4.5] decane)
2. To determine the efficacy of frontalinalin and brocain in monitoring of Coffee Berry Borer

CHAPTER TWO

2.0 REVIEW OF LITERATURE

2.1 Coffee

The importance of coffee in the world economy is overwhelming as it places this tropical crop, as the second largest export commodity in the world after petroleum products and has annual returns exceeding US \$ 70 billion (Vega *et al.*, 2006). The genus *Coffea* comprises of 103 species (Davis *et al.*, 2006) although only two are economically important, namely *Coffea arabica* (arabica) and *Coffea canephora* (robusta) Pierre ex A. Froehner. Coffee is grown in 80 countries in > 10 million hectares of land (FAO, 2013) and an estimated 125 million coffee farmers rely on the crop for subsistence (Lewin *et al.*, 2004). *Coffea arabica* constitute 70% of the coffee that is traded globally (Medina *et al.*, 2006). It is considered to be of higher quality and fetches a higher market value than the better yielding *C. canephora* due to a better aroma and less caffeine levels (Medina *et al.*, 2006).

In Kenya, coffee production is on approx. 170, 000 hectares of land. More than 90% of the coffee acreage is under arabica coffee while the rest is under robusta coffee (Gichimu & Omondi, 2010). Around 700,000 small-holder farmers own 75% of land with coffee whereas the remainder is belong to about 3000 individuals who own medium and large estates (USAID, 2010). Despite this, small holder farmers only contribute only about a half of the total production (Monroy *et al.*, 2013). Yields are higher in estates because of intensive use of fertilizers, irrigation, pesticides, herbicides and fungicides. However, small holder farmers purchase fewer farm inputs and rely on conventional farming practices such as mulching for water preservation and weed control (Monroy *et al.*, 2013). On average, 30% of the total cost of production is spent on pest and disease management (USAID, 2010). Since pesticide sprays is the main control method for pests (Nyambo *et al.*, 1996; USAID, 2010), it is important to search for other alternatives that are safe and cheaper, so as to bring down the cost of production.

2.2 Coffee Berry Borer

2.2.1 Taxonomy and Geographic Distribution

The Coffee Berry Borer, *H. hampei* (Coleoptera: Scolytidae) is the most severe insect pest of coffee throughout the world (Le Pelley, 1968; Jaramillo *et al.*, 2006). Some of the plants where the pest occur include plants such as *Tephrosia sp*, *Phaseolus lunatis*, *Hibiscus sp*, and *Oxycanthus sp* (Le pelley, 1968). However, its primary host is coffee and reproduction does not occur on any other host plants (Vijayalakshmi *et al.*, 2013).

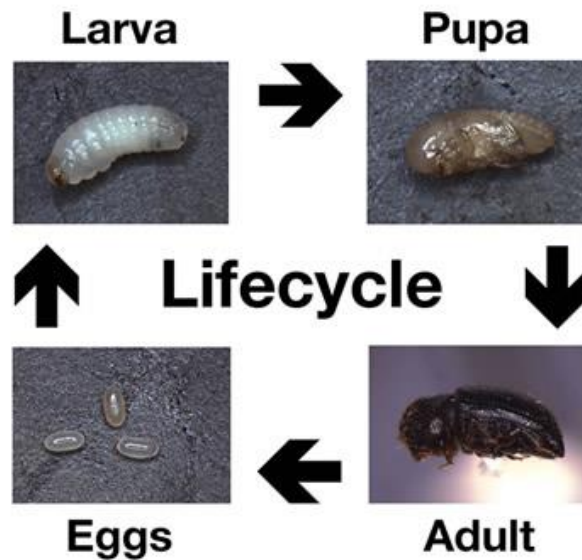
Coffee Berry Borer was first reported in coffee fields in Gabon in 1901, and later in Kenya in 1928 (Vega *et al.*, 1999). Although, the pest origin has not been confirmed yet, it is mostly believed to be native to Africa (Le Pelley, 1968). *Hypothenemus hampei* has since spread to almost all the 80 coffee producing countries in the world (Vega *et al.*, 2009) with the exception of Nepal and Papua New Guinea (Burbano *et al.*, 2011).

2.2.2 Biology of Coffee Berry Borer

Coffee Berry Borer is a small beetle which on average measures 1.4 – 2 mm in length with males slightly smaller than the females. The borer has a skewed sex ratio of 10:1 (female: male) (Brun *et al.*, 1995). Males lack functional wings and cannot fly to colonize new habitats. Therefore, they are not considered a major threat like adult females that attack coffee berries as early as 8 weeks after flowering which may persist until harvest time, 8 months later (Baker, 1999).

During colonization, each *H. hampei* female attack a single berry (Jaramillo *et al.*, 2006). It is not clear the mechanism the colonizing female referred to as pioneer borers use to space and evade sharing of hosts (Jaramillo *et al.*, 2006), which would increase competition for food (Vega *et al.*, 2011). The borer penetrates the coffee berry exocarp, mesocarp and finally reaches the endosperm where it builds galleries for its brood before it can commence egg

laying. Oviposition may start 2 days after infestation and the juvenile stages last for an average of 4, 15, and 7 days at the egg, larval, and pupal stages, respectively at 27 °C (Barrera, 1994). Sibling mating takes place inside the berry and inseminated females often abandon the initial berry and search for new hosts in which they oviposit (Vega *et al.*, 2011). However, the pioneer female and all the male progeny do not abandon the initial berry (Baker *et al.*, 1992). The females can oviposit their entire lifetime (Corbett, 1933), and almost 300 eggs have been reported in a single berry (Jaramillo *et al.*, 2009a). The life span for the males comparable to that of females (282 days) (Damon, 2000) is quite short lasting between 20-87 days (Barrela, 1994).



Source: University of Hawai'i

Plate 2.1: Life cycle of *Hypothenemus hampei*

2.2.3 Damage and economic Importance

Coffee Berry Borer larval and adult stages are responsible for coffee damage through feeding. The pest infestation reduce yield and quality of the berries and in some instances, coffee berries abscise prematurely (Le Pelley, 1968; Murphy & Moore, 1990). Holes created by the borers during penetration also serve as entry points for bacterial and fungal pathogens (Damon, 2001).

According to Baker *et al.* (2002), the conversion factor (i.e., the amount of parchment coffee obtained from a given amount of freshly picked coffee berries after processing) under low *H. hampei* pressure is 5:1. However, high infestation can alter this ratio by up to 17:1, leading to serious economic repercussions for farmers (Baker *et al.*, 2002; Jaramillo *et al.*, 2011). In addition, high *H. hampei* pressure may cause coffee to be prohibited from export due to international marketing policies that restrict export of coffee berries with more than 1.5% damage caused by insect pests (Duque & Baker, 2003).

Worldwide losses incurred due to *H. hampei* damage is estimated at >US \$ 500 million annually and affects more than 20 million households in developing countries (Vega *et al.*, 2003; 2009). Crop losses due to the pest of up to 96% have been reported in some East African countries (Magina, 2005). In Kenya, infestation levels of up to 80% have been reported (Masaba *et al.*, 1985) and the pest is reported to contribute to an on-going decline in coffee production in the country (USAID, 2010)

2.2.4 Management of Coffee Berry Borer

2.2.4.1 Pesticides

Broad-spectrum synthetic insecticides are the main chemical control strategy of the pest, but they are often highly toxic and pose an environmental hazard (Jaramillo *et al.*, 2006). This has led to discontinuation of use of some of the previously renowned pesticides in most coffee growing countries including Kenya. For example, endosulfan an organochlorine insecticide, which was previously extensively used in the control of Coffee Berry Borer has since been banned due to its negative environmental impact (PAN 2008; PCPB, 2011). Development of pesticide resistance in *H. hampei* for the same pesticide was also reported (Brun *et al.*, 1995). The inbreeding nature of Coffee Berry Borers inside berries due to a single colonizing female inhabiting each berry and sibling mating accelerate rapid spread of pesticide resistance when mutation

occurs (Brun *et al.*, 1995). In Kenya, pesticide sprays are prevalent particularly amongst large estates (Nyambo *et al.*, 1996; Monroy *et al.*, 2013;)

2.2.4.2 Mixed cropping

Studies conducted in Kenya, have shown that shading reduce *H. hampei* infestation levels in coffee (Mugo *et al.*, 2013; Jaramillo *et al.*, 2013b). According to Jaramillo *et al.*, (2013a), lower pest population densities in shaded coffee could be due to the fact that, coffee evolved as an understorey forest tree hence, intercropping it with trees mimics its natural setting thereby supporting a healthier crop. Mixed cropping also promotes biodiversity leading to high population of natural enemies and fewer pests (Landis *et al.*, 2000).

2.2.4.3 Sanitation

Sanitation is an important way of managing *H. hampei*. It involves timely and complete harvest of ripe berries and collection of berries that have fallen on the ground so as to get rid of avenues that may harbour *H. hampei* during the off-season. These control strategy is mainly manual making the practice hugely laborious and expensive particularly for small scale coffee growers (Baker, 1999).

2.2.4.4 Biological control agents

Numerous natural enemies of *H. hampei* have so far been reported. They include; predators, parasitoids, entomopathogenic fungi and nematodes (Vega *et al.*, 2009). However, only few natural enemies have so far been commercialised such as bethylid *Prorops nasuta* Waterston (parasitoid) and *Baeuveria bassiana* Vuillemin (entomopathogenic fungus). *Prorops nasuta* is present in many coffee growing countries in Africa (Le Pelley, 1968), including Kenya (Jaramillo & Vega, 2009b). The parasitoid has been artificially introduced to other continents, although only very low parasitism levels have been achieved (Vega *et al.*, 2009). On the other hand, *B. bassiana* causes varied *H. hampei* mortalities ranging less than 1% and 60% (Balakrishnan *et al.*, 1994; Vega *et al.*, 2009). *Baeuveria bassiana* was a common post-harvest control method of *H. hampei* in Colombia, but a survey

by Mejia & Lopez (2002) showed that most farmers were abandoning it due to low efficacy.

Generally, lack of long term coordinated studies to scout for natural enemies in the coffee growing countries have contributed to the insignificant progress in utilization of natural enemies of *H. hampei* as bio-control agents (Vega *et al.*, 2009).

2.2.4.5 Host kairomones and pheromones

Scolytid beetles are known to use olfactory cues from plants (kairomones) and pheromones from conspecifics and heterospecifics to locate and choose the hosts to infest (Atkin, 1966; Zhang and Schlyter, 2004). Such behaviourally active compounds are often utilized for pest management through manipulation of insect pests for surveillance, mass trapping, disrupting mating patterns, repellence among other uses (El-Sayed *et al.*, 2006; Cook *et al.*, 2007).

A 1:1 mixture of methanol: ethanol is currently the only existing commercial attractant of *H. hampei* and is widely accepted as a mass trapping lure (Dufour & Frérot, 2008; Vega *et al.*, 2009). Since *H. hampei* is known to prefer ripe coffee berries whose volatile composition has alcohols as a major component (Ortiz *et al.*, 2004), it is likely that *H. hampei* associates the commercial lure with their host kairomones (Vega *et al.*, 2009). However, the methanol: ethanol capture rates are still relatively low, as they only represent a small proportion of the total insects' population in the field (Vega *et al.*, 2009). Therefore, there is a need improve it and study other semiochemicals that can be utilized as attractants or repellents of *H. hampei* for integration into the pest management program.

2.2.5 Role of spiroacetals in mediation of communication

Spiroacetals refers to a sub-group of semiochemicals, mainly derived from fungal metabolites and insect secretions (Aho *et al.*, 2005; Barluenga *et al.*, 2009). Several spiroacetals are insect pheromones, serving as aggregators, repellents, spacers (host markers) and sex pheromones (Francke & Kitching, 2001).

Although several studies have sought to identify the semiochemicals emitted by coffee and their electrophysiological and behavioural effect on *H. hampei* (Ortiz *et al.*, 2004; Mendesil *et al.*, 2009), the information on the chemical ecology of *H. hampei* is still limited (Vega *et al.*, 2009). However, a recent study by Jaramillo *et al.* (2013a) identified about fifty volatile compounds from healthy *C. arabica* berries, some of which are popular pheromones of scolytid beetles. Several spiroacetals though common in various scolytid species were reported in coffee for the first time including frontaline and brocaol. Although electrophysiological tests with *H. hampei* antennae for both spiroacetals gave strong responses, behavioural assays were inconclusive (Jaramillo *et al.*, 2013a). Most recently, Njihia *et al.* (2014), reported a rise in the amount of frontaline released by Coffee Berry Borer infested *C. arabica* var Ruiru 11. Although frontaline was also found in *H. hampei* frass, it was not clear if the pest synthesises the compound as well. Brocaol amounts were very minimal in the berries and quantification for comparison amongst healthy and *H. hampei* infested berries was not done. Since coffee is associated with hundreds of non-pathogenic bacteria and fungi (Vega *et al.*, 2008), it is important to establish if the production of spiroacetals in coffee is due symbiotic association between coffee berries and micro-organisms (Jaramillo *et al.*, 2013a)

Previous reports implicate frontaline as a multipurpose pheromone that facilitates aggregation in some species such as Red turpentine beetle, *Dendroctonus valens* LeConte and terminates aggregation in others such as mountain pine beetle, *Dendroctonus ponderosae* Hopkins, by signalling approaching conspecifics on the unavailability of enough food in the already attacked host trees (Ryker & Libbey, 1982; Pureswan *et al.*, 2000). Blomquist *et al.* (2010), reported that frontaline could be the spacer (marker) compound that space beetles population amongst hosts. In addition, frontaline was recently reported by Liu *et al.* (2013) as both an aggregation and sex pheromone in *D. valens*, although high concentrations reduced attraction particularly in females. Brocaol was recently reported by Jaramillo *et al.* (2013a) as a new natural compound that is structurally similar to conophthorin which is an attractant of *H. hampei*.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Site

Laboratory studies were carried out at the International Centre of Insect Physiology and Ecology (*icipe*) Duduville campus, Nairobi Kenya (1° 16' 60'' S; 36° 49' 0'' E) whereas the field experiments were conducted in a commercial coffee farm in Kiambu County, Kenya (1° 11' 27.15'' S; 36° 49' 23.03'' E. altitude 1,722 m.a.s.l).

3.2 Coffee Berry Borer

Females of Coffee Berry Borer, *H. hampei* were obtained from a culture reared in *icipe*'s laboratory. The insects were maintained using a technique developed by Jaramillo *et al.* (2009a), which utilizes fresh coffee berries in order to closely mimic field conditions. The borers were reared on 150 days old coffee berries (*C. arabica* var. Ruiru 11) collected from a plot in a privately owned coffee plantation in Kiambu, Kenya. The rearing room was maintained at a room temperature, $25 \pm 1^\circ \text{C}$ and $70 \pm 5\%$ relative humidity (RH) and a 12:12 hr light: dark photoperiod. Infested berries were kept inside plastic containers (40 cm x 40 cm x 20 cm) with perforated lids covered with insect gauze (55 mm diameter). The bottom of the container was layered with 2 cm of mixture of plaster of Paris and activated charcoal, which was watered every 3 days to maintain humidity and to prevent desiccation of the berries and the insects. Coffee Berry Borer females that were two months old were used for the laboratory experiments and were starved for 12 hrs before using them.

3.3 Coffee Berry Borer behavioural assays

Two experiments were conducted to test *H. hampei* females behavioural response to brocain and frontalinal. These were done in a Y-tube olfactometer with an airflow and a petridish arena in still air, both carried out in the laboratory.

3.3.1 Y-tube olfactometer assays

To test the behavioural response of Coffee Berry Borer to different concentrations of frontalin, brocain and blends of the two compounds, a Pyrex glass Y-tube olfactometer (Internal diameter 10 mm; stem 85 mm; arms 75 mm at a 60° angle to the stem) (Analytical Research System INC, Gainesville FL, USA) was used (Plate 3.1). The Y-arms of the olfactometer were connected with a PVC tubing (Masteflex. 06409-15 Tygon mfg. by St. Gobain, Paris, France) to a sealed glass odour source chamber (internal volume 50 ml) supplied with charcoal-filtered and humidified air (90% RH). A treated square filter paper strip size (2.5 x 2.5 cm) (No.1 Whatman Int Ltd. Maidstone, England), was placed inside the odour source chambers to dispense the test compounds. The airflow through each arm of the Y-tube was maintained at 100 ml min⁻¹ by a battery-powered pump (USDA/ARS-CMAVE, Gainesville, FL, USA). Female borers were prevented from escaping through the arms of the olfactometer by a screen mesh (1 mm²) held with Teflon tape across the openings of each arm. A 40-W red fluorescent bulb placed 100 cm above the centre of the olfactometer illuminated the test arena evenly (Plate 3.1). The room where the bioassays were conducted was maintained at 25 ± 1° C temperature and 60 ± 5% RH using either a heater, fan and humidifier accordingly. The trials were run between 10:00 and 17:00 hours in order to coincide with the peak of *H. hampei* female activity in the field (Jaramillo *et al.*, 2013a).

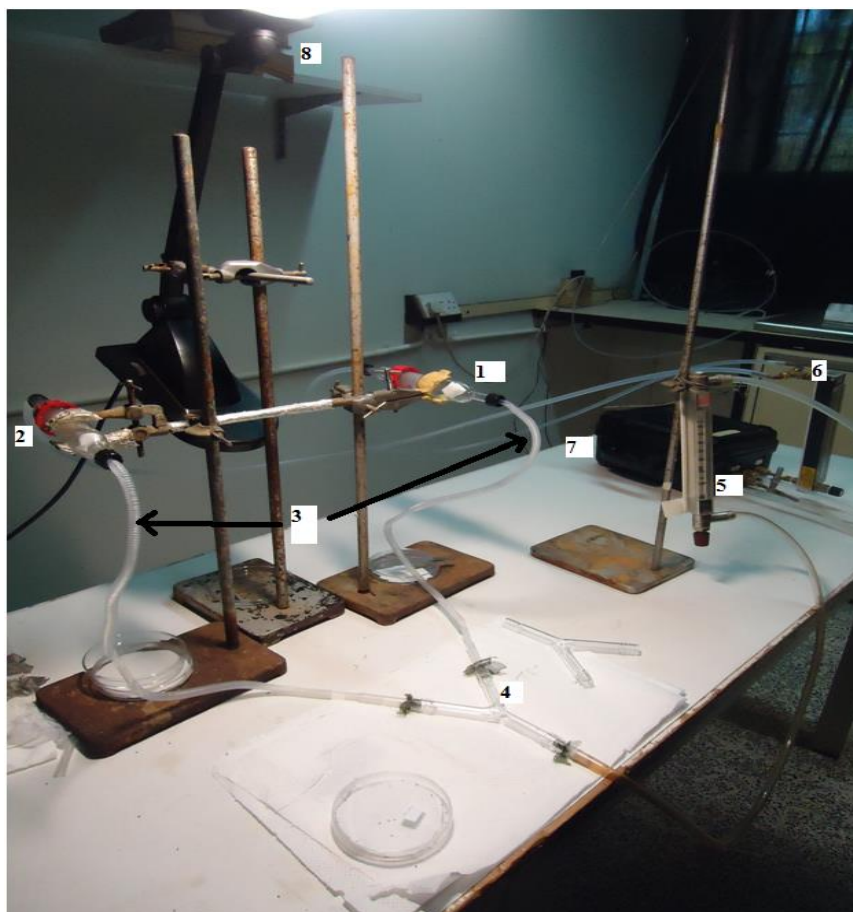


Plate 3.1: Y- tube olfactometer set up. 1 and 2= Odour dispensers (filter papers) placed inside glass chambers; 3= teflon tubes; 4= Y-tube olfactometer; 5= flow meter; 6= air supply; 7= pump; 8 = lamp

3.3.1.1 Bioassays with brocain and frontalinal

Behavioural tests were conducted for nine concentrations of brocain and frontalinal which were prepared from a stock solution of 1 mg ml^{-1} of either spiroacetals formulated in dichloromethane (DCM). The concentrations were made following geometric progression with a multiplication factor of 2 and included; 2.5, 5, 10, 20, 40, 80, 160, 320 and $640 \text{ ng } \mu\text{l}^{-1}$. Tests were conducted by applying $40 \text{ } \mu\text{l}$ of each treatment level (dose) to a square filter paper strip measuring $2.5 \times 2.5 \text{ cm}$. Similarly, $40 \text{ } \mu\text{l}$ of DCM solvent was placed on a separate filter paper to serve as control. After solvent evaporation for 2 min the treatment and control filter paper strips were held in separate glass chambers (internal volume 50 ml) that were connected to the Y – tube olfactory arena (Plate 3.1). The filter paper strips were loaded with new/fresh odour source and

replaced after 30 minutes in order to minimize variability of odour perception among insects introduced at varied times.

Adult *H. hampei* females were individually introduced at the entrance of the main vertical arm of the Y-tube with a fine camel brush and considered to make a choice after walking beyond the Y-tube intersection in ≤ 15 min. Insects that failed to make a choice in 15 minutes were recorded as non-respondents. Fifteen adult females were used for each trial which was replicated five times (N = 75). After each test, the Y-tube were replaced with a clean one and assignment of odour source to each arm of the olfactometer reversed in between tests to eliminate directional bias. After the experiments, glassware was washed with Teepol® (multipurpose detergent. Teepol® products, Kent, UK), rinsed with acetone and then with distilled water and baked in an oven at 80 °C for two hours.

3.3.1.2 Bioassays with blends of brocain and frontalinalin

Preliminary tests were conducted to test *H. hampei* behavioural responses to different formulation and dispensation methods of a sample blend of brocain and frontalinalin. The blend components (40 ng μl^{-1} of brocain and 5 ng μl^{-1} of frontalinalin) were chosen as the reference concentration upon which subsequent blends were formulated. The method of making the blend that caused significant bioactivity in the borers was adopted for use for all other tests involving blends.

In the first method, the sample blend comprising 40 ng μl^{-1} of brocain and 5 ng μl^{-1} of frontalinalin (hereafter referred to as blend A) was formulated by putting the two spiroacetals in a 1.5 ml glass vial. They were subsequently vortexed at 4000 revolutions/minute (Model No. G560E, Scientific industries inc. USA) to obtain a homogenous mixture. In order to test the behavioural response of Coffee Berry Borer females to the blend, a volume of 40 μl of the mixture was applied to a filter paper and an equivalent amount of solvent used as control. The treatment and solvent filter papers were placed in separate odour dispense chambers and tests carried out. Fifteen adult females were used in each replication five times.

In the second method, similar components of blend A above (40 ng μl^{-1} of brocain and 5 ng μl^{-1} of frontalin) were prepared separately and placed in separate 1.5 ml vials. An aliquot of 40 μl of each compound was applied on separate filter papers. The two treated filter papers were subsequently placed inside the same odour source chamber. Similar amount of solvent (40 μl) was placed in a separate chamber to serve as control. Fifteen adult females were used in each replication for five times. All other tests with blends of brocain and frontalin were conducted using this method since bioassays elicited significant behavioural responses in *H. hampei* unlike the former scenario whereby no bioactivity was recorded.

Subsequent blends were formulated by placing frontalin and brocain required concentrations in separate vials and applying them on separate filter papers. The treated filter papers were however placed in the same odour source to dispense both compounds together. Blend A (40 ng μl^{-1} brocain and 5 ng μl^{-1} frontalin) was used as the reference blend upon which dose response studies for the blends were conducted in two ways i.e., by varying high amounts of both compounds in blend, and retaining the dose of brocain while increasing the amount of frontalin (Table 3.1). Lastly, behavioural response of *H. hampei* to the blend that was found to be most attractive was evaluated against its individual components. Five replicates using 15 insects were conducted per test (N= 75).

Table 3.1 Different blend formulations of frontalin and brocain

	Geometric Method		Additive method		
	Brocain (ng μl^{-1})	Frontalin (ng μl^{-1})	Brocain (ng μl^{-1})	Frontalin (ng μl^{-1})	
Blend A	40	5	Blend A	40	5
Blend B	160	20	Blend D	40	10
Blend C	640	80	Blend E	40	20
			Blend F	40	40

3.3.2 Petri dish bioassay

This experiment was conducted to investigate *H. hampei* females infestation levels in coffee berries with brocaïn or frontalín applied on the exoscarp. Fresh berries (*C. arabica* var. Ruiru 11) used in the experiment were obtained from a private farm located in Kiambu County, Kenya. The berries were 150 days old in the yellow orange exoscarp stage which is mostly preferred by Coffee Berry Borers (Ruiz & Baker, 2010). A scalpel (No. 21) was used to cut the berries from the branches without touching their exoscarp to avoid contamination. They were used while fresh the same day of collection from the field.

These tests were conducted in a glass petri dish (14 cm diameter) (Analytical Research System INC, Gainesville FL, USA), which was fitted with a filter paper disc at the bottom of the dish (No.1 Whatman Int Ltd. Maidstone, England). A cylindrical mesh (1 mm²) was fitted between the bottom and top petridish lids to allow for aeration and prevent borers from escaping from the arena. The experiments were conducted at 25 ± 1 °C temperature and 60 ± 5% RH. Coffee borer responses to the spiroacetals was tested in two set ups (Plate 3.2). In both tests, 100 µl of 40 ng µl⁻¹ brocaïn and 80 ng µl⁻¹ frontalín were used to treat each berry. These concentrations had been identified to be the most attractive and repellent respectively from the preceding dose response *H. hampei* olfactory tests. Similar amount of solvent (5% DMSO+95% water) was applied on to the control berries.

In the first experiment, the petri dish was divided into two equal halves (Plate 3.3). A circular area of ~1.5 cm diameter located in the middle of the filter paper was drawn with a pencil, and this served as the point to introduce females. *Hypothenemus hampei* infestation levels of treated berries were compared between: brocaïn and control (solvent only), and frontalín and control (solvent only). Each set of five berries treated with either of the compounds or solvent were placed on each half on the extreme end and was equidistant from the centre and 9 cm separating them. After introducing a batch of 10 insects in the middle they were left for 4 hrs to allow for infestation to occur after which the experiment was stopped. The Petri dish was rotated horizontally at an angle of 90° after every 15 min to minimize positional bias

and after each test the filter paper was replaced. Ten replicates were conducted for all the tests on different days (N=100 insects).

In the second experiment, *H. hampei* infestation levels of brocain, frontalín and control (solvent) treated berries were compared jointly by dividing the Petri dish arena into three equal sections (Plate 3.2). Three berries were separately treated with the test material and they were placed in each section and nine insects introduced together in the middle of the Petri dish. The Petri dish was rotated horizontally at an angle of 120° after every 15 min to randomize treatments positions. Likewise, the filter paper was replaced after each test. This test was replicated 11 times (N = 99) and every trial lasted for 4 hrs. Infestation was considered successful when at least 90% of the insects had fully penetrated the berries in both experiments.

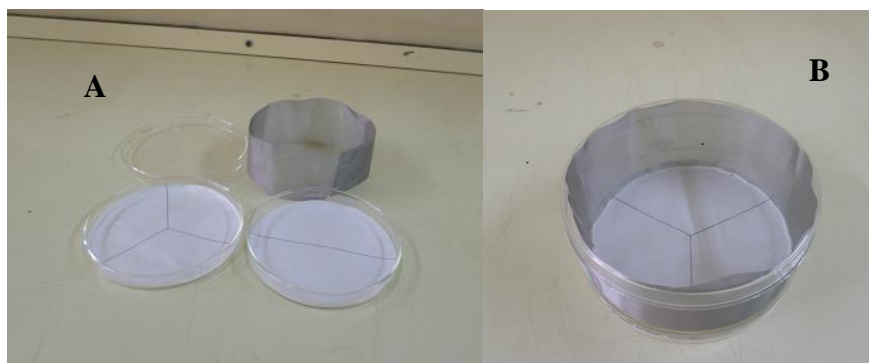


Plate 3.2: A; Petridish arena parts. B; assembled set up.

3.4 Measuring release rates of brocain and frontalín

In order to establish if mixing brocain and frontalín, interfered with the volatility of individual components of the blends, release rates of brocain and frontalín, while emitted as single compounds and in sample blends was tested by solid phase micro-extraction (SPME) method. The experiment was conducted under controlled conditions maintained at $25 \pm 2^\circ\text{C}$ temperature and $60 \pm 5\%$ RH. To determine release rates, SPME fibres (65 μm PDMS - DVB, Bellefonte, USA) were conditioned at 250°C for 30 minutes on an Agilent 7890A GC system. The SPME was subsequently covered with an aluminium

foil to avoid contamination of the fibre. The clean SPME was then inserted in the glass odour source chamber (internal volume 50ml), where the treatments had been placed (Plate 3.3). The various treatments included $40 \text{ ng } \mu\text{l}^{-1}$ brocain and $5 \text{ ng } \mu\text{l}^{-1}$ frontalinalin (Blend A) with both compounds either mixed and dispensed together in one filter paper, or both compounds dispensed separately by two filter papers placed in the same dispense chamber (Plate 3.3). Three and four replicates for the two scenarios were conducted respectively. In addition, release rates of unmixed brocain and frontalinalin were studied separately (exact concentrations comprising blend A were used). This was replicated thrice and served as control. The release rates were determined by exposing a clean SPME fibre to a chamber (internal volume 50 ml) in which 40 μl of each treatment level was placed on a filter paper strip (s). The fibre was removed from the chamber after 15 minutes and replaced with a new one that continued to trap volatiles for further 15minutes. Hence, every experiment lasted a total of 30 minutes.

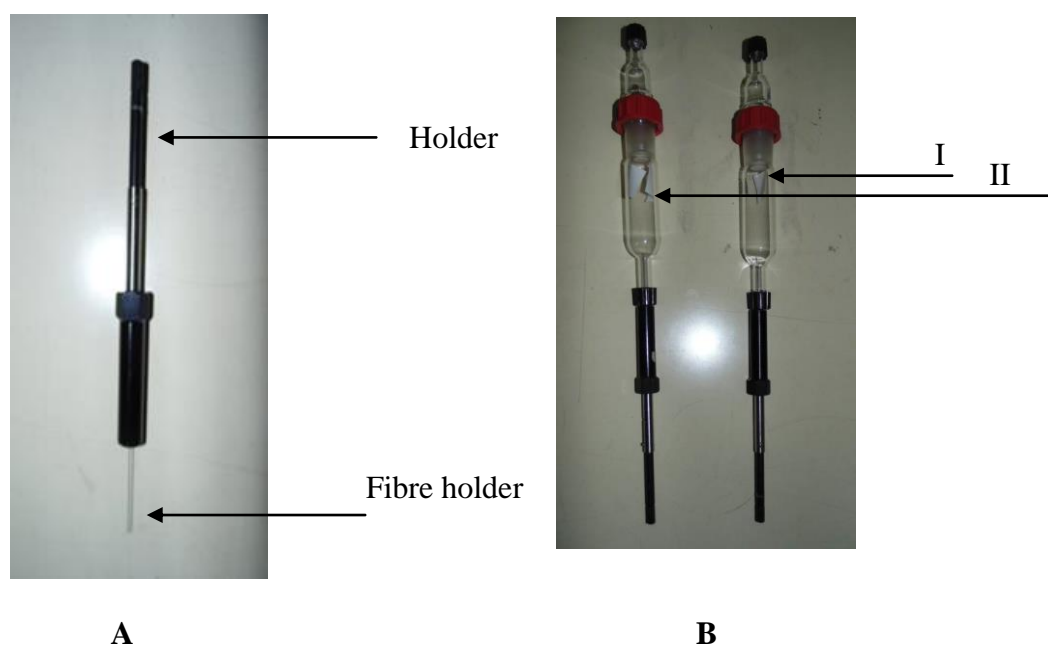


Plate 3.3: A; Solid phase micro-extraction (SPME) fibre. B; SPME used to quantify release rates while; I, Blend A components (frontalinalin and brocain) are combined and dispensed in one filter paper, II, blend A components are not combined and are dispensed by two filter papers.

3.5 GC-MS analysis of frontalín and brocaín

First, the identity and purity of the purchased brocaín and frontalín samples was confirmed by analysing them using coupled gas chromatography-mass spectrometry (GC-MS) on an Agilent Technologies 7890A GC linked to a 5795C MS, equipped with MSD ChemStation E.02.00.493, and Wiley 9th/NIST 2008 MS library and a HP5 MS column (30 m x 0.25 mm iD). The temperature program was 5 min at 35°C, then 10°C/min to 280°C. An aliquot (1 µl) of either compounds was analysed in the splitless mode, using helium as a carrier gas at a flow rate of 1.0 ml/min. Spectra were recorded at 70 eV in the electron impact (EI) ionisation mode, and emission current of 34.6 µA. The samples were identified by comparing their mass spectra with those in the library (NIST/EPA/NIH Mass spectral Library 2005a version V2.od).

Volatile samples attached on SPME during the release rates evaluation tests were desorbed by exposing the SPME fibre in the heated injection port of the GC-MS for two minutes. The temperature program was 5 min at 35°C, then 10°C/min to 130°C for 0 min and then 50°C/min to 250°C for 3.1 min. Samples were analysed in the splitless mode, using helium as a carrier gas at a flow rate of 1.0 ml/min. The amount of brocaín and frontalín sampled was quantified by comparing peak areas with standard equations obtained with known quantities of authentic standards of the compounds.

3.6 Efficacy of brocaín and frontalín under field conditions

Field trials were conducted for 3 weeks between 18th November 2013 and 2nd December 2013 to investigate the response of *H. hampei* to brocaín and frontalín in traps placed in a coffee plantation. The experiment was laid in a completely randomized block design with 3 plots acting as the blocks and compounds incorporated in traps acting as treatments. Each compound was tested alone and in combination with commercial attractant bait of methanol and ethanol mixture (Table 3.2) to investigate any potential improvement or inhibition of the attractant. The experiment was carried out in the same coffee plantation (Kiambu County, Kenya) where berries for the experiments were collected (see section 3.2; 3.7). No pesticides are used in the plantation, and the

main pest control measure was crop sanitation involving timely harvest of ripe berries, collection of fallen berries and weeding. However, no crop sanitation in the target plots was carried out during the time of the field trials.

Brocap® traps (PROCAFE, Santa Tecla, El Salvador, and CIRAD, Montpellier, France) were deployed in unshaded coffee field (Plate 3.4). The traps have been reported to facilitate survey and mass trapping *H. hampei* in coffee farms (Wiriyadiputra *et al.*, 2009; Messing, 2012). The experimental farm was divided into three equal plots based on their proximity to a shaded coffee section of the farm. The specific characteristics of the plots were as follows: plot A was the section adjacent to the shaded plot; plot B was located in the middle, and plot C was at the farthest end from the shaded plot (full sunlight exposure). Each plot had approx. 100 trees (planting density 2 x 2 m) and a distance of 15m was maintained between the plots. Traps were suspended from the coffee trees at approx. 120 cm above the ground and >10 m apart. In the collection bottle of the trap, 500 ml distilled water plus 5 drops of teepol® detergent was added to kill trapped females. The spiroacetals were prepared from a stock solution of 1 mg ml⁻¹ of either brocain or frontalín formulated in 5% DMSO+95% water (solvent). The concentrations used in the field experiment were selected by increasing by eight-fold, the least concentrations of brocain and frontalín that had been found to elicit attraction and repellence in *H. hampei* respectively, from the preliminary olfactory tests. Pure 1:1 methanol: ethanol mixture and 5% DMSO+95% water (solvent) were used as positive and negative controls respectively. A total of seven baits was used in every plot as shown in (Table 3.2). These treatments were put inside the dispensers that were provided together with the traps. A hole (2 mm diameter) was drilled through the lid of all the traps dispensers in order to deliver the compounds. Treatments were replaced with new ones after a week and this was done thrice. Re-randomization of traps in each plot was also done on every visit. Insects recovered in traps were counted and placed in labelled 10 ml vials with 70% ethanol for preservation.

Table 3.2. Various lures used in traps to bait *Hypothenemus hampei*

Compounds/Blends	Concentration (ng/μl)	Volume (ml)
1. 95% distilled water + 5% DMSO (solvent)	-	3
2. 1:1 Ethanol+ methanol mixture (EM)	-	3
3. Brocain+ solvent	320	0.96+3
4. Frontalin+ solvent	320	0.96+3
5. Brocain+ EM	320	0.96+3
6. Frontalin + EM	320	0.96+3
7. Solvent+ EM	-	0.96+3

DMSO = dimethyl sulfoxide ,Solvent = 95% distilled water + 5% DMSO and EM = 1:1 Ethanol+ methanol mixture



Plate 3.4: Brocap® trap suspended on a coffee tree in the field.

3.7 Authentic chemical standards

Authentic standards of the two spiroacetals were used in the experiments. Frontalin (purity $\geq 98\%$) was purchased from ConTech Inc.(USA). Brocain (purity 97%) was provided by Prof. Wittko, Francke, University of Hamburg, Germany. Methanol, ethanol, and dichloromethane and dimethyl sulfoxide solvents (purity $\geq 98\%$) were purchased from Sigma Aldrich chemical company.

3.8 Data analysis

Data on Y- olfactory tests for Coffee Berry Borer females responses to brocain, frontalin and blends were analysed by Chi Square (χ^2) test to compare the numbers of insects making choices between treatment and control, relative to the total number of insects introduced including those that did not respond. Release rates of brocain and frontalin in sample blends were analysed using ANOVA after transformation to normalize data using the formulae $\log(\text{amount sampled by SPME} + 0.0001)$. Chi Square, χ^2 test was done to compare preference levels of *H. hampei* to infest coffee berries that had been treated with frontalin, brocain or solvent (control) on the exoscarp. These tests were performed using R version 2.15.1 software (R Core Team, 2012). Data on field trials were analysed using Analysis of Variance (ANOVA). Averages of *H. hampei* females captured were log transformed before analysis using the formula $\log(\text{number of catches}+1)$. Multiple comparisons for the plots and treatments baited in traps were performed using Student-Newman-Keuls (SNK) procedure. This test was performed using SAS 1999 (SAS, 1999). All tests were performed at 5% significance level.

CHAPTER FOUR

4.0 RESULTS

4.1 Coffee Berry Borer behavioural assays

4.1.1. Y-tube olfactometer tests

4.1.1.1 Behavioural response to frontalin and brocain

No significant differences were found between treatment and control (DCM solvent) for the first four least concentrations of frontalin (2.5, 5, 10, 20 ng μl^{-1}). All concentrations above 40 ng μl^{-1} were significantly repellent to Coffee Berry Borer females (Figure 4.1). The optimal dose was 80 ng μl^{-1} frontalin with only 12% choosing it and 64% opting for the control.

Likewise, as observed for frontalin, responses to concentration between 2.5 and 20 ng/ μl of brocain were not significantly different. However, at 40 ng/ μl of brocain there was a threefold (58%) increase in the number of Coffee Berry Borers preferring the treatment compared to the control (21%). No significant response was observed for 80 ng μl^{-1} . However, all concentrations above 160 ng μl^{-1} were avoided by borers, which preferred DCM solvent (Figure 4.2).

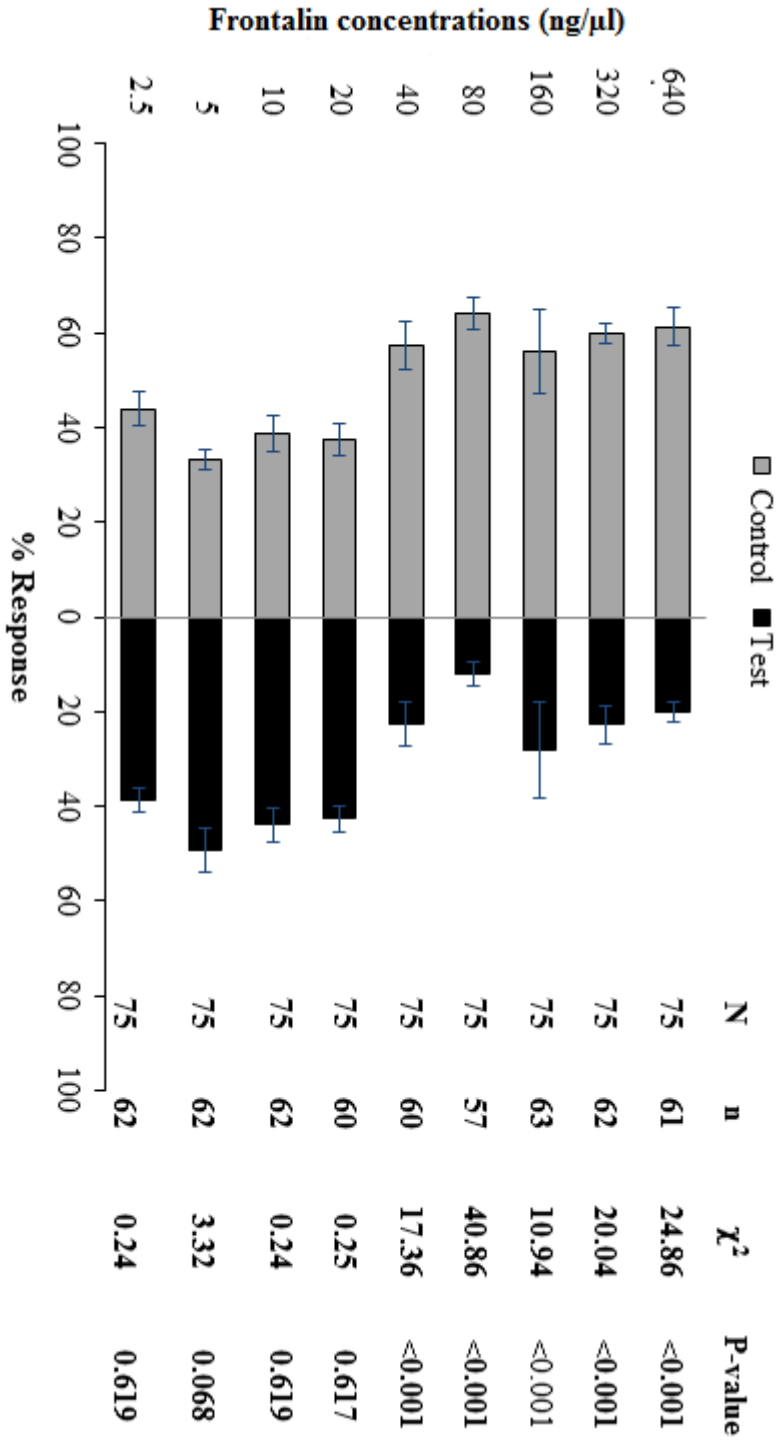


Figure 4.1: Olfactometer responses (mean ± SE) of *Hypothenemus hampei* females to frontalin. N is total number of insects and n is the total respondents (i.e. n=N less non-respondents). Control is DCM solvent only. The percent response for each arm was calculated relative to N.

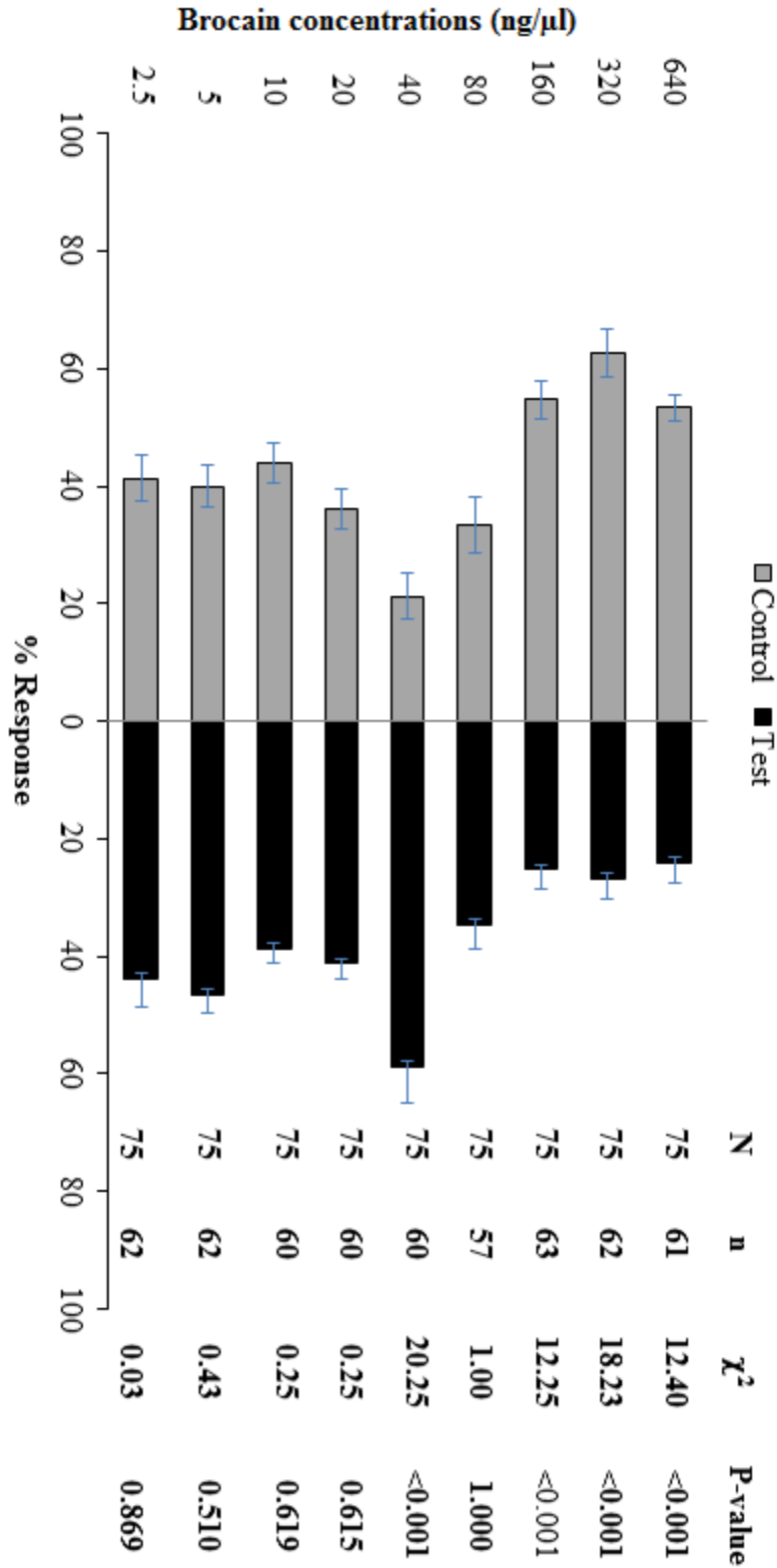


Figure 4.2: Olfactometer responses (mean ± SE) of *Hypothenemus hampei* females to brocaïn. N is total number of insects and n is the total respondents. Control is DCM solvent. The percent response was calculated relative to N.

4.1.1.2 Behavioural response to blends of brocaïn and frontalín

Preliminary blend studies showed that when blend A components (40 ng μl⁻¹ brocaïn and 5 ng μl⁻¹ frontalín) were mixed during formulation and

subsequently dispensed as a mixture in a single filter, the blend failed to elicit any significant behavioural response in *H. hampei* females ($\chi^2_1 = 0.25$, $P < 0.617$). On the contrary, the same components were highly attractive when formulated and dispensed separately in two filter papers which were placed in the same odour dispenser ($\chi^2_1 = 25.46$, $P < 0.001$) (Figure 4.3). Hence, this later method was adopted for use while conducting all other subsequent bioassays with blends of brocain and frontalin.

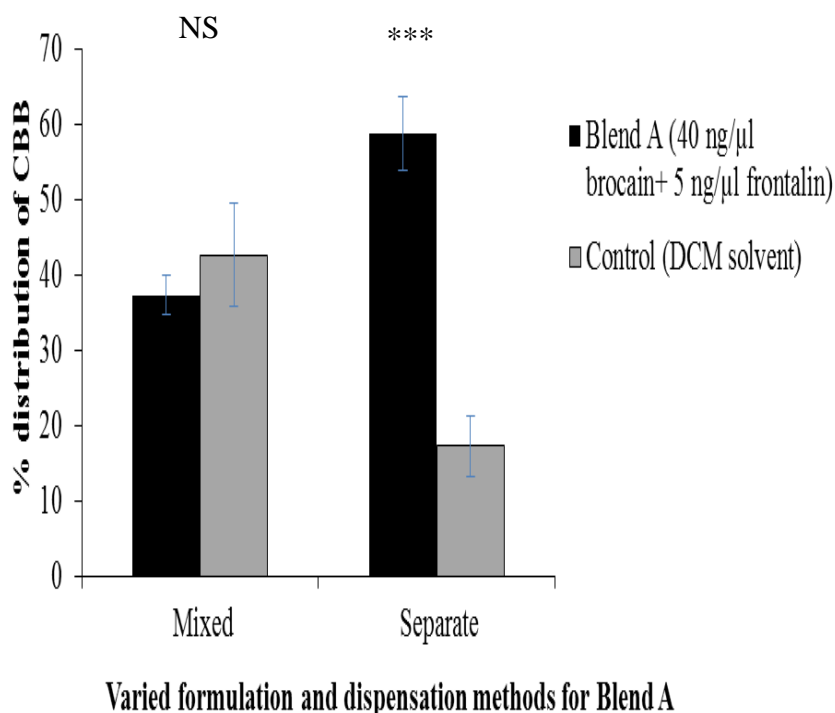


Figure 4.3: Olfactometer responses (mean \pm SE) of *Hypothenemus hampei* females to 40 ng/ μ l brocain and 5 ng/ μ l frontalin, blend A. Mixed refer to brocain and frontalin components of blend formulated and dispensed together but Separate refer to blend A components formulated and dispensed independently. The asterisks indicate the significance levels * = significant at 0.001 and NS = not significant).**

A dose response study whereby, both frontalin and brocain components in blend A were increased by geometric progression showed that all the three tested blends were significantly more attractive to *H. hampei* females than the DCM solvent (control). However, as the concentrations comprising blends were increased, the blends significantly became less attractive to the borers. (Figure 4.4). The ranking of preference by *H. hampei* females follows the order

blend A >blend B > blend C (blend A: +41%, $\chi^2_1=25.47$, $P < 0.001$; blend B: +28%, $\chi^2_1=10.79$, $P < 0.01$; and blend C: +22.7%, $\chi^2_1=7.07$, $P < 0.05$) (Figure 4.4).

Coffee berry borer responses amongst blends A, D, E and F that comprised 40 ng μl^{-1} brocain and 5, 10, 20 and 40 ng μl^{-1} frontalinal respectively, showed that blends with higher concentration of frontalinal were less preferred (blend A: +41%, $\chi^2_1 = 25.47$, $P < 0.001$; blend D: +17%, $\chi^2_1 = 3.94$, $P < 0.05$; and blend E: +10.7%, $\chi^2_1 = 1.36$, $P > 0.05$) (Figure 4.4). Blend F that comprised the highest amount of frontalinal tested significantly repelled the borers (negative response) (blend F: -38%, $\chi^2_1 = 21.29$, $P < 0.001$) (Figure 4.5).

Finally, while comparison of *H. hampei* responses amongst the most attractive blend of all the blends tested (40 ng μl^{-1} brocain + 5 ng μl^{-1} frontalinal, Blend A) and individual components making up this blend was investigated, most insects significantly preferred blend A than DCM solvent and 5 ng μl^{-1} frontalinal, + 41.4%, $\chi^2_1 = 25.46$, $P < 0.001$; + 32%, $\chi^2_1 = 14.69$, $P < 0.001$ respectively). However, 40 ng μl^{-1} brocain attracted significantly more borers than the blend (+ 28%, $\chi^2_1 = 11.31$, $P < 0.001$) (Figure 4.6).

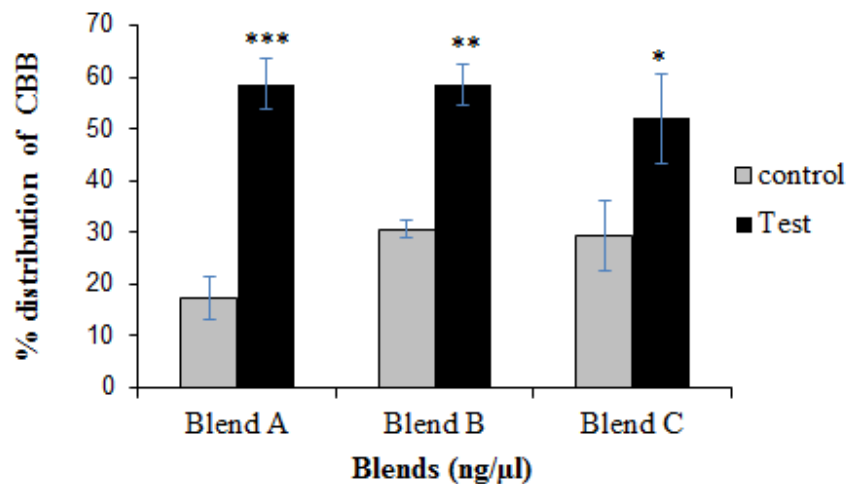


Figure 4.4:Olfactometer responses (mean \pm SE) of *Hypothenemus hampei* females to blends. Blend A: 40 ng/ μl brocain + 5ng/ μl frontalinal, blend B: 160 ng/ μl brocain + 20ng/ μl frontalinal, blend C: 640 ng/ μl brocain + 80ng/ μl frontalinal. The asterisks indicate the significance levels (* = significant at 0.05, ** = significant at 0.01 and *** = significant at 0.001).

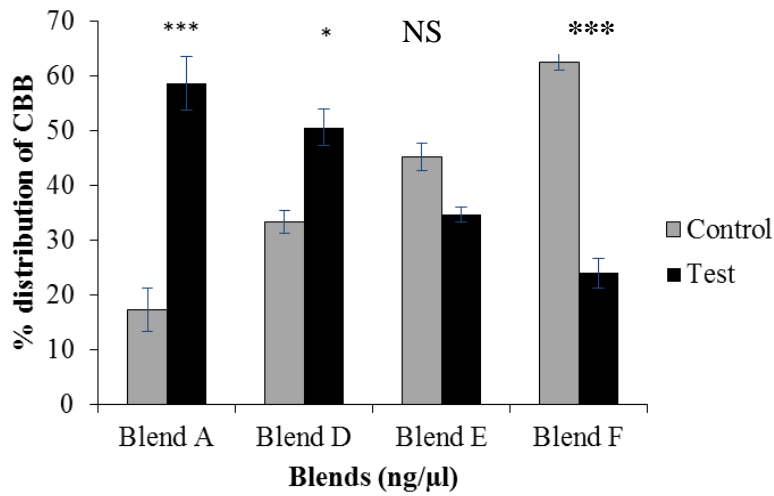


Figure 4.5: Olfactometer responses (mean \pm SE) of *Hypothenemus hampei* females to blends. Blend A: 40 ng/μl brocain + 5 ng/μl frontalin; blend D: 40 ng/μl brocain + 10ng/μl frontalin; blend E: 40 ng/μl brocain + 20 ng/μl frontalin; and blend F: 40 ng/μl brocain + 40 ng/μl frontalin. The asterisks indicate the significance levels (* = significant at 0.05 and * = significant at 0.001).**

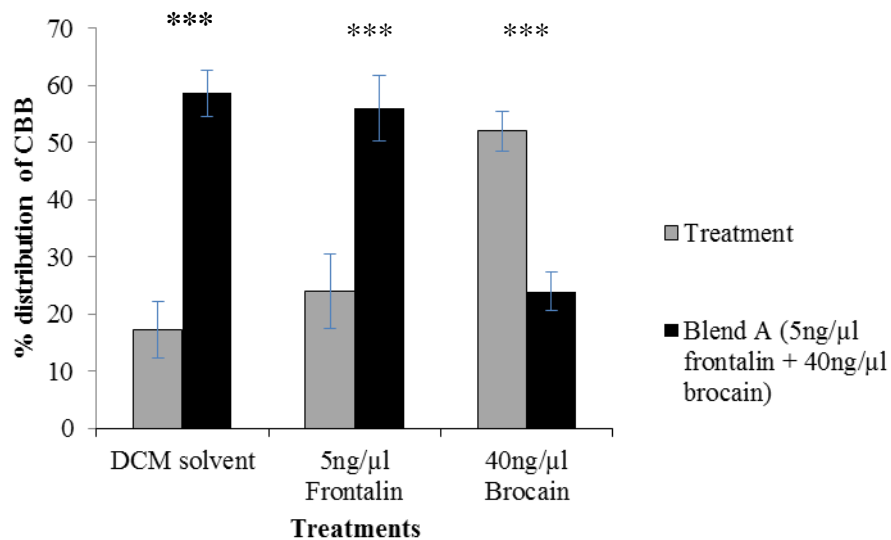


Figure 4.6: Comparison of *Hypothenemus hampei* responses (mean \pm SE) to optimal blend (A) against DCM solvents and individual components of the blend, 5ng/μl frontalin and 40ng/μl brocain respectively. The asterisks indicate the significance levels (*) = < 0.001).**

4.1.2 Petri dish assays

Hypothenemus hampei successful infestation levels were significantly low in berries whose surfaces were treated with frontalin, compared to berries treated with solvent (control) (- 26%, $\chi^2_1 = 4.62$, $P < 0.05$). However, brocain appeared to enhance arrestment in *H. hampei* that led to higher infestation levels in brocain-treated berries than the control (solvent treated berries) (+ 30%, $\chi^2_1 = 7.41$, $P < 0.01$) (Figure 4.7).

Likewise, in multiple-choice assays, there were differential colonization levels by females of berries treated with brocain, frontalin or solvent (Figure 4.8). Significant differences in the level of infestation among berries treated with either compound were recorded ($\chi^2_2 = 44.77$, $P < 0.001$). Frontalin disrupted infestation by 50% while brocain doubled infestation levels compared to the control.

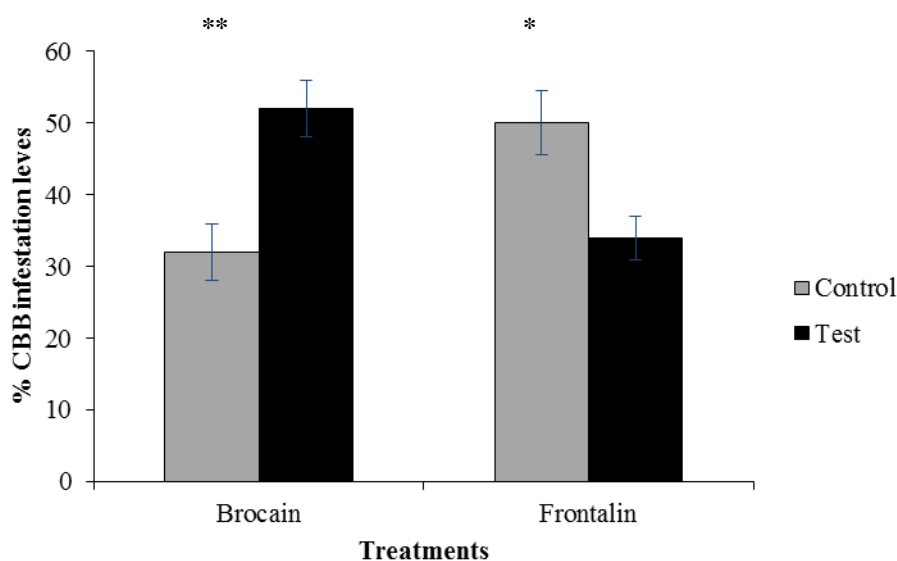


Figure 4.7: Comparison of *Hypothenemus hampei* infestation levels of ripe coffee berries treated with either 40 ng/ μ l brocain or 80 ng/ μ l frontalin versus control (solvent -5% Dms0+95% water) treated berries. The asterisks indicate the significance levels (* = significant at 0.05, ** = significant at 0.01).

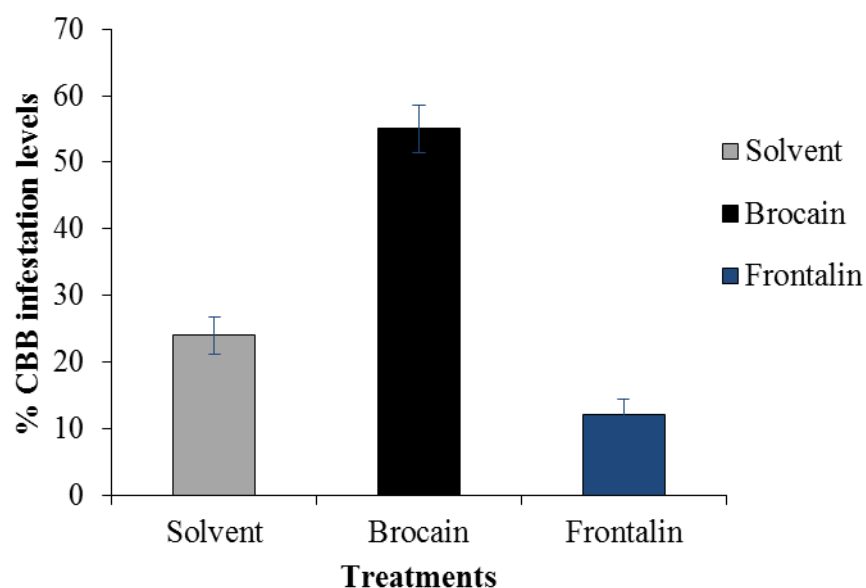
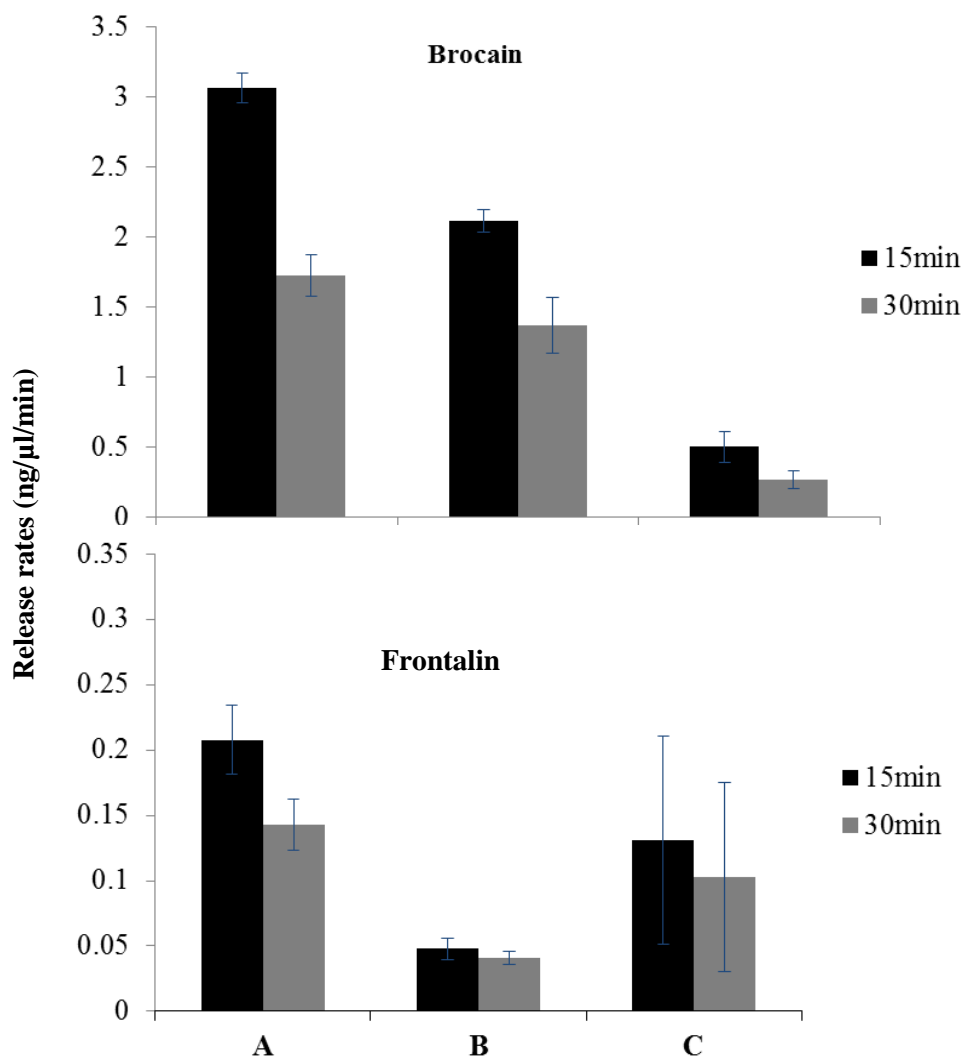


Figure 4.8: Joint comparison of *Hypothenemus hampei* infestation levels amongst ripe coffee berries treated with solvent (5% Dms0+95% water), 40 ng/ μ l brocain or 80 ng/ μ l frontalín.

4.2 Release rates and interaction of brocain and frontalín

The release rate of blend A components (40 ng μ l⁻¹brocain and 5 ng μ l⁻¹frontalín) varied depending on evaluation time and method used to prepare the blend. Release rates of the brocain component significantly diminished over time while tested alone and in blends ($F_{(1,16)} = 16.95$, $p < 0.001$). In contrast, frontalín was relatively stable and its emission rates did not significantly vary over time ($F_{(1,16)} = 1.04$, $p = 0.3241$).The mode of formulating and dispensing blends had a significant effect on the release rates of brocain and frontalín components of the blends ($F_{(2,16)} = 80.51$, $p < 0.0001$ and $F_{(2,16)} = 5.40$, $p < 0.05$ respectively). Their release rates were lower in blends than when presented as individual compounds (Figure 4.9). In preliminary olfactory assays, the brocain component alone (40 ng μ l⁻¹) had been recorded to be more attractive than all the blends (Figure 4.6)



Different modes of compound formulation and dispensation

Figure 4.9 Release rates of blend A components (40 ng/μl brocain and 5 ng/μl frontalin). A; control (individual compounds), B; blend components dispensed together in a single filter paper and C; blend components dispensed in separate filter papers

4.3 Efficacy of brocain and frontalin under field conditions

Coffee Berry Borer captures significantly varied amongst the plots ($F_{2,21} = 5.27$, $P < 0.01$). Plot C had the highest Coffee Berry Borer captures whereas plot A had the least captures. *H. hampei* captures in Plot B were not significantly different from those in both plot A and B (Table 4.1). The seven treatments that were incorporated in traps were significantly different ($F_{6,9} = 22.26$, $P < 0.0001$) (Table 4.2). Frontalin did not catch any borers whereas least

H. hampei catches were observed in brocain and control baited traps. Traps with a commercial attractant, methanol: ethanol (EM) and EM plus solvent/ brocain (mixtures) had the highest numbers of *H. hampei*. The trap loaded with a mixture of frontalinalin and the commercial attractant had low borer captures (Table 4.2).

Table 4.1: *H. hampei* captures (Mean \pm SE) in the three coffee plots

Plots	Average <i>H. hampei</i> captures
A (Adjacent to a shaded plot)	5.10 \pm 1.53a
B (Next to plot A)	8.52 \pm 2.43ab
C (Furthest from shaded plot A)	12.10 \pm 3.12b

Means followed by the same letter are not significantly different (p=0.05, SNK test)

Table 4.2: *H. hampei* captures (Mean \pm SE) of traps baited with different compounds

Treatments	Trap captures (mean \pm SE)
Ethanol: Methanol	19.00 \pm 4.29a
Ethanol: Methanol + brocain (EMB)	17.89 \pm 4.13a
Ethanol: Methanol + solvent (EMS)	16.00 \pm 4.37a
Ethanol: Methanol+ Frontalin (EMF)	4.44 \pm 1.32b
Brocain	1.33 \pm 0.24b
Control	1.33 \pm 0.33b
Frontalin	0.00 \pm 0.00c

Means followed by the same letter are not significantly different (p=0.05, SNK test)

CHAPTER FIVE

5.0 DISCUSSION

5.1 Behavioural response of *H. hampei* to frontalin

In the behavioural assays, frontalin failed to elicit behavioural response at very low concentration. However, the spiroacetal was consistently repellent to *H. hampei* at concentrations 40 ng μl^{-1} and above. Similar dose dependant responses of *Scolytinae* species to semiochemicals have been reported in many studies such as (Faccoli *et al.*, 2005; Erbilgin *et al.*, 2003). Andersson *et al.*, (2009), showed that stimulation of olfactory receptor neurons of *Ips typographus* varied depending on the concentration of semiochemicals. Generally, low concentrations were less stimulants while compared to higher concentrations, although there was a limit above which increase in concentration did not translate to higher stimulation. Faccoli *et al.* (2005), reported that addition of varied concentrations of non-host volatiles (repellents) to spruce bark beetle *Ips typographus* diet led to variation in feeding activity of the pest amongst the treated food and to control (diet with only solvent). Food with high concentrations of repellent compounds elicited higher anti-feedant behaviour than the lower concentrations.

The behavioural findings in this study suggest that frontalin plays a crucial role in Coffee Berry Borer colonization process of berries. The compound is likely produced as a defensive compound by coffee to protect itself from herbivory by providing inhibitory signals to *H. hampei*. Repellent volatiles from plants are common and play a key role in attack density regulation of bark beetles amongst hosts (Byers, 1989). Frontalin was first reported in ripe coffee berries by Jaramillo *et al.*, (2013a). Most recently, Njihia *et al.* (2014) reported that amounts of frontalin in coffee berries varied after infestation with Coffee Berry Borer. In the study, frontalin emission sharply increased after the initial attack by the pest.

Frontalin was recently reported to be present in *H. hampei* frass as well (Njihia *et al.*, 2014). Hence, it is possible that frontalin also acts as a host marker

pheromone by *H. hampei* to space its population in order to avoid conspecific colonizing female competition. According to Francke & Kitching, (2001) spiroacetals are often found in insects excretes. Host marking compounds are also known to reduce the likelihood of pioneer females sharing a host (Nufio & Papaj, 2001), a behaviour that has been observed in Coffee Berry Borers (Jaramillo *et al.*, 2006).

In addition, host marking compounds may cause pioneer females, which happen to share hosts on rare occasions, to allocate less eggs to the shared host as compared to individuals solely attacking a host (Papaj *et al.*, 1990; Nufio & Papaj, 2001). Recent studies by Vega *et al.*, 2011; Njihia *et al.*, 2014 reported that fecundity of *H. hampei* females greatly diminish when colonizing females are forced to co-exist in a host. Since a single berry may accommodate up to approx. 200 eggs of *H. hampei* (Jaramillo *et al.*, 2009a), it appears that this could be an adaptive behaviour of the colonizing female to repel incoming colonizing beetles due to the limited carrying capacity of the berry, as the niche would only suffice for its brood to complete the life cycle.

Colonizing beetles often detect the density of conspecifics amongst potential hosts before landing depending on the type of pheromones released by the beetles already attacking a host and the concentrations (Schlyter *et al.*, 1987; Zhang *et al.*, 1992). Indeed, a study by Kraker (1988) reported that *H. hampei* females were attracted to healthy ripe coffee berries and repelled by borer-infested berries under field conditions.

Past studies depict frontalin as a common multi-functional pheromone, that normally mediate aggregation; anti-aggregation; spacing (host marking) and mating functions in various bark beetles species (Blomsquit *et al.*, 2010; Ryker & Libbey, 1982; Strom *et al.*, 2013; Liu *et al.*, 2013). Frontalin contributes to termination of aggregation of mountain pine beetle, *Dendroctonus ponderosae* by signalling approaching conspecifics on the unavailability of enough food in the already attacked host trees (Ryker & Libbey, 1982). Vega *et al.* (2011), hypothesized that frontalin, could be a host marker compound responsible for spacing *H. hampei* pioneer females.

Due to the findings recently published by Njihia *et al.* (2014), and the current study, we speculate that frontalin plays a dual function in Coffee Berry Borer chemical ecology, both as a defensive compound produced by coffee plant to repel the pest and defend itself and as a host marker pheromone that space *H. hampei* females.

5.2 Behavioural response of *H. hampei* to brocain

Similar findings, of non-response as with frontalin at low concentration were observed with brocain. However, brocain was recorded to be significantly attractive to *H. hampei* at 40 ng μl^{-1} and repellent at high concentrations above 160 ng μl^{-1} . Repellence of *H. hampei* females at higher concentration of brocain could be due to dual function of the compound acting as an attractant at low concentrations and a repellent at high concentrations. Such dual purpose functioning of semiochemicals whereby a low concentration facilitates aggregation and high concentration are antiaggregants has been reported in bark beetles. Examples are endo-brevicommin, myrtenol and verbenone in *Dendroctonus frontalis* Zimm (Rudinsky *et al.*, 1974; Vite *et al.*, 1985)

Brocain was first reported by Jaramillo *et al.* (2013a) as a new compound, structurally very similar to (5*S*,7*S*)-conophthorin which is a Coffee Berry Borer attractant (host kairomone). Since the compound was highly attractive at a relatively low concentration, it is likely that Coffee Berry Borers use the compound to signalling commencement of infestation and infestation termination at higher concentration.

5.3 Interaction of frontalin and brocain

Release rates of both brocain and frontalin in sample blends was found to be inhibited while compared to the control (individual components). In addition, brocain was highly volatile unlike frontalin. Most blends formulations comprising the two spiroacetals, were significantly preferred by *H. hampei* than the control. However, 40 ng μl^{-1} brocain used alone was more preferred than all the blends. Gradual addition of small amounts of frontalin to 40 ng μl^{-1}

brocain significantly reduced attraction of borers to the mixture. Repellence was observed to the mixture that had highest amount of frontalin. Therefore, frontalin seemed to antagonize brocain. Secondary metabolites of plants are known to elicit avoidance behaviour in beetles as well as disrupt attraction of beetles to their aggregative compounds (El-Sayed & Byers, 2000)

5.4 Potential of the two spiroacetals in the control of Coffee Berry Borer

In laboratory assays, *H. hampei* infestation levels varied amongst coffee berries with brocain and frontalin applied on the exocarp. Frontalin-treated berries were only attacked by half the borers that attacked solvent treated berries. On the other hand, two-fold higher infestation levels were recorded in berries treated with brocain as compared to those treated with solvent. The mean attack density of solvent treated berries was one *H. hampei* per berry, just like in field situations where each berry is attacked by an individual borer (Jaramillo *et al.*, 2006). Differential infestation levels of coffee berries depending on the compound applied on the berry exocarp further supports the fact that Coffee Berry Borer not only rely on visual cues for host selection but also on olfactory cues. Faccoli *et al.* (2005), in a similar study reported that addition of attractants to *Ips typographus* author diet enhanced the beetles feeding whereas addition of repellents caused anti-feedant behaviour.

In field assays, semiochemicals used to bait traps significantly affected the number of *H. hampei* found in traps. The commercial attractant of *H. hampei* caught maximum number of the pest, which was similar to its combination with solvent and brocain. However, no trap catches of *H. hampei* were recorded when frontalin was used as lure in the traps. Furthermore, frontalin addition to the commercial attractant disrupted captures of the blend by 77%. These findings show that frontalin is not only able to repel *H. hampei* but also inhibits the activity of *H. hampei* attractants (brocain and methanol: ethanol). Other studies, reported that frontalin could disrupt up to 96% of the usual captures of commercial attractants of Jeffrey pine beetle, *Dendroctonus jeffreyi* Hopkins (Strom *et al.*, 2013). However, Liu *et al.* (2013), reported frontalin both as an aggregation pheromone and sex pheromone of red turpentine beetle,

Dendroctonus valens LeConte, although high concentrations reduced attraction particularly in females.

Trap captures of brocain in the field tests were very low, quite similar to traps baited with solvent (negative control). However, brocain neither inhibited nor improved the commercial attractant captures. Previous results from GC-MS analysis showed brocain to be highly volatile unlike frontalin under laboratory conditions. The extent of brocain vaporization may have been greater in the field leading to the compound diminishing soon after placement in the field before the end of the one week interval after which lures baited in traps were replaced with new ones leading to inconclusive findings with brocain in the field.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, this study established that spiroacetals brocain and frontalín are pivotal in *H. hampei* colonization and infestations processes as illustrated by their influence on *H. hampei* behaviour. This proves that they are potential candidates for Coffee Berry Borer field management. Frontalín may be used to repel *H. hampei* away from coffee fields. Brocain is a potential candidate for monitoring and mass trapping of *H. hampei* although further tests need to be conducted for optimization.

6.2 Recommendations for future research

1. It is important that an elaborate field-testing of the performance of brocain and frontalín is conducted. In the study the following points should be put into account:
 - a. Evaluate the efficacy of the spiroacetals in coffee farms with high *H. hampei* infestation levels since the current study was conducted in a commercial farm with low *H. hampei* populations.
 - b. Screen different concentrations of the spiroacetals in field experimentation
 - c. A slow release dispenser or solvent should be used to ensure that the compounds do not vaporise before replacement.

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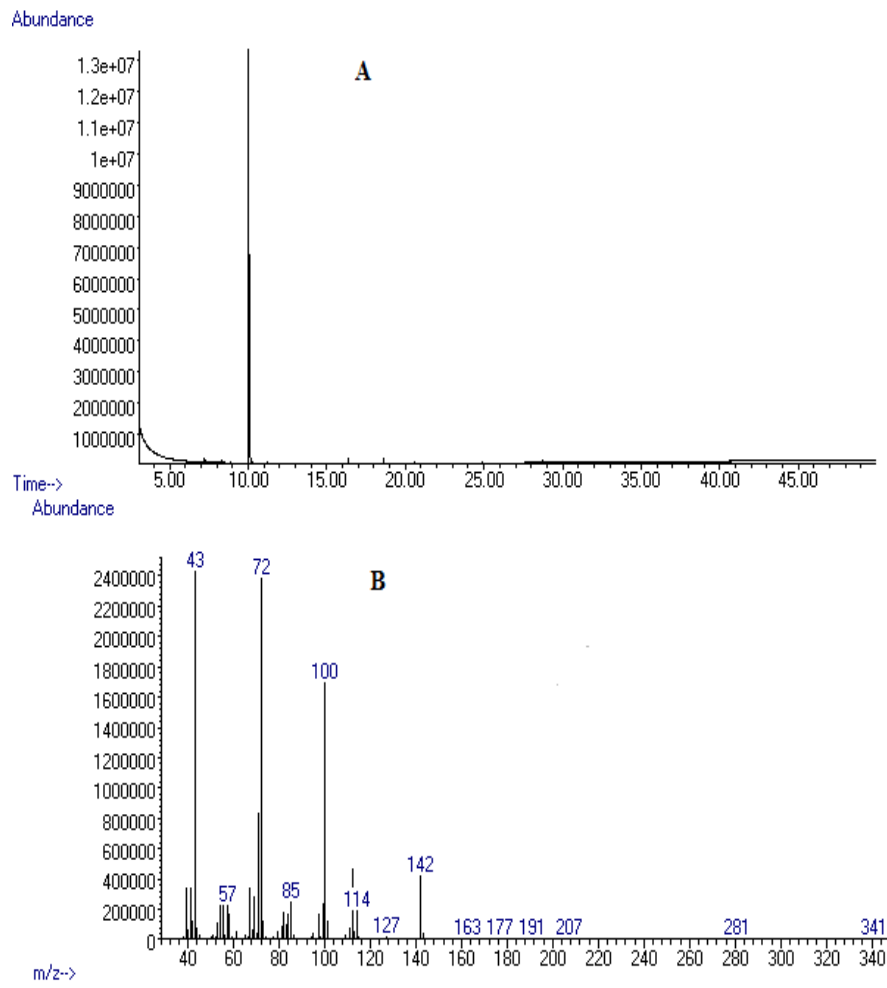
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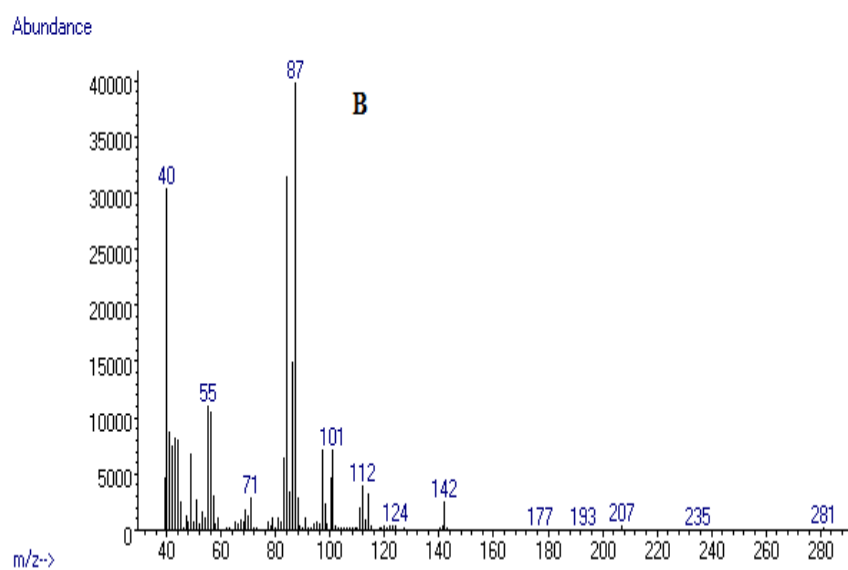
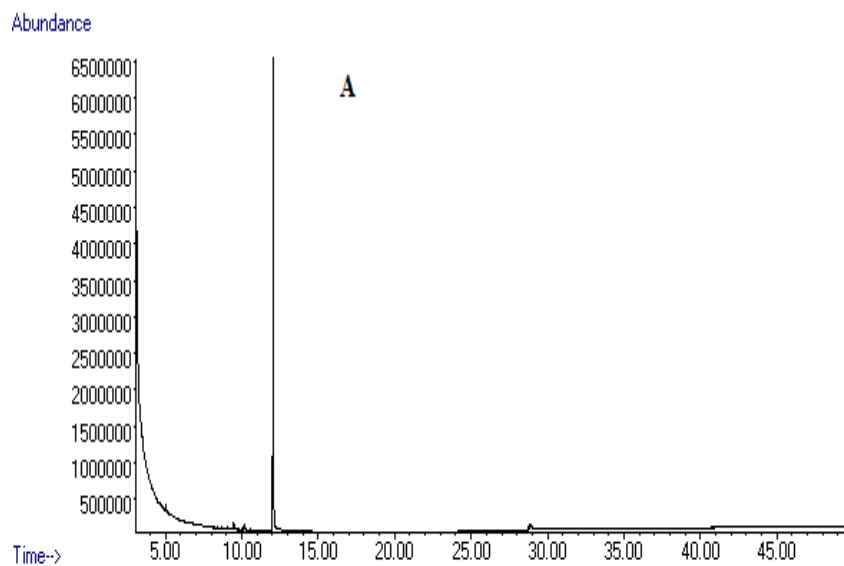
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APPENDICES



Appendix 1. A; GC-MS spectrum of frontalin chromatogram. B; Fragmentation pattern of frontalin



Appendix 2. A; GC-MS spectrum of brocain chromatogram. B; Fragmentation pattern of brocain