Relationship between virulence and repellency of *Metarhizium anisopliae* and *Beauveria bassiana* towards *Macrotermes michaelseni* and chemical identification of the mediating signals

David Mwangi Mburu

A thesis Submitted in Fulfillment for the Degree of Doctor of Philosophy in Zoology in the Jomo Kenyatta University of Agriculture and Technology

2009

DECLARATION

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

Signature……. Date……

David Mwangi Mburu

This thesis has been submitted with our approval as supervisors

Signature …….. Date……

Prof. Linus M. Gitonga

JKUAT, Kenya

Signature ……. Date……

Dr. Mary W. Ndug'u

JKUAT, Kenya

Signature ……. Date……

Prof. Ahmed Hassanali

Emeritus Scientist, ICIPE/KU, Kenya

Signature ……. Date……

Dr. Jean N. K. Maniania

Scientist, ICIPE, Kenya

Signature ……. Date……....

Dr. Peter G. N. Njagi

Scientist, ICIPE, Kenya

DEDICATION

This thesis is dedicated to my late grandma who first took me to school, my tutors at all levels of academics from whom I have acquired knowledge, my parents, siblings, and my family.

Mûûgî nîî mûtaare, hûthû nîyo îywûî, kahora karî indo na njamba ti ikere.

ACKNOWLEDGEMENT

The achievement of this study would not have been possible were it not for thorough supervision by experts from the Departments of Zoology and Chemistry, Jomo Kenyatta University of Agriculture and Technology (JKUAT) and Behavioural and Chemical Ecology Department (BCED) at ICIPE-African Insect Science for Food and Health. I would like to express my sincere gratitude to Prof. Linus M. Gitonga and Dr. Mary. W. Ndung'u, my University supervisors for the enormous pieces of academic advice, encouragement and critically reviewing this thesis. I would also like to thank the Management at *icipe*- African Insect Science for Food and Health for enabling the study.

Special gratitude to Prof. A. Hassanali (Emeritus Scientist-icipe), Chemistry Department, Kenyatta University, for his immense contribution and input to this research from the time of conceptualizing the project, constructive advice during the actual bench work and critical examination of this write-up. More importantly, my heart-felt gratitudes to him for allowing me to sit beside him at his computer's desk in his office on countless occasions, where he guided me on how to write and communicate science. I am deeply indebted to Dr. N. K. Maniania for allowing me to use facilities in his laboratory for virulence bioassays. Special thanks go to Dr. P. G. N. Njagi for valuable guidance in this work and encouragement throughout the study. I wish to thank Dr. B. Torto for facilitating the work in BCED.

My gratitude is also due to the members of staff of the BCED department and especially so, Kaye Wanyama for the interest and support in the work. Tremendous contribution in this work by the staff of Arthropod pathology unit, especially Mr. Richard Rotich, is greatly appreciated. I cannot forget fellow students especially, Dr. W. M. Wanzala, Ms. Fikira S. Kimbokota and Dr. S. W. Wachira with whom we shared students' office at BCED. Other ARPPIS scholars with whom I shared the student room at ICPE during the last corrections of this manuscript, Dr. B. K. Muli, Dr. S. Sande, Dr. A. B. Omondi, Dr. L. M. Nyangarisa, Dr. D. Mailafiya, Dr. R. Musundire, Dr. A. Raleva., Dr. V. Ranaivo, Dr. Y. Abdullahi and Dr. A. Kipkoech are also acknowledged.

The Netherlands-SII through ARPPIS, at ICIPE for the offer of the scholarship for the PhD opportunity is greatly appreciated. Finally yet importantly, glory and honour to the Almighty God for this excellent opportunity and His grace, which is sufficient for those who believe in Him.

TABLE OF CONTENTS

TITLE ……I

DECLARATION ……II

DEDICATION ……III

ACKNOWLEDGEMENT ……IV

TABLE OF CONTENTS……VI

LIST OF TABLES……...XIII

LIST OF FIGURES……...XV

LIST OF PLATES……...XIX

LIST OF ABBREVIATIONS AND ACRONYMS……XXI

ABSTRACT……...XXIII

CHAPTER ONE 1

1.0 INTRODUCTION AND LITERATURE REVIEW 1

1.1 INTRODUCTION 1

1.2 LITERATURE REVIEW 4

1.2.1 Biology of termites 4

1.2.2 Termite distribution and diversity 5

1.2.3 Eusociality and altruism in termites 6

1.2.4 Termite nests 7

1.2.5 Chemical communication in termites 8

1.2.6 Strategies for control of subterranean termites 10

1.2.6.1 Chemical control 11
1.2.6.2 Cultural control 11
1.2.6.3 Predators and parasitoids of termites 13
1.2.6.4 Biological control 13
1.2.6.5 Entomopathogenic fungi 14
1.2.6.6 Infection processes 14
1.2.6.6.1 Conidial attachment to the host cuticle 15
1.2.6.6.2 Spore germination 15
1.2.6.6.3 Penetration of the cuticle to the integument 16
1.2.6.6.4 Proliferation in the host haemocoel 17
1.2.6.6.5 Death of host and saprophytic development of the fungi 17
1.2.6.6.6 *Beauveria bassiana* 18
1.2.6.6.7 *Metarhizium anisopliae* 19
1.2.7 Factors influencing efficacy of fungi as biological control agents 20
1.2.7.1 Relative humidity 20
1.2.7.2 Temperature 21
1.2.7.3 Solar radiation 21
1.2.7.4 Pathogen properties 22
1.2.7.5 Population of host 22
1.2.8 Strategies for control of insect pests using microbial agents 23
1.2.8.1 Introductions 23
1.2.8.2 Conservation 24

1.2.8.3 Augmentation....24
1.2.8.4 Autoinoculation....25
1.3 Safety of entomopathogenic fungi....26
1.4 Chemical ecology for sustainable pest control 27
1.5 JUSTIFICATION 28
1.6 HYPOTHESES 30
1.7 GENERAL OBJECTIVE....30
1.7.1 Specific objectives....30
CHAPTER TWO....32
2.0 GENERAL MATERIALS AND METHODS 32
2.1 Study sites....32
2.2 Termites....32
2.3 Isolates of fungi....35
2.3.1 Preparation of conidial suspension for mortality responses 35
2.3.2 Conidial germination tests....37
2.3.3 Mycoses tests for virulence bioassays....39
2.4 Scaled-up production of fungal conidia for repellency bioassays 41
2.5 Protocol for olfactometric bioassays....41
2.6 Collection of volatiles of isolates of fungi....44
2.7 Elution of volatiles of isolates of fungi 45

CHAPTER THREE **48**

3.0 PATHOGENICITY OF DIFFERENT ISOLATES OF *METARHIZIUM ANISOPLIAE* AND *BEAUVERIA BASSIANA* TO TERMITE *MACROTERMES MICHAELSENI* **48**

3.1 INTRODUCTION **48**

3.2 MATERIALS AND METHODS **49**

3.2.1. Study sites, termites, conidial suspensions, germination and mycoses 49

3.2.2. Initial screening of isolates of fungi for time-mortality responses 49

3.2.3. Dose-response mortality experiments 52

3.3 DATA ANALYSES **53**

3.4 RESULTS **54**

3.4.1 Initial screening of isolates of fungi for their virulence on the termite 54

3.4.2. Dose-mortality responses 56

3.5 DISCUSSION **58**

CHAPTER FOUR **62**

4.0 REPELLENCY OF DIFFERENT ISOLATES OF *METARHIZIUM ANISOPLIAE* AND *BEUVERIA BASSIANA* TOWARDS TERMITES *MACROTERMES MICHAELSENI* **62**

4.1 INTRODUCTION **62**

4.2 MATERIALS AND METHODS **63**

4.2.1 Study sites, worker termites, germination, mycoses and production of conidia 63

4.2.2 Dose-repellency bioassays 64

4.3 DATA ANALYSES 66

4.4 RESULTS 67

4.4.1 Repellency-dose responses 67

4.4.2 Correlation between virulence and repellency 67

4.5 DISCUSSION 72

CHAPTER FIVE 76

5.0 IDENTIFICATION OF REPELLENT CONSTITUENTS OF ISOLATES OF *METARHIZIUM ANISOPLIAE* AND *BEAUVERIA BASSIANA* AGAINST TERMITE *MACROTERMES MICHAELSENI* 76

5.1 INTRODUCTION 76

5.2 MATERIALS AND METHODS 79

5.2.1 Study area, conidial mass production and collection of volatiles from fungi 79

5.2.2 Gas chromatography (GC) 79

5.2.3 Gas chromatography coupled mass spectrometry (GC-MS) 81

5.3 DATA ANALYSIS 83

5.4 RESULTS 83

5.5 DISCUSSION 94

CHAPTER SIX 97

6.0 REPELLENCY OF DIFFERENT BLENDS OF SYNTHETIC CONSTITUENTS OF *METARHIZIUM ANISOPLIAE* AND *BEAUVERIA BASSIANA* TOWARDS *MACROTERMES MICHAELSENI* 97

6.1 INTRODUCTION 97

6.2 MATERIALS AND METHODS 100

6.2.1 Study sites and worker termites 100

6.2.2 Analyses of blend effects of synthetic constituents of fungi on termites 100

6.2.3 Olfactometric set-up of the bioassays 100

6.2.4 Comparative repellency tests of blends of synthetic constituents 100

6.4 RESULTS 103

6.5 DISCUSSION 116

CHAPTER SEVEN 120

7.0 BEHAVIOUR OF HEALTHY TERMITES *MACROTERMES MICHAELSENI* ADJACENT TO INFECTED CONSPECIFICS 120

7.1 INTRODUCTION 120

7.2 MATERIALS AND METHODS 122

7.2.1 Termites 122

7.2.2 Preparation of conidial suspensions 122

7.2.3 Germination of conidia and mycoses tests 122

7.2.4 Infection of termites with conidia of fungi 122

7.2.5 Olfactometric bioassays 122

7.3 DATA ANALYSES 123

7.4 RESULTS 124

7.5 DISCUSSION 130

CHAPTER EIGHT **134**

8.1 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS **134**

REFERENCES **136**

LIST OF TABLES

Table 2.1 Fungal isolates used against the termite *Macrotermes michaelseni* for virulence, repellency and behavioural bioassays under laboratory conditions. 36

Table 3.1 Lethal Time (LT_{50}) values of time-mortality responses of various isolates of *Metarhizium anisopliae* and *Beauveria bassiana* against *Macrotermes michalseni* at a concentration of 10^7 conidia/ ml^{-1}.....55

Table 3.2 Lethal concentration (LC_{50}) values of dose-mortality responses of various isolates of *Metarhizium anisopliae* and *Beauveria bassiana* against *Macrotermes michaelseni*.....57

Table 4.1 The RD_{50} values of repellency-dose responses of various strains of *Metarhizium anisopliae* and *Beauveria bassiana* against *Macrotermes michaelseni* when exposed to varying doses of dry conidia (conidia g^{-1}).....70

Table 5.1 Compounds identified from emission of the most (ICIPE 51) and the least (ICIPE 69) repellent isolates of *Metarhizium anisopliae*.....86

Table 5.2 Compounds identified from emission of the most (ICIPE 276) and the least (ICIPE 278) repellent isolates of *Beauveria bassiana*.....89

Table 6.1 Major identified components (n = 10) in the volatiles of the most repellent (ICIPE 51) and the least repellent (ICIPE 69) isolates of *Metarhizium anisopliae* and their relative proportions used in test blends. 105

Table 6.2 Major identified constituents (n = 10) of the most (ICIPE 276) and the least (ICIPE 278) repellent isolates of *Beauveria bassiana* and their relative proportions in tested blends. 107

Table 6.3 Mean percent repellency of blends of the major components at doses equivalent to the relative proportion of each constituent at 50% repellency-dose (RD_{50}) of the full blends of isolates of *Metarhizium anisopliae* towards termite, *Macrotermes michaelseni*.....112

Table 6.4 Mean percent repellency of blends of the major components at doses equivalent to the relative proportion of each constituent at 50% repellency-dose (RD_{50}) of the full blends of isolates of *Beauveria bassiana* towards termite, *Macrotermes michaelseni*.....114

LIST OF FIGURES

Figure 2.1 Diagram of the specially designed Y-olfactometer used in testing the repellency of the fungal isolates and blends of compounds identified from the isolates and attraction of healthy termites, *Macrotermes michaelseni* towards infected conspecifics. "A", "B" and "C" are the three compartments of the Y-olfactometer. 43

Figure 4.1 Mean repellency (± SE) of highly repellent and least repellent isolates of *Metarhizium anisopliae*, ICIPE 30 (---♦---) and ICIPE 69 ---■---, respectively, at different doses against *Macrotermes michaelseni*. Mean repellency values of *Metarhizium anisopliae* were significantly greater (α = 0.05, P = 0.0001, SNK test) than those of *Beauveria bassiana*.....69

Figure 4.2 Relationship between virulence (LC_{50}, conidia/ml) and repellency (RD_{50}, g) of *Metarhizium anisopliae*, towards the termite *Macroterms michaelseni*. Fungal isolates: a, (ICIPE 51), b, (ICIPE 30), c, (ICIPE 18), d, (ICIPE 49), e, (ICIPE 60), f, (ICIPE 20), g, (ICIPE 21), h, (ICIPE 41), and i (ICIPE 69). 71

Figure 5.1 Representative total ion chromatograms of Super Q volatile collection of the most repellenct isolate (ICIPE 51) of *Metarhizium anisopliae* 21 days post harvest. IS indicates the peak for methyl salicylate (MS), the internal standard. 90

Figure 5.2 Representative total ion chromatograms of Super Q volatile collection of the least repellenct isolate (ICIPE 69) of *Metarhizium anisopliae* 21 days post

harvest. IS indicates the peak for methyl salicylate (MS), the internal standard. 91

Figure 5.3 Representative total ion chromatograms of Super Q volatile collection of the most repellenct isolate (ICIPE 276) of *Beauveria bassiana* 21 days post harvest. IS indicates the peak for methyl salicylate (MS), the internal standard. 92

Figure 5.4 Representative total ion chromatograms of Super Q volatile collection of the least repellenct isolate (ICIPE 278) of *Beauveria bassiana* 21 days post harvest. IS indicates peak for methyl salicylate (MS), the internal standard. 93

Figure 6.1 Representative gas chromatogram of the most repellent isolate of *Metarhizium anisopliae* (ICIPE 51). Numbered peaks are the major components used in evaluating blend effects in the subtraction assays; 1 (hexanol), 2 (3-octanone), 3 (acetic acid), 4 (1-octene), 5 (2-nonanone), 6 (2-nonanol), 7 (phenylethylalcohol), 8 (3-nonen-2-one), 9 (α-borneol), 10 (4,5-dihydro-5-pentyl-2-(3H) furanone) on the chromatogram. Some of the peaks that are unnumbered appeared on the control chromatogram. IS indicates the peak for methyl salicylate (MS), the internal standard. 108

Figure 6.2 Representative gas chromatogram of the least repellent isolate of *Metarhizium anisopliae* (ICIPE 69). Numbered peaks are the major components used in evaluating blend effects in the subtraction bioassays: 1 (hexanol), 2 (ethylacetamide), 3 (butyrolactone), 4 (1-ethyl-2-methylbenzene), 5 (1-octen-3-ol), 6 (3-octanol), 7 (2-propyl-1-pentanol), 8 (2-pyrrolidinone), 9

(phenylethylalcohol), 10 (cedrene) on the chromatogram. Some of the peaks that are unnumbered appeared on the control chromatogram. IS indicates the peak for methyl salicylate (MS), the internal standard. 109

Figure 6.3 Representative gas chromatogram of the most repellent isolate of *Beauveria bassiana* (ICIPE 276). Marked peaks are the major components used in evaluating blend effects in the subtraction bioassays: 1 (2-methyl-1-butanol), 2 (1-pentanol), 3 (hexanol), 4 (butyrolactone), 5 (phenol), 6 (3-octenol), 7 (4-nonanone), 8 (2-nonanone), 9 (α-borneol), 10 (dihydro-5-pentyl- 2-(3H) furanone) on the chromatogram. Some of the peaks that are unnumbered appeared on the control chromatogram. IS indicates the peak for methyl salicylate (MS), the internal standard.....110

Figure 6.4 Representative gas chromatogram of the least repellent isolate of *Beauveria bassiana* (ICIPE 278). Numbered peaks are the major components used in evaluating blend effects in the subtraction bioassays: 1 (2-methyl-1-butanol), 2 (1-Pentanol), 3 (hexanol), 4 (butyrolactone), 5 (3-octenol), 6 (2-nonanone), 7 (camphor), 8(α-borneol), 9 (E)-α-bergamotene), 10 (dihydro-5-pentyl-2-(3H)furanone) on the chromatogram. Some of the peaks that are unnumbered appeared on the control chromatogram. IS indicates the peak for methyl salicylate (MS), the internal standard.....111

Figure 7.1 Attraction of healthy worker termites *Macrotermes michaelseni* towards conspecifics infected with conidia of the most pathogenic isolate (ICIPE 51) of *Metarhizium anisopliae* at different concentrations of conidia. 126

Figure 7.2 Attraction of healthy worker termites *Macrotemes michaelseni* towards conspecifics infected with conidia of the least pathogenic isolate (ICIPE 69) of *Metarhizium anisopliae* at different concentrations of conidia. 127

Figure 7.3 Attraction of healthy worker termites *Macrotermes michaelseni* towards conspecifics infected with conidia of the most pathogenic isolate (ICIPE 276) of *Beauveria bassiana* at different concentrations of conidia. 128

Figure 7.4 Attraction of healthy worker termites *Macrotermes michaelseni* towards conspecifics infected with conidia of the least pathogenic isolate (ICIPE 278) of *Beauveria bassiana* at different concentrations of conidia. 129

LIST OF PLATES

Plate 2.1 Photograph illustrating trapping of termites, *Macrotermes michaelseni* from their mounds in the field at 1612 m asl, S 01° 13.366' E 036° 53.766' Kasarani, Nairobi, Kenya. 33

Plate 2.2 Photograph of incubation and acclimatization processes of the termites used in the study.....34

Plate 2. 3 Germinated (A) and non-germinated (B) conidia of *Meterhizium anisopliae* at x 400.....38

Plate 2.4 Photographs of mycosed cadavers of *Macrotermes michaelseni* by fungi, *Metarhizium anisopliae* (i) and *Beauveria bassiana* (iii) and non-mycosed (controls) (ii, iv) under laboratory conditions.....40

Plate 2.5 Air entrainment system used to collect volatiles from isolates of *Metarhizium anisopliae* (ICIPE 51, ICIPE 69) and *Beauveria bassiana* (ICIPE 276, ICIPE 278). 46

Plate 2.6 A photograph of the set-up used in elution of volatile collections from the filter traps with the adsorbent (Super Q) into vials placed under ice where nitrogen gas was flushed through the filters.....47

Plate 3.1 Burgerjon spray tower used to spray conidial suspension onto termites 51

Plate 5.1 Gas chromatographs, attached to an autosampler and coupled to a HP (Dell) monitor, used for analyses of volatiles.....80

Plate 5.2 Gas chromatography linked mass spectrometer equipped with an autosampler and coupled to a HP monitor used for GC-coupled mass spectrometric analyses of volatile collections. 82

LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
APU	Arthropod pathology unit
ARPPIS	African Regional Program in Insect Science
ARS	Agricultural Research Section
BCED	Behavioural and Chemical Ecology Department
DF	Digrees of Freedom
DRC	Democratic Republic of Congo
GC	Gas Chromatography
GC-MS	Gas Chromatography - Mass Spectrometry
GLM	General Linear Model
GEE	Generalized Estimating Equation
GENMOD	Generalized Model
ICIPE	International Centre of Insect Physiology and Ecology
RT	Retention time
SAS	Statistical Analysis Systems
SNK	Student –Newman Keuls
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KU	Kenyatta University
SNK	Student-Newmann-Keuls
SE	Standard Error
SAD	Sabouraud Dextrose Agar

RH Relative Humidity

SII The Netherlands International Institutes Programme

ABSTRACT

It is not well understood how termites survive in hemiedaphic habitats with diverse array of potentially infective fungi. In the present study, it was initially found that *Macrotermes michaelseni* detected a virulent isolate of *Metarhizium anisopliae* from some distance in a specially designed Y-olfactometer and avoided direct physical contact through olfaction. The overall objective of the study was to evaluate the relationship between virulence and repellency of different isolates of *M. anisopliae* and *Beauveria bassiana* towards the termite and identify possible mediating signals. The results show an interesting co-evolutionary phenomenon in which the termite's response to either *M. anisopliae* or *B. bassiana* is directly related to the potential harm which these fungi can inflict on the insect and that the virulent strains are more likely to be recognized from some distance and be avoided.

Volatile organic compounds emitted by the most and the least repellent isolates of *M. anisopliae* and *B. bassiana* were collected with the use of Super Q as an adsorbent and analysed through GC-MS. Identifications of the compounds were based on the interpretation of the mass spectral fragmentation followed by comparisons with spectral data from authentic samples, which were coupled with computer searches in HP Mass spectral library NIST98 Wiley. Where necessary, reference compounds were also co-chromatographed to confirm GC retention times. Olfactometric bioassays were used to confirm repellency of the selected constituent blends in their respective proportions and amounts present at 50% lethal dose of the respective fungal isolates. There were

qualitative and quantitative differences in the volatile profiles of the most and the least repellent isolates among the fungal species. Six to seven major components of volatiles from *M. anisophliae* and *B. bassiana*, were found to be largely responsible for the repellency action of the blends against termite, *M. michaelseni.* The results with the other components in blends indicated that the repellent action of the different components were due to the combined effects of the different components. The significance of the results and their implications in screening and use of entomopathogens and their 'entomochemicals' for control and management of termites are highlighted.

A number of workers have explored possible tactics used by termites to mitigate high risks of fungal transmission within their colony. How healthy individuals of these insects respond to infected conspecifics at different levels of infection at different doses have not been known until the results of this study. Healthy termites may be attracted to conspecifics that are freshly infected with fungal conidia to offer assistance to deal with infections. We compared these for the most and the least virulent (repellent) isolates of the two fungi after 4, 24, 48 and 72 hours post-infection. The results showed a switch from high attraction (freshly infected members) to low attraction (72 hours post-infection by all fungal isolates) towards the termite suggesting mediation of chemical signals produced by infected termites in the communication process. Further research is needed to characterize the underlying mechanisms mediating the "cry for help" phenomena by freshly infected termite *M. michaelseni*.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Social insects perform collective activities that result in the formation of complex spatio-temporal patterns in which worker castes are able to work together and collectively tackle far beyond the abilities of any one individual (Camazine *et al*. 2001). Some of these patterns include large mounds and extensive networks of underground galleries, which are several million times the size of an individual social insect (Theraulaz *et al*., 2003). Among the most impressive nest structures are those produced by the African termites of the subfamily Macrotermitinae (Nutting, 1999). A mature nest of a *Mactermes* species usually reaches six to seven metres high and may have a basal area of $50m^2$ (Theraulaz *et al*., 2003). Mounds appears only when the colony is sufficiently mature and the extensive network of galleries radiating from each mound (Darlington and Dransfield, 1987) are used to access foraging areas and transport forage back to the nest (Darlington, 1991). The foraging can extend up to 50 m from the epicenter of the mound (Dangerfield *et al*., 1998). In light of the tunneling behaviour of *Macrotermes* species such as *Macrotermes michaelseni* Sjölstedt (Isoptera: Macrotermitidae), they are considered as ecosystem engineer since they influence topography and soil physical properties (Dangerfield *et al*., 1998).

Generally, termites are eusocial insects that are classified in the order Isoptera and have different castes living in a colony. They inhabit hemiedaphic habitats, help in the

degradation of organic matter essential for improving soil fertility (Collins, 1981) and play an important role in the recycling of nutrients (Peterson *et al.*, 2008), energy flow (Singh and Singh, 1981) and soil formation (Watson and Gay, 1991). Termites are able to modify their habitat to their own preferences thereby influencing the local vegetation and microclimate (Glover *et al*., 1964; Butt, 1990).

Metarhizium michaelseni have developed cellulose-digesting capabilities with both symbiotic (Matsuura, 2003) and endogenous fungi (Watanabe *et al*., 1998) that produce cellulose digesting enzyme β-glucosidase allowing digestion of plants as a source of food. Thus, different species of termites are able to digest cellulose from a range of food sources including dry and dump wood, soil and grass. In this sense, all termites have high affinity for cellulose and this makes them a serious pest. They damage tree seedlings in managed forests (Milner and Staples, 1996), literally eat their way through linoleum, fence posts and all types of timber at homesteads both in urban and rural areas. Thus, termites are a major constraint to reforestations (Cowie and wood, 1989) and aforestation. They also attack buried reticulation systems in electrical power posts, telecommunication posts and cables and piping in irrigation (Milner and Staples, 1996). The damage, which they cause to crops both in the field and in storage as well as other structures in relatively short time periods, is enormous (Sands, 1973; Wood and Sand, 1978; Cowie *et al*., 1989). This is due to the large numbers of workers, capacity to penetrate a variety of materials and ability to consume a wide range of wood varieties. Therefore, termites restrict horticultural development and plantation forestry (Nair and Varma, 1985; Logan and El-

Bakri, 1990). Several species of termite cause serious damage to pasture especially in the semi-arid and sub-humid tropics, causing significant yield losses (Johnson and Gumel, 1981). Thus, they destroy the livelihood of tropical farmers and even threaten statues of cultural heritage and ancient books (Korb, 2007). However, termites are a source of food to various communities in some parts of the world especially in Africa due to their being rich in calories and proteins (Pearce, 1997).

In view of their pest status, there is need to control and manage termite populations through sound tactics in effective and sustainable strategies given the global consensus to reduce the chemical input in agriculture, horticulture and forest systems (Strasser *et al*., 2000; El-Sayed *et al*., 2006). Among the control strategies being sought are entomopathogenic fungi such as *Metarhizium anisopliae* (Metschnikoff) Sorokin (Ascomycetes: Hypocreales) and/or *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycetes: Hypocreales). The contact mode of action of the fungi coupled with the social behaviour of the termite makes it a potential candidate for possible horizontal transmission of the pathogen within the termite communities. However, epizootics in mounds in the field as expected have shown mixed results (Rath, 2000). It is not well understood how they evade these infective entomopathogenic fungi. It is believed that fungal conidia from these fungi are repellent to the termite but the chemical constituents in the volatile blends of different isolates of these fungi that may modify the behaviour of the termite towards the infective fungi have not been reported. It would be important to

evaluate the repellency levels of each of the constituent compounds individually and in blends.

1.2 Literature review

1.2.1 Biology of termites

Termites are soft-bodied, terrestrial, social insects that have individuals of varying morphology (castes), belonging to the order Isoptera and live together in a colony (Krishna and Weesner, 1970; Nutting, 1990). The order Isoptera constitutes five families of lower termites and one family of higher termites with over 2700 described species (Krishna and Weesner, 1970). The lower termites rely on protozoa for digestion while the higher termites rely on enzymes from microorganisms such as *Termitomyces* Heim (Basidiomycetes: Agaricatidae), besides protozoa. Termites have different groups of individuals called castes living together in a colony. The tremendous ecological fitness of eusocial insects such as termites is due to the optimization of division of labour between different castes, which is one of the key organizational elements of complex societies (D'Ettorre, 2007). The largest individual in a colony is the queen, whose function is to lay eggs. A king is always by her side to mate and fertilize the eggs. Mating is only once in her life time and the sperms are stored in its spermatheca. The soldiers have large heads with powerful jaws while the workers make up the majority in the colony. In *M. michaelseni*, minor workers are females whereas major workers are males (Pearce, 1997). The worker termites are responsible for most of the labour, whereas the soldier castes play a defensive role in termite colonies.

In all species of termites, there is a separation into two developmental pathways, the sexual line and the apterous path. The sexual line has wing buds while the latter leads to individuals that function as workers (Thorne, 1997). Winged reproductive members (allates) swarm out of the nests, locate new niche, shed their wings and pair up as males and females to begin new colonies (Pearce, 1997). They vary in colour from black to pale brown and the wings are opaque grey to black. The timing of swarming varies depending on species but usually occur after sufficient rainfall. Environmental conditions and a complex genetic inheritance pattern control termite caste (Hayashi *et al*., 2007). All individuals carry developmental instructions for all castes such that hormonal and other stimuli induce particular pathways of differentiation (Lefebvre and Thorne, 1984). Each worker and soldier caste consists of major and minor groups. According to Okot-Kotber (1981), the major and minor workers of *M. michaelseni* have three nymphal instars. The minor soldiers develop directly from the third instar female nymph and major soldiers from the fourth instar female nymph. Allates develop from first instar larvae followed by five nymphal instars.

1.2.2 Termite distribution and diversity

The ability of all social species in the animal kingdom to recruit members to new sources of food, to defend the territory or to protect colonies against enemies is crucial to their success, survival and colonization of diverse and vast habitats (Wyatt, 2006). This ability is one of the most important factors behind the extraordinary ecological dominance of social insects such as termites in many habitats (Wyatt, 2006). Besides the basic understanding of the biology of termites, knowledge of their diversity is a prerequisite for

gauging adequacy and efficacy of pest management strategies (Logan *et al.*, 1990). These insects are diverse in their behaviour and nutritional ecology (Wood and Johnson, 1986) and inhabit nearly two-thirds of the earth's land surface lying between 45º N and S latitude (Wood and Sand, 1978). In tropical and subtropical regions, termite population density exceeds 600 individuals per square meter (Collins and Wood, 1984). Termite numbers, species and nest variety increase from the higher latitudes towards the equator. Temperature and rainfall influence their distribution (Pearce, 1997).

Among the termite species, there are lower termites and the evolutionary advanced higher ones. The former include the families, Mastotermitidae, Kalotermitidae, Termopsidae and Rhinotermitidae, while the latter comprises the family Termitidae. Termites' in the subfamily Macrotermitinae, such as *M. michaelseni*, are agriculturalists of the fungus garden, *Termitomyces sp.* that enable them to digest cellulose and lignin in their guts (Müeller and Gerardo, 2002). Termite families such as Macrotermitidae and Rhinotermitidae consist mostly of the pest species. The Rhinotermitidae are the major pests in America, Europe and Asia while Macrotermitidae are a major pest in Africa and Asia (Pearce, 1997).

1.2.3 Eusociality and altruism in termites

Eusociality is a term used for the highest level of social organization in a hierarchical classification where some members of a group have reproductive division of labour (with or without sterile castes), cooperate in caring for the young ones and have overlapping generations (Michener, 1969; Crespi, 1992). Eusociality groups in termites are diploid in

both sexes unlike haplodiploid in Hymenoptera (Thorne, 1997). In eusocial groups, a limited number of individuals such as queens and kings are fertile and fecund, but most workers and soldiers are sterile. The Cooperation in the care of the brood within the nest and the overlap of adult generations results in the workers taking various chores for the benefit of the whole colony. In termites, *M. michaelseni* the workers forage for food, feed and rear their siblings and/or offspring of reproductives in their parent's generation. In addition, the worker caste is involved in building as well as mending broken parts of the nest. Evolutionary and developmental dynamics have led the worker castes in many social orders of the insects such as termites to have "opportunity cost" on fertility and cooperate to help the overall well being of the entire population. This phenomenon is referred to as altruism (Lehmann and Keller, 2006).

1.2.4 Termite nests

Termites are among the most impressive builders in nature, constructing elaborate nest structures, which in some species are up to 30m in diameter (Bonabeau *et al*., 1997). Subterranean termites construct tunnels, tens to hundreds of feet underground. This enables them to reach feeding sites and to transport food items to their nest (Lee *et al*., 2008). Some of these nests are about 10^4 to 10^5 times bigger than the individual termite workers in terms of body size (Bonabeau *et al*., 1998). However, there is no termite architect with a plan directing the workers who work literally and figuratively in the dark (Wyatt, 2006). Instead, the structure emerges from simple responses of individual termites to local cues. Most species of termites in the family Macrotermitidae including *M. michaelseni*, constructs large epigeal nests and extensive underground gallery systems

in both the tropics and subtropics (Dangerfield *et al*., 1998). They use soil pellets impregnated with pheromone to build pillars. The pellets are deposited at random and if enough pellets collect, the termites respond by building a pillar or stripe. Termites sometimes fumigate their nest with volatile antiseptic chemicals of unknown origin (Chen *et al*., 1998) and enrich their wall-building material with faeces that have antimicrobial properties (Rosengaus *et al*., 1998).

1.2.5 Chemical communication in termites

Although insects are protected from the external surroundings by a cuticular structure impermeable to chemical sensation, they do detect signals from their surroundings (Chapman, 1998). Foraging, defense, nest building and alarm trails in termite societies involve semiochemicals, albeit at different concentrations (Pasteels and Bordereau, 1998). The most complex animal societies so far described are found among the social insects including termites (Wyatt, 2006). The individuals in these societies interact through a complex web of semiochemical cues (Dani *et al*., 2005). These complex mixtures reflect the overlaying of many different messages (Wyatt, 2006). Termites live in the dark thus sensory and chemical communications are very important (Pearce, 1997; Wilson, 1971). Being social insects, termites may have evolved a way of coding chemical messages on their cuticle (Wyatt, 2006). Cuticular lipids are often involved in insect-insect interactions (Howard and Blomquist, 1982; Smith *et al*., 2009). These include information about their species, colony, caste, age and gender (Wyatt, 2006). When disturbed, termites communicate alarm mechanically via vibrations (Röhring *et al*., 1999). These insects are capable of kin distinction and worker castes departing from the

nest to their foraging areas tend to form working parties with their kin (Kaib *et al.*, 1996). Recently, Šobotník *et al.* (2008) observed that pseudergates and soldiers of termite *Prorhinotermes canalifrons* Sjöstedt (Isoptera: Rhinotermitidae) exposed to (E, E)-α-farnesene exhibited an alarm reaction that resulted into rapid walking of the activated castes. Peppuy *et al*. (2001) showed that (*Z*)–Dodec-3-en-1-ol is a major component of the trail following pheromone from the sternal gland extracts of workers of *Macrotermes annandalei* Silvestri (Isoptera: Macrotermitidae).

Termites, like other insects are able to detect variations in various microclimatic factors such as variation in humidity, temperature and airflow (Pearce, 1997). This is attributed to the complexity of their sensory system that facilitates execution of both simple and complex behaviours (Johnston, 1989). The insect's cuticle is modified at some point in the head to give rise to sensory organs for detection of external stimuli (Chapman, 1998). Many sensory organs are located in hair-like structures known as sensilla that protrude from the cuticle (Hansson *et al*., 1996). Sensilla vary in number, morphology, function, and distribution according to an insect's sensory requirements. These structures are involved in detection of mechanical, thermal or chemical stimuli and convey these messages to the central nervous system (CNS) via the sense organs viz mechanoreceptors, thermoreceptors and chemoreceptors, respectively (Hansson *et al*., 1996). The fully integrated signals in the CNS together with internal stimuli modulate appropriate behaviours of the insect including posture, foraging, nest building, detection and avoidance of risks in their habitats. Chemical information usually coded in molecules

in gaseous phase is detected through the olfactory system (Hansson *et al.*, 1996). *Macrotemes michaelseni* spend most of their life in hemiedaphic and dark habitats where they may have developed strategies for chemical communication. It would be necessary to elucidate the underlying mechanisms involved in defense against infective microbes such as *M. anisopliae* and *B. bassiana* by the temite *M. michaelseni*

1.2.6 Strategies for control of subterranean termites

The first principle of pest control is to decide whether control is both desirable and economically feasible (Cowie *et al*., 1989). The estimates of loss of yield had in the 80s had been rarely assessed in details for the termite control in Africa (Wood *et al*., 1980). This is partly due to lack of organized service industry providing control of termites in the tropics and subtropics where the majority of destructive subterranean termite species are found (Su and Scheffrahn, 1998). For instance, in rural areas of many developing countries, damage to structures by several termite species is often tolerated. Comparatively, in the developed nations like the USA, the presence of a single destructive species such as the Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae), can sustain a multimillion-dollar termite control industry (Su and Scheffrahn, 1998). It is probably true that the tolerance threshold for this pest is zero in countries with relatively high standards of living. The cost of control is considered relatively small in comparison to the value of a house and damage potential by termites (Su and Scheffrahn, 1998). The control measures differ depending on the species of termite involved due to differential ecology of a given termite species.

1.2.6.1 Chemical control

Termite control and management have relied more or less exclusively on the use of synthetic chemicals (Culliney and Grace, 2000). The insecticides used for ground treatments in 1930s included sodium arsenide, trichlorobenzene, pentachlorophenol and DDT (Beeson, 1941). These chemicals are highly toxic to humans, livestock and are hazardous to the environment. In agriculture and in forestry in particular, the control of termites has almost exclusively relied on persistent organochlorine insecticides since their development in the 1940's (Sands, 1973, Sen-Sarma, 1986). Other cyclodienes such as heptachlor, aldrin and dieldrin soon followed and dominated the termite control industry until mid 1980s (Su and Scheffrahn, 1998). Conventionally, termiticide barriers have been put in the soil around the roots to prevent damage to plants by subterranean termites (Logan and Abood, 1990). Environmental persistence and public health concern, however, led to withdrawal of cyclodienes from the market in the late 1980s. Organophosphate (chlorpyrifos), pyrethroids (permethrin) and nicotinoid (imidacloprid) are the compounds in the market for termite control (Wright *et al*., 1991). The latter termiticides are less persistent in the soil but expensive to most people in developing countries.

1.2.6.2 Cultural control

Cultural control methods include procedures that help to maintain or enhance plant vigour and which are good agricultural practices (Epila and Ruyooka, 1988). Termites may sometimes damage healthy plants, but unhealthy and stressed plants are generally more susceptible to attack (Sen-Sarma, 1986; Sands, 1973). Drought, disease, weeds,

lack of fertilizer, transplant shock, mechanical and fire damage may increase the likelihood of termite attack (Nair and Varma, 1985). Maintenance of plant good vigor reduces termite attacks. It includes good quality seeds, healthy seedlings, appropriate transplanting procedures, crop rotation and destruction of plant and crop residues (Logan *et al*., 1990). Good quality seeds, sound nursery practices and selection of vigorously growing seedlings are more likely to produce healthy plants (Harris, 1971).

Further, deficiency or excess water may stress plants and encourage attacks since levels of termite foraging activity are related to soil moisture contents. In India, termite attack on tea is greater during the rainy season than during the dry seasons (Srivastava and Butani, 1987). Monocropping for long periods reduces soil fertility and under such conditions, plants are less vigorous and susceptible to attack. This has been suggested as causative factor in *Hodotermes mossambicus* Hagen (Isoptera: Rhinotermitidae) attack on cotton in South Africa and in *Macrotermes subhyalinus* Sjölstedt (Isoptera: Macrotermitidae) damage on maize in Ethiopia (Cowie and Wood, 1989). Both dry wood and subterranean termites frequently gain access to living plant tissues through wounds or fire damage (Harris, 1971). The removal of wooden debris from the land before planting trees or plantation crops reduces subsequent termite attacks (Srivastava and Butani, 1987). Thus, cultural control methods may play an integral role in the prevention of termite attack to crops but its reliability on control and management of this pest has not sufficiently been documented.

1.2.6.3 Predators and parasitoids of termites

A few parasitoids of termites are known but their potential for regulating termite populations seems negligible (Culliney and Grace, 2000; Schmid and Hempel, 1998). Termites have a wide range of predators, both opportunist and specialist invertebrate and vertebrates that attack the alate reproductives or foraging workers outside the nest (Logan *et al*., 1990). However, a few predators attack termites within the nest (Sheppe, 1970). Of all the invertebrate animals the ants, *Pheidole megacephala* Fabricius (Hymenotera: Formicidae) are the most efficient predators of termites and may have a considerable impact on termite populations in some parts of the world (Deligne *et al*., 1981; Culliney and Grace, 2000). Among the vertebrates, the aardvark *Orycteropus afer* Linnaeus (Tubulidentata: Orycteropodidae) is the greatest predator of termites (Milewski *et al*., 1994). Most of these predators have a varied diet and termites are a minor or at least not a necessary part of it (Sheppe, 1970). However, the influence and impacts of these predators on termite population dynamics is relatively negligible (Wood and Johnson, 1986).

1.2.6.4 Biological control

Biological control constitutes a more environmentally acceptable alternative to convectional chemical control measures (Mauldin and Beal, 1989; Culliney and grace, 2000). Classical biological control of termites is difficult due to its cryptic ecology of the insect (Milner *et al*., 1998a; Rath, 2000). Reports of viruses and protozoa appear to offer no serious prospects for effective management of termite (Milner and Staples, 1996). Although *Serratia marcescens* Bizio (Enterobacteriales: Enterobacteridae) and *Bacillus*

thuringiensi Berliner (Bacillales: Bacillidae) have been shown to kill termites in the laboratory (Khan *et al*., 1977a, b), the exotoxin from *B. thuringiensis* is hazardous to mammals, and *S. marcescens* is an opportunistic human pathogen (Milner and Staples, 1996). Most research has been directed towards the use of nematodes and fungi (Milner and Staples, 1996). However, nematodes were unable to effect useful levels of control under field conditions in the US (Mauldin and Beal, 1989).

1.2.6.5 Entomopathogenic fungi

The ancestors of fungi are believed to have been simple aquatic forms with flagellated spores (James *et al*., 2006). For about 130 years, entomopathogenic fungi especially *M. anisopliae*, have been used for biocontrol of pest insects (Zimmermann, 2007a). In addition, *B. bassiana* has also been used throughout the world mainly for inundative control of insects (Zimmermann, 2007b). About 750 species of entomopathogenic fungi are distributed almost equally throughout the whole kingdom of fungi (Ferron, 1978). Despite this great number, only about 90 species have received more attention and studied intensively for use against pests of agricultural and medical importance (Gillespie and Moorhouse, 1990). These species are mostly Deuteromycetes and Entomophthorales (Charnley, 1989). Entomopathogenic fungi have been proposed as the most promising biological control agents of termites due to their enclosed habitat (Zoberi and Grace, 1990; Storey and McCoy, 1992; Rath, 2000).

1.2.6.6 Infection processes

Entomopathogenic fungi attack host insects through contact with the body integument of the insect (Robert and Humber, 1981; Talaei-hassanloui *et al*., 2007) with the exception

of the genus *Culicinomyces*. In this genus, the germ tubes from ingested conidia penetrate the midgut of mosquito larvae into the haemocoel (Inglis *et al*., 2001). Some Hyphomycetes have also been reported to infect their host through the alimentary canal and the respiratory system (Hall and Papierok, 1982).

1.2.6.6.1 Conidial attachment to the host cuticle

The attachment of the conidia on the insect cuticle is a prerequisite to infection and pathogenesis. This process is initiated by secretion of enzymes and the hydrophobic bonding between the epicuticle of the insect host and the spore surface (Gabriel, 1968; Boucias and Latgé, 1988). Secretions of adhesive mucus as the conidia swell during pre-germination supplement the initial hydrophobic interactions between the conidia and the cuticle surface (Hajek and St. Leger, 1994). The surface of the spore varies in different fungi and the spores of many members of the entomophthorales are covered with mucus glycoprotein that interacts with and modifies epicuticular waxes on the insect cuticle.

1.2.6.6.2 Spore germination

Spore germination on the cuticle has been reported to be mostly dependent on the microclimatic factors, especially temperature and humidity (Ferron, 1981). Nutritional requirements for germination and growth are not complex and require utilizable source of carbon and nitrogen for continued hyphal growth and lysis (Smith and Grula, 1981). Many fungal spores require saturated humidity for germ tube development (Newman and Carner, 1975; Ferron, 1985). A water film maintains cell turgor and provides a medium for the uptake of dissolved nutrients, including the breakdown products of the cuticle (Butt, 1990). Due to respiration through the spiracles, microclimate on the insect

epicuticle may enable germ tube formation when a low humidity is recorded. For example, *B. bassiana* infected adult bean weevil *Acanthoscelides obtectus* Say (Coleoptera: Brucidae) irrespective of relative humidity (Ferron, 1977). Pre-germinated conidia of *B. bassiana* were reported to produce esterase, lipase and N-acetylglucosaminidase activities when deposited on the cuticle of wax moth *Galleria mellonella* Linaeus (Lepidoptera: Pyralidae). The spore germinates into a germ tube (Butt, 1990; Huxham *et al*., 1989). Germination cues both chemical and topographical signals invoke the germination process (Smith and Grula, 1981). The microenvironment on the surface of the cuticle is also essential for germination since these areas form pockets with higher levels of humidity (Butt *et al*., 1990; Bidochka *et al*., 2000). The insect cuticle that hardens by sclerotization (Hopkin and Kramer, 1992; Chapman, 1998) is the most formidable barrier that a fungus must transgress and the germ tubes have to penetrate directly into the cuticle (Zacharuk, 1970 a, b, c; St.Leger *et al*., 1986).

1.2.6.6.3 Penetration of the cuticle to the integument

In most species of entomopathogenic fungi, such as *M. anisopliae* and *B. bassiana*, cuticular penetration is frequently preceded by the formation of appresorial structure (Hall and Papierok, 1982). The appressorium has been reported to facilitate anchorage during penetration, softening of the cuticle and concentration of cytoplasmic components involved in penetration (Ferron, 1985). Penetration of the hyphae through the cuticle is either by infection pegs produced from the underside of appressoria or by direct entry of germ tubes (Hajek and St. Leger, 1994). Enzymes such as lipases, proteases and chitinases are produced for the degradation of epicuticular waxes, protein and chitin

matrices, respectively (St.Leger *et al.*, 1997). Most entomopathogenic fungi penetrate the cuticle of host insect due to their ability to counter the presence of inhibitory compounds such as phenols and lipids on the surface of the cuticle (Hall and Papierok, 1982).

1.2.6.6.4 Proliferation in the host haemocoel

After penetration, hyphae develop colonies around the point of penetration and produce hyphal bodies or blastospores. The fungus usually grows in the haemocoel as yeast-like hyphal bodies that multiply by budding (Ferron, 1978). They circulate in the hemolymph and give rise to secondary hyphae that colonize host tissues (Praserthon and Tanada, 1968). Zhao *et al*. (2007) using overlay gel analysis showed that there was trehalose-hydrolysing activity in the haemolymph of *Locusta migratoria* Linnaeus (Orthoptera: Acrididae) when the locust was inoculated with conidia of *M. anisopliae*. The ability of the fungus to develop within the haemocoel depends on its capacity to overcome the immunodefensive mechanisms of the insect. For less or nonpathogenic isolates, encapsulation, phagocytocysis and melanisation of the fungal propagules occur upon penetration into the haemocoel. In highly pathogenic isolates, fungi overcome encapsulation and free-living blastospores are present in the haemolymph within 24-48 hours. Nutrient rich hemolymph allows for production of secondary metabolites such as oxalic and citric acids (Vey and Gotz, 1986).

1.2.6.6.5 Death of host and saprophytic development of the fungi

After the insect dies, the fungus grows saprophytically and spreads to tissues of the cadaver (Burnet, 1980). The saprophytic phase begins by the invasion of tissues and ends with the formation of reproductive organs, sexual or asexual (Inglis *et al.*, 2001). When

the atmosphere reaches saturation, the mycelia emerge through the integument and develop conidiophores and production of infective units, conidia ensues (Zimmermann, 2007 a, b). Competition between the mycelial colonization of the host tissues and the surrounding flora occurs. The mycerial colonization of the host cadaver leads to transformation of the host cadaver into mummy, which hardens due to absorption of humoral liquids by the fungi (Ferron, 1985). Each cadaver constitutes an infective focus, which by multiplying the quantity of inoculum thus ensures propagation of the disease.

1.2.6.6.6 *Beauveria bassiana*

Aristotle in his book Historia Animalium, first mentioned that bees suffered a fungal "disease" but it was Bassi (1834) who empirically demonstrated for the first time that *B. bassiana* was the cause of white muscardine disease in silkworm. The fungus has also been isolated from termite, *Reticulitermes flavipes* Kollar (Isoptera: Rhinotermitidae) (Zoberi, 1995) but data on the fungus infections on *M. michaelseni* are limited. The fungus is made of characteristic white, later yellowish or occasionally reddish colonies (Zimmermann, 2007a). This species is the most widely distributed species of the genus and has been isolated from the surface of trees, soil and infected insects in different insect orders (Zimmermann, 2007a). Despite the presence of *B. bassiana* in large numbers of arthropods, most isolates of this fungus have restricted host range (Goettel *et al*., 1990; Ferron *et al*., 1991). The genus *Beauveria* produces beuvericin, bassianolid and oospoein as metabolites. The latter is a red antibiotic pigment that colours the insect host cadaver and curbs bacterial growth (Inglis *et al*., 2001).

1.2.6.6.7 *Metarhizium anisopliae*

Metchnikoff conducted the first field trial in 1884 after developing a facility to mass-produce the spores of *M. anisopliae* for the control of larvae of sugar beet curculio *Gieonus punctiventris* (Germar) (Coleptera: Curculionidae) (Zimmermann, 2007a). To date, many attempts have been made to develop entomopathogenous fungi as a biopesticide for the control of a wide range of pests (Ferron, 1978; Zimmermann, 1993; Lacey *et al*., 2001). The fungus is widely used for biocontrol of insect pests and many commercial products such as Green Guard™ and Bio-Blast™ are in the market in Australia (Milner, 2000) and the USA (Rath, 2000), respectively, or under development (Zimmermann, 2007b).

Metarhizium anisopliae is a generalist entomopathogen that infects a good number of non-social insect hosts. There are also some records of this fungus infecting social insects, though for *M. michaelseni*, the data is limited. For instance, *M. anisopliae* is virulent to most genus of termites such as *Coptotermes lacteus* Froggatt (Isoptera: Rhinotermitidae) (Staples and Milner 2000) and *Nasutitermes exitiosus* Hill (Isoptera: Nasutidae) (Milner *et al*., 1998a). However, it does not infect humans or higher animals (Rath, 2000; Sun *et al*., 2002). This species of the fungus is easy to mass-produce due to robust conidia and is believed to be well adapted to the soil ecosystem (Milner and Staples, 1996).

The conidia of *M. anisopliae* are arranged in cylindrical or slightly ovoid chains and are green in colour (Zimmermann, 2007b). The conidia can survive for more than 18 months in termite nests and are effective even at a high temperature of 36˚C (Milner and Staples, 1996). The desication of the fungal spores and destruction by UV radiation and temperature extremes are mitigated in the soil matrix (Delate *et al*., 1995). Thus, termites are considered as potentially good candidates for control with fungal pathogens because they have social interactions and live in humid and crowded environment conducive for fungal growth, survival and ultimate infection on termites (Delate *et al*., 1995; Creffield, 1996; Vargo *et al*., 2003). Most species of the genus *Metarhizium* produce destruxins, afrapeptins and oosporein metabolites that serve to overcome the insect host immune mechanisms (Strasser *et al*., 2000; Vey *et al*., 2001).

1.2.7 Factors influencing efficacy of fungi as biological control agents

1.2.7.1 Relative humidity

This is considered one of the most important environmental factors influencing the development of terrestrial entomopathogenic fungi (Lacey *et al*., 2001). Saturated or near-saturated air or water film around the insect host cuticle and the surrounding is critical for spore germination and the infection of the host (Benz, 1987). For most insect hosts epizootics normally occur during periods of high relative humidity. For instance, many species in the genus *Entomophthora* need saturated or near saturated air to discharge their conidia (Wilding, 1981).

1.2.7.2 Temperature

Temperature directly affects the progression of disease and the time of death of the host insects (Müller and Schimid-Hempel, 1993; Inglis *et al*., 2001). Temperature is one of the critical factors affecting the efficacy of entomopathogenic fungi both under controlled conditions and in nature. According to Inglis *et al*. (2001), the optimum temperature for most entomopathogenic Hyphomycetes is between 20° and 25°C. Infection and disease spread can, however, occur at temperatures ranging between 15° and 30°C. The authors state that for temperatures above 30°C, the vegetative growth of most taxa is inhibited and growth ceases at about 37°C. Environmental temperature range of the geographical origin of some isolates also has been reported to influence viability at various temperature regimes (Vidal *et al*., 1997). Watson *et al*. (1992) reported that houseflies, *Musca domestica* Linnaeus (Diptera: Muscidae) elevated their body temperatures when infected by *Entomophthora muscae* (Cohn) Fresenius (Zygomycetes: Entomophthorales) resulting in the reduction of disease severity.

1.2.7.3 Solar radiation

Solar radiation is one of the most important factors affecting persistence of propagules in the environment. This is because conidia, hyphal bodies and hyphae of all entomopathogenic fungi are susceptible to damage by ultra violet (UV) radiation (Braga *et al*., 2001a). This may account for the relatively short persistence of the conidia in environments where they are directly exposed to ultraviolet radiation. Braga *et al*. (2001b) reported wide variability in UV-B tolerance among divergent *M. anisopliae* isolates. However, positive stimulation of pathogens by light has been reported. For

instance in *B. bassiana*, mycelial growth, intensity of sporulation and germination have been shown to be stimulated by light (Benz, 1987).

1.2.7.4 Pathogen properties

Pathogenicity and virulence of a pathogen are essential elements in the selection of a suitable candidate for microbial control (Tanada and Fuxa, 1987). These terms are used in many disciplines including medicine, epidemiology, microbiology and insect pathology. Pathogenicity is the ability of an organism to infect and cause disease in a host while virulence is the intensity of the disease of a pathogen within the host organism (Thomas *et al*., 2004). Most fungal pathogens are considered highly virulent relative to other pathogenic organisms due to among other factors short incubation periods, production of copious amounts of secondary inocula and can cause rapid increase in disease prevalence (Carruthers *et al*., 1991). Intraspecific differences in pathogenic among isolates of a given fungal species have been reported (Hall and Papierok, 1982). Thus, some fungal isolates may have high virulence in some species of insect hosts although they may have no virulence in others.

1.2.7.5 Population of host

Factors such as population density, age, nutrition, and exposure to injury can influence the susceptibility of insects to entomopathogenic fungi (Inglis *et al*., 2001). Further, there are variations in the susceptibility of different developmental stages of a given insect to entomopathogenic fungi. Young larvae of *Ostrinia nubilalis* Hubner (Lepidoptera: Pyralidae) were more susceptible than older larvae to pathogenic isolate of *B. bassiana* (Feng *et al*., (1985). Vestergaard *et al.* (1995) reported that adult western flower thrips,

Frakliniella occidentalis Pergande (Thysanoptera: Thripidae), were more susceptible to *Verticilium lecanii* Zimmermann (*Deuteromycetes*: *Moniliaceae*) than their conspecific larvae. The behaviour of the host also influences disease development and proliferation or even epizootics. For termites, social behaviour allows for contact among conspecifics in the colony community, which make these insects suitable target candidates for control with entomopathogenic fungi (Wells *et al*., 1995).

1.2.8 Strategies for control of insect pests using microbial agents

Five strategies have been employed in the use of entomopathogenic fungi for biological control of insect pests, which include introductions, inudative, augmentation, inoculation and conservation of natural enemies (Fuxa and Tanada, 1987; Fuxa, 1997; Goettel and Hajek, 2001). According to Inglis *et al*. (2001), the use of entomopathogenic fungi in the control of edaphic insects has utilized both inundative and inoculation strategies. Autodissemination has also been used for the control of a wide range of pests (Vega *et al*., 2000).

1.2.8.1 Introductions

New species of entomopathogens or more virulent strains of the species that already exist are placed in new habitats with the intension that they become established and provide self-perpetuating and eventually have long-term control of the target pest (Inglis *et al*., 2001). For instance, the fungus *Entomophaga maimaiga* Dustan (Zygomycetes: Entomophthorales) was introduced in North America from Japan in 1910-1911 for the management of the forest defoliator *Lymantria dispar* Linnaeus (Lepidoptera: Lymantriidae) and became a principal mortality factor for the pest (Goettel and Hajek,

2001). There have been some attempts to establish exotic fungi for the control of various pests in the field. For instance, Dustan (1923) attempted to introduce *Entomophthora erupta* Dustan (Zygomycetes: Entomophthorales) for the control of green apple bug *Lygocoris communis* (Neolygus) Knight (Hemiptera: Miridae) and *Zoophthora radicans* (Brefeld) Batko (Zygomycetes: Entomophthorales) for the control of the spotted alfalfa aphid, *Aphis craccivola* Fabricius (Homoptera: Aphididae) (Milner *et al*., 1983).

1.2.8.2 Conservation

This strategy involves manipulation of the habitat of the natural enemies, including fungi, so that their activity and effectiveness as biocontrol agents are enhanced (Goettel and Hajek, 2001). The development and proliferation of diseases have been accomplished through altering cultural practices and the management of agricultural practices such as irrigation or use of pesticides. Intensification of cropping systems has reduced the effectiveness of indigenous natural enemy populations thus appropriate manipulation of the environment can compensate for this. Brown and Nordin (1986) developed some early harvesting strategy that maximized the development of and spread of *Z. erynia* even when the alfalfa weevil *Hypera postica* Gyllenhal (Coleoptera: Bruchidae) populations were lower than the critical levels for epizootic development. This was accomplished by harvesting the crop when mycoses of the larvae were observed.

1.2.8.3 Augmentation

In this strategy, pathogen density inocula is increased through the development of microbial insecticides, which are applied like aerial insecticides with the aim of causing high acute host mortality but also initiating epizootics (Ferron *et al*., 1991). Fungi are

introduced in either low (inoculative) or in very large numbers (inudative) (Fuxa, 1997) and the disease development and proliferation depend on the ability of the pathogen to become established in the environment and produce a secondary inocula that is capable of polycyclic infection (Inglis *et al*., 2001).

1.2.8.4 Autoinoculation

Autoinoculation is a relatively recent strategy that involves disseminating pathogens among target pest populations using devices that attract the insect pests into the foci of the pathogens (Vega *et al*., 2000). This strategy exploits manipulation of the behaviour of the pest to enhance the spread of a pathogen to susceptible conspecifics earlier in the season than they would normally be targeted (Furlong *et al*., 1995). Thus, disease epizootics is able to establish, spread and decimate small populations before the crop is damaged (Inglis *et al*., 2001). The devices used in autodissemination of the pathogens are structured according to the behaviour of the target insect pest. For instance, Pell *et al*. (1993) reported that male diamond back moths were attracted to a sex pheromone trap inoculated with *Z. radicans* conidia. Maniania (1998) designed a trap for the dissemination of *M. anisopliae* among tsetse flies, *Glossina spp*. (Diptera: Glossinidae) populations in the field. The approach was also used by Klein and Lacey (1999) for the control of Japanese beetle *Popillia japonica* Newman (Coleoptera: Scarabaeidae) with *M. anisopliae*.

Scolte *et al*. (2005) reported horizontal transfer of conidia of *M. anisopliae* among male and female *Anopheles gambiae s.s.* (Diptera: Culicidae) during mating under laboratory

conditions. Vega *et al*. (1995) used an autodissemination device for the control of sap beetle *Carpophilus lugubris* Murray (Coleoptera: Nitidulidae) with *B. bassiana*. The advantage of this strategy is that it is specific to a target pest and only little amount of fungal inocula are used thereby limiting economic problems of mass production, formulation and storage. Inside the trap device, the fungal conidia are protected from UV (Inglis *et al*., 2001). However, a number of workers have reported that termites, *M. michaelseni* included have developed behavioural and physiological mechanisms of avoiding spread of microbial disease within their communities (Rosengaus *et al*., 1998; Rosengaus, 1999a; Rosengaus *et al*., 2000; Cremer *et al*., 2007).

1.3 Safety of entomopathogenic fungi

Although pesticide application leads to instant death of the target pests, there are undesirable effects that include insecticide resistance, outbreaks of secondary pests, contamination of the environment and safety risks to both humans and non-target animals (Colborn *et al*., 1997) that come along with it. In recent years, progress has been made in the development of fungal biocontrol agents for control and management of insect pests, weeds and plant diseases (Goettel, 1984) as alternatives to conventional use of chemicals for pest control and management (Strasser *et al*., 2000; Jackson *et al*., 2000). Mycoinsecticides have features that provide more ecologically sound pest control than chemical pesticides (Goettel *et al*., 1990; Moore and Prior, 1993). They are generally safe to humans and other non-targets, selective to varying degrees and suitable for integrated pest control programs. In addition, they may provide extended periods of control by remaining in the environment and becoming established permanently besides being

biodegradable (Goettel and Johnson, 1992; Lacey *et al*., 2001). Nevertheless, Hajek and Butler (2000) point out that toxins from fungal biocontrol agents may pose some risks of direct infection or allergies to non-target organisms. Thus, a lot of research is still needed to assess their safety (Goettel and Hajek, 2001).

1.4 Chemical ecology for sustainable pest control

Chemical ecology involves the study of the origins, functions and significance of natural chemicals mediating interactions between organisms (Pickett *et al*., 1997; Miller *et al*., 2006a). Chemical ecology is not panacea for the many problems associated with the use of pesticides. However, the study of this diverse field provides impetus for exploration and understanding of other semiochemicals such as pheromones for alternatives of broad-spectrum toxicants (Hummel and Miller, 1984; Pikett *et al*., 1997). However, if the potential of semiochemicals such as sex pheromones in crop protection is to be realized, a greater understanding of the natural chemicals mediating interactions among insect pests and their hosts (plants and animals) is essential (Miller *et al*., 2006b). Insect olfactory systems present models to study interactions between invertebrates and the environment (Witzgall *et al*., 2007). They have evolved fast processing of specific odorant blends for general chemical monitoring (De Bruyne and Baker, 2008).

Semiochemicals such as sex pheromones when used alone often give ineffective or insufficient pest control results (Howse *et al*., 1996). Thus, for optimum results in pest control, they should be combined with other strategies in integrated pest management (Pickett *et al*., 1997) for optimum pest control results. For example, in push-pull

strategies, crops are protected by combination of host-masking agents, repellents, antifeedants or oviposition deterrents (Pickett *et al*., 1997) and the push and pull components are generally nontoxic (Cox *et al*., 2007). Sex pheromones have been used most successfully in mating disruptions in Lepidoptera while aggregation pheromones have been used in trap lures of Coleoptera (Howse *et al*., 1996).

Recent advances in techniques for elucidating structures of chemical compounds mediating in insect pest-host interactions have led to collaborations with a strong interdisciplinary association between chemists and biologists. It is expected that interdisciplinary interactions will continue to accelerate and provide insights into the understanding of similar or different interactions involving a wide range of insect species in nature.

1.5 JUSTIFICATION

Macrotermes michaelseni is one of the most serious pests in sub-saharan Africa, which requires sustainable control and management strategies. For decades the control and the management of termites have relied almost exclusively on the use of conventional chemicals. This has resulted in increased public concern over pollution to the environment, risk of safety to human and animal health. Thus, many countries in the world either have banned or have placed severe restrictions on the use of synthetic chemicals in termite control. Termite resistance to chemical pesticides has in any case further complicated the problem. However, currently there are very few non-chemical

options for termite control (Rath, 2000). Thus, there is urgent need for the development of alternative begnin tools and tactics for the management of the pest.

Fungi that are pathogenic to insects differ from other groups of insect pathogens in their ability to invade a host by penetrating its cuticle. The use of infective fungi against termites is an option worthy of study because the social interactions between termites have potential to spread the virulence of the fungi throughout a colony (Rath, 2000; Cremer *et al*., 2007). However, termites have evolved a range of behavioural and physiological tactics to avoid contact with infection by fungi (Rosengaus *et al*., 1999a; Myles, 2002; Rosengaus *et al*., 2004). One such tactic involves olfactory detection of potential harmful fungi and subsequent avoidance from a distance. Repellency of fungal conidia eliciting avoidance response by termites is not well understood. Documented information on the virulence and repellency of different isolates of *M. anisopliae* and/or *B. bassiana* towards *M. michaelseni* and any positive correlation between these parameters have been scant. Moreover, in the existing body of knowledge, the identity of the volatile blends emitted from these fungi and possible olfactory activity on *M. michaelseni* is lacking. Identification of repellent(s) from highly virulent isolates may provide models for the development of potent repellent products for the management of this termite and perhaps, other termite species.

1.6 HYPOTHESES

Based on the preceding information, the following hypotheses were tested:

i. Different isolates of fungi have different levels of infection /differe in virulence to the termite *Macrotermes michaelseni.*

ii. There is a relationship between pathogenicity and repellency between different isolates of *M. anisopliae* as well as *B. bassiana* with different infectivity levels to termites.

iii. The repellency results from blends of some major components of the volatile emissions of isolates of the fungi.

iv. There are qualitative and quantitative differences in the composition of blends from different isolates of fungi.

v. Being social insects, a specific set of responses guide healthy termite members to ensure that infection is not transmitted to the rest of the colony.

1.7 GENERAL OBJECTIVE

The general objective of the study was to elucidate the mechanisms underlying avoidance behaviour of the termite, *M. michaelseni* species to *M. anisopliae* and *B. bassiana* and to identify the mediating signals.

1.7.1 Specific objectives

i. To determine different levels of lethal time values of different isolates of *M. anisopliae* and *B. bassiana* against termites, *M. michaelseni*.

ii. To determine the relationship between pathogenicity and repellency of different isolates of the fungi to the termite.

iii. To identify the major and minor components of the repellent blends of the fungal isolates from trapped volatiles.

iv. To compare qualitative and quantitative differences in the compositions of volatile emissions of the selected fungal isolates and correlate these with repellency.

v. To establish the behaviour of healthy termites adjacent to infected conspecifics at different stages of infection.

CHAPTER TWO

2.0 GENERAL MATERIALS AND METHODS

2.1 Study sites

The laboratory experiments were carried out at both Arthropod Pathology Unit and Department of Behavioural and Chemical Ecology of the International Center of Insect Physiology and Ecology, Duduville Campus in Nairobi, Kenya.

2.2 Termites

Worker castes of termites *M. michaelseni* used for pathogenicity bioassays were trapped overnight using a modified method described by Tamashiro *et al*. (1975) from three different mounds located at (1) 1612 m asl, S 01° 13.366' E 036° 53.766', (2) 1610 m asl, S 01° 13.068, 036° 53.823', and (3) 1618 m asl, S 01° 13.144' E 036° 53.717' at Kasarani, Nairobi, Kenya (Plate 2.1). The trap consisted of a plastic bucket with perforations (2 cm in diam.) at the base, which allowed the movement of the termites from the mounds. Pieces of wood were put inside the bucket as well as soil crumps from the mounds. The collected termites were covered with dark polyethylene plastic sheet, transported to the laboratory and placed on similar plastic sheets. The termites were individually picked with a pair of soft forceps and placed on Petri dishes (9 cm diam.) lined with wet filter paper (Whatman No. 1, 9 cm in diameter). They were then transferred into an incubator (26 ± 2 °C and 90 ± 5% RH in the dark) (Plate 2.2) and kept for 20 minutes for acclimatization before being used in the bioassays. The relative humidity in the incubator was controlled using stable saturated solution of K_2SO_4 (Supelco, Sigma-Aldrich, United Kingdom).

Plate 2.1 Photograph illustrating trapping of termites, *Macrotermes michaelseni* from their mounds in the field at 1612 m asl, S 01° 13.366' E 036° 53.766' Kasarani, Nairobi, Kenya.

Plate 2.2 Photograph of incubation and acclimatization processes of the termites used in the study.

2.3 Isolates of fungi

2.3.1 Preparation of conidial suspension for mortality responses

Fungal isolates used in this study were obtained from ICIPE's Arthropods Germplasm Centre, Arthropod Pathology Unit and were isolated from different substrates (Table 2.1). The fungal isolates were cultured on Sabouraud Dextrose Agar (SDA) medium (Oxoid, Basingstoke, Hampshire, England). Initially the conidia of these isolates were harvested from two-three weeks old surface cultures by scrapping. They were suspended in 10 ml sterile distilled water containing 0.05% Triton X-100 (Fluka, Sigma Aldrich) and 3 mm glass beads in universal bottles. The suspensions were homogenized in a Vortex (Genie 2, Scientific Industries, Bohemia, New York) for five minutes. Different concentrations were obtained through serial dilutions and use of a neuber haemocytometer (Hausser, scientific Horsham, USA) as described by Goettel and Inglis (1997).

Table 2.1 Fungal isolates used against the termite *Macrotermes michaelseni* for virulence, repellency and behavioural bioassays under laboratory conditions.

Species isolates	**Origin of isolates of fungi and locality**	**Year of isolation**
Metarhizium anisopliae		
ICIPE 51	Soil, Kitui, Kenya	2005
ICIPE 30	Lepidoptera (*Busseola fusca*), Migori, Kenya	1989
ICIPE 18	Soil, Mbita, Kenya	1989
ICIPE 56	Tree, Nairobi, Kenya	1990
ICIPE 47	Soil, Kitui, Kenya	1990
ICIPE 62	Soil, Kinshasa, DRC	
ICIPE 7	Soil, Matete, DRC	1990
ICIPE 95	Sandfly (*Lutzomyia sp*.) Baringo, Kenya	1996
ICIPE 44	Forest soil, Meru, Kenya	1990
ICIPE 49	Soil, Mt. Kenya, Kenya	
ICIPE60	Soil, Kakelo-Seme, Kenya	1990
ICIPE 20	Soil, Migori, Kenya	1989
ICIPE 21	*Schistocerca gregaria*, Port Sudan, Sudan	1999
ICIPE 41	Soil, Kitui, Kenya	1990
ICIPE 69	Soil, Matete, DRC Congo	1990
Beauveria bassiana		
ICIPE 276	Soil, Mbita, Kenya	2004
ICIPE79	Tick (*Rhipicephalus appendiculatus*), Rusinga Island, Kenya	1996
ICIPE 278	Soil, Kericho, Kenya	2005

2.3.2 Conidial germination tests

Germination tests were performed on all isolates of the two fungi species used in the mortality and repellency tests. The viability of conidia was determined for each isolate by spread-plating 0.1 ml of conidial suspension at 3×10^6 conidia ml^{-1} on Petri dishes containing SDA. Four sterile cover slips (22 × 22 mm) were placed individually on different locations on each plate. The plates (n = 6 per replicate) were sealed with parafilm and incubated at 26 ± 2ºC and 90 ± 5% RH and examined between 15-18 hours under phase contrast microscope. The percentage germination of conidia was determined from 100 spore counts under cover slips at ×40 magnification. Only those conidia with germ tubes with the same length as the diameter of the conidia were designated as having germinated (Plate 2.3).

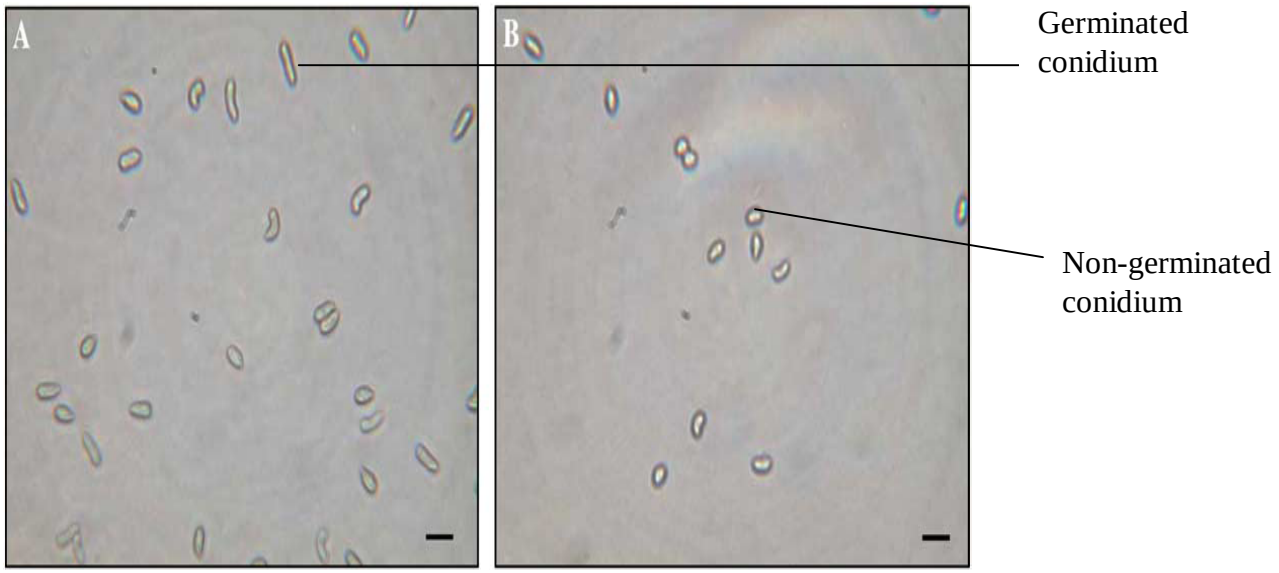
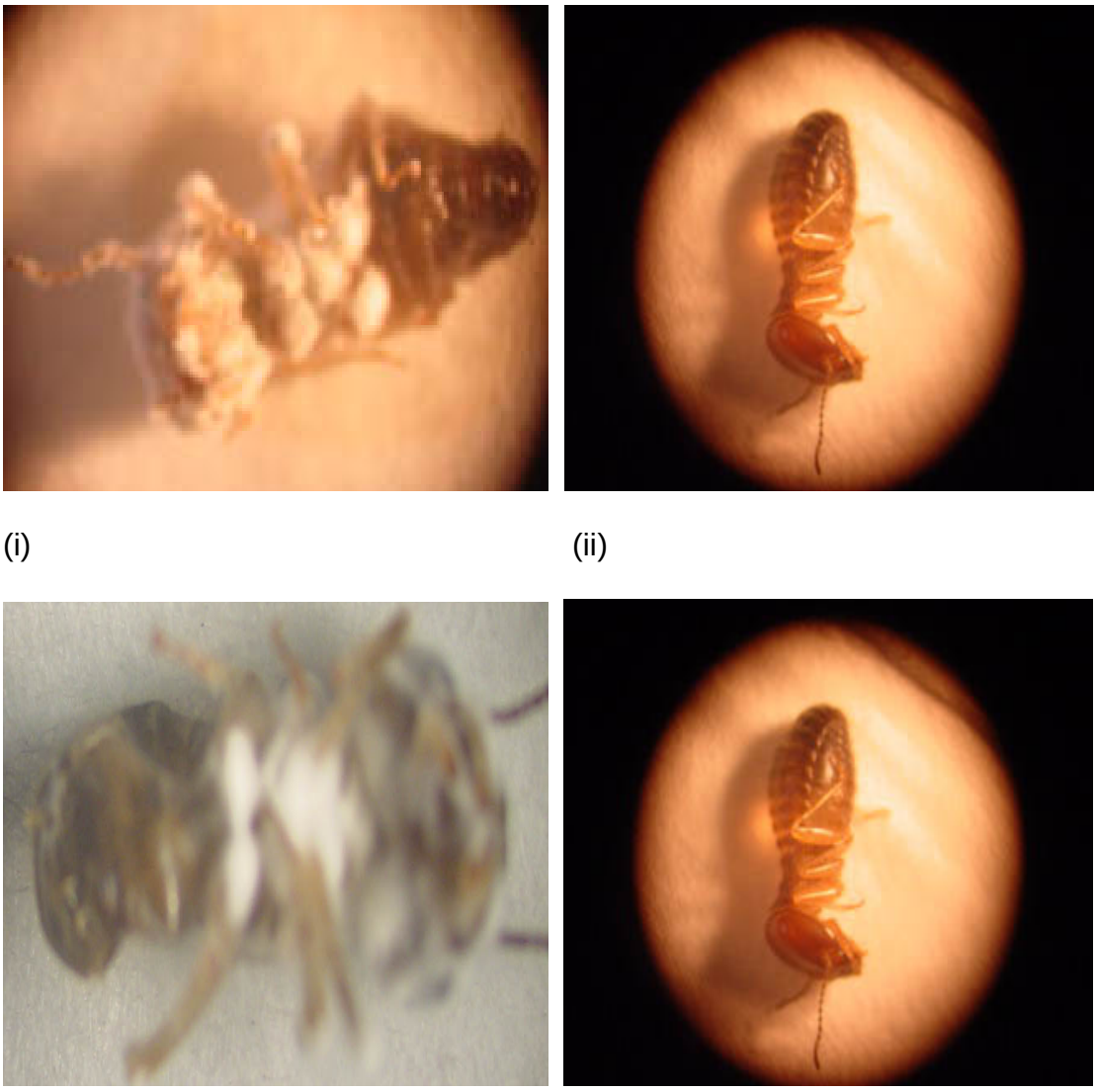


Plate 2. 3 Germinated (A) and non-germinated (B) conidia of *Meterhizium anisopliae* at x 400

2.3.3 Mycoses tests for virulence bioassays

To confirm that mortality in the virulence bioassays was due to fungal infection, the termites cadavers were removed from treatments, surface-sterilized in 1% sodium hypochlorite solution and then in 70% alcohol (Supelco, Sigma Aldrich, UK) for three seconds in each solution and rinsed for three minutes in sterile distilled water. They were then placed onto Petri dishes lined with filter papers (Whatman, 9 cm in diam.), which were then moistened with sterile distilled water. The Petri dishes were then covered with their lids, the edges of which were sealed with Parafilm. This procedure was also followed for control replicates. Mycoses were confirmed by daily microscopic examination of hyphae and spores (Plate 2.4 i and iii) at a magnification of ×400. The tests for time-mortality and dose-mortality relationships were terminated after 14 days post infection.



(i) (ii)

(iii) (iv)

Plate 2.4 Photographs of mycosed cadavers of *Macrotermes michaelseni* by fungi, *Metarhizium anisopliae* (i) and *Beauveria bassiana* (iii) and non-mycosed (controls) (ii, iv) under laboratory conditions.

2.4 Scaled-up production of fungal conidia for repellency bioassays

Dose-repellency bioassays required substantial numbers of dry conidia. This was achieved by growing conidia on long white rice substrates following the technique described by Goettel (1984) and modified by Maniania *et al*. (2003). Two kilograms of rice (Pishori) were soaked in sterilized distilled water for 10 min, rinsed three times and transferred to steel trays (33 cm × 25 cm × 13 cm). The trays were wrapped with polyethylene autoclave bags and sterilized for one hour at 121ºC. The substrates were left to cool at room temperature after which they were inoculated with three-day-old cultures of blastospores (50 ml) and thoroughly mixed for complete coverage of the rice with the inocula. The cultures were incubated in a controlled temperature room (26 ± 2°C and 60-70 % RH). After 21 days, the conidial substrates were allowed to dry overnight at room temperature (22-25°C). The conidia were harvested by sifting the substrate through a sieve (295-µm mesh size) into hazard polyethylene bags, which were then sealed. Conidia were stored in a refrigerator (4-6°C) before use and only up to one month-old dry conidia were used in the repellency bioassays.

2.5 Protocol for olfactometric bioassays

The Worker termites were trapped overnight and the protocol of handling them were similar to the one described above (section 2.1.2). Repellency of the selected isolates towards the termite was evaluated in dual-choice Y-tube olfactometers constructed from glass, each consisting of three compartments, “A”, “B” and “C” (Fig. 2.1). “A” served as the release site of the termites, and “B” and “C” served as source of test treatment or control. Nylon gauze (40 mesh size) was attached with an adhesive to the floor of each

olfactometer to facilitate easy movement of the insects on the floor of the device. A tygon tube (5.3 mm id, 6.35 od) (Supelco, North Harrison Road, Bellefonte, USA) connected to a vent (6 mm in diam.) at the junction facilitated airflow from each compartment to the Y-junction and then to an aspirator. The setup ensured that the air from the three arms did not mix until at the Y-junction. A flowmeter (Cole Parmer, Chicago, USA) connected to the tygon tube helped to regulate the airflow at 5 mls^{-1}. Compartment "A" was illuminated with two florescent bulbs (220 V, 13A, AC). The rest of the olfactometer, including compartments "B and "C", were shielded with a dark cotton cloth (shaded part in Fig. 2.1). The combination of brightness and darkness acted as a 'push-pull' set of visual stimuli to induce the termites to move away from the release area (compartment A) toward the treated and control compartments. The set-up was carried out in a fume hood.

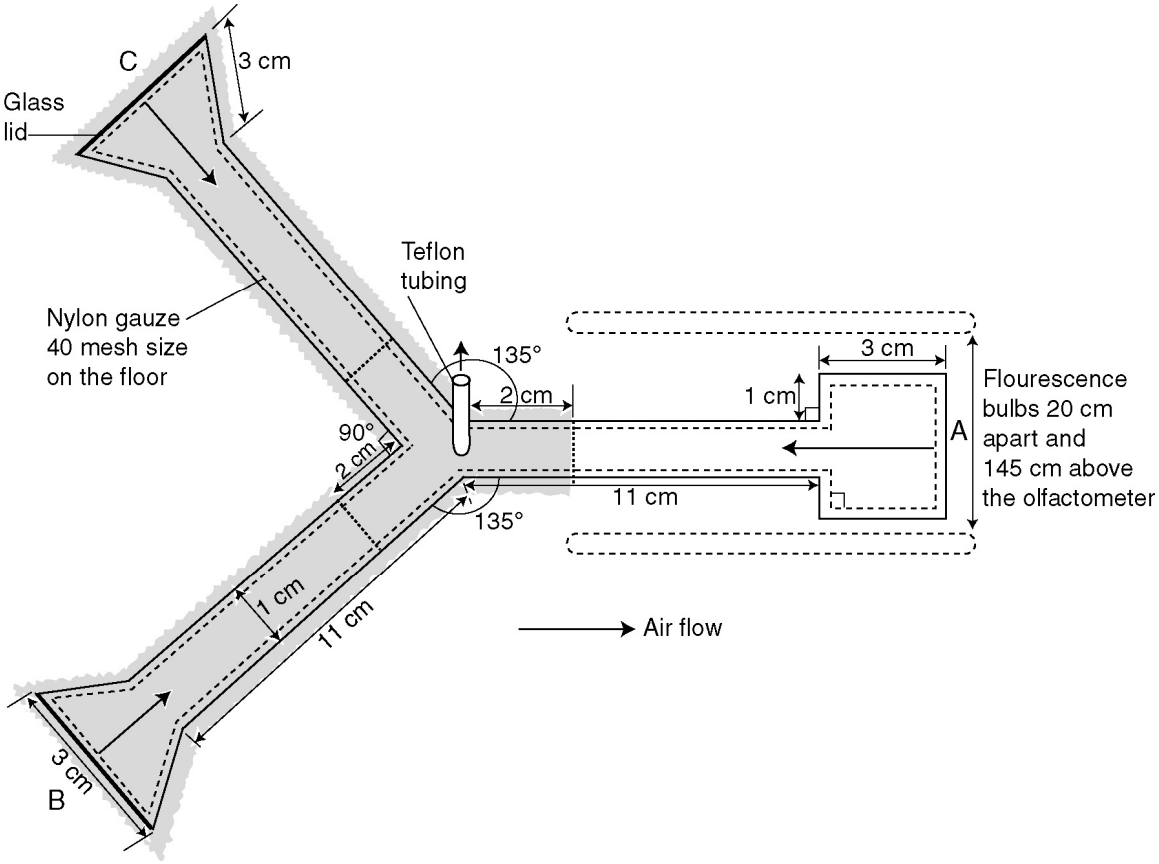


Figure 2.1 Diagram of the specially designed Y-olfactometer used in testing the repellency of the fungal isolates and blends of compounds identified from the isolates and attraction of healthy termites, *Macrotermes michaelseni* towards infected conspecifics. "A", "B" and "C" are the three compartments of the Y-olfactometer.

2.6 Collection of volatiles of isolates of fungi

Dry conidia of the most repellent (ICIPE 51) and the least repellent (ICIPE 69) isolates of *M. anisopliae* and most repellent (ICIPE 276) and least repellent (ICIPE 278) of *B. bassiana* were produced as described in section 2.1.3.1. Germination tests of the conidia were done as described in section 2.1.3.3.2 to test for their viability. Volatiles were collected from two-three weeks old isolates of these fungi. Forty grams of dry conidia of respective isolates were weighed on a balance (Mettler AT 261 Delta, Listers 2000, USA) and put in volatile collection jars (ARS, Gainesvaille, USA) (Plate 2.5) which were then tightly closed with lids.

Head-space volatile emissions from dry conidia of selected isolates were collected in a 3-cm-long filter trap made of Teflon tubes packed with 3mg of Super-Q polymer (80–100 mesh) as an adsorbent (ARS, Gainesvail, USA) held in place between two plugs of glass wool. Each trap was cleaned by flushing 1ml of dichloromethane (HPLC grade, Fluka 99.9 %) through the adsorbent before headspace trapping. Traps were then dried by passing purified nitrogen (BOC gases, Kenya) through each of the traps at a rate of 3 ml/minute and then each was sealed with Teflon thread tape on both ends to prevent contamination. For storage before use, traps were wrapped with dry aluminium foils.

To collect volatiles using the air entrainment system, a continuous flow at 3ml/minute of purified medical air (BOC gases, Kenya) was further cleaned by passing through a carbon filter (ARS, Gainesvail, USA) (Plate 2.5) and ultimately into the volatile collecting jars,

each containing a different fungal isolate. A flowmeter (Aalborg, Orangeburg, NY, USA) regulated airflow into each jar (Plate 2.5). The adsorbent traps were each then firmly held in place by Teflon screw caps that were connected to a Teflon tube at the top of each volatile collecting jar. The volatile collecting jars were sealed with their lids to prevent contamination. Air was sucked from the closed system at a rate of 3 ml/minutes at one end of each of the filter traps through a Teflon tube connected to a diaphragm vacuum pump (Wertheim, Germany) (Plate 2.5). Trapping of the volatile blends was done for 12 hours in the dark.

2.7 Elution of volatiles of isolates of fungi

Traps from section 2.6, were then removed from the system and adsorbent eluted into 2 ml vials (Sigma, Aldrich) with 100 µl of dichloromethane under ice by flushing purified nitrogen through the filter traps (Plate 2.6). The vials with the volatile extract were then tightly corked with their caps and then sealed with Teflon tapes. In gas chromatographic analyses (GC) an equivalent volume of 0.2262 µg of methyl salicylate (Sigma, USA) was added as an internal standard to 40 µl of each sample of ICIPE 69 (*M. anisopliae*), ICIPE 276 (*B. bassiana*) and ICIPE278 (*B. bassiana*) and the amount was doubled for ICIPE 51(*M. anisopliae*). One microliter of each of the sample of the isolates was injected for all the GC and coupled GC- mass spectrometer (GC-MS) anayses as described in chapter five. In the control set-ups, the same protocol as described above was followed with exclusion of the fungus in the volatile collecting jar. To replicate trapping of volatiles, adsorbent traps were thoroughly cleaned as above and re-used. The experiment was replicated four times.

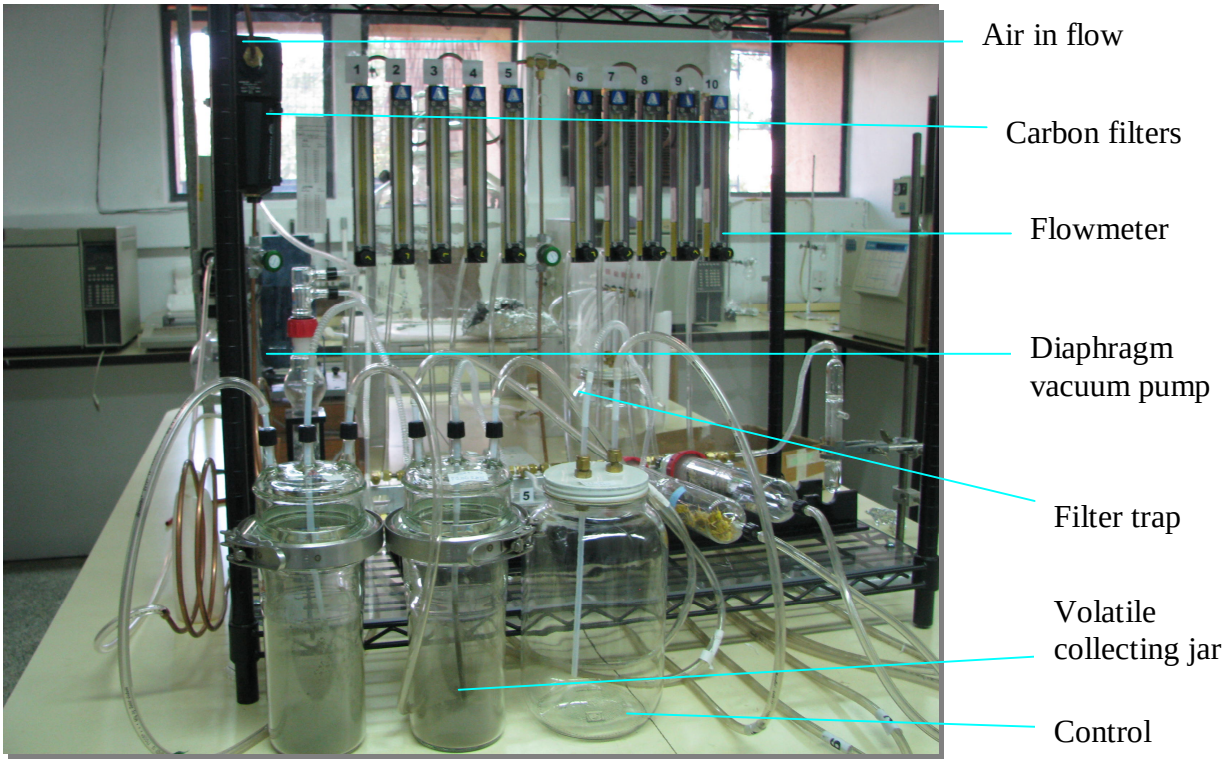


Plate 2.5 Air entrainment system used to collect volatiles from isolates of *Metarhizium anisopliae* (ICIPE 51, ICIPE 69) and *Beauveria bassiana* (ICIPE 276, ICIPE 278).

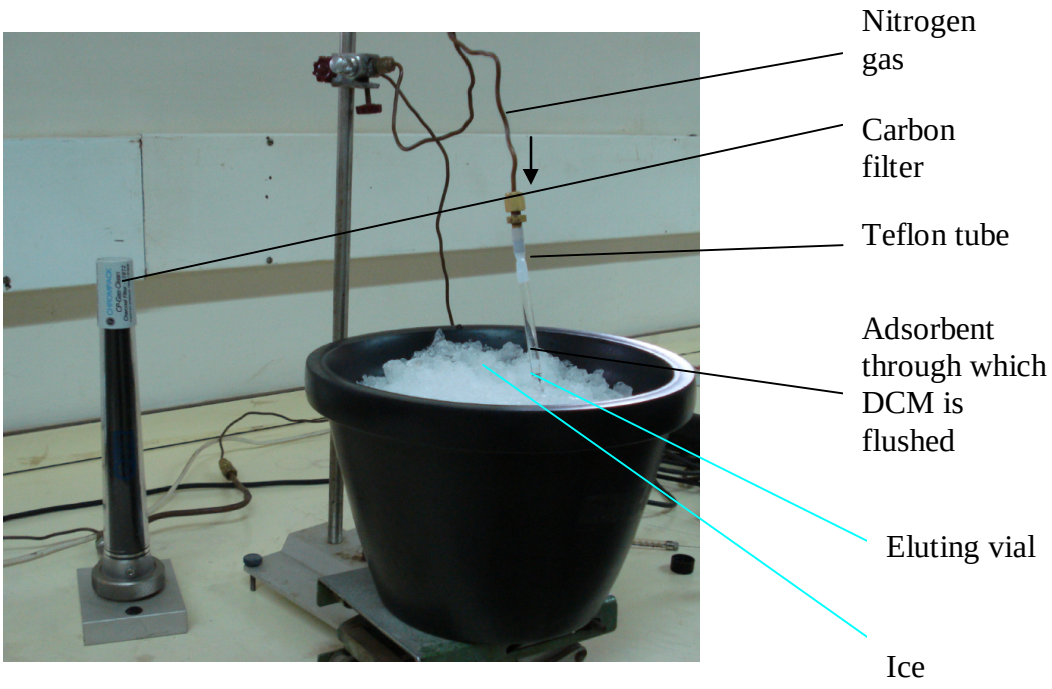


Plate 2.6 A photograph of the set-up used in elution of volatile collections from the filter traps with the adsorbent (Super Q) into vials placed under ice where nitrogen gas was flushed through the filters.

CHAPTER THREE

3.0 PATHOGENICITY OF DIFFERENT ISOLATES OF *METARHIZIUM ANISOPLIAE* AND *BEAUVERIA BASSIANA* TO TERMITE *MACROTERMES MICHAELSENI*

3.1 INTRODUCTION

Termite, *M. michaelseni* live in concealed habitat in mounds and the nest ecology makes the pest unsuitable target for control by application of insecticides. It has been proposed that infectious pathogens such as *M. anisopliae* and *B. bassiana* can potentially spread more easily between group members that live at high densities characterized by frequent social contact as happens in termite, *M. michaelseni* compared to solitary living individuals (Shykoff and Schmid-Hempel, 1991; Cremer *et al*., 2007). In addition, group members are often close "relatives" and thus susceptible to the same pathogenic infections (Cremer *et al*., 2007).

To make progress in the understanding of fungal virulence, and to develop more efficient biopesticides for sustainable management of termites, it is important to study the virulence of fungi in the laboratory under standard conditions. The specific objectives of this study were (i) to screen different isolates of *M. anisopliae* and *B. bassiana* for their time-mortality effects on the termite species and (ii) to carry out mortality-dose responses on selected isolates of these fungi under standard conditions.

3.2 MATERIALS AND METHODS

3.2.1. Study sites, termites, conidial suspensions, germination and mycoses

The laboratory used for the experiments were as described chapter two section 2.1. Female worker castes of *M. michaelseni* that were used for pathogenicity bioassays were trapped overnight and handled using the protocol described in Chapter two (section 2.2). Preparation of conidial suspension of isolates of fungi used for time-mortality responses in this study was carried out using the protocol described in Chapter two (section 2.3.1). Conidial germination and mycoses tests were carried out as described in Chapter two sections 2.3.2 and 2.3.3, respectively.

3.2.2. Initial screening of isolates of fungi for time-mortality responses

Fifteen isolates of *M. anisopliae* and three of *B. bassiana*, which were initially isolated from different substrates (Table 2.1), were obtained from the *icipe*'s culture collection. For each treatment and control groups, six Petri dishes each containing 20 worker termites were used. The control samples were sprayed with sterile distilled water containing 0.05% Triton X-100 (Fluka, Sigma Aldrich, UK) (without conidia) before inoculating conidia onto the termite using a Burgerjon spray tower (Plate 3.1) (Burgerjon, 1956). For treatment groups (20 workers), six Petri dishes per treatment containing termites were altogether sprayed with 10 ml of a standard concentration of 10^7 conidia ml^{-1}. The spray tower was cleaned with 90% alcohol and sterile distilled water between treatments. The experiment was repeated on three different occasions under similar laboratory conditions.

To maintain social cohesion within the group (Sun *et al*., 2003), two soldier termites were also added into each Petri dish after conidial inoculation. A piece of wet cotton wool was used to maintain high humidity in each Petri dish throughout the experiment. The lids of the Petri dishes had five aeration holes (2 mm in diam.) to ensure free flow of air. Two pieces of sterile cypress wood, *Cupressus lusilanica* Dallimore, (approximately 50 × 30 × 1.5 mm) and 0.5 g of fungal garden, *Termitomyces sp.* from the termite mounds were provided as shelter and for food, respectively, after applications of conidia. The fungal gardens were from respective termite mounds. The groups of termites were maintained as described in Chapter two section 2.2. Mortalities were recorded daily for calculation of LT_{50} values (time needed to within 50% mortality during screening) of each of the fungal isolates. Mycoses tests were carried out as described in Chapter two section 2.3.3.

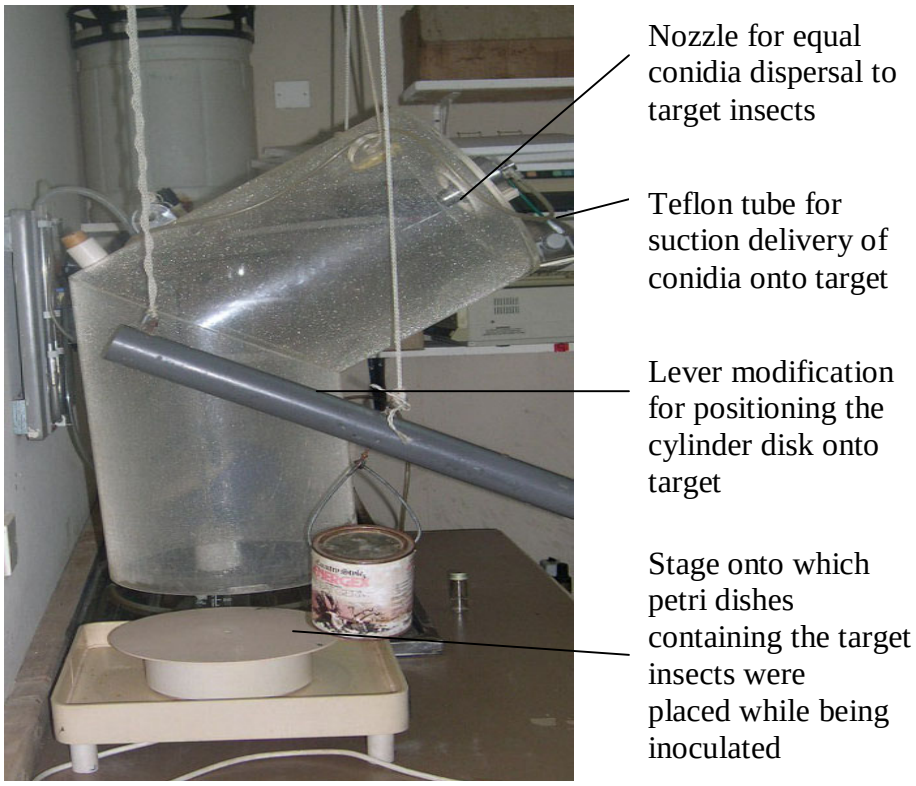


Plate 3.1 Burgerjon spray tower used to spray conidial suspension onto termites

3.2.3. Dose-response mortality experiments

After the initial screening nine isolates of *M. anisopliae* and three of *B. bassiana* were used for dose-response studies. The isolates of *M. anisopliae* were selected on the basis of their LT_{50} values achieved after the initial screening and ranged from most virulent (ICIPE 51, ICIPE 30, and ICIPE 18), moderately virulent (ICIPE 49, ICIPE60, and ICIPE 20), to least virulent (ICIPE 21, ICIPE 41 and, ICIPE 69). For *B. bassiana*, all the three isolates (ICIPE 276, ICIPE 79 and ICIPE 278) were used. Each treatment and control Petri dish contained 20 worker termites. Termite control groups (six replicates) were sprayed before the treatment groups using Burgerjon spray tower as described above. For treatment groups, six conidial concentrations were used (10^5, 3×10^5, 10^6, 3×10^6, 10^7 and 3×10^7 conidia ml^{-1}) for each isolate. The Burgerjon spray tower was cleaned between treatments as described earlier. Ten ml of respective conidial concentration was sprayed onto six replicate Petri dishes at one time. The treatment and control experiments were repeated on three different occasions under similar conditions as described for time-mortality response experiments. Experimental procedure remained similar as described for time-mortality responses (Chapter three section 3.2.2). Cumulative data for four days post-infection were used for analyses of the dose-mortality responses for both treatment and control replicates.

3.3 DATA ANALYSES

In all the tests, data for mortality for the different isolates towards the termite were individually pooled before analyses. Mortality data were corrected for natural mortality in the controls using Abbott's formula (Abbott, 1925) and arcsin-transformed to normalize the data before invoking repeated measures analysis of variance (ANOVA) using Proc Mixed of SAS version 9.1 (SAS Institute, 2003). Means were separated by Student-Newman-Keuls (SNK) test. Lethal time to 50% mortality (LT_{50}) and lethal concentration to 50% mortality (LC_{50}) were estimated with repeated measures logistic regression via generalized estimating equations (GEE) (Throne *et al*., 1995; Stokes *et al*., 2000). These analyses were carried out using GENMOD procedure of SAS version 9.1 (SAS Institute, 2003). The level of significance was set at 5% for all analyses to achieve the respective confidence intervals used to identify significant differences among the values of LT_{50}, and LC_{50} for the different isolates of the fungi.

3.4 RESULTS

3.4.1 Initial screening of isolates of fungi for their virulence on the termite

Results from initial screening of isolates showed that there were no significant differences in mortalities among the treated termites from the three termite mounds ($F_{df\ (2,\ 30)} = 0$, $P = 1$, n = 33, SNK test). Accordingly, in all tests, the mortality data from different mounds were pooled before analyses. Percentage mean mortalities of worker termites caused by isolates of *M. anisopliae* were significantly greater than those caused by isolates of *B. bassiana* at a concentration of 10^7 conidia ml^{-1} ($F_{df\ (17,\ 3366)} = 285.4$, $P = 0.0001$, n = 3564, SNK test). There were significant time effects on mortalities among isolates of the two species of fungi ($F_{df\ (10,\ 3366)} = 5827.8$, $P = 0.0001$, n = 3564, SNK test). There were significant interactions between the fungal treatments and time effects on mortalities of termite groups at a concentration of 10^7 conidia ml^{-1} ($F_{df\ (170,\ 3366)} = 39.6$, $P = 0.0001$, n = 3564, SNK test). The variations in LT_{50} values of isolates of *M. anisopliae* (n=15) and *B. bassiana* (n=3) are shown in Table 3.1. There were significant differences in mycoses among isolates of the two fungi ($F_{df\ (17,\ 306)} = 9.3$, P = 0.0001, n = 324). Percentage mean mortalities in the control groups were significantly less than in all the fungal treatments ($P<0.05$, SNK test) (Table 3.1). Among the control replicates the percentage mean mortalities were not significantly different ($F_{df\ (2,\ 15)} = 3.6$, $P > 0.035$, n = 18, SNK test).

Table 3.1 Lethal Time (LT_{50}) values of time-mortality responses of various isolates of *Metarhizium anisopliae* and *Beauveria bassiana* against *Macrotermes michalseni* at a concentration of 10^7 conidia/ ml^{-1}.

Isolates of fungi	***% mortality (Mean ± SE)***	***% mycoses on cadavers***	***LT_{50} values/CLs***	***Slope ± SE***
Control	13.1 ± 0.8	0 ± 0		
M. anisopliae				
ICIPE 51	85.4 ± 2.3^{a}	99.4 ± 0.4^{a}	2.0^{a} (2-2.1)	7.1 ± 0.3
ICIPE 30	84.6 ± 2.3^{ab}	98.6 ± 0.5^{a}	2.1^{b} (2.1-2.2)	7.2 ± 0.4
ICIPE 18	83.1 ± 2.4^{bc}	97.8 ± 1^{ab}	2.3^{c} (2.2-2.4)	7.7 ± 0.2
ICIPE 56	82.6 ± 2.4^{bcd}	97.8 ± 1.1^{ab}	2.3^{c} (2.2-2.4	6.2 ± 0.2
ICIPE 47	81.7 ± 2.5^{cde}	96.7 ± 1.2^{ab}	2.4^{c} (2.3-2.5)	5.8 ± 0.3
ICIPE 62	80.8 ± 2.5^{def}	94.4 ± 1.3^{abc}	2.5^{c} (2.4-2.6)	6.9 ± 0.2
ICIPE 7	80 ± 2.5^{edf}	93.1 ± 1.4^{abcd}	2.5^{c} (2.4-2.7)	5.8 ± 0.3
ICIPE 95	79.7 ± 2.5^{ef}	91.7 ± 1.5^{bcd}	2.6^{cd} (2.5-2.7)	6.8 ± 0.1
ICIPE 44	78.8 ± 2.6^{ef}	91.7 ± 1.1^{bcd}	2.6^{cd} (2.6-2.8)	6.7 ± 0.5
ICIPE 49	77.1 ± 2.7^{fg}	90.8 ± 1.7^{cde}	2.8^{cd} (2.7-3)	6.1 ± 0.4
ICIPE60	77 ± 2.7^{gh}	90.8 ± 1.2^{de}	2.9^{cd} (2.7-3.2)	5.6 ± 0.6
ICIPE 20	76.2 ± 2.7^{gh}	90.3 ± 1.6^{de}	2.9^{cd} (2.9-3)	7.5 ± 0.2
ICIPE 21	70.6 ± 3^{i}	90.3 ± 1.5^{de}	3.6^{e} (3.2-4)	9 ± 0.5
ICIPE 41	70.2 ± 2.^{8i}	90.3 ± 1.2^{de}	4.1^{e} (3.9-4.3)	7.3 ± 0.4
ICIPE 69	65.5 ± 3.1^{j}	90 ± 1.6^{de}	4.4^{e} (4.6-4.6)	4.2 ± 0.9
B. bassiana				
ICIPE 276	60.5 ± 3^{k}	89.7 ± 1.6^{de}	4.5^{e} (4.1-4.9)	4.3 ± 0.3
ICIPE79	60.4 ± 2.9^{k}	88.6 ± 1.3^{de}	4.6^{f} (4.5-4.4.7)	5.8 ± 0.3
ICIPE 278	47.7 ± 2.3^{l}	85.8 ± 1.4^{e}	5.8^{g} (5.5-6.2)	4.4 ± 0.3

Percentage mortality (mean ± SE) and percentage mycoses (mean ± SE) followed with the same letters within a column are not significantly different (α = 0.05, p = 0.0001, SNK test). LT_{50} values followed by the same letters within a column are not significantly different (Proc GENMOD, α = 0.05, p = 0.0001, SNK test,). 95% confidence limits of the LT_{50} values are shown in brackets in the column labeled LT_{50}. Failure of 95% confidence limits to overlap was used as the criteria for identifying significant differences among

LT50 values (Proc GENMOD and the significance was also tested (α = 0.05, p = 0.0001, SNK test).

3.4.2. Dose-mortality responses

There were significant differences in percentage mean mortalities among the treatment groups ($F_{df\ (5,\ 1224)}$ = 540.3, P = 0.0001, n = 1296, SNK test) of different isolates of the two fungi. The mean percentage mortalities among the termite groups were directly proportional to the conidial concentrations and differed significantly among fungal isolates ($F_{df\ (5,\ 1224)}$ = 646.9, P = 0.0001, n = 1296, SNK test) (Table 3.1). There were significant interactions between the treatment groups and the concentrations of conidia ($F_{df\ (55,\ 1224)}$ = 7.8, P = 0.0001, n = 1296, SNK test) on the mortalities of the termite groups. The variations among the nine LC_{50} values of isolates of *M. anisopliae* and the three of *B. bassiana* are shown on Table 3.2. Percentage mean mortality in the control groups (13.1 ± 1.1, mean ± SE) were not significantly different among one another ($F_{df\ (2,\ 15)}$ = 4.3, n=18, P = 0.33, SNK test) but were significantly less than in all the fungal treated termite groups (P < 0.05, SNK test) (Table 3.2). Percentage mean mortalities in time-mortality and dose-mortality control groups were also not significantly different from one another ($F_{df\ (1,\ 34)}$ = 1.6, n = 36, P = 0.22, SNK test).

Table 3.2 Lethal concentration (LC_{50}) values of dose-mortality responses of various isolates of *Metarhizium anisopliae* and *Beauveria bassiana* against *Macrotermes michaelseni*.

Isolates of fungi	***LC_{50} Values***	***95% Confidence limits***	***Slope ± SE***
M. anisopliae	**(conidia ml^{-1})**		
ICIPE 51	$1.5 \times 10^{4\,a}$	$(1.0\text{-}2.1)10^4$	1.1 ± 0.06
ICIPE 30	$1.9 \times 10^{4\,a}$	$(1.4\text{-}2.7)10^4$	0.6 ± 0.03
ICIPE 18	$3.9 \times 10^{4\,b}$	$(2.7\text{-}5.5)10^4$	0.6 ± 0.05
ICIPE 49	$7.9 \times 10^{4\,c}$	$(6.3\text{-}10)10^4$	0.7 ± 0.03
ICIPE 60	$11.8 \times 10^{4\,c}$	$(10.3\text{-}13.6)10^4$	0.6 ± 0.03
ICIPE 20	$12.7 \times 10^{4\,cd}$	$(10.5\text{-}16.6)10^4$	0.5 ± 0.03
ICIPE 21	15.4×10^{4cd}	$(10.6\text{-}23.3)10^4$	0.4 ± 0.04
ICIPE 41	$46.4 \times 10^{4\,e}$	$(42\text{-}51.9)10^4$	0.5 ± 0.02
ICIPE 69	149.3×10^{4f}	$(111.5\text{-}199.8)10^4$	0.4 ± 0.03
B. bassiana			
ICIPE 276	$240.0\times 10^{4\,g}$	$(206.1\text{-}279.5)10^4$	0.3 ± 0.01
ICIPE79	$591.1 \times 10^{4\,h}$	$(451.2\text{-}774.5)10^4$	0.3 ± 0.01
ICIPE 278	1140.9×10^{4i}	$(932.8\text{-}94.5)10^4$	0.3 ± 0.01

LC_{50} values followed by the same letter within a column are not significantly different (Proc GENMOD, α = 0.05, p = 0.0001, SNK test,). Failure of 95% confidence limits to overlap was used as the criteria for identifying significant differences among LC_{50} and were tested for significance (α = 0.05, p = 0.0001, SNK test). The dose-mortality response data were pooled for four days post infection.

3.5 DISCUSSION

All the isolates of *M. anisopliae* and *B. bassiana* were pathogenic to the termite, *M. michaelseni*. Different isolates of the two species of fungi had different pathogenicity levels in terms of time-mortality responses on the worker termite under standard conditions (Table 3.1). After screening, the pathogenicity of the nine selected isolates of *M. anisopliae* showed mortality-dose responses as illustrated in Table 3.2. These show that the two fungi species have the capacity to inflict damage to the termites, particularly the more pathogenic isolates. This is evident from successful germination of the germ tubes of the conidia of different isolates of the two fungal species and their subsequent penetration into the insect integuments into the haemocoel (Plate 2.4 i and iii). The resultant effect was the death of the infected individual worker termites. Of particular interest are the varying levels of pathogenicity of the different fungal isolates to the termite, *M. michaelseni*. The different hosts from which they were isolated may account for this differential pathogenicity, which may have arisen because of the different demands of different integuments during evolutionary interactions with different insects (Hajek and St. Leger, 1994).

The use of entomopathogenic fungi in the control and management of pests of agricultural importance (Ferron, 1978) and disease vectors has long been appreciated (Maniania *et al.*, 1994; Maniania, 1998). Results on time-mortality and dose-mortality responses obtained in this study colloborate reports that many termite species are susceptible to different isolates of *M. anisopliae* and *B. bassiana* with varying levels of

infections under laboratory conditions. For example, worker termite, *Reticulitermes flavipes* Kollar (Isoptera: Rhinotermitidae) died within one to three days post-exposure to a strain of *B. bassiana* isolated from *R. flavipes* workers collected from a street tree in Toronto, Canada (Zoberi and Grace, 1990). A strain of *M. anisopliae* in china has been reported to cause 100% mortality to termite, *Odontotermes formosanus* Shiraki (Isoptera: Termitidae) at a concentration of 3×10^8 conidia ml^{-1} under laboratory conditions (Dong *et al*., 2007). Laboratory evaluation of the fungus, *M. anisopliae* against *Odontotermes guptai* Roonwal and Bose (Isoptera: Termitidae) resulted to LC_{50} of 86.84 hours at 2×10^7 conidia/g soil (Swaran and Varma, 2003). Sun *et al*. (2002) evaluated the sporulation of 22 isolates of *M. anisopliae* and *B. bassiana* on cadavers of *C. formosanus* 11 days post-death and found out that isolates of *B. bassiana* could be categorized into groups with high total sporulation (11 days) and low quick sporulation on second and third day. However, isolates of *M. anisopliae* fell into a group with high quick sporulation and low total sporulation. The results lead to the conclusion that isolates of *M. anisopliae* have an advantage over *B. bassiana* in termite control due to the termite defensive social behaviour.

In the present study, all the isolates of *M. anisopliae* were more virulent than those of *B. bassina*. A commercial isolate of *M. anisopliae*, DAT F-001 applied to the dorsal surface of termite *Coptotermes acinaciformis* Froggatt (Isoptera: Rhinotermitidae) at 8.1×10^7 spores ml^{-1} resulted to 100% mortality within four days (Rath and Tidbury, 1996). Myles (2002) isolated a virulent strain of *M. anisopliae* from subterranean termite, *R. flavipes*

after “cup tests” with 1,000 termite workers were filled with autoclaved brick sand with a wood stake wrapped in cardboard. Before dying, the termites came to the surface and mycelium gradually grew over their bodies and eventually sporulated into dark green conidiophores. There were variations in time-mortality and dose-mortality responses among the isolates used in the present study, thus indicating different activity spectra of the fungal isolates against the termite species.

The screening of 15 isolates of *M. anisopliae* resulted in additional information of the presence of a lethal isolate of fungus (ICIPE 51) with high leathal time value (LT_{50} = 2 days) to kill 50% of termites, *M. michaelseni*, which had not been tested among termites. The isolate was obtained from a soil of a mound of termite (Table 2.1) suggesting that there could be species of *M. anisopliae* whose potential vilurence towards the termite studied and other termite species remain unknown. The study was mainly focused on the use of *M. anisopliae* towards termites and the three *Beauveria* species, which were included in the bioassays, were stable under laboratory conditions at the time. There is need to carry out further screening of fungal isolates inorder to isolate, culture and maintain perhaps potentially more virulent isolates of *M. anisopliae* and *B. bassiana*. In this study, different time-mortality (LT_{50}) and dose-mortality (LC_{50}) values obtained from the different isolates of fungi enabled the selection of the most pathogenic (ICIPE 51, ICIPE 30 and ICIPE 18), relatively pathogenic (ICIPE 49, ICIPE 60 and ICIPE 20) and the least pathogenic (ICIPE 21, ICIPE 41 and ICIPE 69) isolates of *M. anisopliae*. These isolates were subsequently tested for repellency-dose (RD_{50}) responses towards the

termite in order to correlate the relationship between virulence and repellency (Chapter four). From the results obtained in this study, ICIPE 51 and ICIPE 30 were selected as possible biological control agents that can be developed for use in the framework of IPM programs for the management of the termite, *M. michaelseni*. However, evalution of how these isolates perform in field conditions towards the termite and perhaps other termite species is required.

CHAPTER FOUR

4.0 REPELLENCY OF DIFFERENT ISOLATES OF *METARHIZIUM ANISOPLIAE* AND *BEUVERIA BASSIANA* TOWARDS TERMITES *MACROTERMES MICHAELSENI*

4.1 INTRODUCTION

Insects are among the most successful organisms in the animal kingdom (Chapman, 1998). Their capacity to survive and reproduce depends greatly on their ability to identify and respond selectively to enemy-specific semiochemicals from a heterogeneous environment (Dicke and Grostal, 2001). Chemical and behavioural ecology provide a framework of understanding the origins, functions and importance of natural chemicals mediating interactions between organisms (Pickett *et al*., 1997). Such relationships, often adaptive, comprise the oldest of communication systems in terrestrial environments (Pickett *et al*., 1997). Detection and avoidance of volatile cues of virulent pathogens to social insects such as termite, *M. michaelseni*, play an integral part on the fitness of these social insects in their hemiedaphic habitats. Chemical cues emanating from infective pathogens such as fungi are some of the factors, which could be involved in these interactions. Entomopathogenic fungi such as *M. anisopliae* and *B. bassiana* are some of the most potent biological control agents whose volatile constituents are not well understood.

Possible mediation of olfactory signals in the avoidance of contact with entomopathogenic fungi by termites has been demonstrated in several studies. For example, Kramm *et al*. (1982) found that healthy members of *Reticulitermes virginicus* (Banks) (Isoptera: Rhinotermitidae) did not contact their cadavers infected with *M. anisopliae* suggesting involvement of semiochemical signals. Hänel and Watson (1983) infected Australian termite, *N. exitiosus* with entomopathogenic fungus, *M. anisopliae* by both dusting and spraying in the field near Canberra. These two workers concluded that "unknown factors" were involved in inhibiting complete development of the fungus in nest colony. In the present study, a specially designed choice Y-olfactometer was used to measure the responses of *M. michaelseni* to volatile blends from different isolates of *M. anisopliae* and *B. bassiana* with different levels of virulence. The objective of the study was to (i) determine whether the termite is able to detect a virulent fungus by olfaction and whether it is repelled from a distance and (ii) to find out the relationship between virulence and repellency of the different isolates of the two fungi to the termite studied.

4.2 MATERIALS AND METHODS

3.2.1 Study sites, worker termites, germination, mycoses and production of conidia

The laboratory sites used for the experiments were as described in Chapter two section 2.1. Termites were trapped overnight and the protocol of handling them remained as described in Chapter two (section 2.2). Mass production of conidia of isolates of fungi used in this study followed the protocol described in Chapter two (section 2.4). Conidial germination and mycoses tests were carried out as described in Chapter two sections 2.3.2 and 2.3.3, respectively.

4.2.2 Dose-repellency bioassays

This study was carried out in a fume-hood using a Y-tube olfactometer (Fig. 2.1) described in Chapter two section 2.5. To standardize the bioassay system, a highly virulent isolate of *M. anisopliae* (ICIPE 30) was used to test for repellency against the termite. Steam sterilized sawdust (0.5 g) from cypress wood, *C. lusilanica* dried at 85 ± 1˚C for 1 hr, was mixed with varying amounts of dry conidia (0.005, 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 g) held in folded aluminium foil and placed in treatment compartment ("B"). The other compartment ("C") functioned as the control with aluminium foil containing only sterilized sawdust (also 0.5 g). Folded foil wrapping served to prevent direct physical contact of test materials by the test temites but allowed diffusion of volatiles through the folds into the compartments. Glass lids were loosely placed at the openings of all the three compartments of the olfactometer to avoid escape of termites, while facilitating airflow into the olfactometer. Groups of worker termites (20) were put in compartment "A". The number of termites in the treated and control compartments together with those in their respective arms were recorded at an interval of 10 minutes for 90 minutes to give nine readings for each replicate. During scoring of the data, the edge of the black cloth at the two compartments was briefly (< 30 seconds) lifted to avoid extended exposure of the termites to light. Termites that did not move beyond the 2cm mark after the Y-junction were treated as non-responders.

The assay was replicated ten times. The treatment and control arms were swapped after every replicate to eliminate any asymmetric bias of either the olfactometer or the

surroundings. The above procedure was followed to determine the repellency-dose (RD_{50}) values of different amounts of dry conidia (0.0125, 0.025, 0.05, 0.1 and 0.2 g) of all the nine isolates of *M. anisopliae* and the three of *B. bassiana*. Each dose of each isolate was replicated twelve times. For the control assay, both arms of the olfactometer were untreated. After each replicate test, the olfactometer was sterilized with 70% alcohol to kill the conidia. Pure acetone was used to rinse the olfactometers to eliminate any possible conidial odours. The device was then thoroughly washed with residue-free liquid soap detergent in sterilized distilled water and dried in an oven (45^{o}C).

4.3 DATA ANALYSES

Repellency to the termite was calculated using the formula:

$$\frac{P_{nc} - P_{nt}}{P_{nc} + P_{nt}} \times 100$$

where P_{nc} and P_{nt} represent the average percentage of worker termites in control and treatment arms, respectively (Wanzala *et al*., 2004). In all the tests, data for repellency were individually pooled before analyses, arcsin transformed to normality before invoking repeated measures analysis of variance (ANOVA) using Proc Mixed of SAS version 9.1 (SAS Institute, 2003). Means were separated by Student-Newman-Keuls (SNK) test.

The repellency doses to 50% (RD_{50}) were estimated with repeated measures logistic regression via generalized estimating equations (GEE) (Throne *et al*., 1995; Stokes *et al*., 2000). These analyses were carried out using GENMOD procedure of SAS version 9.1 (SAS Institute, 2003). The relationship between virulence and repellency of the fungal isolates towards the termite was established through non-linear regression analysis. The level of significance was set at 5% for all analyses to identify significant differences among the values of LT_{50}, LC_{50} and RD_{50} for the different isolates of the fungi.

4.4 RESULTS

4.4.1 Repellency-dose responses

In the push-pull olfactometer set-up, worker termites showed a propensity to move towards the control arm (fungus-free) of the olfactometer. Those that entered into the arm of the compartment that contained conidia of different isolates did not move close to the odour sources and with higher doses of the more virulent fungi, they rapidly retreated from the arm. Results of the initial assay with a highly virulent isolate (ICIPE 30) and least virulent (ICIPE 69) indicated clear dose-response relationships ($r = 0.98$, $R^2 = 0.99$, $F_{df\ (8, 81)} = 22$, $P = 0.0001$, n = 90, SNK test) and ($r = 0.99$, $R^2 = 0.99$, $F_{df\ (8, 81)} = 22$, $P = 0.0001$, n = 90, SNK test), respectively (Fig. 4.1). There also were significant differences in repellency between isolates ($F_{df\ (11, 6420)} = 504.2$, $P = 0.0001$, n = 6480, SNK test) and between the doses of conidia of a given isolate ($F_{df\ (4, 6420)} = 574.7$, $P = 0.0001$, n = 6480, SNK test). There were significant interaction effects between isolates and doses on repellency of the fungi against the termite groups (in Petri dishes) ($F_{df\ (44, 6420)} = 6.6$, $P < 0.0001$, n = 6480, SNK test). The repellency indices of *M. anisopliae* and *B. bassiana* as measured by RD_{50} values are shown in Table 4.1.

4.4.2 Correlation between virulence and repellency

There was a strong positive correlation ($r = 0.919$, $R^2 = 0.85$, $P = 0.0001$) between virulence and repellency of the nine isolates of *M. anisopliae* (Fig. 4.2). A similar relationship ($r = 0.99$, $R^2 = 0.99$, $P = 0.0001$) was found for isolates of *B. bassiana*. Both

virulence and repellency of the isolates of *M. anisopliae* were significantly greater than those of *B. bassiana* ($P = 0.0001$, SNK test).

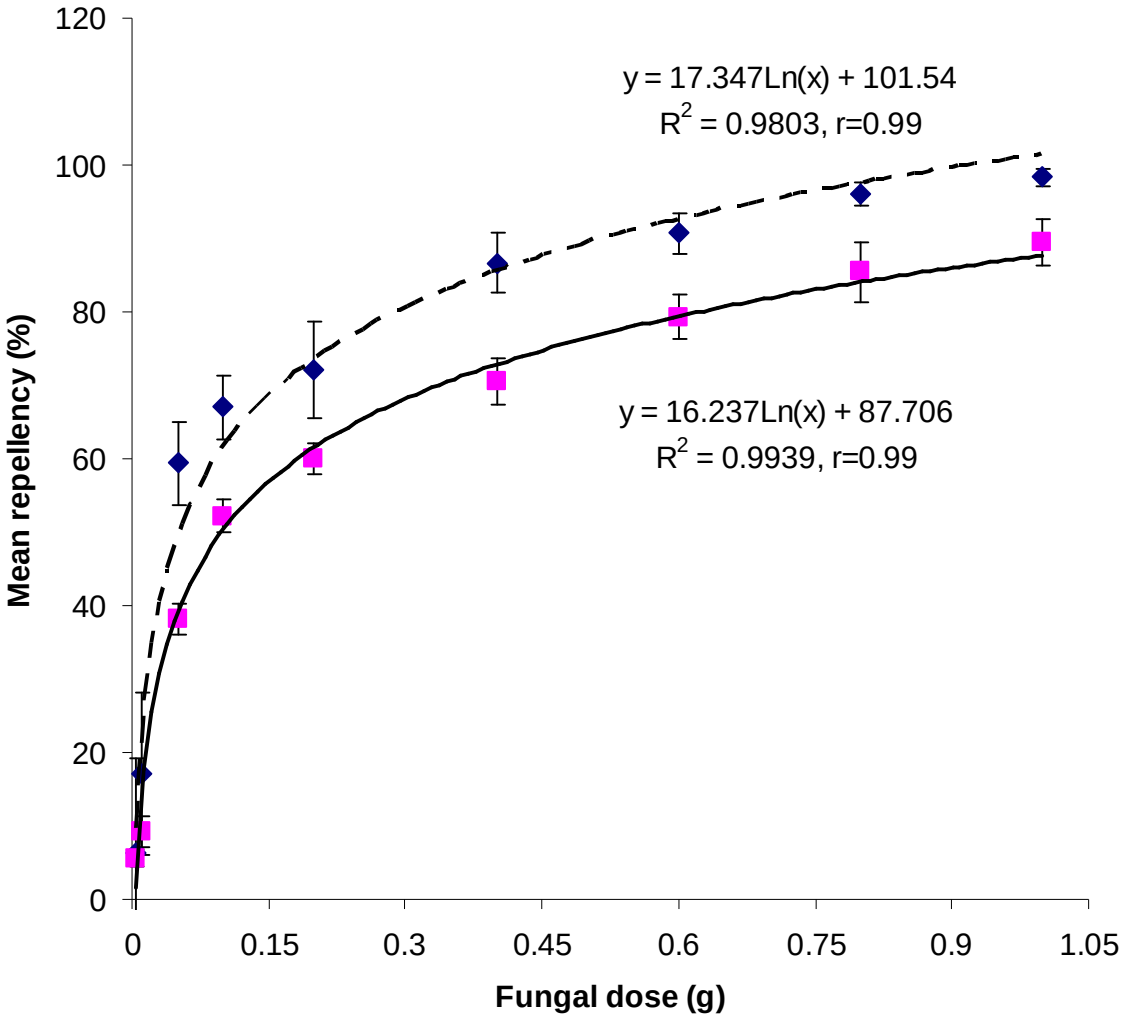


Figure 4.1 Mean repellency (± SE) of highly repellent and least repellent isolates of *Metarhizium anisopliae*, ICIPE 30 (---♦---) and ICIPE 69 ---■---, respectively, at different doses against *Macrotermes michaelseni*. Mean repellency values of *Metarhizium anisopliae* were significantly greater (α = 0.05, P = 0.0001, SNK test) than those of *Beauveria bassiana*.

Table 4.1 The RD_{50} values of repellency-dose responses of various strains of *Metarhizium anisopliae* and *Beauveria bassiana* against *Macrotermes michaelseni* when exposed to varying doses of dry conidia (conidia g^{-1}).

Isolates of fungi	Repellency dose (RD_{50}, g)	95% Confidence limits	Slope ± SE
M. anisopliae			
ICIPE 51	1.9×10^{-2} [a]	$(1.7 – 2.1) \times 10^{-2}$	0.7 ± 0.04
ICIPE 30	2.2×10^{-2} [a]	$(2 – 2.4) \times 10^{-2}$	0.6 ± 0.03
ICIPE 18	3.2×10^{-2} [b]	$(2.9 – 3.7) \times 10^{-2}$	0.6 ± 0.03
ICIPE 49	5.2×10^{-2} [c]	$(4.9 – 5.6) \times 10^{-2}$	0.5 ± 0.04
ICIPE 60	8.2×10^{-2} [d]	$(7.4– 9.2) \times 10^{-2}$	0.5 ± 0.02
ICIPE 20	9.3×10^{-2} [e]	$(8.8 – 9.8) \times 10^{-2}$	0.4 ± 0.01
ICIPE 21	20.7×10^{-2} [f]	$(16.9 – 25.3) \times 10^{-2}$	0.5 ± 0.03
ICIPE 41	37.4×10^{-2} [g]	$(31.1 – 45) \times 10^{-2}$	0.4 ± 0.04
ICIPE 69	57.3×10^{-2} [h]	$(48. – 68.4) \times 10^{-2}$	0.4 ± 0.02
B. bassiana			
ICIPE 276	101.86×10^{-2} [i]	$(80.9 - 128.2) \times 10^{-2}$	0.5 ± 0.03
ICIPE 79	102.19×10^{-2} [i]	$(83.4- 125.2) \times 10^{-2}$	0.6 ± 0.03
ICIPE 278	140.07×10^{-2} [j]	$(108.8- 180.4) \times 10^{-2}$	0.6 ± 0.03

RD_{50} values followed by the same letter within a column are not significantly different (Proc GENMOD, $P > 0.05$, $\alpha = 0.05$, $p = 0.0001$, SNK test). Failure of 95% confidence limits to overlap was used as the criteria for identifying significant differences among RD_{50} and were tested for significance ($\alpha = 0.05$, $p = 0.0001$, SNK test,). The dose-mortality response data were for four days post infection.

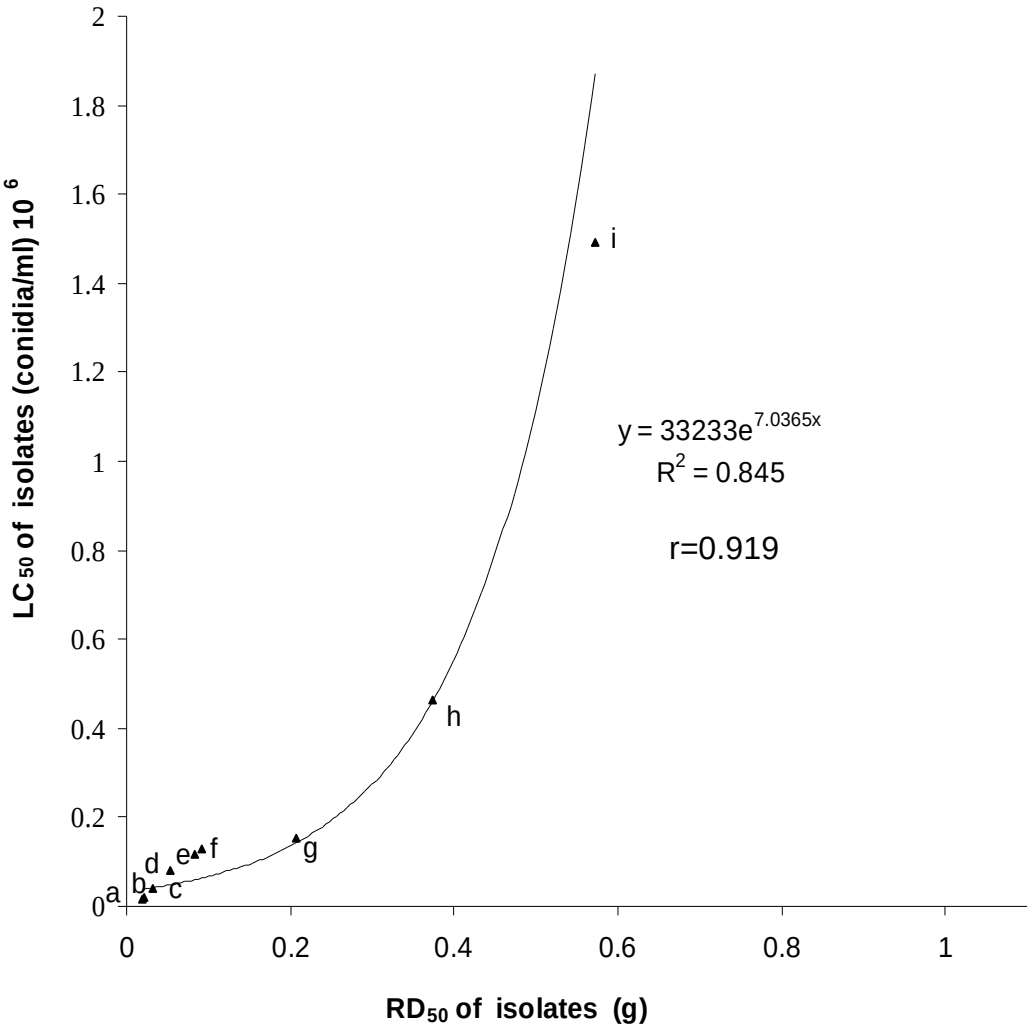


Figure 4.2 Relationship between virulence (LC_{50}, conidia/ml) and repellency (RD_{50}, g) of *Metarhizium anisopliae*, towards the termite *Macroterms michaelseni*. Fungal isolates: a, (ICIPE 51), b, (ICIPE 30), c, (ICIPE 18), d, (ICIPE 49), e, (ICIPE 60), f, (ICIPE 20), g, (ICIPE 21), h, (ICIPE 41), and i (ICIPE 69).

4.5 DISCUSSION

In the current assays, termites that entered the arm of the olfactometer attached to the compartment with higher doses of more virulent isolates came to a standstill some distance away from the odour sources before retreating. This shows that the termite has capacity to detect the potentially harmful concentrations of fungi by olfaction and avoid direct physical contact. The flow rate of air deployed in the olfactometer was 5 ml min^{-1} and there was no evidence of conidia being drawn from the folded aluminium foils into the treatment arm upwind. This was confirmed by determining the weights of the foils with conidia before and after each assay, which showed no loss in weight. In addition, close microscopic inspection at × 400 magnifications on the test termites after the bioassays, showed no evidence of contamination with conidia.

Previously, Staples and Milner (2000) tested the effect of conidia of different isolates of *M. anisopliae* incorporated into sand placed at the base of a 50ml agar tube on the degree of tunneling by *C. lacteus.* In the presence of some highly virulent isolates of the fungus, the insect retreated from the substrate after the initial contact with the infected sand and sealed off the tunnels thus preventing further contact. However, their experimental set-up did not clearly differentiate between avoidance behaviour resulting from contact and that emanating from olfaction, which may account for the failure by termites in some experiments to demonstrate evasive behaviour. The Y-olfactometer set-up in the present study, confirms the mediation of volatile signals emanating from the fungi in the response

behaviour of the insect. Olfactory detection and avoidance of contact with the potentially infective fungi may have been an important evolutionary trait in *M. michaelseni* and probably other termite species to counter the challenges of semi-edaphic habitats. When infection does occur to members of some termite species, several other behavioural mechanisms have been shown to limit possible social exchanges of infectious conidia within the community. These mechanisms include increased rate of mutual grooming (Rosengaus *et al*., 1998), walling off infected areas of a colony (Milner and Staples, 1996) and removal of the diseased individuals from nests (Traniello *et al*. 2002). There is need to test, which, if any, of these mechanisms also operate in *M. michaelseni* as part of its defensive repertoire against fungal infections.

The differential repellency of fungi suggest qualitative and/or quantitative variations in the composition of volatile blends emitted by different strains. These may be used as signatures of communicatin by termites to detect and avoid pathogenic strains and warn con-specifics of the presence of potent risks. From the foregoing, there arose the need to evaluate the composition of these volatiles using gas chromatographic analyses (GC) and coupled gas chromatogram mass-spectroscopy (GC-MS) and bioassays to identify and confirm, respectively the active constituents of the volatiles. Olfactometer assays of these compounds individually and in mixtures would aid in the identification of those constituents that contribute to the repellency of the natural repellent blends. Differences in proportions of the different components in blends may also account for the differences

in the repellency of the different fungal isolates. These are reported in Chapter five described below.

In the olfactometer set-ups, the volatile blends are supposedly concentrated since the flow of air suction is directed to the oncoming groups of termites in the enclosed push pull system. In the field, it remains unknown, however, whether these activity-patterns of repellency could persist where odour intensity varies rapidly and unpredictably as it often occurs in nature. In the field, even if enormous dilutions occur due to open space it is expected that repellency may still be high close to the source. Thus, close to the fungi a sharp gradient of the repellent blends can be expected, which could guide the termites to avoid direct contact. Study to explore the behaviour of the insect in such situations needs to be undertaken.

Defensive adaptations of the termite to the conidia of *M. anisopliae* and *B. bassiana* present a serious constraint to the successful utilization of these fungi as mycoinsecticides. It may be possible to adjust the conidia dosage level below alarm thresholds. It may also be possible to overcome the repellency by incorporating conidia with attractants or a proprietary masking compound in a bait (Rath and Tidbury, 1996). While the strong repellency of conidia of fungi may pose problems for inducing colony pathogenesis, this repellency might be exploited as an anti-termite agent for wood preservation (Myles, 1994). Highly repellent conidia may also be incorporated as

additives in synthetic building products such as rigid foam boards, which are highly susceptible to termite damage (Myles, 1992).

The results of repellency-dose responses in the present study have made it clear that termite, *M. michaelseni* and perhaps other termite species detect and avoid conidia of infective fungi such as *M. anisopliae* and *B. bassiana* through olfactory responses. The objectives of the study validate the hypothesis that there is a relationship between pathogenicity and repellency between different isolates of *M. anisopliae* as well as *B. bassiana* with different infectivity levels to the termite. Follow-up experiments on the efficacy of fungi against termites in the field are needed before their full potential, as 'mycorepellent' agent can be determined.

CHAPTER FIVE

5.0 IDENTIFICATION OF REPELLENT CONSTITUENTS OF ISOLATES OF *METARHIZIUM ANISOPLIAE* AND *BEAUVERIA BASSIANA* AGAINST TERMITE *MACROTERMES MICHAELSENI*

5.1 INTRODUCTION

Chemical cues emitted by respective host plants have shown to be of paramount importance in the olfactory system in insects as they forage for their food resources (Bernays and Chapman, 1994; Angioy *et al*., 2003). Consequently, the identification of the particular tpes and amounts of volatile chemicals emitted by host-plant tissue used by insects as guide for foraging play a critical role in understanding the patterns of interactions between the insects and food resources (Piñero and Dorn, 2007). This may helps in development of more efficient monitoring and/or control tactics (Cook *et al*., 2007). Blends of volatiles compounds emitted by host-plants are known to mediate the attraction of gravid female herbivores to oviposition sites, but in most cases, the role and the identity of individual odour components are not known (Piñero and Dorn, 2007). For instance, evidence suggests that mixtures of host plant-derived compounds are required to elicit appropriate levels of response in adult moths, *Cydia* (*Grapholita*) *molesta* Busck (Lepidoptera: Tortricidae) (Natale *et al*., 2003; Tasin *et al*., 2006).

Chemical cues emitted by the different isolates of fungi were previously shown to play significant role in eliciting avoidance behaviour in the termite, *M. michaelseni* (Chapter

four). Consequently, identification of the particular types and amounts of volatile chemicals emitted by isolates of fungi that the termite detect as signatures for avoidance of risks is crucial for a better understanding of termite-fungi interactions. The first step in identifying semiochemicals that elicit a behavioural response in a given insect is the observation of the behaviour that appears to be mediated by the chemicals in an appropriate bioassay (Wyatt, 2006). A key feature of any bioassay is that it should be a reliable measure of the behaviour ultimately to be assessed. Chemical cues provide possible spatial pieces of information unlike visual cues (Wyatt, 2006). The hypothesis that volatile constituents from isolates of *M. anisopliae* and *B. bassiana* elicit olfactory response in the termite, *M. michaelseni* can only be supported if the identities of these constituents are known. Subsequently, identification of these compounds would facilitate bioassays of the different chemical mixtures in blends from the different isolates of these fungi. GC-MS has been widely used in the identification of chemical compounds (Dean and Heinrich, 1984). Although identification of some Volatile Organic Compounds (VOCs) from fungi has been achieved (Fischer, 2000; Filer *et al*., 2001), to date, knowledge on the identity of possible volatile organic constituents released by *M. anisopliae* and *B. bassiana* have been limited. Possible interactions between the termite and potent major and minor components of the VOCs from these fungi have not been previously reported.

Thus, there is need to improve our knowledge and understanding of the chemistry of the possible constituents of VOCs emitted from the isolates of *M. anisophliae* and *B.*

bassiana and their role in mediating avoidance behaviour of the insect. In this study, the hypotheses were that the repellency of the fungal isolates to the termite results from blends of some major and minor components of the volatile emissions and that there are qualitative and/or quantitative differences between the isolates. The objectives of the study were to test validity of these hypotheses leading to the identification of natural repellent components from the most and the least repellent isolates of *M. anisophliae* and *B. bassiana* that had been tested in olfactometric assays. Incorporation of these compounds in novel termite control and management strategies, for example in "push" or "push pull" tactics, cannot be feasible without the understanding of their nature under laboratory conditions prior to their evaluation in the field.

5.2 MATERIALS AND METHODS

5.2.1 Study area, conidial mass production and collection of volatiles from fungi

The study sites remained as described in Chapter two section 2.1. The protocol of scaled-up production of conidia and collection of volatiles from isolates of fungi were as described in Chapter two sections 2.4 and 2.6, respectively.

5.2.2 Gas chromatography (GC)

For gas chromatographic analyses of the volatile collections, a Hewlett-Packard HP5890 A Series II GC (Plate 5.1) equipped with a Flame Ionization Detector (FID) and an autosampler injector HP 7673 were used to separate the eluted volatile collections. The GC was interfaced to a HP computer monitor (Dell Optiplex X 520) (Plate 5.1) via 3365 MSD ChemStation software (G1701EA E.02.00.493), on the screen of which the chromatograms of each analysed blend appeared. A HP1 methyl silicone non-polar capillary column measuring 30 m (Length) x 0.25 mm (internal diam.) and 0.25 µm (film thickness) was employed for separation with sample injections done in splitless mode. The oven temperature program was set at 35ºC for three minutes followed by a temperature rise of 10ºC per minute up to 280ºC and maintained at this final temperature for 10 minutes. The injector and detector temperatures were set at 280ºC. Nitrogen (BOC gases, Kenya) was used as the carrier gas at a flow rate of 1.2 ml min^{-1}. The FID had a mixture of clean (medical) air and hydrogen gas (from a generator, Dorminick, USA) flow at 31 ml min^{-1} and 405 ml min^{-1}, respectively. A delay of 0.5 minutes before injection purging was maintained.

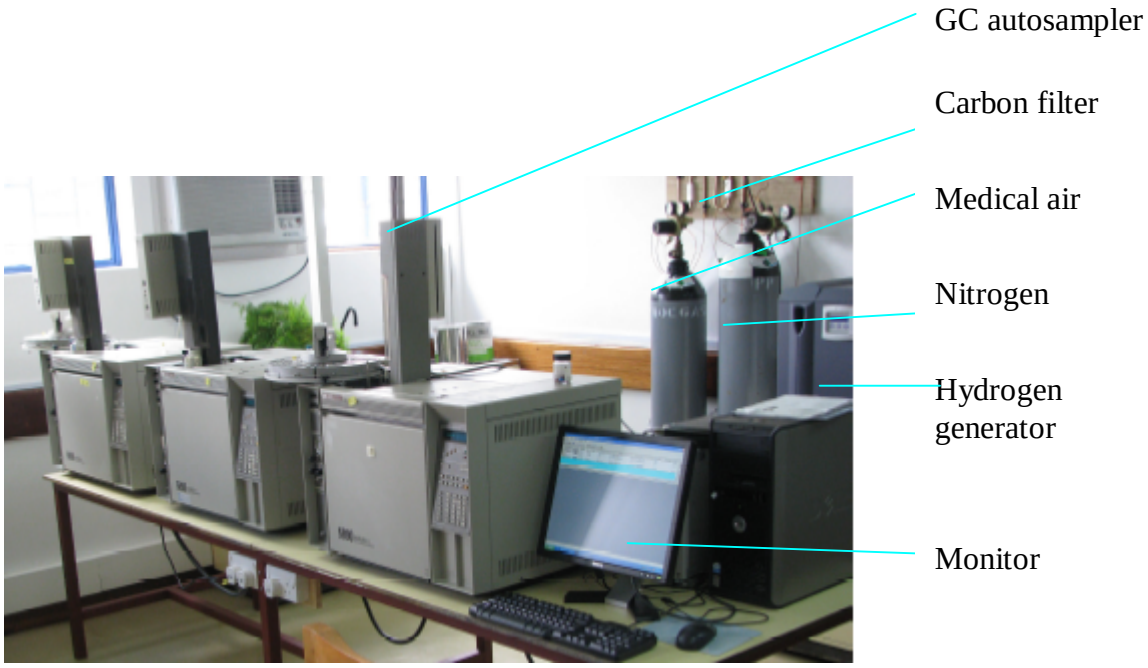


Plate 5.1 Gas chromatographs, attached to an autosampler and coupled to a HP (Dell) monitor, used for analyses of volatiles.

5.2.3 Gas chromatography coupled mass spectrometry (GC-MS)

Sample eluates of fungal volatiles were also analyzed by coupled GC-MS using a Hewlett Packard (HP) 7890 A series GC (Agilent technologies, Wilmington, DE, USA) coupled to a 5975 C series mass spectrometer fitted with an 7683 B series autosampler (Agilent technologies, Wilmington, DE, USA) and a Triple Axis Detector (Plate 5.2). The GC was equipped with a nonpolar capillallary column (HP5 MS 5% with phenylmethyl silicone) measuring 30 m (length) x 0.25 µm (i.d.) and 0.25 µm (film thickness) for separation of chromatographic peaks. The GC was coupled to a HP monitor (L1710) for displaying of chromatographic data which were acquired and evaluated by 3365 MSD ChemStation software (G1701EA E.02.00.493).

Oven temperature programming was the same as that described for the GC analyses above and the splitter was off for 0.5 min. The carrier gas was helium at a constant flow rate of 1.2 ml min^{-1}. For Electron Impact (EI) of the MS, the ionization voltage was 70 eV and temperature of the ion source and the interface were 230ºC and 150ºC, respectively. Identification of the constituent compounds was based on the interpretation of the mass spectral fragmentation patterns obtained from the John Wiley and NIST MS data libraries (Hewlett Packard, USA) followed by comparisons with spectral data of synthetic samples (Sigma Aldrich). The identity of each component was confirmed by co-injections with synthentic compounds on Hewlett Packard 5890 gas chromatograph described above.

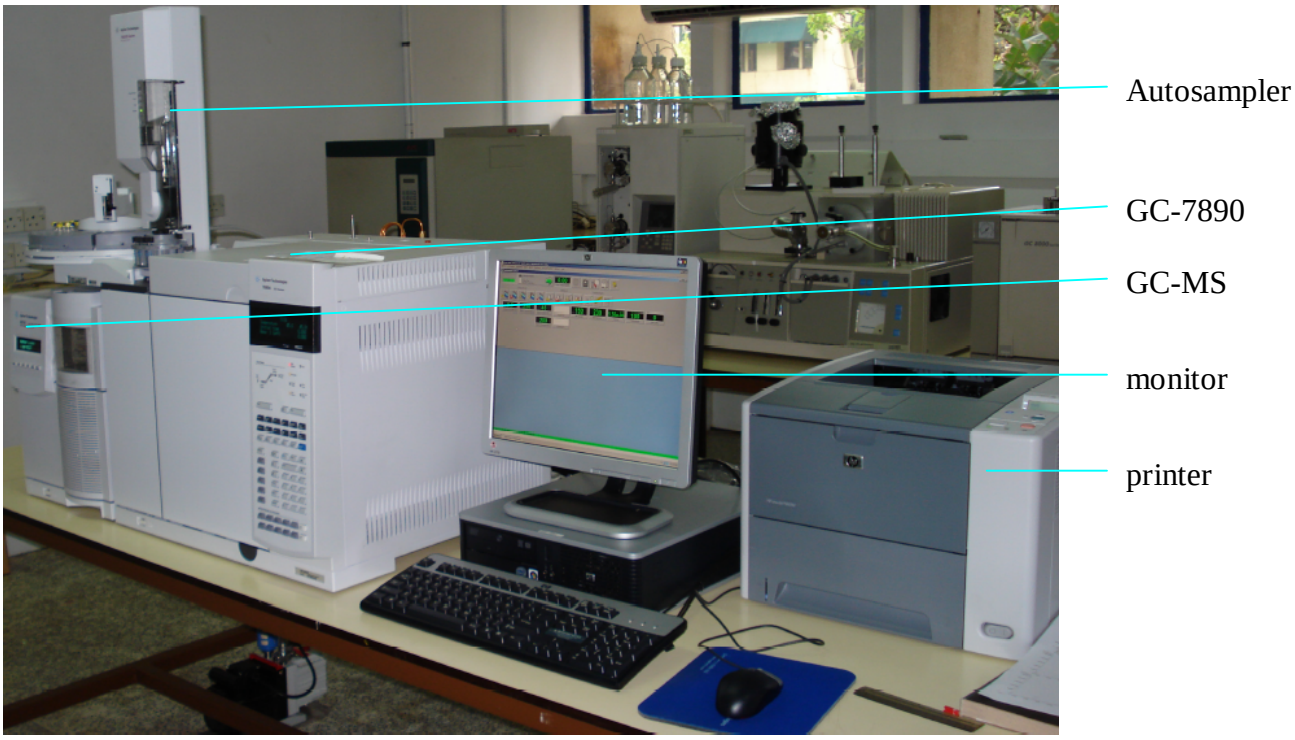


Plate 5.2 Gas chromatography linked mass spectrometer equipped with an autosampler and coupled to a HP monitor used for GC-coupled mass spectrometric analyses of volatile collections.

5.3 DATA ANALYSIS

Data was analysed as described in section 5.2.2 and 5.2.3 above.

5.4 RESULTS

In this chapter, details of gas chromatograms and constituent compounds of the most and the least repellent isolates of *M. anisopliae* and *B. bassiana* are reported. Variations in relative amounts of the individual compounds present in the most and the least repellent isolates are shown in Tables 5.1 (*M. anisopliae*) and 5.2 (*B. bassiana*). GC profiles of the individual headspace odour collections from the most repellent (ICIPE 51) isolate of *M. anisopliae* and the least repellent one (ICIPE 69) are shown in Figures 5.1 and 5.2, respectively. Similarly, for *B. bassiana*, the odour profiles of the most repellent isolate (ICIPE 276) and the least repellent one (ICIPE 278) are shown in Figures 5.3 and 5.4, respectively. Identification of the compounds by coupled GC-MS and confirmation by peak enhancement using synthentic standards revealed that there were qualitative differences in the compositions of the volatile blends collected from the least and the most repellent isolates of these species of fungi. In total, there were 43 and 26 compounds identified from the volatile emissions of the most repellent (ICIPE 51) and the least repellent isolate (ICIPE 69) of *M. anisopliae*, respectively. For the most and the least repellent isolate of *B. bassiana*, 19 and 15 compounds were identified, respectively.

Volatile collection from the most repellent isolate of *M. anisopliae* (ICIPE 51) was made up of alcohols (12), ketones (9), terpenoids (5), Ethers (2), aldehydes (2), straight chain aliphatic hydrocarbons (4), branched aliphatic hydrocarbons (3), cyclic aliphatic

hydrocarbon (1), branched aromatic hydrocarbon (1), steroids (2), acids (2) and an ester (Table 5.1). Volatiles from the least repellent isolate (ICIPE 69) consisted of alcohols (6), ketones (5), terpenoids (2), alkylated benzenes (3), branched aromatic hydrocarbon (4), staright chain aliphatic hydrocarbons (2), an acid, an amine and an ether. From the foregoing, one of the major differences between the two isolates of *M. anisopliae* was that steroids and aldehydes were absent from the volatile blends of the least repellent isolate of *M. anisopliae* while alkylated benzenes were absent from the most repellent isolate. In both isolates alcohols and ketones were more in number than the other groups of compounds. There were also quantitative differences in the constituent compounds between the most and the least repellent isolates of these fungi.

There were 19 and 15 compounds identified from the most and the least repellent isolates of *B. bassiana*, respectively. The most (ICIPE 276) and the least (ICIPE 278) repellent isolate of this fungus also showed qualitative and quantitative differences in volatile profiles (Table 5.2). For example, the volatile blend from the most repellent (ICIPE 276) consisted of alcohols (8), ketones (6) and terpenoids (4). In contrast, volatile profiles of the least repellent ICIPE 278 had fewer alcohols (6) and ketones (4). In addition, it had terpenoids (4) and a straight-chain aliphatic hydrocarbon.

From the chromatographic profiles and GC-MS analyses of volatile collections from the most and the least repellent isolates of *M. anisopliae*, a large proportion of these constituents was only present in one of the isolates. For example, stigmasterol (2.11µg),

acetic acid (12.09µg) and 3-octanone (3.7µg) were present only in the most repellent isolate (ICIPE 51) of *M. anisopliae* while 2-pyrrolidinone (6.6µg), ethylacetamide (2.87µg) and 1-ethyl-2-methylbenzene (2.3µg) were present only in the least repellent isolate (ICIPE 69) of this fungus. Similarly, 2-heptanone (2.03µg), 4-nonanone (4.19µg) and phenol (7.13µg) were only present in the most repellent isolate (ICIPE 276) of *B. bassiana* while tridecane was only present in the least repellent isolate (ICIPE 278) of this fungus. Overall, it was evident that the composition of volatiles from the isolates of the two fungi differed qualitatively and quantitatively. The implications of these results are discussed.

Table 5.1 Compounds identified from emission of the most (ICIPE 51) and the least (ICIPE 69) repellent isolates of *Metarhizium anisopliae*

		Isolates of *Metarhizium anisopliae*			
Compounds (cpd)	**Peak**	**ICIPE 51**		**ICIPE 69**	
	No. †	**area % of total, (µg)**	**RT**	**area % of total (µg)**	**RT**
1-Chrolopentane	1	0.58 (1.0)	4.297	-	-
2- Methyl-1-butanol	2	1.05 (1.76)	4.386	-	-
1-Pentanol	3	0.54 (0.9)	5.269	-	-
Ethylbenzene	4	-	-	0.98 (0.72)	7.844
Hexanol	5	13.88(24.16)	8.211	6.99 (5.16)	8.137
Acetic acid,pentyl ester	6	1.053 (1.83)	8.369	-	-
N-Ethylacetamide	7	-	-	3.9 (2.87)	8.537
2-Heptanone	8	1.19 (2.08)	8.651	-	-
Nonane	9	-	-	0.98 (0.72)	8.836
2-Heptanol	10	1.61 (2.78)	8.894	-	-
2- Butoxy ethanol	11	0.79 (1.37)	9.0	2.45 (1.8)	8.986
Butylolactone	12	1.07 (1.86)	9.132	9.18 (6.76)	9.175
Heptanoic acid	13	0.74 (1.28)	9.23	-	-
α- Pinene	14	0.79 (1.37)	9.573	-	-
Camphene	15	0.53 (0.93)	9.886	2.49 (0.8)	11.031
2,5-Dimethyl-tetrahydrofuran	16	0.42 (0.73)	10.025	-	-
1-ethyl-2-methylbenzene	17	-	-	3.13 (2.3)	10.198
1- Heptanol	18	1.19 (2.07)	10.407	-	-
1-Octen-3-ol	19	-	-	2.7 (1.99)	10.577
3-Octanone	20	2.12 (3.7)	10.726		
3-Octanol	21	-	-	4.73 (3.49)	10.734
1,2-Dimethylhydrazine	22	1.49 (2.59)	11.021	-	-
1,4-Dichloro benzene	23	-	-	1.59 (1.17)	11.178
Acetic acid	24	6.95 (12.09)	11.25	-	-
2-Propyl-1-pentanol	25	-	-	7.98 (5.89)	11.53
5-Ethyldihydro-2-(3H)furanone	26	0.65 (1.13)	11.987	1.78 (1.34)	11.983
1-Octene	27	2.39 (4.15)	12.298	-	-
2-Pyrrolidinone	28	-	-	8.96 (6.6)	12.314
2-Heptene	29	0.54 (0.94)	12.455	-	-
4-Ethyl-1,2-dimethyl benzene	30	-	-	1.1 (0.81)	12.542
2-Nonanone	31	4.61 (8.02)	12.648		

		Isolates of *Metarhizium anisopliae*			
Compounds (cpd)	**Peak**	**ICIPE 51**		**ICIPE 69**	
	No. †	**area % of total, (µg)**	**RT**	**area % of total (µg)**	**RT**
2-Nonanol	32	2.29 (3.96)	12.799	-	
Phenyl ethyl alcohol	33	8.47 (14.75)	13.04	11.46(8.45)	13.02
3-Nonen-2-one	34	1.75 (3.04)	13.404	-	-
α-Camphor	35	1.18 (2.05)	13.565	-	-
4-Methyl-2,7- octadiene	36	-	-	1.45 (1.07)	13.899
Borneol	37	4.92 (8.56)	13.912	-	-
4-Methyl-2-pentyl acetate	38	1.36 (2.36)	14.466	-	-
5-Butyldihydro-2-(3H) furanone	39	0.56 (2.36)	15.246	-	-
Cyclododecane	40	0.59 (0.97)	15.413	-	-
1-Hexadecyne	41	0.41 (1.03)	15.514	-	-
1-Methyl-naphthalene	42	-	-	0.97 (0.71)	15.808
2-Methyl naphthalene	43	0.28 (0.48)	15.81	4.75 (3.06)	16.718
4,5-Dihydro-5-pentyl-2-(3H) furanone	44	3.91 (6.81)	16.728	-	-
2-Butyl-2-octenal	45	0.71 (1.24)	16.848	-	-
7-Methanoazulene	46	0.56 (0.98)	17.491	-	-
3-Tert-Butyl-4-hydroxyanisole	47	0.31 (0.54)	18.12	-	-
9-Acetylphenanthrene	48	-	-	0.31 (0.23)	17.724
5,6-Dihydro-6-pentyl-2(H)-pyra-2-one	49	0.16 (0.28)	18.236	-	-
α-Cedrene	50	1.03 (1.8)	18.389	1.75 (1.29)	17.944
Hexadecane	51	-	-	0.38 (0.28)	18.419
Propanoic acid	52	-	-	0.55 (0.41)	19.636
Hexadecanoic acid	53	0.43 (0.75)	25.149	-	-
Tetracosane	54	0.48 (0.83)	27.353	-	-
Heptadecane	55	0.52 (0.9)	28.144	-	-
Octadecane	56	0.35 (0.61)	29.653	-	-
Stigmasterol	57	1.21 (2.11)	36.772	-	-
Sitosterol	58	1.42 (2.47)	37.956	-	-

In the Table, the values in brackets are the amounts (µg) of the respective compounds. Quantification was done using methyl salicylate as an internal standard. The numbers in

the column reading, “peak no.[†]” corresponds to the constituent compounds of the isolates labeled in chromatograms in Figures 5.1 and 5.2.

Table 5.2 Compounds identified from emission of the most (ICIPE 276) and the least (ICIPE 278) repellent isolates of *Beauveria bassiana*

		Isolates of *Beauveria bassiana*			
Compounds	**Peak**	**ICIPE 276**		**ICIPE 278**	
	no. †	**area % of total**	**RT**	**area % of total**	**RT**
2- Methyl-1-butanol	1	2.44 (4.24)	4.381	4.299 (2.98)	4.308
1-Pentanol	2	7.93(13.78)	5.281	7.918 (5.48)	5.291
2,3-Dimethyl-1-butanol	3	1.59 (2.77)	6.992	1.436 (0.99)	6.997
Hexanol	4	32.22 (56)	8.185	23.7 (16.41)	8.171
2-Heptanone	5	1.17 (2.03)	8.653	-	-
Butylolactone	6	2.8 (4.87)	9.139	3.46 (2.4)	9.13
Phenol	7	4.1 (7.13)	10.654	-	-
3-Octanol	8	2.55 (4.44)	10.903	8.09 (5.6)	10.902
5-Ethyldihydro-2-(3H)furanone	9	1.46 (2.53)	11.979	-	-
4-Nonanone	10	2.41 (4.19)	12.298	-	-
2-Nonanone	11	3.8 (6.62)	12.639	2.79 (1.93)	12.639
2-Nonanol	12	1.84 (3.19)	12.775	-	-
Phenyl ethyl alcohol	13	1.43 (2.48)	13.016	1.41 (0.98)	13.017
3-Nonen-2-one	14	1.54 (2.68)	13.423	1.39 (0.96)	13.423
Camphor	15	1.45 (2.52)	13.555	1.75 (1.21)	13.555
Borneol	16	7.56 (13.14)	13.894	9.1 (6.3)	13.895
4,5-Dihydro-5-pentyl-2-(3)furanone	17	3.3 (5.73)	16.716	2.25 (1.56)	16.719
Tetradecane	18	-	-	1.55 (1.07)	17.156
7-Methanoazulene	19	1.14 (1.99)	17.494	1.62 (1.12)	17.495
(E)-α-Bergamotene	20	1.44 (2.5)	18.399	3.05 (2.11)	18.402

Values in brackets are the amounts (µg) of the respective compounds. Quantification was done using methyl salicylate as an internal standard. The numbers in the column reading, "peak no.†" corresponds to the constituent compounds of the isolates labeled in chromatograms in Figures 5.3 and 5.4.

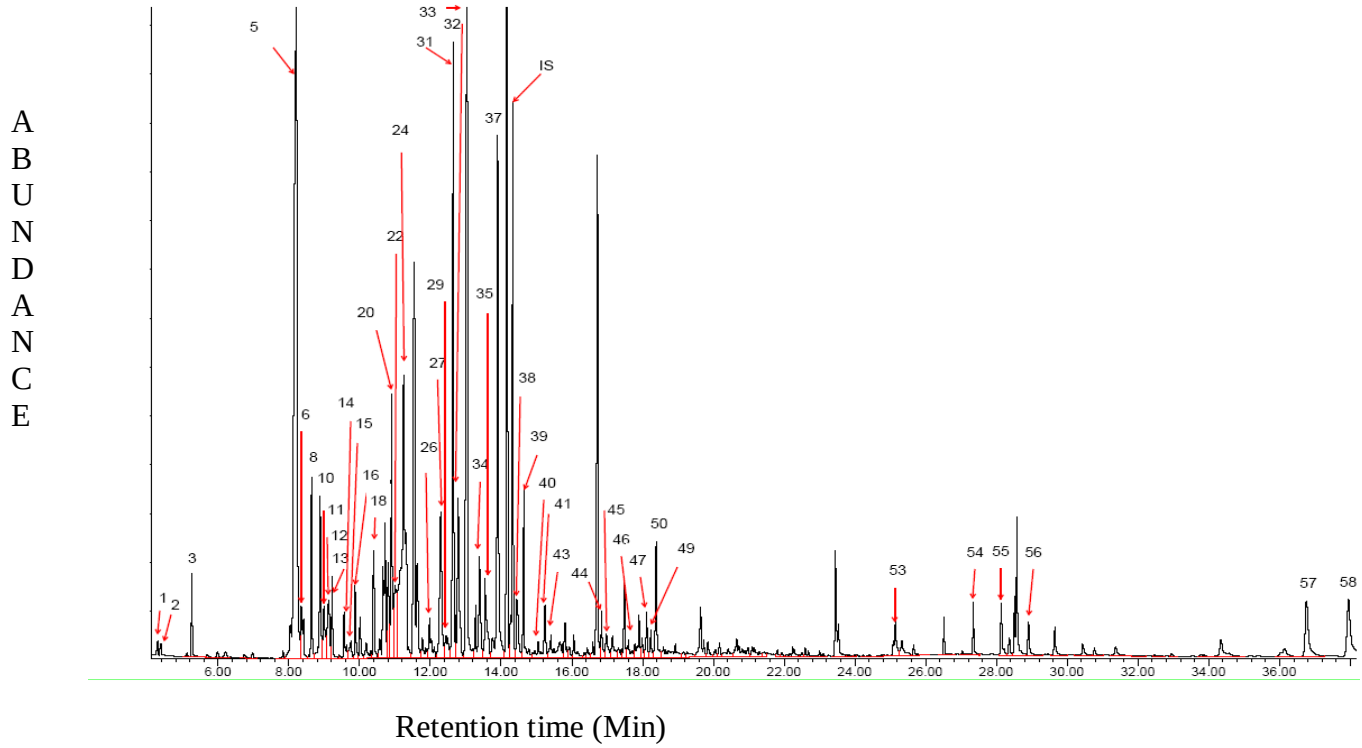


Figure 5.1 Representative total ion chromatograms of Super Q volatile collection of the most repellenct isolate (ICIPE 51) of *Metarhizium anisopliae* 21 days post harvest. IS indicates the peak for methyl salicylate (MS), the internal standard.

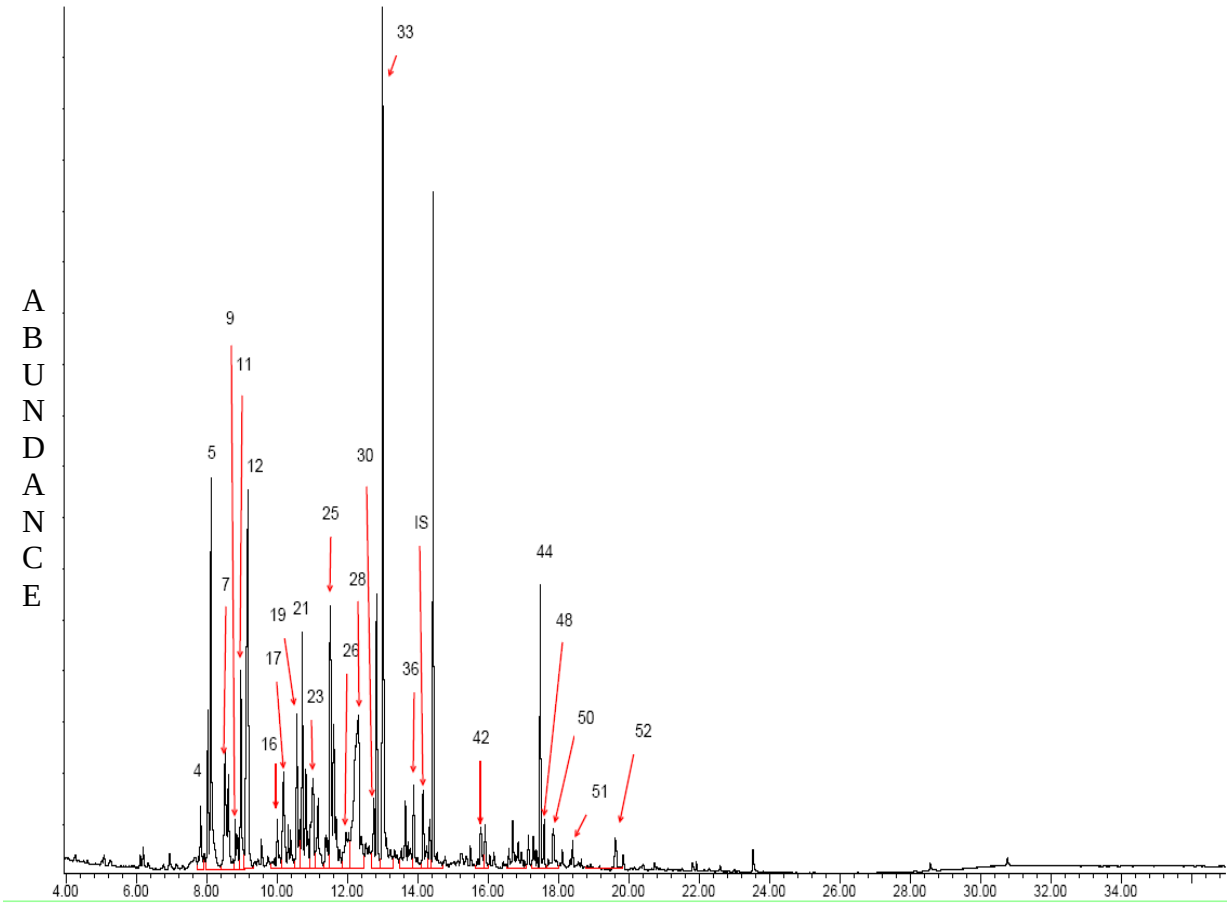


Figure 5.2 Representative total ion chromatograms of Super Q volatile collection of the least repellenct isolate (ICIPE 69) of *Metarhizium anisopliae* 21 days post harvest. IS indicates the peak for methyl salicylate (MS), the internal standard.

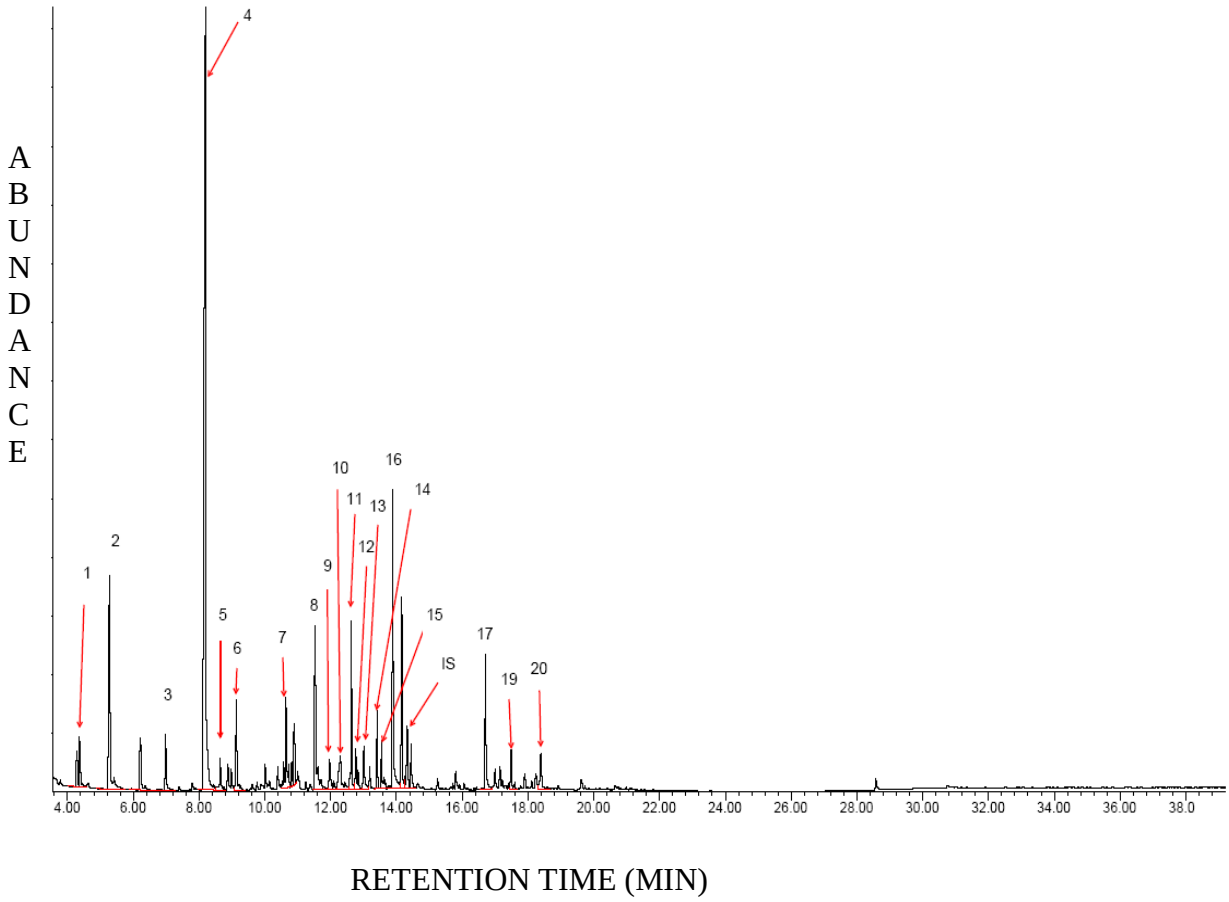


Figure 5.3 Representative total ion chromatograms of Super Q volatile collection of the most repellenct isolate (ICIPE 276) of *Beauveria bassiana* 21 days post harvest. IS indicates the peak for methyl salicylate (MS), the internal standard.

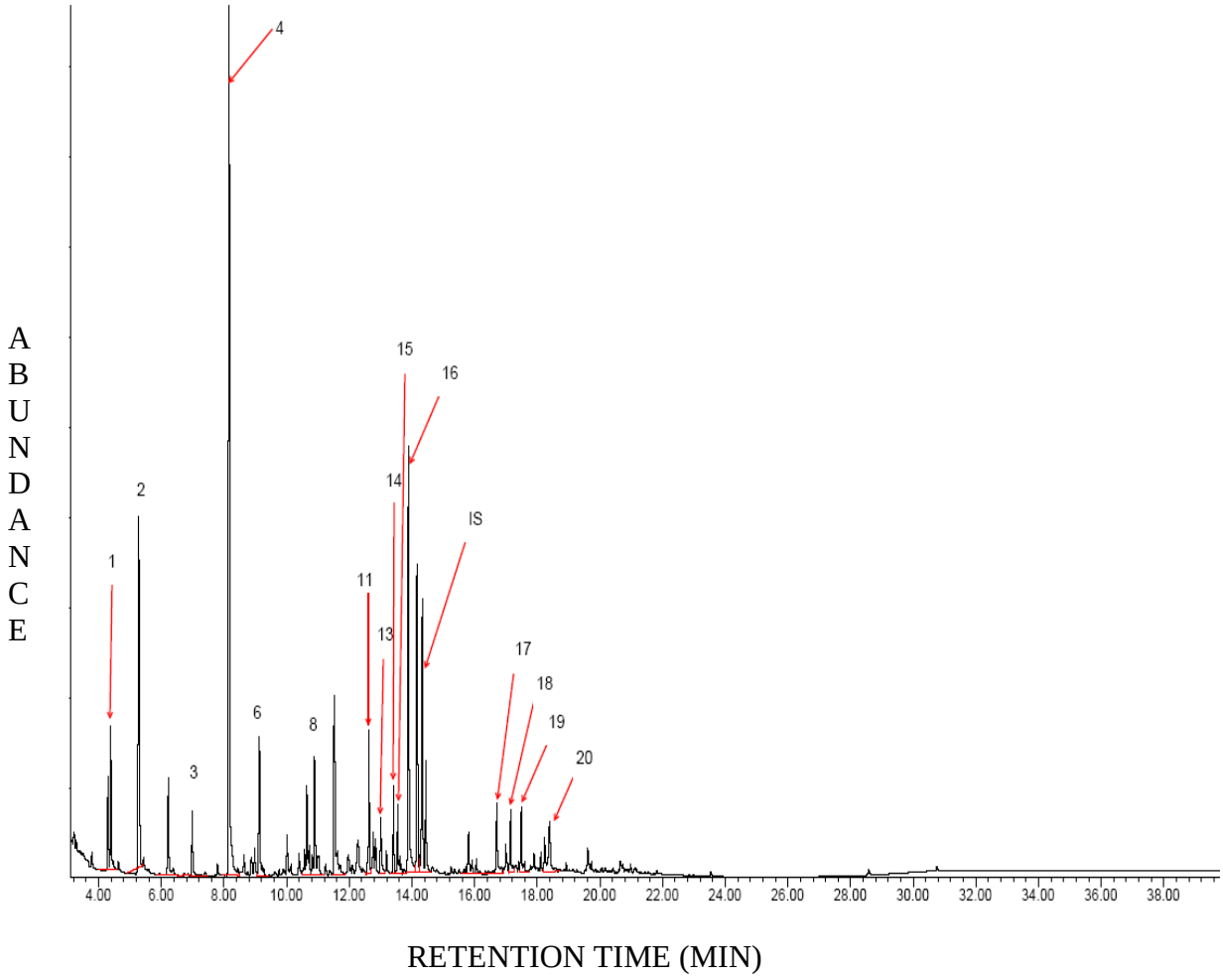


Figure 5.4 Representative total ion chromatograms of Super Q volatile collection of the least repellenct isolate (ICIPE 278) of *Beauveria bassiana* 21 days post harvest. IS indicates peak for methyl salicylate (MS), the internal standard.

5.5 DISCUSSION

A large number of VOCs are produced by the conidia of the most and the least repellent isolates of *M. anisopliae* as well as *B. bassiana* suggesting that the compositions of volatile blends from the different isolates of these fungi are strain specific. However, there is also an appreciable number of compounds that are present in all the isolates of the fungal isolates. The results confirm that different isolates of the two fungi produce volatile mixtures that are qualitatively and quantitatively different. Earlier, olfactometric assays showed that termites are able to detect pontentially infective fungi through olfactory mechanisms from a distance and avoid them (Chapter four). The qualitative and quantitative differences in the constituent compounds of volatile blends from the different isolates of fungi may partly explain the different levels of repellency spectra obtained (Chapter four) among the different isolates towards the termite, *M. michaelseni*.

Social insect communities like the termite, *M. michaelseni* live in groups at high densities (Cremer *et al*., 2007) with varying levels of chemical interaction. The results confirm and document the presence of repellent conidia of the isolates of fungi studied, which had been believed to be repellent (Milner and Staples, 1996; Rosengaus *et al*., 1999b). If the spores repel termites, a barrier utilizing a formulation of highly repellent spores may be possible around or beneath a house, resulting in protection from termite invasion (Milner *et al*., 1997). However, the use of the most repellent isolates of the fungi may also mean that there will be less horizontal transmission of the spores from infected or contaminated termites to the other nest mates (Rath, 2000). A ‘recruitment stimulus”

may be needed in bait to overcome the repellency (Grace *et al*., 1996). There is need to identify the key behaviourally active constituents responsible for the observed repellency spectra of different fungal isolates. These include those that have intrinsic repellent activity and those that synergise the repellent action of specific constituents or the repellent blend. In this regard, Chapter six below outlines the relative repellent actions of different blends of the identified constituents of the selected isolates of *M. anisopliae* and *B. bassiana* in the Y-shaped olfactometer.

Characterization of such behaviourally active blends is expected to broaden the understanding on the relationship between these profiles and avoidance behavioural response of *M. michaelseni*. This may aid in the development of semiochemicals as mass repellents (push factor) in integrated pest management to protect wooden structures such as linouleum, crop seedlings and stored cereals from invasion by the *M. michaelseni* and perhaps other termite species. Natural repellents have been shown to be useful in the protection of crops against post-harvest insect pests such as lesser grain borer, *Rhyzopertha dominica* Fabricius (Coleoptera; Bostrichoidae) (Bekele and Hassanali, 2001). In push pull strategies, natural repellents have been utilized in Integrated Pest Management (IPM) of *Helicoverpa armigera* Hubner (Lepidoptera: Nuctuidae) in cotton (Duraimurugan and Regupathy, 2005) and IPM of cereal stem borer, *Chilo partellus* Swinhoe (Lepidoptera: Pyralidae) and *Striga harmonthica* (Del.) Benth in western Kenya (Khan *et al*., 2006; Hassanali *et al*., 2008; Khan *et al*., 2008). In view of the results obtained in this study, it is evident that the repellency of the fungal isolates to the termite,

M. michaelseni results from blends of major and minor components of the volatile emissions and that are different qualitatively and/or quantitatively between the isolates. The objectives of this aspect of the study thus validated the set hypotheses leading to the identification of natural repellent components from the most and the least repellent isolates of *M. anisophliae* and *B. bassiana* that had been tested in olfactometric assays in Chapter four.

CHAPTER SIX

6.0 REPELLENCY OF DIFFERENT BLENDS OF SYNTHETIC CONSTITUENTS OF *METARHIZIUM ANISOPLIAE* AND *BEAUVERIA BASSIANA* TOWARDS *MACROTERMES MICHAELSENI*

6.1 INTRODUCTION

Odour plumes of volatile chemicals form gaseous phases of molecules that disperse from their sources (Murlis *et al*., 1992). Structures of the odours are complex, much like that of smoke plumes carried in wind. As in the case of many herbivores (Bernays and Chapman, 1994), insects experience highly complex blends of volatile chemicals from a multitude of sources as they forage to locate food, mates and oviposition sites under field conditions (Bartelt and Zilkowski, 1998). The odour structure and the rate of dispersion are some of the important factors that determine the probability of an insect contacting the volatile blends at different locations downwind (Murlis *et al*., 1992).

Different infochemical blends mediate communication within and between different trophic levels (Mayer *et al*., 2008). Understanding how some insect pests utilize the coded information in volatile chemicals has been a challenge of practical importance in pest management (Bartelt and Zilkowski, 1998), with pheromones being the most intensively studied of these chemicals (Pickett *et al*., 1997). Blends of volatile compounds emitted by host plants are known to mediate the attraction of mated female, *Cydia (Grapholita) molesta* Busck (Lepidoptera: Tortricidae) (Piñero and Dorn, 2008) to

oviposition sites. Volatile odour emissions from some fungi species such as *Fomitopsis pinicola* Swinhoe (Aphyllophorales: Polyporeaceae) have been shown to attract females of generalist beetles such as *Malthodes fuscus* Waltl (Coleoptera: Cantharidae) (Faldt *et al*., 1999). Repellent odour emissions from infective isolates of *M. anisopliae* and *B. bassiana*, were implicated to have invoked avoidance behaviour towards termite, *M. michaelseni* (Chapter four and five). However, the role of the individual odour constituents is still not understood. The hypothesis that particular components of the volatile emissions from isolates of the two fungi species invoke avoidance repertoire in the termite, as observed in Chapter four, can only be ascertained, if the synthentic constituents of the isolates are tested against the insect in subtraction assays of the blends.

The ability to control and measure the delivery of crude and synthetic volatiles has contributed significantly to the current understanding of the involvement of chemical volatiles in insect communication (Bartelt and Zilowiski, 1998). In particular, quantification of these constituents during experiments play a role in understanding how they (crude, synthetic) function and/or influence the behaviour of insects including termites. The first objective of this study was to evaluate the repellency indices of different blends of selected constituents of the most and the least repellent isolates of *M. anisopliae* and *B. bassiana* towards the termite *M. michaelseni*. The second objective was to compare the repellency indices of the different blends and identify candidate constituents responsible for conferring the differential biological activities. It was envisaged that the information obtained would help guide the selection and use of

appropriate blends of constituent components of the isolates of the fungi in an integrated control and management of *M. michaelseni*.

6.2 MATERIALS AND METHODS

6.2.1 Study sites and worker termites

The study was carried out at ICIPE (Chapter two, section 2.1) and the protocol for handling worker termite *M. michaelseni* used remained as described in Chapter two section 2.2.

6.2.2 Analyses of blend effects of synthetic constituents of fungi on termites

The relative amounts of the constituents of the blends were quantified using methyl salicylate as an internal standard in the GC-MS analyses as described in Chapter five, section 5.2.2 and 5.2.3. The synthentic constituents in blends of each of the isolates of fungi were assayed in relative amounts present in their natural proportions (GC-MS). These compounds were commercially available (Sigma Aldrich).

6.2.3 Olfactometric set-up of the bioassays

Repellency of different blends of the synthetic contituents towards worker termite, *M. michaelseni* were evaluated in Y-tube olfactometer set-up (Fig. 2.1) described in Chapter four section 4.2.2. However, nylon gauze (40-mesh size) separated each of the compartments A and B from their respective arms. The gauze prevented the contact of the temites with the filter papers containing the odour blends.

6.2.4 Comparative repellency tests of blends of synthetic constituents

Six serial dilutions of each of the chemical constituents (Table 5.1 and Table 5.2) identified in the most and the least repellent isolates of *M. anisopliae* (ICIPE 51 and ICIPE 69) and of *B. bassiana* (ICIPE 276 and ICIPE 278) studied were prepared in 40µl of acetone (97-99%, Sigma Aldrich). Different blends of the synthentic components

(Table 5.1 and Table 5.2), in relative amounts present in natural blends (GC-MS analses) were prepared in acetone. At the onset of the bioassays, Whatman No. 1 filter papers (2 cm × 2 cm) were loaded with 50µl of each of the blend mixtures and were placed in either compartment "B" or "C" of the Y-olfactometer (Plate 2.5). The other compartment functioned as the control with the Whatman filter paper containing only acetone (50µl). Escape of the termites and aeration of the set-up were as described in section 4.2.2 of Chapter four. Acetone was allowed to evaporate (three minutes) before introduction of each of the groups of 20 worker termites into compartment "A". Compartment A and the arm attached to it were illuminated by light as described in section 2.5 of Chapter two (Fig. 2.1). The number of termites in the treated and the control compartments, together with those in their respective arms (past the two cm score line off the intersection, (Fig. 2.1), were recorded at an interval of 10 min for 60 min to give six readings for each replicate dose. Protocols of handling the black cloth, non-respondents (during the bioassays) and cleaning of the olfactometer (after the bioassays) were as described in Chapter four section 4.2.2.

The above protocol was repeated with each component excluded from each of the blends at RD_{50} values of 0.227 $mgcm^{-2}$ and 0.427 $mgcm^{-2}$ for the most (ICIPE 51) and the least (ICIPE 69) repellent isolates of *M. anisopliae*, respectively. Simillary, the protocol was followed with the exclusion of the individual components from the blends of the most (ICIPE 276) and the least (ICIPE 278) repellent isolates of *B. bassiana* at RD_{50} of 0.925

$mgcm^{-2}$ and 2.687 $mgcm^{-2}$, respectively. In all the subtraction assays, the experiments consisted of 15 replicates.

6.3 DATA ANALYSES

Repellency indices of the different blends of the full blends and those with each constituent missing were calculated using the formula:

$$\frac{P_{nc} - P_{nt}}{P_{nc} + P_{nt}} \times 100$$

where P_{nc} and P_{nt} represent the average percentage of worker termites in control and treatment arms, respectively (Wanzala *et al*., 2004).

In all the tests, data for repellency were individually pooled before analyses, arcsin transformed to normality before invoking repeated measures analysis of variance (ANOVA) using Proc Mixed of SAS version 9.1 (SAS Institute, 2003). Means were separated by Student-Newman-Keuls (SNK) test. The repellency doses (RD_{50}) that are required to give 50% repellency indices) of each of the blend were estimated with repeated measures logistic regression via generalized estimating equations (GEE) (Throne *et al*., 1995; Stokes *et al*., 2000). These analyses were carried out using GENMOD procedure of SAS version 9.1 (SAS Institute, 2003). The level of significance was set at 5% for all analyses to identify significant differences among the values of RD_{50} for the different blends of synthetic constituents.

6.4 RESULTS

In this study, details of repellency of full blends and subtractions of the synthetic constituent compounds identified from the most and the least repellent isolates of *M. anisopliae* and *B. bassiana* are reported. The 10 constituents of each of the most (ICIPE 51) and the least (ICIPE 69) repellent of *M. anisopliae* were more than 1.7% and 2.5% of the blends, respectively (Table 6.1). For *B. bassiana*, the 10 constituents were based on 2% and 1.7% for the most (ICIPE 276) and the least (ICIPE 278) repellent isolates, respectively (Table 6.2). The major identified constituents of the volatiles from the most (ICIPE 51) and the least (ICIPE 69) repellent isolates of *M. anisopliae* are shown in Table 6.1. For *B. bassiana*, the most (ICIPE 276) and the least (ICIPE 278) repellent isolates are shown in Table 6.2. The corresponding GC profiles of the volatiles of *M. anisopliae* are shown in Figures 6.1 (ICIPE 51) and 6.2 (ICIPE 69), respectively. Those of *B. bassiana*, are indicated in Figures 6.3 (ICIPE 276) and 6.4 (ICIPE 278), respectively.

The RD_{50} values of the blend mixtures of the 10 synthentic constituents of the most (ICIPE 51) and the least repellent (ICIPE 69) isolates of *M. anisopliae* were at 0.227 mg cm^{-2} and 0.427 mg cm^{-2}, respectively. Those of *B. bassiana* were 0.925 mg cm^{-2} and 2.687 mg $cm^{-}2$ for the most (ICIPE 276) and the least repellent (ICIPE 278) isolates, respectively. There were significant variations in the mean percent repellency of the different synthetic blends in subtraction assays of odours from the most ($F = 11.5_{df\ (10, 154)}$, n = 165, p= 0.0001, SNK, test) and the least ($F = 10.9_{df\ (10, 154)}$, n = 165, p = 0.0001,

SNK, test) repellent isolates of *M. anisopliae* (Table 6.3). Similarly, significant variations were obtained in the mean percent repellency indices of the resulting blend mixtures of the nine constituents of the most ($F = 9.5_{df\ (10,\ 154)}$, n = 165, $p = 0.0001$, SNK, test) and the least ($F = 4.2_{df\ (10,\ 154)}$, n = 165, $p = 0.0001$, SNK, test) repellent isolates of *B. bassiana* (Table 6.4). The repellecy indices of full blends of isolates of fungi species were significantly different ($F = 19.24_{df\ (3,\ 56)}$, n = 60, $p = 0.0001$, SNK, test).

Variations in percent mean repellency indices resulting from the subtraction assays of each odour of blends of nine constituents of isolates of *M. anisopliae* and *B. bassiana* are shown in Tables 6.3 and 6.4, respectively. Subtraction of most ketones and terpenes from full blends of the synthentic constituents significantly reduced ($p < 0.05$, SNK test) the mean percentage repellency of each of the resulting blends. For example, exclusion of 4,5-dihydro-5-pentyl-2-(3H) furanone reduced the repellency indices of the full blends of synthentic compounds of ICIPE 51, ICIPE 276 and ICIPE 278 by 2.25, 1.38 and 1.42 times, respectively. Similarly, exclusion of 2-pyrrolidinone from the full blend of ICIPE 69 made the resulting blend repel the termite by 1.49 times less than the full blend. Significant difference ($F = 11.79_{df\ (3,\ 56)}$, n = 60, $p = 0.0001$, SNK, test) among blend mixtures of the first six constituents that contributed most to the repellency of odours from each of the isolates was obtained (Table 6.3 and 6.4). A combination of these resulted to higher repellency indices than that of the corresponding full blends by 1.21, 1.16, 1.39 and 1.81 times for ICIPE 51, ICIPE 69, ICIPE 276 and ICIPE 278, respectively.

Table 6.1 Major identified components (n = 10) in the volatiles of the most repellent (ICIPE 51) and the least repellent (ICIPE 69) isolates of *Metarhizium anisopliae* and their relative proportions used in test blends.

Most repellent isolate of *M. anisopliae*			**Least repellent isolate of *M. anisopliae***		
ICIPE 51			**ICIPE 69**		
[*]Peak No.	**Constituents in blends**	**Component %, (µg[†])**	**[*]Peak No.**	**Constituents in blends**	**Component %, (µg[†])**
1	Hexanol	27.6, (24.2[†])	1	Hexanol	11.1, (5.2[†])
2	3-Octanone	3.2, (2.8[†])	2	Ethylacetamide	6.2, (2.9[†])
3	Acetic acid	13.8, (12.1[†]	3	Butylolactone	14.5, (6.8[†])
4	1-Octene	4.7, (4.1[†])	4	1-Ethyl-2-methylbenzene	4.9, (2.3[†])
5	2-Nonanone	9.1, (8[†])	5	1-Octen-3-ol	4.3, (2[†])
6	2-Nonanol	4.6, (4[†])	6	3-Octanol	7.5, (3.5[†])
7	Phenylethyl alcohol	16.9, (14.8[†])	7	2-Propyl-1-pentanol	12.6, (5.9[†])
8	3-Nonen-2-one	3.4, (3[†])	8	2-Pyrrolidinone	14.1, (6.6[†])
9	Borneol	9.8, (8.6[†])	9	Phenylethylalcohol	18.2, (8.5[†])
10	4,5-Dihydro-5-pentyl-2(3H)furanone	6.8, (6[†])	10	Cedrene	6.6, (3.1[†])

[*]The peak numbers are as marked in gas chromatograms on Figures 6.1 and 6.2 below for ICIPE 51and ICIPE 69, repellent isolates of *M. anisopliae*, respectively. The numbers indicated with a symbol [†] are the actual amounts (µg) of the constituents as they occurred in GC-MS analyses. These compounds were more than 1.7% and 2.5% for ICIPE 51 and

ICIPE 69, respectively, of the total compound counts after the GC-MS analysis. Percentages shown in the column of components correspond to the relative abundance of each compound in the mixture.

Table 6.2 Major identified constituents (n = 10) of the most (ICIPE 276) and the least (ICIPE 278) repellent isolates of *Beauveria bassiana* and their relative proportions in tested blends.

Most repellent isolate of *B. bassiana*			**Least repellent isolate of *B. bassiana***		
ICIPE 276			**ICIPE 278**		
***Peak No.**	**Constituents**	**Component %, (ug†)**	***Peak No.**	**Constituents**	**Component %, (ug†)**
1	2-Methyl-1-butanol	3.5, (4.2†)	1	2-Methyl-1-butanol	6.5, (3†)
2	1-Pentanol	11.5, (13.8†)	2	1-Pentanol	12, (5.5†)
3	Hexanol	46.6, (56†)	3	Hexanol	35.7, (16.4†)
4	Butylolactone	4.1, (4.9†)	4	Butyrolactone	5.2, (2.4†)
5	Phenol	5.9, (7.1†)	5	3-Octanol	12.2, (5.6†)
6	3-Octanol	3.7, (4.4†)	6	2-Nonan-2-one	4.1, (1.9†)
7	4-Nonanone	3.5, (4.2†)	7	Camphor	2.6, (1.2†)
8	2-Nonanone	5.5, (6.6†)	8	Borneol	13.7, (6.3†)
9	Borneol	11.1, (13.4†)	9	(E)-alpha-Bergomotene	2.25, (1.6†)
10	4,5-Dihydro-5-pentyl- 2- (3H) furanone	4.7, (5.7†)	10	4,5-Dihydro-5-pentyl- 2- (3H) furanone	4.6, (2.1†)

*The peak numbers are as marked in the gas chromatograms on Figures 6.3 and 6.4 below for ICIPE 276 and ICIPE 278 repellent isolates of *M. anisopliae*, respectively. The numbers indicated with a symbol † are the actual amounts (µg) of the constituents as they occurred in GC-MS analyses. These compounds were more than 2% and 1.7% for ICIPE 276 and ICIPE 278, respectively, of the total compound counts after the GC-MS analysis.

Percentages shown in the column of components correspond to the relative abundance of each compound in the mixtures.

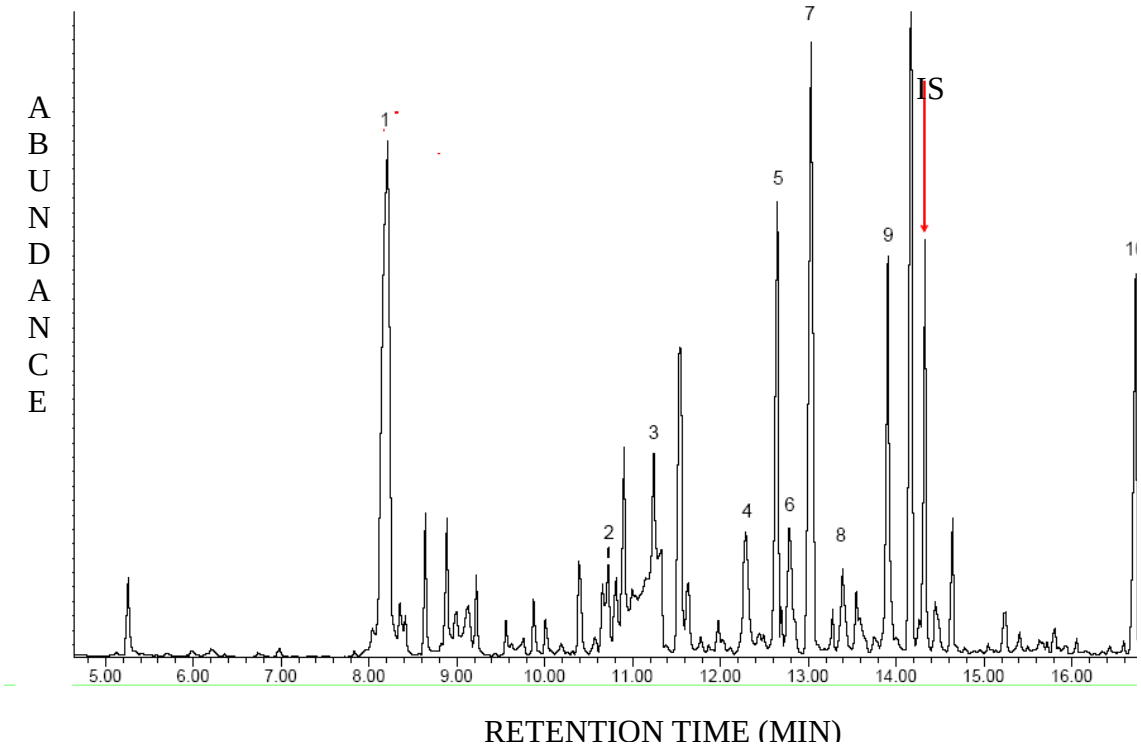


Figure 6.1 Representative gas chromatogram of the most repellent isolate of *Metarhizium anisopliae* (ICIPE 51). Numbered peaks are the major components used in evaluating blend effects in the subtraction assays; 1 (hexanol), 2 (3-octanone), 3 (acetic acid), 4 (1-octene), 5 (2-nonanone), 6 (2-nonanol), 7 (phenylethylalcohol), 8 (3-nonen-2-one), 9 (α-borneol), 10 (4,5-dihydro-5-pentyl-2-(3H) furanone) on the chromatogram. Some of the peaks that are unnumbered appeared on the control chromatogram. IS indicates the peak for methyl salicylate (MS), the internal standard.

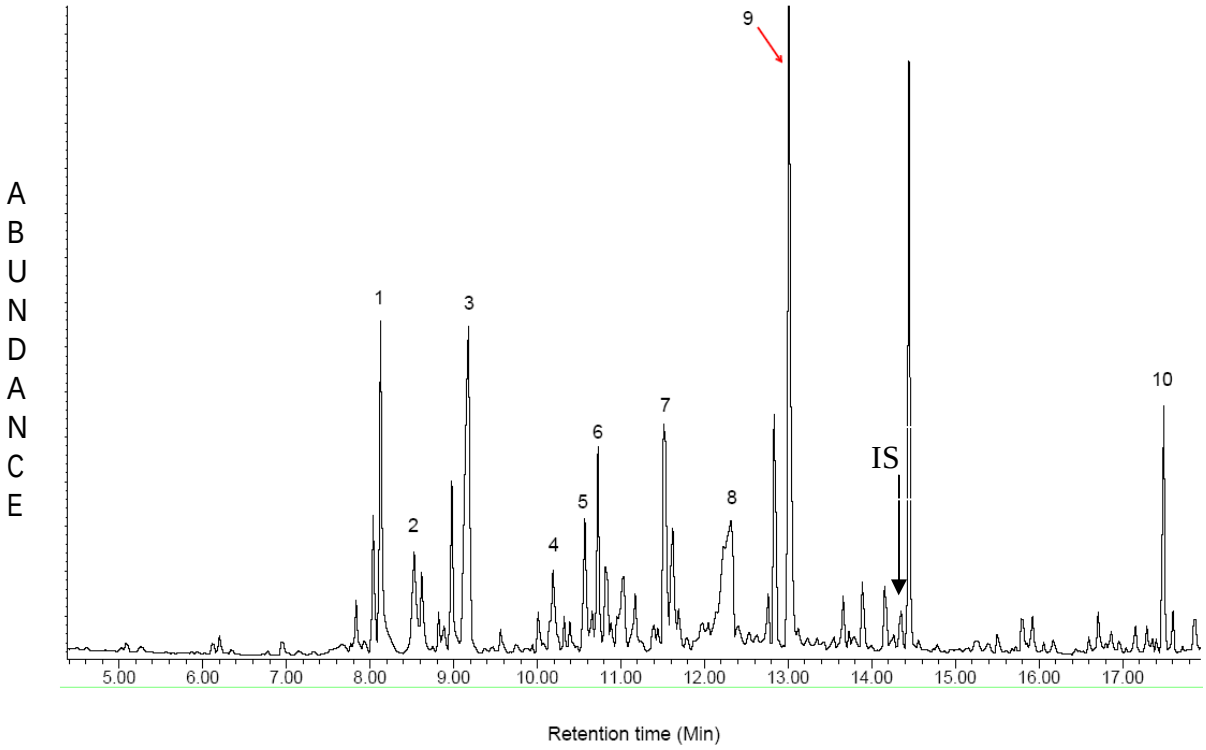


Figure 6.2 Representative gas chromatogram of the least repellent isolate of *Metarhizium anisopliae* (ICIPE 69). Numbered peaks are the major components used in evaluating blend effects in the subtraction bioassays: 1 (hexanol), 2 (ethylacetamide), 3 (butyrolactone), 4 (1-ethyl-2-methylbenzene), 5 (1-octen-3-ol), 6 (3-octanol), 7 (2-propyl-1-pentanol), 8 (2-pyrrolidinone), 9 (phenylethylalcohol), 10 (cedrene) on the chromatogram. Some of the peaks that are unnumbered appeared on the control chromatogram. IS indicates the peak for methyl salicylate (MS), the internal standard.

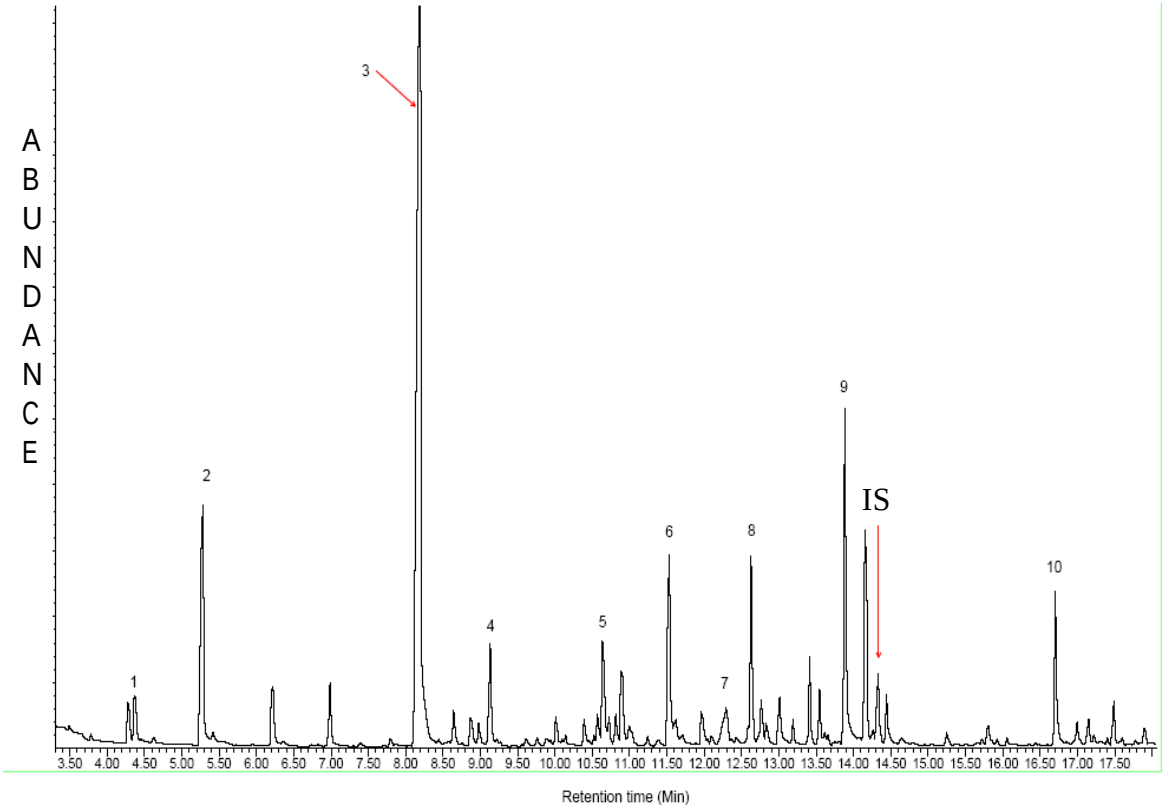


Figure 6.3 Representative gas chromatogram of the most repellent isolate of *Beauveria bassiana* (ICIPE 276). Marked peaks are the major components used in evaluating blend effects in the subtraction bioassays: 1 (2-methyl-1-butanol), 2 (1-pentanol), 3 (hexanol), 4 (butyrolactone), 5 (phenol), 6 (3-octenol), 7 (4-nonanone), 8 (2-nonanone), 9 (α-borneol), 10 (dihydro-5-pentyl- 2-(3H) furanone) on the chromatogram. Some of the peaks that are unnumbered appeared on the control chromatogram. IS indicates the peak for methyl salicylate (MS), the internal standard.

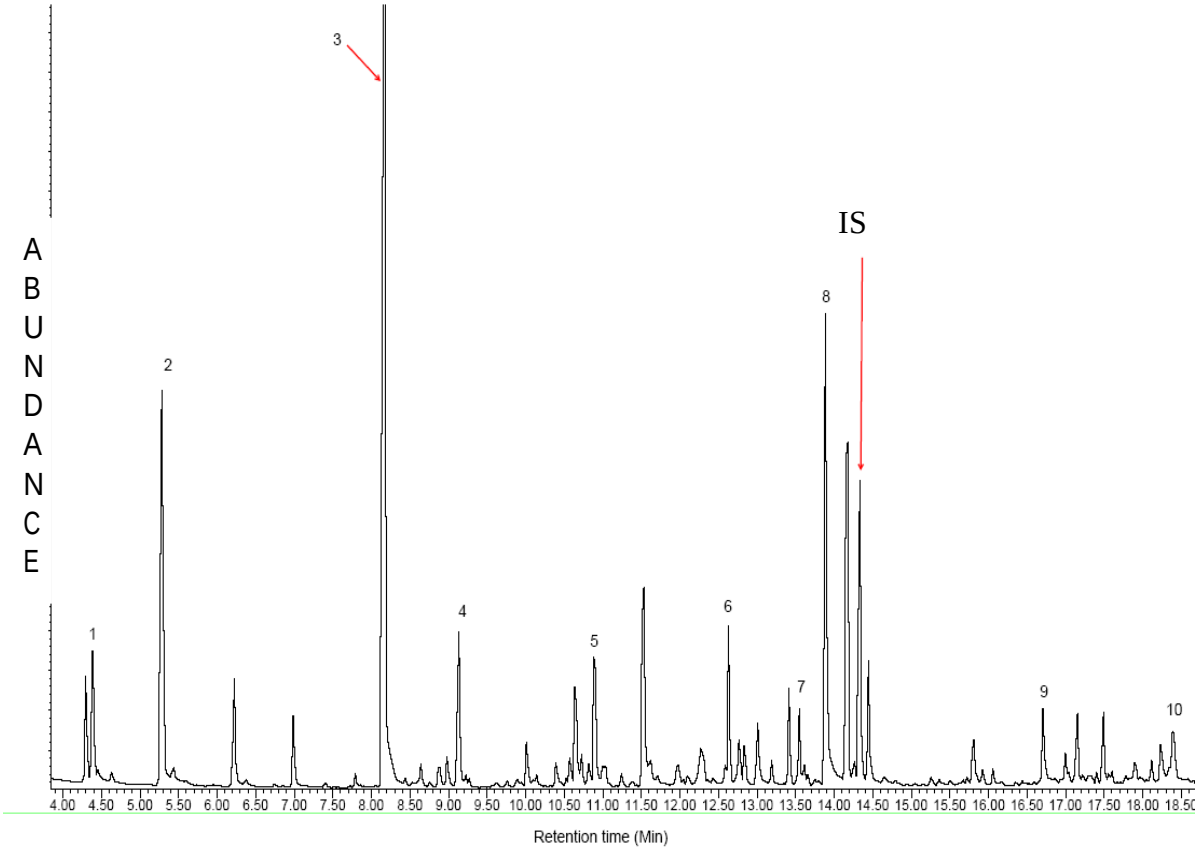


Figure 6.4 Representative gas chromatogram of the least repellent isolate of *Beauveria bassiana* (ICIPE 278). Numbered peaks are the major components used in evaluating blend effects in the subtraction bioassays: 1 (2-methyl-1-butanol), 2 (1-Pentanol), 3 (hexanol), 4 (butyrolactone), 5 (3-octenol), 6 (2-nonanone), 7 (camphor), 8(α-borneol), 9 (E)-α-bergamotene), 10 (dihydro-5-pentyl-2-(3H)furanone) on the chromatogram. Some of the peaks that are unnumbered appeared on the control chromatogram. IS indicates the peak for methyl salicylate (MS), the internal standard.

Table 6.3 Mean percent repellency of blends of the major components at doses equivalent to the relative proportion of each constituent at 50% repellency-dose (RD_{50}) of the full blends of isolates of *Metarhizium anisopliae* towards termite, *Macrotermes michaelseni*.

Most repellent isolate of *M. anisopliae* (ICIPE 51)		**Least repellent isolate of *M. anisopliae* (ICIPE 69)**	
	% repellency		**% repellency**
Constituents in blends	**Mean ± SE**	**Constituents in blends**	**Mean ± SE**
Full Blend* (FB)	80.1 ± 3.1^{ab}	Full Blend* (FB)	71.1 ± 3.5^{ab}
FB without 1	86.6 ± 3.1^{ab}	FB without 1	73.1 ± 2.5^{ab}
FB without 2	78.0 ± 5.8^{ab}	FB without 2	71.5 ± 2.4^{ab}
FB without 3	74.5 ± 3.8^{ab}	FB without 3	71.0 ± 2.5^{ab}
FB without 4	70.9 ± 3.2^{ab}	FB without 4	70.1 ± 3.4^{ab}
FB without 5	67.9 ± 3.8^{b}	FB without 5	61.3 ± 2.5^{c}
FB without 6	67.8 ± 3.3^{b}	FB without 6	59.4 ± 1.8^{c}
FB without 7	65.6 ± 3.7^{b}	FB without 7	58.9 ± 2.4^{c}
FB without 8	65.0 ± 2.7^{b}	FB without 8	58.8 ± 2.2^{c}
FB without 9	63.6 ± 3.6^{b}	FB without 9	56.8 ± 1.8^{c}
FB without 10	35.6 ± 6.4^{c}	FB without 10	47.6 ± 2.1^{d}
5-10 combined	96.7 ± 2.0^{a}	5-10 combined	92 ± 2^{a}

Constituents of ICIPE 51 involved in blends are numbered 1 (1-octene), 2 (Hexanol), 3 (2-Nonanol), 4 (phenylethylalcohol), 5 (3-nonen-2-one), 6 (acetic acid), 7 (3-octanone), 8 (2-nonanone), 9 (borneol) and 10 (4,5-dihydro-5-pentyl-2-(3H)furanone). For ICIPE 69, the constituents involved are numbered 1 (1-octene), 2 (3-octanol), 3 (hexanol), 4 (1-ethyl-2-methylbenzene), 5 (2-Propyl-1-pentanol), 6 (phenylethylalcohol), 7 (cedrene), 8 (ethylacetamide), 9 (butyrolactone) and 10 (2-pyrrolidinone). *Full blend consisted of 10-constituents of corresponding isolate. Mean (± SE) percent repellency of the resulting

blends with different letters within a column are significantly different ($p = 0.0001$, SNK test) at RD_{50} of 0.227 mg cm^{-2} and 0.427 mg cm^{-2} for ICIPE 51 and ICIPE 69, respectively. Doses tested were equivalent to the relative proportion of each in GC-MS analyses.

Table 6.4 Mean percent repellency of blends of the major components at doses equivalent to the relative proportion of each constituent at 50% repellency-dose (RD_{50}) of the full blends of isolates of *Beauveria bassiana* towards termite, *Macrotermes michaelseni*.

***Beauveria bassiana* (ICIPE 276) (Most repellent)**		***Beauveria bassiana* (ICIPE 278) (Least repellent)**	
	% repellency		**% repellency**
Constituents in blends	**Mean ± SE**	**Constituents in blends**	**Mean ± SE**
Full blend* (FB)	63.1 ± 1.9^{ab}	Full blend* (FB)	51.1 ± 2.6^{ab}
FB without 1	68.7 ± 3^{ab}	FB without 1	57.6 ± 1.8^{ab}
FB without 2	65.8 ± 2.7^{ab}	FB without 2	53.8 ± 2.5^{ab}
FB without 3	62.4 ± 1.2^{ab}	FB without 3	48.0 ± 2.4^{abc}
FB without 4	57.5 ± 1.9^{bc}	FB without 4	47.6 ± 2.9^{abc}
FB without 5	57.5 ± 3.0^{bc}	FB without 5	45.7 ± 3.5^{abc}
FB without 6	55.4 ± 2.1^{bcd}	FB without 6	45.6 ± 3.2^{abc}
FB without 7	49.2 ± 4.1^{cd}	FB without 7	45.2 ± 2.0^{bc}
FB without 8	48.7 ± 2.8^{cd}	FB without 8	43.3 ± 3.3^{bc}
FB without 9	46.7 ± 1.8^{cd}	FB without 9	37.3 ± 3.1^{c}
FB without 10	45.6 ± 3.0^{cd}	FB without 10	36.0 ± 3.2^{c}
5-10 combined	87.6 ± 1.7^{a}	5-10 combined	83.1 ± 1.7^{a}

Constituents of ICIPE 276 and ICIPE 278 involved in blends are numbered 1(3-octanol), 2 (2-methyl-1-butanol), 3 (hexanol), 4 (1-pentanol), 5 (phenol), 6 (butyrolactone), 7 (4-nonanone), 8 (2-nonanone), 9 (borneol), 10 (4,5-dihydro-5-pentyl-2-(3H)furanone and for ICIPE 278 are numbered 1(3-octanol), 2 (2-methyl-1-butanol), 3 (hexanol), 4 (1-pentanol), 5 (butyrolactone), 6 (2-nonanone), 7 (camphor), 8 (borneol) ((E)-α-bergamontene), 10 (4,5-dihydro-5-pentyl-2-(3H)furanone). *Full blend consisted of 10-

constituents of corresponding isolate. Mean (± SE) percent repellency of the resulting blends with the same letters within a column are not significantly different ($p = 0.0001$, SNK test) at RD_{50} values of 0.925 mg cm^{-2} and 2.687 mg cm^{-2} for ICIPE 276 and ICIPE 278, respectively. Doses tested were equivalent to the relative proportion of each con in GC-MS analyses.

6.5 DISCUSSION

On filter papers in olfactometric assays, the RD_{50} (giving 50% repellency indices) of odour of blends of 10 major synthetic constituents identified from ICIPE 278 and ICIPE 51 were found to be the highest (2.687 mg cm^{-2}) and the lowest (0.227 mg cm^{-2}), respectively, confirming the greater sensitivity of the termite *M. michaelseni* towards odour emissions of the latter. Of interest is the different ways in which the major constituents of isolates of the two fungi species contribute to the mean percent repellency indices to the termite in blend subtraction assays (Table 6.3 and 6.4). The results of subtraction assays of each of the components from the corresponding full blends give insight into the relative contribution of these components to avoidance behaviour of the termite. The absence of 4,5-dihydro-5-pentyl-2-(3H)-furanone in the blends of odour of 10 major synthetic constituents identified from ICIPE 51, ICIPE 276 and ICIPE 278 resulted in the largest drop in the mean percentage repellency indices of the resulting blends towards the termites. Similarly, exclusion of the 2-pyrrolidinone from the odour of blend of 10 major synthetic constituents identified from ICIPE 69 resulted in the largest drop in the mean repellency index of the resulting blend towards the termite, thus identifying these compounds as the most important components of the corresponding active blends.

Of the individual major components subtracted from the corresponding blends in which they occurred hexanol, 1-octene, 2-nonanol, 1-octen-3-ol, 3-octanol, 1-ethyl-2-methylbenzene and 2-methyl-1-butanol did not significantly affect the repellency indices

of the resulting blend towards the termite (Table 6.3 and 6.4). On the other hand, exclusion of 3-nonen-2-one, 2-nonanone, 4-nonanone, 3-octanone, acetic acid, 3-nonanone, borneol, ethylacetamide, cedrene, 2-propyl-pentanol, camphor, phenol and (E)-α-bergamontene in the corresponding blends where they occurred, significantly reduced the repellency indices of the resulting blends (Table 6.3 and 6.4). Moreover, combinations of six best repellents identified in subtraction assays, significantly resulted in higher repellency indices than those of the corresponding full blends, indicating special potency of some of the mixtures (Table 6.3 and 6.4). Thus, two variants of blend effects may be noted from this study: enhancement of the activity of inherent active components by less active constituents and synergism between moderately potent repellent compounds to produce a mixture that is more active than a linear summation of the individual activities of the constituents. The first variant is illustrated by the high repellency indices of different odours of the 10 major constituents of the corresponding isolates of *M. anisopliae* and *B. bassiana*. The second variant and probably the most expected, is illustrated by combination of the first six best major repellent components identified in the subtraction assays, which resulted to significantly higher repellency indices than that of the corresponding full blends by 1.21, 1.29, 1.39 and 1.63 times for ICIPE 51, ICIPE 69, ICIPE 276 and ICIPE 278, respectively. The responses of *M. michaelseni* to these synthetic constituents individually or in blends had not been determined until the results obtained from this study.

Indeed, some of the repellent compounds including 3-octanone, 2-nonanone, camphor, borneol, cedrene and acetic acid, have been identified from volatile blends of various fungi *spp*. and have been shown to be repellent to insects (Börjesson *et al*., 1992). For instance, 3-nonanone, camphor and borneol were shown to be repellent to adult mosquito, *Aedes aegypti* Linnaeus (Diptera: Culicidae) (Hwang *et al*. 1985). Sesquiterpene ketone, 5,6-dimethyl-8-isopropenylbicyclo-4-4-decen-3-one and cedrene were tested for their efficacy to disrupt food recruitment by *C. formosanus* as soil barriers (Maistrello *et al*., 2001). It was found out that after 21 days, wood consumption and survival by termites were significantly lower in the ketone-treated soil compared to that treated with cedrene.

Previously, 3-octanol was shown to be one of the specific constituents of volatiles from fungus *Verticillium bulbillosum* Zimmermann (Deuteromycetes: Moniliaceae) that stimulates the preference of soil Collembola, *Onychiurus armatus* Tullberg (Collembola: Onychiuridae) towards the fungus (Bengtsson *et al*., 1991). Electrophysiological experiments reported that 1-octen-3-ol, is a mild attractant for *Glossina fuscipes fuscipes* Gambiense (Diptera: Glossinidae) and *Glossina morsitan morsitan* Gambiense (Diptera: Glossinidae) (Van der Goes *et al*., 1996; Gikonyo *et al*., 2002) and a potent olfactory attractant for adult mosquitoes, *Anopheles gambiae* Gambiense (Diptera: Culicidae) in the field (Ditzen, *et al*., 2008). 2-Methyl-1-butanol is a constituent of volatile composition from yeast and attracts flying beetle, *Carpophilus humeralis* Fabricius (Coleoptera: Nitidulidae) (Nout and Bartelt, 1998).

The avoidance behaviour of the termite in the Y-olfactometer in the presence of the blend mixtures suggests that the termite has olfactory sensory neurons to detect these repellent constituents. However, the insects' odorant receptor complex (Ditzen *et al*. 2008) that code information of detection of repellent signals from these 'entomochemicals' are unknown. The insect deciphers the coded information from the volatile signatures and behavourally respond for its adaptive fitness. The results stress the importance of evaluating the components in blends to elucidate their individual full potency in a given bioactivity (Bekere and Hassanali, 2001).

The study collates and accummulates important information as evidence of the presence of diverse entomochemicals which play a role in ecological interaction between the termite, *M. michealseni* and infective fungi. Different blends of identified constituents of the selected fungal isolates validate the hypothesis that repellency of *M. anisopliae* and *B. bassiana* to the termite results from blends of some major components of the volatile emissions. There is need to determine the interactions between the termite and the repellent constituents (and perhaps other termite species) in push-pull tactics in nature.

CHAPTER SEVEN

7.0 BEHAVIOUR OF HEALTHY TERMITES *MACROTERMES MICHAELSENI* ADJACENT TO INFECTED CONSPECIFICS

7.1 INTRODUCTION

Termites such as *M. michaelseni* share a long history of association with symbiotic and infective fungi in various habitats. Division of labour in the termite as demarcated by different castes is a trade off in ultruism for these eusocial insects where some members, mainly workers willingly forego some individual privileges to work for the benefit of other castes in a colony community (Thorne, 1997). Group living has many benefits when compared to a solitary lifestyle. For instance, cooperation between the different castes can increase the efficiency of brood care, foraging (for workers) or anti-predator defenses (for soldiers) (Cremer *et al*., 2007). Termites, however, are also exposed to high risks of potent horizontal and vertical transmission of pathogens (Rich *et al*., 2007) with contact mode of action such as fungi. To mitigate the high risks of transmission of infective fungi such as *M. anisopliae* and *B. bassiana* within a given colony, termites have evolved several behavioural and physiological adaptations (Rosengaus *et al*., 2004; 2007) to counter the challenges of fungal horizontal transmission.

Behavioral mechanisms include the use of vibratory displays to warn nestmates about the presence of lethal fungal concentrations (Rosengaus *et al*., 1999b; Myles, 2002). Increase in the rate of mutual grooming in dampwood termite, *Zootermopsis angusticollis* Hagen (Isoptera: Termopsidae) when exposed to pathogens has been

reported (Rosengaus *et al*., 1998) and walling-off conidial infected areas of a colony by *R. flavipes* (Milner and Staples, 1996; Milner *et al*., 1998b). Physiological mechanisms include fungistatic secretions associated with exocrine glands and faecal pellets of the termite *N. exitiotus* that reduces microbial growth within nest chambers (Rosengaus *et al*., 2000, 2004), enhancement of resistance to infection through cellular and humoral immune responses in dampwood termites, *Z. angusticollis* (Rosengaus *et al*., 1999a; Traniello *et al*., 2002). Lamberty *et al*. (2000) reported the presence of antibiotic peptides in salivary gland secretions of termites. These defensive mechanisms demand cooperation between members of social groups or altruistic behaviours of some colony members for the benefit of the whole colony (Müller and Schmid-Hempel, 1993; Cremer *et al*., 2007).

The behavioural responses of healthy termite *M. michaelseni* adjacent to infected conspecifics have not been determined. The objective of the study was to determine the response of healthy members of the termite to conspecifics infected with conidia of the least virulent and the most virulent isolates *M. anisopliae* and *B. bassiana* at various levels of infection under standard conditions. The study would help to determine whether being social insects, a specific set of responses guide healthy members to ensure that infection is not transmitted to the rest of healthy colony members.

7.2 MATERIALS AND METHODS

7.2.1 Termites

The worker castes needed for the study were trapped overnight and maintained under laboratory conditions as described in Chapter two section 2.2.

7.2.2 Preparation of conidial suspensions

Conidial suspensions used in this experiment were of the most (ICIPE 51), the least (ICIPE 69) pathogenic isolates of *M. anisopliae*, and the protocol of their preparation remained as described in Chapter two section 2.3.1.

7.2.3 Germination of conidia and mycoses tests

Germination and mycoses tests were performed as described in sections 2.3.2 and 2.3.3, respectively in Chapter two.

7.2.4 Infection of termites with conidia of fungi

Five concentrations of conidia of each of the isolates (3×10^5, 10^6, 3×10^6, 10^6, 3×10^7) were prepared as described in section 2.3.1 in Chapter two and inoculated to groups of 20 worker termites using the protocol described in section 3.2.2 in Chapter three. Each treatment dose consisted of five replicates that were repeated four times. The protocol of handling the control groups remained as described in Chapter three section 3.2.2.

7.2.5 Olfactometric bioassays

Behaviour response of healthy worker termites adjacent to volatiles from conspecifics infected with fungi was evaluated using Y-tube olfactometer set-up (Fig. 2.1) and experimental protocol described in Chapter two section 2.5. In the treatment compartments, a group of 10 infected worker termites at various levels of conidial

infection of each of the five doses (3, 24, 48, 72 and 96 hours post-infection) were used. Control compartment had a group of 10 healthy termites. The data was recorded after every five minutes for 25 minutes and repeated four times to give 20 readings for each replicate dose.

7.3 DATA ANALYSES

Data on attraction behaviour of healthy termites towards infected conspecifics were calculated using the formula: $\frac{P_{nt} - P_{nc}}{P_{nt} + P_{nc}} \times 100$

where P_{nt} and P_{nc} represent the average percentage of worker termites in the treatment (arms containing infected termites) and control (arms with healthy termites), respectively (Wanzala *et al.*, 2004). In all the tests, data for attraction were individually pooled within the infection time levels before analyses. In attraction behavioural tests, data were arcsin transformed to normality (Gomez and Gomez, 1984) before invoking repeated measures analysis of variance (ANOVA) using Proc Mixed of SAS version 9.1 (SAS Institute, 2003). Means were separated by Student-Newman-Keuls ($\alpha = 0.05$, SNK) test.

7.4 RESULTS

In the Y-olfactometric "push-pull" system, the majority of healthy worker termites showed propensity to move towards the arm that contained infected conspecifics thus indicating that they were attracted to the volatiles emerging from the infected members. The attraction indices for the most and the least pathogenic (repellent) isolates of *M. anisopliae* did not vary significantly ($F = 0.92_{df\ (1,\ 779)}$, n = 800, $P = 0.34$, SNK, test) between the two isolates (ICIPE 51 and ICIPE 69). However, there were significant differences in attraction between the different dose levels ($F = 5.67_{df\ (4,\ 779)}$, n = 800, $P = 0.0002$, SNK, test) and different periods of infection ($F = 156.68_{df\ (4,\ 779)}$, n = 800, $P = 0.0001$, SNK, test) of these two isolates. Interaction between the period of infection and dose levels were significantly different ($F = 4.19_{df\ (4,\ 779)}$, n = 800, $P = 0.0001$, SNK, test) between the two isolates of *M. anisopliae*. There also were trade-off interaction in attraction indices at the different dose levels after 48 hours of infection for both ICIPE 51 and ICIPE 69 as shown in Figures 7.1 and 7.2, respectively.

Attraction indices of the most and the least pathogenic (repellent) isolates of *B. bassiana* (ICIPE 276 and ICIPE 278) did not vary significantly ($F = 0.03_{df\ (1,\ 779)}$, n = 800, $P = 0.86$, SNK, test). However, there were significant differences among the dose levels of conidial treatment ($F = 4.81_{df\ (4,\ 779)}$, n = 800, $P = 0.0008$, SNK, test) and the different levels of infection as depicted by the different periods ($F = 113.07_{df\ (3,\ 779)}$, n = 800, $P = 0.0001$, SNK, test) for ICIPE 276 and ICIPE 278. Interaction between the infection periods and dose levels between the two isolates were also significant ($F = 3.08_{df\ (12,\ 779)}$,

n = 800, P = 0.0003, SNK, test). As observed with the different dose levels of the two isolates of *M. anisopliae*, there were trade-off interactions in the attraction indices at the different dose levels after 48 hours of infection for both ICIPE 276 and ICIPE 278 as shown in Figures 7.3 and 7.4, respectively.

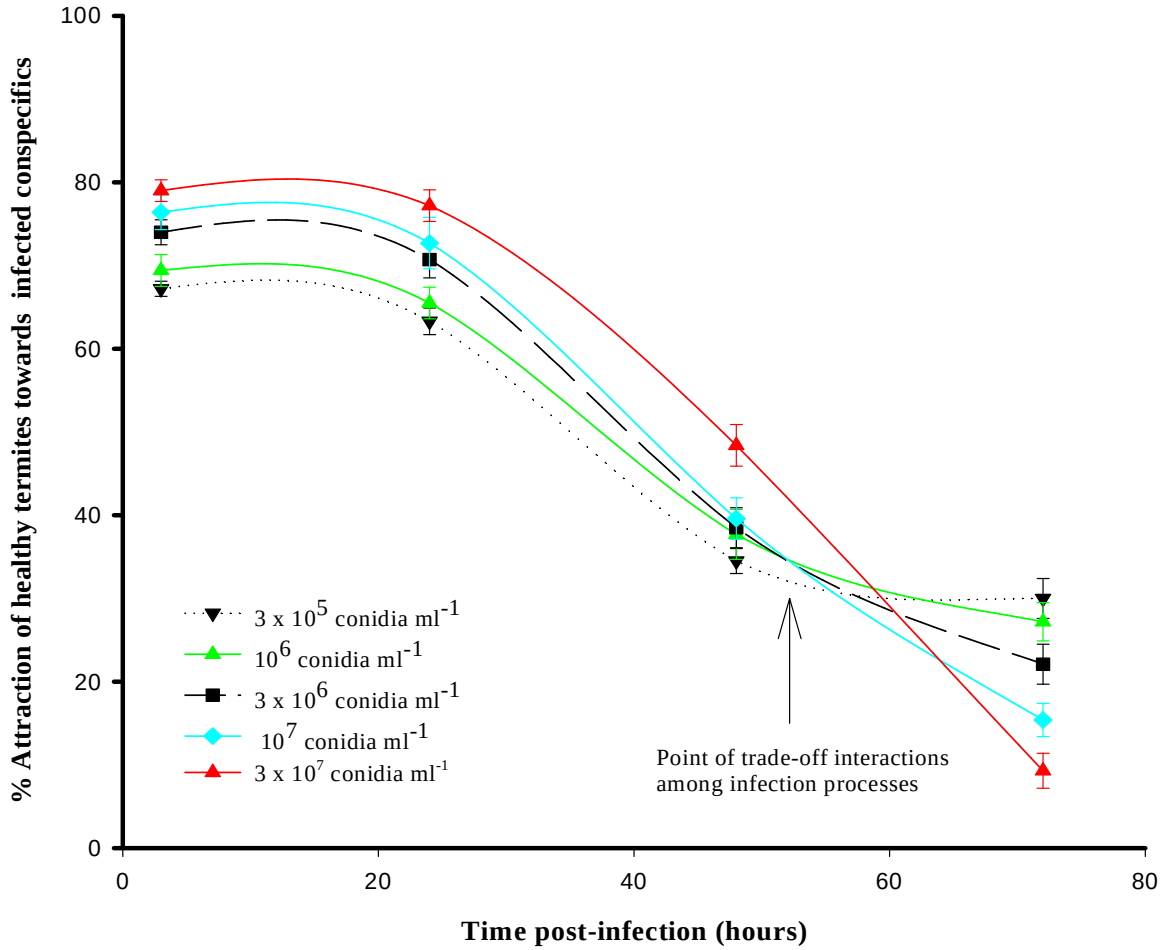


Figure 7.1 Attraction of healthy worker termites *Macrotermes michaelseni* towards conspecifics infected with conidia of the most pathogenic isolate (ICIPE 51) of *Metarhizium anisopliae* at different concentrations of conidia.

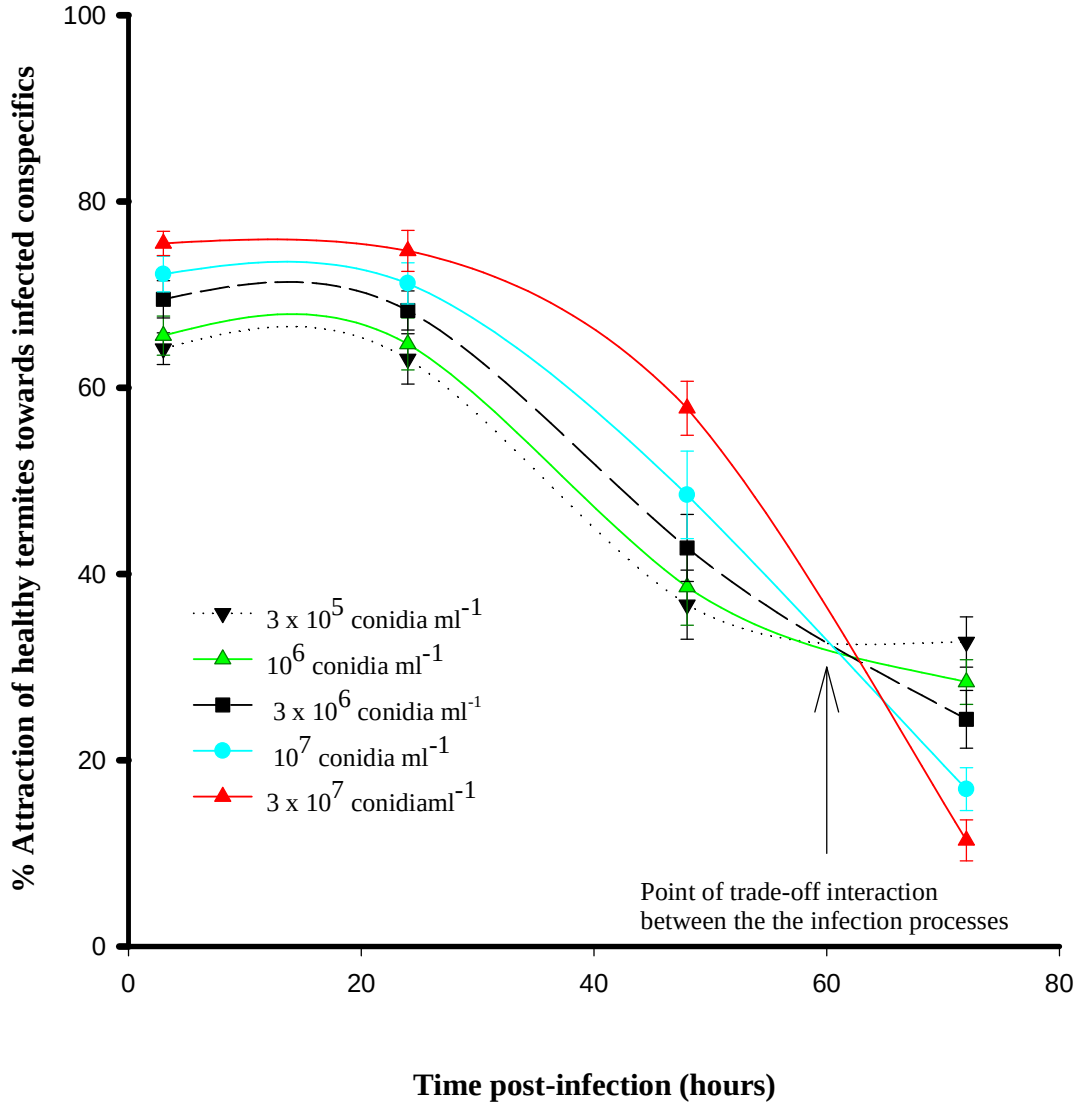


Figure 7.2 Attraction of healthy worker termites *Macrotemes michaelseni* towards conspecifics infected with conidia of the least pathogenic isolate (ICIPE 69) of *Metarhizium anisopliae* at different concentrations of conidia.

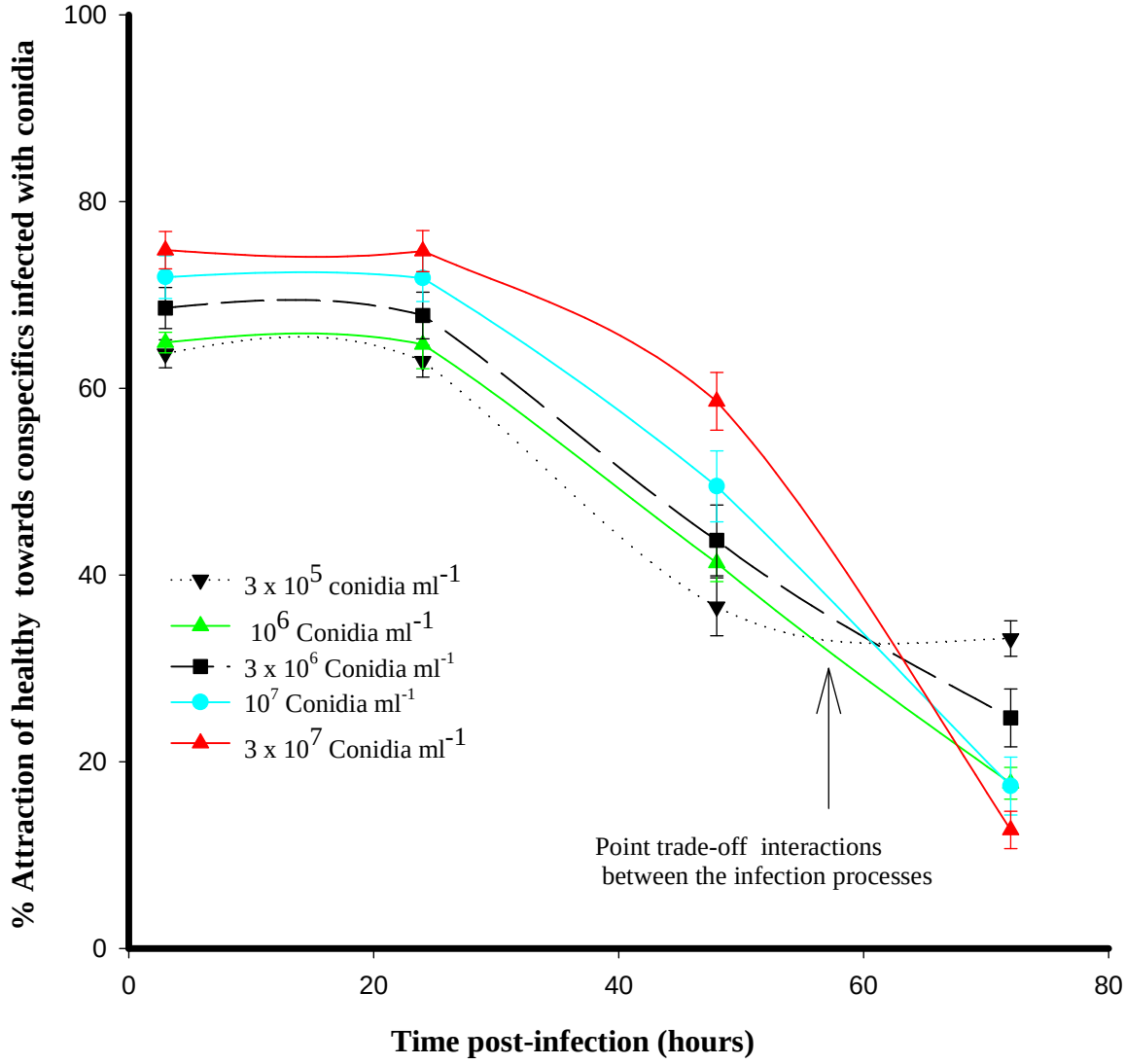


Figure 7.3 Attraction of healthy worker termites *Macrotermes michaelseni* towards conspecifics infected with conidia of the most pathogenic isolate (ICIPE 276) of *Beauveria bassiana* at different concentrations of conidia.

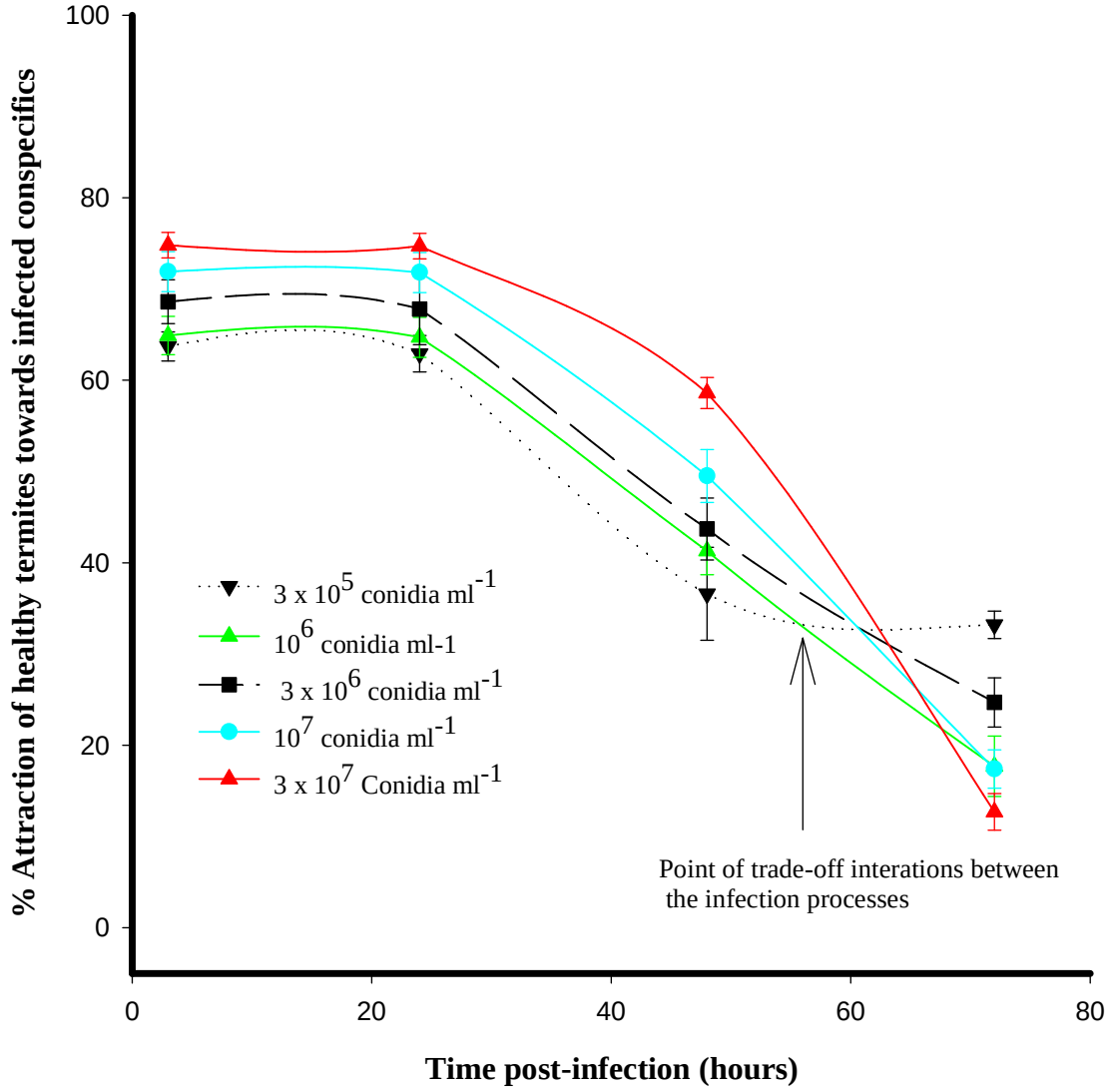


Figure 7.4 Attraction of healthy worker termites *Macrotermes michaelseni* towards conspecifics infected with conidia of the least pathogenic isolate (ICIPE 278) of *Beauveria bassiana* at different concentrations of conidia.

7.5 DISCUSSION

Healthy termite groups were attracted to freshly infected conspecifics with isolates of *M. anaisopliae* and *B. bassiana* at different conidial concentrations. This suggests that there was mediation of chemical signals emanating from the infected termites in the interaction between the infected conspecifics and the healthy termite members. The underlying mechanisms invoking the attraction behaviour of healthy termites towards the infected ones are, however, not known. Of particular importance is the lack of significant differences in attraction indices of the most and the least pathogenic isolates in both *M. anaisopliae* and *B. bassiana*. This suggests that so long as a conspecific member of the termite community is under risk, the healthy members of the colony will make vigorous effort to mount a "rescue operation" irrespective of the species of fungi that abide within the premises of the mound.

There were significant differences in the attraction indices at different dose levels and at different levels of infection thus suggesting that, the underlying mechanism is both dose and time dependent relative to the infection process. In addition, a conidial concentration at a higher dose is expected to infect and cause virulence to a given infected insect at a higher rate than the lower doses. Thus, the underlying mechanisms of attraction of the health members by the infected conspecifics are reduced at a higher rate by the higher doses than the lower doses of the conidia. This suggestion is somewhat confirmed by the trade-off in the attraction indices after 48 hours post-infection and became clear after 72 hours of termite infection (Fig. 7.1, 7.2, 7.3 and 7.4). The attraction behaviour may have

an important evolutionary trait in these altruistic insects to “cry for help” to counter the risks of spread of the disease pathogens from the infected members in the nests. It is also not known whether this “cry for help” mechanism does occur in other termite species as part of defense repertoire against the possible horizontal transmission of infectious pathogens among termite populations.

Several other behavioural mechanisms have been shown to limit possible social exchanges of infectious fungi within the nest community in termites when infection does occur to members of some termite species. Rosengaus *et al*. (1998) reported that dry wood termites, *Z. angusticolis* increased the rate of mutual grooming during and after exposure to the conidia of *M. anisopliae* while Milner and Staples (1996) reported walling off infected areas of a colony by *C. flavipes* and removal of diseased individuals from their nest (Traniello *et al*. 2002). The results from the current study, may partly explain the behaviour that has been observed in a termite nest such as the removal of infected member from a colony, the increased rate of grooming in the presence of pathogen-infected memebers (Rath, 2000) and walling-off areas contaminated with a given infective fungi. It would be expected that when few members of a termite colony are infected with conidia such as *M. anisopliae* or *B. bassiana*, the infected conspecific would trigger an inherent mechanisms that may lead to the production of chemical signals that act as signatures of communication. Thus, the healthy members decode the information from the chemical signals and direct the inherent energy to specific sets of behaviours in order to keep the colony clean (Traniello *et al*. 2002; Rosengaus *et al*., 2003). The attraction behaviour may implicate an important evolutionary trait in these

eusocial insects to “cry for help” to their conspecifics to counter the risk of spread of disease pathogen within the nests.

The differential attraction of healthy termites by infected conspecifics suggests qualitative and/or quantitative change in the composition of chemical signals emanating from the treated insects over time. Characterization of the chemical composition of the volatiles at varying durations of fungal infections that mediate the observed behaviour would further the understanding of the nature of the signatures. Olfactometric assays of the identified compounds individually and in mixtures would help to characterize those that contribute to the initial attraction and what compositional changes occur leading to the observed decline over time of infection for the different fungal isolates. The chemicals that invoke the attraction behaviour in healthy termite, *M. michaelseni*, towards infected conspecifics may be used in push pull tactics (as pull factor) in integrated control and management of the termite and perhaps other termite species. They could also be incorporated in baits for mass trapping of termites.

The study gives an insight that being social insects, a specific set of information, which is, coded as signals from infected conspecifics guide healthy members in making eusocialial decisions important for adaptive fitness and survival (Pie *et al*. 2005) in microbial rich subterranean niche. Chemical components responsible for the attraction of healthy termites to infected conspecifics require further investigation for qualitative and quantitative identification. Thus, the objective of the study was met and the results break

into a new area of study in the termite research. The results give strong indications to the validity of the hypothesis that being social insects, a specific set of responses guide healthy members to ensure that infection is not transmitted to the rest of the colony.

CHAPTER EIGHT

8.1 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

Fifteen isolates of *M. anisopliae* and three of *B. bassiana* were evaluated for virulence against the termite *M. michaelseni* from the results obtained from this study, it is clear that, the insect was susceptible to all the isolate albeit at different levels. Nine selected isolates of *M. anisopliae* and the three of *B. bassiana* were studied and showed differential repellency indices towards the termite *M. michaelseni*. Detection and avoidance of repellent isolates of fungi by termites through olfaction was demonstrated for the first time. There was a strong positive correlation between virulence and repellency of the selected isolates of the two fungi towards the termite *M. michaelseni* suggesting that termites *M. michaelseni*, have developed inherent repertoire to detect and avoid infective fungi in the hemiedaphic habitats.

Mycochemical emissions from *M. anisopliae* and *B. bassiana* mediate the observed avoidance behaviour in termite *M. michaelseni*, in a Y-olfactometer set-up. The mycochemical signals differ qualitatively and quantitatively among the most and the least repellent isolates of *M. anisopliae* and *B. bassiana* and the diffences may account for the differential repellency indices reported in Chapter four. The synthetic constituents of the identified components from odour emission from the isolates of the fungi also showed differential repellency to the termite in Y-olfactometer. The synthetic constituents were tested in proportions they occurred in nature (GC-MS

analyses). This confirmed the repellent nature of the fungal isolates. This is the first time that entomochemical repellents to the termite *M. michaelseni* have been identified.

Healthy termites *M. michaelseni* are attracted to conspecifics freshly infected with conidia of isolates of *M. anisopliae* and *B. bassiana* in a Y- Olfactometer set-up. The attraction significantly decreases over the period of infection. This is the first time the the termite attraction behaviour is being reported and suggests mediation of volatiles which are produced from freshly infected termites. The identification of the chemical signals involved in the attraction behaviour could facilitate bioassays with synthetic components in Y-olfactometer.

In view of the foregoing, further research is recommended. The relationship between virulence and repellency of infective fungi species could be tested on other termite species under laboratory conditions. Further research could be done to evaluate the repellency of fungi and the performance of the identified repellent constituents in the field be evaluated under natural conditions. Chemical signals emitted by termites *M. michaelseni* infected with conidia of *M. anisopliae and B. bassiana* that attracts healthy conspecifics need to be investigated, assayed under laboratory and natural conditions for possible utilization in mass trapping of *M. michaelseni* and perhaps other termite species. The information on the attractive and chemical signals, repellency and virulence of the isolates of *M. anisopliae* and *B. bassiana* can further be explored for potential utilization

in pull, push and kill tactics, respectively, in IPM strategies against the *M. michaelseni* and perhaps other termite species.

REFERENCES

Abbott, W. S. (1925). A method for computing the effectiveness of an insecticide. *Journal of Economic Entomology*, **18**: 265-267.

Angioy, A. M., Desogus, A. Barbarossa, I. T., Anderson, P. and Hansson, B. S. (2003). Extreme sensitivity in an olfactory system. *Chemical Senses*, **28**: 279-284.

Barnays, E. A. and Chapman, R. F. (1994). Host-plant selection by phytophagous insects. Comtemporary topics in entomology 2. Chapman and Hall, New York, USA. pp. 268-367.

Bartelt, R. J., and Zilkowski, B. W. (1998). A versatile and quantitative volatile-delivery system for laboratory bioassays. *Journal of Chemical Ecology*, **24** (3): 535-558.

Bassi, A. (1834). Del Mal del segno calicinaccio o moscardino malattia che affligei bachi da seta. Parte 1. *Teorial Lodi*. pp. 67.

Beeson, C. F. C. (1941). A guide to the control of termites for forest officers. *Indian forest records (New series), Entomology*, **4**: 44-90.

Bekere, J. and Hassanali, A. (2001). Blend effects in the toxicity of the essential oil constituents of Ocimum kilimandscharicum and Ocimum kenyense

(Labiateae) on two post-harvest insect pests. *Phytochemistry*, **57 (3):** 385-391.

Bengtsson, G., Hedlund, K., and Rundgren, S. (1991). Selective odor perception in the soil Collembola, *Onychiurus armatus*. *Journal of Chemical Ecology*, **11**: 2113 – 2125.

Benz, G. (1987). Environment. In: Fuxa, J. R. and Tanada, Y. (Eds.). Epizootiology of insect diseases. John Wiley, New York. pp. 177-214.

Bernays, E. A. and Chapman, R. F. (1994). Host-plant selection by phytophagous insects. Chapman and Hall, New York. pp. 245-279

Bidochka, M. J., Kamp, A. M. and Amritha De Croos, J. N. (2000). Insect pathogenic fungi: from genes to populations. In: Kronstand, J. W. (Ed.), Fungal pathology. Kluwer Academic Publishers, The Netherlands. pp. 171-193.

Bonabeau, E., Theraulaz, G., Deneubourg, J. L., Aron, S. and Camezine, S. (1997). Self-organization in social insects. *Trends in Ecology and Evolution*, **12**: 188-193.

Bonabeau, E., Theraulaz, G., Deneubourg, J. L., Franks, N. R., Rafelsberger, O., Joly, J. L. and Blanco, S. (1998). A model for the emergence of pillars, walls and royal chambers in termite nests. *Philosophical Transactions of the Royal Society of London Series B*: *Biological Sciences*, **353**: 1561-1576.

Börjesson, T., Stöllman, U. and Schnürer, J. (1992). Volatile metabolites produced by six fungal species compared with other indicators of fungal growth on

cereal grains. *Applied and Environmental Microbiology*, **58** (8): 2599-2605.

Boucias, D. G. and Latgé, J. P. (1988) Nonspecific induction of germination of *Conidiobolus obscurus* and *Nomuraea rileyi* with host and non-host cuticle extract. *Journal of Invertebrate Pathology*, **51**: 186-171.

Braga, G. U. L., Flint, S. D., Messias, C. L., Anderson, A. J. and Roberts, D. W. (2001a). Effects of UV-B irradiance on conidia and germinants of the entomopathogenic hypomycetes *Metarhizium anisopliae*: a study of reciprocity and recovery. *Photochemistry and Photobiology*, **73**: 140-146.

Braga, G. U. L., Flint, S. D., Messias, C. L., Anderson, A. J., Roberts, D. W. (2001b). Effects of UV-B on conidia and germlings of the entomopathogenic hypomycete *Metarhizium anisopliae*. *Mycological Research*, **105**: 874-882.

Brown, G. C. and Nordin, G. L. (1986). Evaluation of an early harvest approach for induction of *Erynia* epizootics in alfalfa weevils populations. *Journal of the Kansas Entomological Society*, **59**: 446-453.

Burgerjon, A. (1956). Pulverisation de poudrage au laboratoire par des preparations pathogens insecticides. *Annals of Epiphytology*, **4**: 677-688.

Burnet, P. J. C. (1980). The metabolism of the aromatic amino acids concerned in the cross liking of insect cuticle. *Insect Biochemistry*, **10**: 476-500.

Butt, T. M. (1990). Fungal infection process: A mini review. In: Proceedings and abstract 5th International colloquium on Invertebrate Pathology and Microbial Control. Adelaide, Australia. pp. 121-124.

Butt, T. M., Beckett, A. and Wilding, N. (1990). A historical study of the invasive and developmental process of the aphid pathogen, *Erynia neoaphidis* (Zygomycotina: Entomophthorales) in the pea aphid *Acyrthosiphon pisum*. *Canadian Journal of Botany*, **68**: 2158-2163.

Camarine, S., Deneubourg, J. L., Franks, N., Sneyd, J., Theraulaz, G. and Bonabeau, E. (2001). Self-organization in biological systems. Princeton, N. J., Princeton University Press. pp. 239-345.

Carruthers, R. I., Sawyer, A. J. and Hural, K. (1991). Use of fungal pathogens for biological control of insect pests. In: Sustainable agriculture research and education in the field: A proceeding national academic press. Washington DC. USA. pp. 417-

Chapman, R. F. (1998). The Insect: Structure and Functions. 4th Ed. Cambridge University press, UK. pp. 3-18.

Charnley, A. K. (1989). Mycoinsecticides; present use and future prospects. In: Progress and Prospects in insect control. *BCPC Monograph*, **43**, pp. 165-181.

Chen, J., Henderson, G., Grimm, C. C., Lloyd, S. W. and Laine, R. A. (1998). Termites fumigate their nests with naphthalene. *Nature*, **392**: 558–559.

Colborn, T., Dumanoski, D. and Myers, J. P. (1997). Our Future; Are we threatening our fertility, intelligence and survival? A scientific detective story. Plume/ Penguin, Ringwood. UK. pp. 219-256.

Collins, N. M. (1981). The role of termites in the decomposition of wood and leaf litter in the southern Guinea savanna of Nigeria. *Oecologia*, **51**: 389-399.

Collins, N. M. and Wood, T. G. (1984). Termites and atmospheric gas production. *Science*, **224**: 84-86.

Cook, S. M. Khan, Z. R. and Pickett, J. A. (2007). The use of push-push strategies in integrated pest management. *Annual Review of Entomology*, **52**: 375-400.

Cowie, R. H. and Wood, T. G. (1989). Damage to crops, forestry and rangeland by fungus-growing termites (Termitidae: Macrotermitinae) in Ethiopia. *Sociobiology*, **15**: 139-153.

Cowie, R. H., Logan, J. W. M. and Wood, T. G. (1989). Termite (Isoptera) damage and control in tropical forestry with special reference to Africa and Indo-Malaysia: A review. *Bulletin of Entomological Research*, **97**: 173-184.

Cox, S. M., Khan, Z. R. and Pickett, J. A. (2007). The push-pull strategies in integrated pest management. *Annual Review of Entomology*, 52: 375-400.

Creffield, J. W. (1996). Wood-destroying insects, wood borers and termites. CSIRO publishing, Collinwood, Australia. pp. 44.

Cremer, S., Armitage, S. A. O. and Schmid-Hempel, P. (2007) Social immunity. *Current Biology*, **17**: 693-702.

Crespi, B. J. (1992). Eusociality in Australian gall thrips. *Nature*, **359**: 724-726.

Culliney, T. W. and Grace, J. K. (2000). Prospects for the biological control of subterranean termites (Isoptera: Rhinotermitidae), with special reference to *Coptotermes formosanus*. *Bulletin of entomological Research*, **90**: 9-21.

D'Ettorre, P. (2007). Evolution of sociality: You are what you learn. *Current Biology*, **17** (17): 766-768.

Dangerfield, J. M., McCarthy, T. S. and Ellery, W. N. (1998). The mound-building termites *Macrotermes michaelseni* as an ecosystem engineer. *Journal of Tropical Ecology*, **14** (4): 507-520.

Dani, F. R., Jones, G. R., Corsi, S. Beard, R., Pradella, D. and Turillazzi, S. (2005). Nestmate recognition cues in the Honeybee: Differential importance of cuticular alkanes and alkenes. *Chemical Senses*, **30**: 477-489.

Darlington, J. P. E. C. (1991). Turnover in the populations within mature nests of the termite *Macrotermes michaelseni* in Kenya. *Insectes Sociaux*, **38**: 251-262.

Darlington J. P. E. C. and Dransfield R. D. (1987). Size relationships in nest populations and mound parameters in the termite *Macrotermes michaelseni* in Kenya. *Insectes Sociaux*, **34**: 165-180.

De Bruyne, M. and Baker, T. C. (2008). Odour detection in insects: Volatile codes. *Journal of Chemical Ecology*, **34**: 882 – 897.

Dean, L. S. and Heinrich, A. (1984). Combined Gas Chromatography and Electroantennogram recording of insect olfactory responses. In: Hummel, H. E. and Miller, T. A. (Eds.). Techniques in pheromone research. Springer-Verlag. New York. pp. 161-162.

Delate, K. M., Grace, K. J. and Tome, C. H. M. (1995). Potential use of pathogenic fungi in baits to control the Formosan subterranean termite (Isoptera: Rhinotermitidae). *Journal of Applied Entomology*, **119**: 429-433.

Deligne, J., Quennedey, A. and Blum, M. S. (1981). The enemies and defense mechanisms of termites. In: Hermann, H. R. (Ed.). Social insects. Vol. 2. New York, Academic Press. pp. 1-76.

Dicke, M. and Grostal, P. (2001) Chemical detection of natural enemies by arthropods: An ecological perspective. *Annual Review of Ecology and Systematics*, **32**: 1-23.

Ditzen, M., Pellegrino, M. and Vosshall, L. B. (2008). Insect odorant receptors are molecular targets of the insect repellent DEET. *Science*, **319**: 1838-1842.

Dong, C., Zhang, J., Chen, W. and Huang, H. (2007). Characterization of a newly discovered China variety of *Metarhizium anisoplaie* (*M. anisopliae* var. dcjhyium) for virulence to termites, isoenyme, and phylogenic analysis. *Mycological Research*, **162**: 53-61.

Duraimurugan, P and Regupathy (2005). Push-pull strategy with trap crops, neem and nuclear polyhedrosis virus for insecticide resistance management in *Helicoverpa armigera* (Hubner) in cotton. *American Journal of Applied Science*, **2**:1042–48.

Dustan, A. G. (1923). Studies on a new species of Empusa parasitic on the green apple bug (*Lygus communis* var. *novascotiensis*) in the Annapolis Valley. *Proceedings of the Canadian Entomological Society*, **9**: 14.

EL-Sayed, A. M., Suckling, D. M., Wearing, C. H. and Byers, J. A. 2006. Potential of mass trapping for long-term pest management and eradication of invasive species. *Journal of Economic Entomology*, **99**: 1550–1564.

Epila, J. S. O., and D. B. A. Ruyooka. (1988). Cultural methods of controlling termites attack on cassava (*Manihot esculanta*) with *Vitex doniana*. A preliminary study. *Sociobiology*, **14**: 291-297.

Faldt, J. Jonsell, M., Nordlander, G. and Borg-Karlson, A. K. (1999). Volatiles of bracket fungi *Fomitopsis pinicola* and *Fomes fomentarius* and their functions as insect attractants. *Journal of Chemical Ecology*, **25** (3): 567-589.

Feng, Z., Carruthers, R. I., Roberts, D. W. and Robson, D. S. (1985). Age-specific dose-mortality effects of *Beauveria bassiana* on the European corn borer, *Ostrinia nubilalis*. *Journal of Invertebrate Pathology*, **46**: 259-264.

Ferron, P. (1977). Influence of relative humidity on the development of fungal infection caused by *Beauveria bassiana* (Fungi imperfecti, Moniliales) in emergence of *Acanthoscelides obtectus* (Coleoptera: Brucidae). *Entomophaga*, **22**: 393-396.

Ferron, P. (1978). Biological control of insect pests by entomopathogenous fungi. *Annual Review of Entomology*, **23**: 409-442.

Ferron, P. (1981). Pest control by the fungi *Beauveria* and *Metarhizium*. In: H. D. Burges (Ed.). Microbial control of pests and plant diseseases 1970-1980. Academic Press, London. pp. 465-476.

Ferron, P. (1985). Fungal control. In: G. A. Kerkut and Gilbert, L. I. (Eds.). Comprehensive insect physiology, Biochemistry and Pharmacology, Vol. 12,. Oxford, London, UK. pp. 313-346

Ferron, P. Fargues, J. and Riba, G. (1991). Fungi as microbial insecticides against pests. In: Arora, D. K, Ajello, L. and Mukerji, K. G. (Eds.). Handbook of Applied Mycology: Human, Animals and Insects. Vol.2. Marcel Dekker Inc. New York. pp. 665-706.

Filer, K., Schutz, E. and Geh, S. (2001). Detection of microbial volatile organic compounds (MVOCs) produced by moulds on various materials. *International Journal of Hygiene and Environmental Health*, **204** (2-3): 111-121.

Fischer, G., Muller, T., Schwalbe, R., Ostrowski, R. and Dott, W., (2000). "Species-specific profiles of mycotoxins produced in cultures and associated with conidia of airborne fungi derived from biowaste. *International Journal of Hygiene and Environmental Health*, **203** (2): 105-116.

Furlong, M. J., Pell, J. K., Choo, O. P. and Rahman, S. A. (1995). Field and Laboratory evaluation of a sex-pheromone trap for the autodissemination of the fungal entomopathogen *Zoophthora radicans* (Entomophthorales) by the diamond-back moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae). *Bulletin of Entomological Research*, **85**: 331-337.

Fuxa, J. R. (1997). Microbial control of insects: status and prospects for IPM. In: Upsdhyay, R. K., Mukerji, K. G. and Rajak, R. L. (Eds.). IPM system in

agriculture Vol. 2. Biocontrol in emerging biotechnology. ADTYA Books Ltd. New Delhi, India. pp. 57-104.

Fuxa, J. R. and Tanada, Y. (1987). Epidemiological concept applied to insect epizootiology. In: Fuxa, J. R. and Tanada, Y. (Eds.). Epizootiology of insect diseases. John Wiley and Sons, New York. pp. 3-21.

Gabriel, B. P. (1968). Histochemical studies of the insect cuticle infected by the fungus *Entomophthora coronota*. *Journal of Invertebrate Pathology*, **11**: 82-89.

Gikonyo, N. K., Hassanali, A., Njagi, P. G. N., Gitu, M. P. and Midiwo, J. O. (2002). Odor composition of preferred (Buffalo and Ox) and nonpreferred (Waterbuck) hosts of some Savanna Tsetse flies. *Journal of Chemical Ecology*, **28** (**5**): 969-981.

Gillespie, A. T. and Moorhouse, E. R. (1990). The use of fungi to control pests of agricultural and horticultural importance. In: Whipps, J. M. and Lumsden, R. D. (Eds.). Biotechnology of fungi for improvement of plant growth. Cambridge University Press, London, UK. pp. 313.

Glover, P. E., Trump, E. C. and Water-bridge, L. E. D. (1964). Terminaria and vegetation patterns on the Loita plains of Kenya. *Journal of Ecology*, **52**: 367-377.

Goettel, M. S. (1984). A simple method for mass culturing entomopathogenic hyphomycetes fungi. *Journal of Microbiological Methods*, **3**: 15-20.

Goettel, M. S., and Johnson, D. L. (1992). Environmental impact and safety of fungal biocontrol agents. In: Lomer, C. J. and Prior, C. (Eds.). Biological control of Locusts and grasshoppers. CABI International, Wellingford. UK. pp. 356-361.

Goettel, M. S., Leger, R. J. S., Bhairi, S., Jung, M. K., Oakley, B. R. Roberts, D. W. and Staples, R. C. (1990). Pathogenicity and growth of *Metarhizium anisopliae* stably transformed to benomyl resistance. *Current Genetics*, **17**: 129–132.

Goettel, M. S., and Inglis, G. D. (1997). Fungi: Hyphomycetes. In: L. A. Lacey, Ed., Manual of Techniques in Insect Patholgoy. Academic Press San Diego, USA. pp. 213-249.

Goettel, M. S. and Hajek, A. E. (2001). Evaluation of Non-target effects of pathogens use for management of arthropods. In: Wajnberg, E., Scott, J. K. and Quimby, P. C. (Eds.). Evaluating indirect ecological effects of biological control. CABI Publishing, Montpelier, France. pp. 81-97.

Gomez, K. A. and Gomez, A. A. (1984). Statistical procedures for agricultural research. Wiley, New York. pp. 3-15.

Grace, J. K., Tome, C. H. M., Shelton, T. G., Oshiro, R. J. and Yates, J. R. (1996). Baiting studies and considerations with *Coptotermes formosanus* (Isoptera: Rhinotermitidae) in Hawaii. *Sociobiology*, **28**: 511-520.

Hajek, A. E. and St. Leger, R. J. (1994). Interactions between fungal pathogens and insect hosts. *Annual Review of Entomology*, **39**: 293-322.

Hajek, A. E. and Butler, L. (2000). Predicting host range of entomopathogenic fungi. In : Follet, P. A. and Duan, J. J., (Eds.). Non-target effects of biological control. Kluwer Academic Press, Netherlands. pp. 263-276.

Hall, R. A. and Papierok, B. (1982). Fungi as biological control agents of arthropods of agricultural and medical importance. *Parasitology*, **84**: 205-240.

Hänel, H. and Watson, J. A. L. (1983) Preliminary field tests on the use of *Metarhizium anisopliae* for control of *Nasututermes exitiosus* (Hill) (Isoptera: Termitidae). *Bulletin of Entomological Research*, **73**: 305-313.

Hansson, B. S., Ochieng', S. A., Grosmaitre, X., Anton, S. and Njagi, P. G. N. (1996). Physiological responses and central nervous projections of antennal olfactory receptor neurons in the adult desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae). *Journal of Comparative Physiology*, **179**: 157-167.

Harris, W. V. (1971). Termites: Their recognition and control. 2nd Ed. Longmans, London, UK. pp. 186.

Hassanali, A., Herren, H., Khan, Z. R., Pickett, J. A. and Woodcock, C. M. (2008). Integrated pest management: the push–pull approach for controlling insect pests and weeds of cereals, and its potential for other agricultural systems including animal husbandry. *Philosophical of Transactions of Royal Society Series B.* **363**: *611– 621.*

Hayashi, Y., Lo, N., Miyata, H. and Kitade, O. (2007). Sex-Linked genetic influence on caste determination in a termite. *Science*, **318**: 985-987.

Hopkin, T. L. and Kramer, K. J. (1992). Insect cuticle scrotization. *Annual Review of Entomology*, **37**: 273- 302.

Howard, R. W. and Blomquist, G. J. (1982). Chemical ecology and biochemistry of insect hydrocarbons. *Annual Review of Entomology*, **27**: 149-172.

Howse, P., Stevens, L. and Jones, O. (1996). Insect pheromones and their use in pest management. Chapman and Hall publishers, London, UK. pp. 256.

Hummel, H. E. and Miller, T. A. (1984). Techniques in pheromone research. Springer-Verlag. New York. pp. 134.

Huxham, I. M., Samuels, K. D. K., Haele, J. B. and McCorkindale, N. J. (1989). In vivo and in vitro assays for pathogenicity of wild type and mutant strains of *Metarhizium anisopliae* for three insect species. *Journal of Invertebrate Pathology*, **53**: 143-151.

Hwang, Y., Wu, K., Kumamoto, J. Axelrod, H. and Mulla, M. S. (1985). Isolation and identification of mosquito repellents in *Artemisia vulgaris*. *Journal of Chemical Ecology*, **11** (9): 1297-1306.

Inglis, G. D., Goettel, M. S., Butt, T. M. and Strasser, H. (2001). Use of hyphomycetous fungi for managing insect pests. In: Butt, T. M., Jackson, C. and Magan, N. (Eds.). Fungi as biocontrol agent: Progress, problems and potential. CABI. Wellingford, UK. pp. 27-69.

Jackson, T. A., Alves, S. B., Pereira, R. M. (2000). Success in biological control of soil-dwelling insects by pathogens and nematods. In: Gurr, G., Wratten, S.

(Ed.). Biological control. A measure of success. Kluwer Academic. USA. pp. 271-296.

James, T. Y., Kauff, F., Schoch, C. L., Matheny, P. B., Hofstetter, V., Cox, C. J., Celio, G., Gueidan, C., Fraker, E., Miadlikowska, J., Lumbsch, H. T., Rauhut, A., Reeb, V., Arnold, E. A., Amtoft, A., Stajich, J. E., Hosaka, K., Johnson, D., O'Rourke, B., Crockett, M., Binder, M., Curtis, J. M., Slot, J. C., Wang, Z., Wilson, A. W., Arthur, S., Longcore, J. E., O'Donnell, K. Standridge, S. M., Porter, D., Letcher, P. M., Powell, M. J., Taylor, J. W., White, M. M., Griffith, G. W., Davies, D. R., Humber, R. A., Morton, J. B., Sugiyama, J., Rossman, A. Y., Rogers, J. D., Pfister, D. H., Hewitt, D., Hansen, K., Hambleton, S., Shoemaker, R. A., Kohlmeyer, J., Volkmann-Kohlmeyer, B., Spotts, R. A., Serdani, M., Crous, P. W., Hughes, K. W., Matsuura, K., Langer, E., Langer, G., Untereiner, W. A., Lucking, R., Burkhard, B. Geiser, D. M., Aptroot, A. Diederich, P., Schimitt, I., Schultz, M., Yahr, R., Hibbett, D. S., Lutzoni, F., McLaughlin, D. J., Spatafora, J. W., Vilgaly, R. (2006). Reconstructing the early evolution of fungi using a six-gene phylogeny. *Nature*, **443**: 818-822.

Johnson, R. A. and Gumel, M. H. (1981). Termite damage and crop loss studies in Nigeria-the incidence of termite-scarified groundnut pods and resulting kernel contamination of field and market samples. *Tropical Pest Management*, **27**: 342-350.

Johnston, R. E. (1989). Pheromones, the vomeronasal system, and communication: From hormonal responses to individual recognition. *Annals of the New York Academy of Sciences*, **855**: 333-348.

Kaib, M., Husseneder, C., Epplen, C., Epplen, J. T. and Brandl, R. (1996). Kin-biased foraging in a termite. *Proceeding of the Royal Society of London Series B, Biological Sciences*, **263**: 1527-1532.

Khan, K. I., Fazal, Q. and Jafri, R. H. (1977a). Pathogenicity of locally discovered *Bacillus thuringiensis* strain to the termites: *Heterotermes indicola* (Wassman) and *Microcerotermes championi* (Syder). *Pakistan Journal of Scientific Research*, **29**: 12-13.

Khan, K. I., Fazal, Q, Jafri, R. H. and Ahmed, M. (1977b). Susceptibility of various species of termites to *Serratia marcescens*. *Pakistan Journal of Scientific Research*, **30**: 117-119.

Khan, Z. R., Midega, C. A. O., Hutter, N. J., Wilkins, R. M. and Wadhams, L. J. (2006). Assessment of the potential of Napier grass (*Pennisetum purpureum*) varieties as trap plants for management of Chilo partellus. *Entomologia Experimentalis et Applicata*, **119**: 15–22.

Khan, Z. R. Amudavia, D. M., Midega, C. A. O., Wanyamaa, J. M., Pickett, J. A. (2008). Farmers' perceptions of a 'push–pull' technology for control of cereal stemborers and Striga weed in western Kenya. *Crop Protection*, **27**: 976 – 987.

Klein, M. G. and Lacey, L. A. (1999). An attractant trap for the autodissemination of entomopathogenic fungi into populations of the Japanese beetle, *Popillia japonica* (Coleoptera: Scarabaeidae). *Biocontrol Science and Technology*, **9**: 151-158.

Korb, J. (2007). Termites. *Current Biology*, **17** (23): 995-999.

Kramm, K. R, West, D. F. and Rockenbach, P. G. (1982). Termite pathogens: transfer of entomopathogen, *Metarhizium anisopliae* between *Reticulitermes* sp. termites. *Journal of Invertebrate Pathology*, **40**: 1-6.

Krishna, K. and Weesner, F. M. (1970). Biology of Termites. Vol. 2. Academic Press, New York. pp. 3-22.

Lacey, L. A., Frutos, R., Kaya, H. K. and Vail, P. (2001). Insect pathogens as biological control agents: Do they have a future? *Biological Control*, **21**: 230-248.

Lamberty, M., Zachary, D., Lanot, R., Borderau, C., Robert, A., Hoffmann, J. A., Bullet, P. (2000). Constitutive expression of a cystein-rich antifungal and linear antibacterial peptide in a termite insect. *Journal of Biological Chemistry*, **276**: 4085-4092.

Lee, H. S., Bardunias, P. and Su, Y. N. (2008). Rounding a corner of a bent termite tunnel and tunnel traffic efficiency. *Behavioural Processes*, **77**: 135-138

Lefebvre, P. and Thorne, B. L. (1984). Nymphal-soldier intercastes in *Nasutiterme lujae* and *Nasutiterme columbicus* (Isoptera: Termitidae). *Canadian Journal of Zoology*, **62**: 959-964.

Lehmann, L. and Keller, L. (2006). The evolution of cooperation and altruism- a general framework and a classification of models. *Journal of Evolutionary Biology*, **19**: 1365-1376.

Logan, J. W. M. and Abood, F. (1990). Laboratory trials on the toxicity of hydramethylnone (Amdro; AC217, 300) to *Reticulitermes santonensis* Feytaud (Isoptera: Rhinotermitidae) and *Microtermes lepidus* Sjöstedt (Isoptera: Termitidae). *Bulletin of Entomological Research*, **80**: 19-26.

Logan, J. W. M. and El Bakri, A. (1990). Termite damage to date palms (*Phoenix dactylifera*) L. in Sudan with particular reference to Dongola district. *Tropical Science*, **30**: 95-108.

Logan, J. W. M, Cowie, R. H. and Wood, T. G. (1990). Termite (Isoptera) control in agriculture and forestry by non-chemical methods: a review. *Bulletin of Entomological Research*, **80**: 309-330.

Maistrello, L., Heenderson, G. and Laine, R. A. (2001). Efficacy of vetiver oil and nookatone as soil barriers against Formasan Subterranean termite (Isoptera: Rhinotermitidae). *Journal of Economic Entomology*, **94** (6): 153-1537.

Maniania, N. K., Okello, R. O., Oluoch, R. O. and Odero, T. A. (1994) Potential of entomopathogenic hyphomycetes (Deuteromycotina) fungi for the control of the stem borer *Chilo partellus* (Lepodoptera: Pyralidae). *Journal of African Zoology*, **108**: 529-536.

Maniania, N. K. (1998). A device for influencing adult Tsetse flies, *Glossina spp*., with an Entomopathogenic fungus in the field. *Biological Control*, **11**: 248-254.

Maniania, N. K., Sithanantham, S., Ekesi, S., Ampong-Nyarko, S., Baumgärtner, B., Löhr, B., Matoka, C. M., (2003). A field trail of the entomogenous fungus *Metarhizium anisopliae* for control of onion thrips, *Thrips tabaci*. *Crop Protection*, **22**: 553-559.

Matsuura, K. (2003). Symbionts affecting termite behaviour. In: Insect symbiosis. (Bourtzis, K. and Miller, T. A.). Boca Raton, CRC Press. pp. 131-143.

Mauldin, J. K. and Beal, R. H. (1989). Entomopathogenous nematodes for control of subterranean termites, *Reticulitermes spp*. (Isoptera: Rhinotermitidae). *Journal of Economic Entomology*, **82**: 1638-1642.

Mayer, C. J, Vilcinskas, A. and Gross, J. (2008). Pathogen-induced release of plant allomone manipulates vector insect behavior. *Journal of Chemical Ecology*, **34**:1518–1522

Michener, C.D. (1969). Comparative social behavior of bees. *Annual Review of Entomology*, **14**: 299-342.

Milewski, A. V., Abensperg-Traun, M. and Dickman, C. R. (1994). Why are termites and ant-eating mammals smaller in Australia than in South Africa: history or ecology? *Journal of Biogeography*, **21**: 529-543.

Milner, R. J., Staples, J. A., and Lutton, G. G. (1983). Effect of temperature on *Zoophthora radicans* (Brefeld) Batko: An introduced microbial control

agent of the spotted alfalfa aphid *Therioaphis trifolii* (Monell) *F. maculata*. *Journal of the Australian Entomological Society*, **22**: 167.

Milner, R. J. and Staples, J. A. (1996). Biological control of Termites: Results and experiences within a CSIRO project I Australia. *Biocontrol Science and Technology*, **6**: 3-9.

Milner, R. J., Staples, J. A. and Lutton, G. G. (1997). The effect of humidity on germination and infection of termites by the Hyphomycete, *Metarhizium anisopliae*. *Journal of Invertebrate Pathology*, 69: 64-69.

Milner, R. J., Staple, J. A. and Lutton, G. G. (1998a). Occurrence of *Metarhizium anisopliae* in nests and feeding sites of Australian termites. *Mycological Research*, **10**: 216-220.

Milner, R. J., Staples, J. A., and Lutton, G. G. (1998b). The selection of an isolate of the hyphomycete fungus, *Metarhizium anisopliae*, for control of termites in Australia. *Biological Control*, **11**: 240-247.

Milner, R. J. (2000). Current status of *Metarhizium* as mycoinsecticide in Australia. *Biocontrol News and Information*, **21**: 47-50.

Miller, J. R., Gut, L. J., De lame, F. M., and Stelinski, L. L. (2006a). Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part 1): Theory. *Journal of Chemical Ecology*, **32**: 2089–2114.

Miller, J. R., Gut, L. J., Delame, F. M. and Stelinski, L. L. (2006b). Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth

sexual communication by point sources of sex pheromone (Part 2): Case Studies. *Journal of Chemical Ecology*, **32**: 2115–2143.

Moore, D., Bridge, P. D., Higgins, P. M., Bateman, R. P., and Prior, C. (1993). Ultra-violet radiation damage to *Metarhizium flavoviride* conidia and the protection given by vegetable and mineral oils and chemical sunscreens. *Annals of Application of Biology*. **122**: 605–616.

Müller, C. B. and Schmid-Hempel, P. (1993). Exploitation of cold temperatures as defense against parasitoids in bumblebees. *Nature*, **363**: 65-67.

Müeller, U. G. and Gerardo, N. (2002). Fungus-farming insects: multiple origins and diverse evolutionary histories. *Proceedings of the National Academy of Science of the USA*, **99**: 15247–15249.

Murlis, J. S., Elkinton J. S. and Cardé, R. T. (1992). Odor plumes and how insects use them. *Annual Review of Entomology*, **37**: 505–532.

Myles, T. G. (1992). Non-chemical termite control. *Construction Canada*, **7**: 13-18.

Myles, T. G. (1994). Use of disodium octaborate tetrahydrate to protect aspen waferboard from termites. *Journal of Forest Product*, **44** (9): 33-36.

Myles, T. G. (2002). Review of termite biocontrol agents with methods of isolation and culture, and dosing of the fungal pathogen, *Metarhizium anisopliae*. *Sociobiology*, **39**: 277-280.

Natale, D., Mattiacci, L., Hern, A. Pasqualini, E. and Dorn, S. (2003). Response of female *Cydia molesta* (Lepidotera: Tortricidae) to plant derived volatiles. *Bulletin of Entomological research*, **93**: 335-342.

Nair, K. S. S. and Varma, R. V. (1985). Some ecological aspects of the termite problem in young eucalypt plantations in Kerala, India. *Forest Ecology and Management*, **12**: 287-303.

Newman, G. G., and Carner, G. R. (1975). Environmental factors affecting conidial sporulation and germination of *Entomophthora gammae*. *Entomologia Experimentalis et Applicata*, **15**: 155-159.

Nout, M. J. R. and Bartelt, R. J. (1998). Attraction of a flying Nitidulid (*Carpophilus humeralis*) to volatiles produced by yeasts grown on sweet coral on and a corn-based medium. *Journal of Chemicl Ecology*, **24** (7): 1217-1239.

Nutting, W. L. (1990). Insecta: Isoptera. In: Dindal, D.L., (Ed.). Soil Biology Guide. John Wiley and Sons, New York. pp. 997-1032.

Okot-Kotber, B. M. (1981). Instars and polymorphism in castes in *Macrotermes michaelseni* (Isoptera: Macrotermitidae). *Insectes Sociaux*, **28**: 233-246.

Pasteels, J. M. and Bordereau, C. (1998). Releaser pheromones in termites. In: Vander Meer, R. K., Winston, M. L., Espelie, K. E. (Eds.), Pheromone communication in Social insects. Westview press, Boulder, C.O. UK. pp. 193-215.

Pearce, M. J. (1997). Termites, Biology and Pest management, CABI International, Kent, UK. pp. 15-39.

Pell, J. K., Wilding, N., Player, A. L. and Clark, S. J. (1993). Selection of an isolate of *Zoophthora radicans* (Zygomycetes: Entomophthorales) for biocontrol

of the diamond back moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae). *Journal of Invertebrate Pathology*, **61**: 75-80.

Peppuy, A., Robert, A., Semon, E., Ginies, C., Lettere, M., Bonnard, O., and Bordereau, C. (2001). (Z)-Dodec-3-en-1-ol, a novel termite trail pheromone identified after solid phase micro-extraction from *Macrotemes annandalei*. *Journal of Insect Physiology*, **47**: 445-453.

Peterson, C. J., Gerard, P. D. and Wagner, T. L. (2008). Charring does not affect wood infestation by subterranean termites. *Entomologia Experimentalis et Applicata*. **126**: 78-84.

Pickett, J. A., Wadhams, L. J. and Woodcock, C. M. (1997). Developing sustainable pest control from chemical ecology. *Agriculture, Ecosystems and Environment*, **64**: 149-156.

Pie, M. R., Rosengaus, R. B., Calleri, D. V. and Traniello, J. F. A. (2005). Density and disease resistance in group-living insects:do eusocial species exhibit density-dependent prophylaxis. *Ethology Ecology and Evolution*, **17**: 41-50.

Piñero, J. C. and Dorn, S. (2007). Synergism between aromatic compounds and green leaf volatiles derived from the host plant underlies female attraction in the oriental fruit moth. *Entomologia Experimentalis et Applicata*, **125**: 185-194.

Praserthon, S. and Tanada, Y. (1968). The formation and circulation in Galleria of hyphal bodies of entomopathogeneous fungi. *Journal of Invertebrate Pathology*, **11**: 260-280.

Rath, C. A. (2000). The use of entomopathogenic fungi for control of termites. *Biocontrol Science and Technology*, **10**: 563-581.Rath, A. C. and Tidbury, C. A. (1996). Susceptibility of *Coptotermes acinaciformis* (Isoptera: Rhinotermitidae) to entomopathogenic fungi in Texas. *Sociobiology*, **28**: 67-72.

Rich, W. N., Staurt, R. J. and Rosengaus, R. B. (2007). Susceptibility and behavioural responses of the dampwood termite *Zootermopsis angusticollis* to the entomopathogenic nematode *Steinernema carpocapsae*. *Journal of Invertebrate Pathology*, **95**: 17-25.

Robert, D. W. and Humber, R. A. (1981). Entomogenous fungi. In: Cole, G.T., Kendrick, B., (Ed.). Biology of conidial fungi. Vol. 2. Academic press, New York. pp. 201-236.

Röhrig, A. Kirchner, W. H. and Leuthold, R. H. (1999). Vibrational alarm communication in the African fungus-growing termite genus *Macrotermes* (Isoptera: Termitidae). *Insectes sociaux*, **46**: 71-77.

Rosengaus, R. B., Maxmen, A. B., Coates, L. E. and Traniello, F. A. (1998). Disease resistance: a benefit of sociality in the dampwood termite *Zootermopsis angusticollis* (Isoptera: Termopsidae). *Behavioural Ecology and Sociobiology*, **44**: 125-134.

Rosengaus, R. B, Traniello, J. F. A., Chen, T., Brown, J. J. and Karp, R. D. (1999a). Acquired immunity in a social insect. *Naturwissenschaften*, **86**: 588-591.

Rosengaus, R. B, Jordan, C., Lefebvre, M. L., Traniello, J. F. A. (1999b). Pathogen alarm behaviour in a termite: a new form of communication in social insects. *Naturwissenschaften*, **86**: 544-548.

Rosengaus, R. B., Lefebvre, M. L. and Traniello, J. F. A. (2000). Inhibition of fungal spore germination by *Nasutitermes*: Evidence for a possible antiseptic role of soldier defensive secretions. *Journal of Chemical Ecology*, **26** (1): 21-39.

Rosengaus, R. B., Moustakas, J. E., Calleri, D. V, and Traniello, J. F. A. (2003). Nesting ecology and cuticular microbial loads in dampwood, *Zootermopsis angusticollis and* drywood termites, *Incisitermes minor, I. Schwarzi, Cryptotermes cavifrons*. *Journal of Insect Science*, **3**: 1-6.

Rosengaus, R. B., Traniello, J. F. A., Lefebvre, M. L. and Maxmen, A. B. (2004). Fungistatic activity of the sternal gland secretion of the dampwood termite *Zootermopsis angusticollis*. *Insect Science*, **51** (3): 259-264.

Rosengaus, R. B., Cornelisse, T., Guschanski, K., Traniello, J. F. A. (2007). Inducible immune proteins in dampwood termite *Zootermopsis angusticollis*. *Naturwissenschaften*, **94**: 25-33.

Sands, W. A. (1973). Termites as pest of Tropical food crops. *Pest Article and News Summaries*, **19**, 167-177.

SAS Institute. (2003). SAS user's Guide: Statistic. Version 9.1. SAS Institute, Cary, N.C. USA.

Schmid-Hempel, P. (1998). Parasites in Social Insects. Pricenton University Press, UK. pp. 273-282.

Scholte, E. J., Ng'habi, K., Kihonda, J., Takken, W., Paaijmans, K., Abdulla, S., Killeen, G. F. and Knols, B. G. J. (2005). An entomopathogenic fungus for control of adult African malaria mosquitoes. *Science*, **308**: 1641-1642.

Sen-Sarma, P. K. (1986). Economically important termites and their management in the Oriental region. In: Vinson, S.B., (Ed.). Economic impact and Control of Social Insects. Praeger. New York. pp. 69-102.

Sheppe, W. (1970). Invertebrate predation on termites of the African savanna. *Insectes Sociaux*, **17**: 205-218.

Shykoff, J. A and Schmid-Hempel, P. (1991). Parasites and the advantage of genetic variability within social insect colonies. *Proceedings of Royal Society of London Series B*, **243**: 55-58.

Smith, R. J. and Grula, E. A. (1981). Nutritional requirements for conidial germination and hyphal growth of *Beauveria bassiana*. *Journal of Invertebrate Pathology*, **37**: 222-230.

Smith A. A., Hölldober, B. and Liebig, J. (2009). Cuticular hydroca rbons reliably identify cheaters and allow enforcement of altruism in a social insect. *Current Biology*, **19**: 78-81.

Singh, U. R and Singh, J. S. (1981). Population structure and mound architecture of the termites of a tropical deciduous forest of Varansi, India. *Paedobiologia*, **22**: 213-223.

Šobotník, J. Hanus, R., Kaliná, B., Piskorski, R., Cvačka, J., Bourguignon, and Roisin, Y. (2008). (E, E)-α-Farnesene, an alarm pheromone of the termite *Prorhinotermes canalifrons*. *Journal of Chemical Ecology*, **34**: 478-486.

Srivastava, K. P. and Butani, D. K. (1987). Insect pests of tea in india and their control. *Pesticides*, **21** (2): 16-21.

Staples, J. and Milner, R. J. (2000). A laboratory evaluation of the repellency of *Metarhizium anisopliae* conidia to *Coptotermes lacteus* (Isoptera: Rhinotermitidae). *Sociobiology*, **36**: 133-147.

St. Leger, R. J. Charnley, A. K. and Cooper, R. M. (1986). Cuticle degrading enzymes of entomopathogenic fungi: Mechanisms of interaction between pathogen enzymes and insect cuticle. *Journal of Invertebrate Pathology*, **47**: 295-302.

St. Leger, R. J., Joshi, L. and Roberts, D. W. (1997). Adaptation of proteases, carbohydrase of saprophytic, phytopathogenic, and entomopathogenic fungi to the requirements of their ecological niches. *Microbiology*, 143: 1983-1992.

Stokes, M. E., Davis, C. S. and Koch, G. G., 2000. Categorical data analysis using the SAS system (2^{nd} ed.). SAS Institute Inc., Cary, NC, USA.

Storey, G. K. and McCoy, C. W. (1992). Potential for biological control of soil insects using microbial pesticides in the Caribbean. *Florida Entomologist*, **75**: 533-539.

Strasser, H., Vey, A., and Butt, T. M. (2000). Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium*, *Tolypocladium* and *Beauveria* species. *Biocontrol Science and Technology*, **10**: 717-735.

Su, N. Y. and Scheffrahn, R. H. (1998). A review of subterranean termite control practices and prospects for integrated pest management programs. *Integrated Pest Management Review*, **3**: 1- 13.

Sun, J., Fuxa, J. R. and Henderson, G. (2002). Sporulation of *Metarhizium anisopliae* and *Beauveria bassiana* on *Coptotermes formosanus* and in Vitro. *Journal of Invertebrate Pathology*, **81**: 78-85.

Sun, J., Fuxa, J. R. and Henderson, G. (2003). Effects of virulence, Sporulation, and temperature on *Metarhizium anisopliae* and *Beauveria bassiana* laboratory transmission in *Coptotermes formosanus*. *Journal of Invertebrate Pathology*, **81**: 78-85.

Swaran, P. R. and Varma, R. V. (2003). Loboratory evaluation of the entomopathogenic fungus, *Metarhizium anisopliae* var. *major* against the subterranean termite, *Odontotermes guptai* Roonwal and Bose. *Journal of Biological Control*, **17**(2): 147-151.

Talaei-hassanloui, R., Kharazi-pakdel, A., Goettel, M. S., Little, S. and Mozaffari, J. (2007). Germination polarity of *Beauveria bassiana* and its possible correlation with virulence. *Journal of Invertebrate Pathology*, **94**: 102-107.

Tamashiro, M., Fijii, J. K. and Lai, P. Y. (1975). A simple method to observe, trap and prepare large numbers of subterranean termites for laboratory and field experiments. *Environmental Entomology*, **2** (4): 721-722.

Tanada, Y. and Fuxa, J. R. (1987). The pathogen population. In: Fuxa, J. R. and Tanada, (eds.), Epizootiology of insect Diseases. New York: John Wiley and Sons. pp. 113-157.

Tasin, M. Backman, A. C. and Bengtsson, M. Ioriatti, C. and Wizgall, P. (2006). Essential host plant cues in the grapevine moth. *Naturwissenschaften*, 93: 141-144.

Theraulaz, G., Gautrais, J., Camazine, S. and Deneubourg, J. L. (2003). The formation of spatial patterns in social insects ; from simple behaviours to complex structures. *Philosophical Transactions of Royal Society of London series A*, **361**: 1263-1282.

Thomas, S. R., Stephen, R. and Elkinton, S. J. (2004). Pathogenicity and virulence. *Journal of Insect Physiology*, **85**: 146-151.

Thorne, B. (1997). Evolution of Eusociality in termites. *Annual Review of Ecology and Systematics*, **28**: 27-54.

Throne, J. E., Weaver, D. K. and Baker, J. E. (1995). Probit analysis of correlated data: Multiple observations over time at one concentration. *Journal of Economic Entomology*, **88**: 1510-1512.

Traniello, J. F. A., Rosengaus, R. B. and Sovoie, K. (2002). The development of immunity in social insects: Evidence for the group facilitation of disease resistance. *Proceedings of National Academy of Science of the USA*, **99**: 6839-6842.

Van Der Goes, M. W., Naters, V., Bootsma, L., Den Otter, C. J. and Belemtougri, R. G. (1996). Search for Tsetse attractants: A structure-activity study on 1-Octen-3-ol in *Glossina fuscipes fuscipes* (Diptera: Glossinidae). *Journal of Chemical Ecology*, **22** (2): 343-355.

Vargo, E. L., Husseneder, C. and Grace, J. K. (2003). Colony and population genetic structure of the Formosan subterranean termites, *Coptotermes formosanus*, in Japan. *Molecular Ecology*, **12** (10): 2599-2608.

Vega, F. E., Dowd, P. F. and Bartelt, R. J. (1995). Dissemination of microbial agents using an autoinoculative device and several insect species as vectors. *Biological Control*, **5**: 545-552.

Vega, F. E., Dowd, P. F., Lacey, L. E., Pell, J. K., Jackson, D. M. and Klein, M. G. (2000). Dissemination of beneficial microbial agents by insects. In: Lacey, L. E and Kaya, H. K. (Eds.). Field manual techniques in invertebrate pathology: Application and evaluation of pathogens for control of insets

and other invertebrate pests. Kluwer Academic, Dordrecht. The Netherlands. pp. 153-177.

Vestergaard, S., Butt, T. M., Gillespie, A. T., Schreiter, G. and Eilenberg, J. (1995). Pathogenicity of the Hyphomycetes fungi *Verticillium lecanii* and *Metarhizium anisopliae* to the western flower thrips, *Frankliniella occidentalis*. *Biocontrol Science and Technology*, **5**: 185-192.

Vey, A. and Gotz, P. (1986). Antifungal cellular defense mechanisms in insects. In: Gupta, A. P. (Ed.). Hemocytic and humoral immunity in arthropods. John Wiley, New York, USA. pp. 89-115.

Vey, A., Hoagland, R. and Butt, T. M. (2001). Toxic metabolites of fungal biocontrol agents: In: Butt, T. M. Jackson, C. and Magan, N. (Eds.). Fungal Biocontrol agents-Progress, problems and potential. CABI. Wellingford. UK. pp. 311-346.

Vidal, C., Fargues, J. and Lacey, L. A. (1997). Intraspecific variability of *Paecilomyces fumosoroseus*: Effect of temperature on vegetative growth. *Journal of Insect Physiology*, **70**: 18-26.

Wanzala, W., Sika, K. F. N., Gule, S. and Hassanali, A. (2004). Attractive and repellent host odours guide ticks to their respective feeding sites. *Chemoecology*, **14**: 229-232.

Watanabe, H., Noda, H., Tokuda, G. and Lo, N. (1998). A cellulose gene for of termite origin. *Nature*, **394**: 330-331.

Watson, J. A. L. and Gay, F. J. (1991). Isoptera, In: Naumann, I. D., (E. d). The insects of Australia. 2nd (ed.). Melbourne University Press, Melbourne, Australia. pp. 330-347.

Watson, D. W., Mullens, B. A. and Petersen, J. J. (1992). Behavioural fever response of Musca domestica (Diptera: Muscidae) to infection by *Entomophthora muscae* (Zygomycetes: Entomophthorales). *Journal of Invertebrate Pathology*, **61**: 10-16.

Wells, J. D. Fuxa, J. R. and Henderson, G. (1995). Virulence of four pathogens to *Coptetermes formosanus* (Isoptera: Rhinotermitidae). *Journal of Entomological Science*, **30** (2): 208-215.

Wilding, N. (1981). Pest control by Emtomophthoarales. In: Burges, H.D (Ed.). Microbial control of pests and plant diseases 1970-1980. Kew, UK. pp. 539-551.

Wilson, E. O. (1971). The Insect Societies (Cambridge, Massachussetts: Harvard University Press). pp. 45-57.

Witzgall, P., Steilinski, L., Gut, L. and Thomson, D. (2007). Codling moth management and chemical ecology. *Annual Review of Entomology*, **53**: 502-522.

Wood, T. G. and Sand, W. A. (1978). The role of termites in ecosystems. In: Brian, M.V., (Ed.). Production ecology of ants and termites. Cambridge University Press, Cambridge, UK. pp. 245-292.

Wood, T. G., Johnson, R. A. and Ohiagu, C. E. (1980). Termite damage and crop loss studies in Nigeria-a review of termites (Isoptera) damage to maize and estimation of damage, loss in yield and termite (Microtermes) abundance at Makwa. *Tropical Pest Management*, **26**: 241-253.

Wood, T. G. and Johnson, R. A. (1986). The biology, physiology, and ecology of termites. In: Vision, S. B. (Ed.). Economic impact and control of social insects. Praeger, New York. pp. 1-68.

Wright, C. G., Leidy, R. B. and Dupree, H. E. (1991). Chloropyrifos in the air and soil of the houses four years after its application for termite control. *Bulletin of Environmental Contamination and Toxicology*, **46**: 686-689.

Wyatt, T. D. (2006). Pheromones and animal behaviour. Communication by smell and taste. University of Oxford. Cambridge University press. pp.15-75.

Zacharuk, R. Y. (1970a). Fine structure of fungus *Metarhizium anisopliae* infecting three species of larvae Elateridae. I. Dormant and germinating conidia. *Journal of Invertebrate Pathology*, **15**: 63-80.

Zacharuk, R. Y. (1970b). Fine structure of fungus *Metarhizium anisopliae* infecting three species of larvae Elateridae. II. Conidial germ tubes and appressoria. *Journal of Invertebrate Pathology*, **15**: 81-91.

Zacharuk, R. Y. (1970c). Fine structure of fungus *Metarhizium anisopliae* infecting three species of larvae Elateridae. II. Penetration of the host integument. *Journal of Invertebrate Pathology*, **15**: 372-396.

Zhao, H., Kangwang, Z., Pingyin, Y., Lingli, Y., Lunli, Z., Peng, G. X. and Xia, Y. X. (2007). Trehalose and trehalose-hydrolyzing enzyme in the haemolymph of *Locusta migratoria* infected with *Metarhizium anisopliae* strain CQ Ma102. *Insect Science*, **14**: 277-282.

Zimmermann, G. (1993). The entomopathogenic fungus, *Metarhizium anisopliae* and its potential as a bicontrol agent. *Pesticide Science*, **37**: 375-379.

Zimmermann, G. (2007a). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Science and Technology*, **17** (5/6): 553-596.

Zimmermann, G. (2007b). Review on safety of the entomopathogenic fungi *Metarhizium anisopliae*. *Biocontrol Science and Technology*, **17** (5/6): 1-55.

Zoberi, M. H. (1995). *Metarhizium anisopliae*, a fungal pathogen of *Reticulitermes flavipes* (Isopatera: Rhinotermitidae). *Mycologia*, **87**, 354- 359.

Zoberi, M. H. and Grace, J. K. (1990). Isolation of the pathogen *Beauveria bassiana* from *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). *Sociobiology*, **16**: 289-296.