POTENTIAL CONTROL OF THE SEED BUG *Elasmolomus pallens* BY ENTOMOPATHOGENIC FUNGI AND ASSESSMENT OF ITS RISKS FOR THE TRANSMISSION OF AFLATOXINS

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FACULTY OF SCIENCE
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KUALA LUMPUR

2021

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THE TRANSMISSION OF AFLATOXINS

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THESIS SUBMITTED IN FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITI MALAYA
KUALA LUMPUR

2021

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Matric No: **SHC150060**

Name of Degree: **DOCTOR OF PHILOSOPHY**

Title of Thesis ("this Work"):

POTENTIAL CONTROL OF THE SEED BUG *Elasmolomus pallens* BY ENTOMOPATHOGENIC FUNGI AND ASSESSMENT OF ITS RISKS FOR THE TRANSMISSION OF AFLATOXINS

Field of Study:

ENVIRONMENTAL MICROBIOLOGY

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POTENTIAL CONTROL OF THE SEED BUG *Elasmolomus pallens* BY ENTOMOPATHOGENIC FUNGI AND ASSESSMENT OF ITS RISK FOR THE TRANSMISSION OF AFLATOXINS

ABSTRACT

Elasmolomus pallens is a post-harvest insect pest which causes yield loss of peanuts and sesame in Africa and Asia. In view of mitigating the use of synthetic insecticides on the environment, this study evaluated the potential of entomopathogenic fungi for the biological control of *E. pallens* and assessment of the bug rostrum for its risks of aflatoxins transmission. Adults bugs were dipped briefly in different conidial concentrations of the isolates and observed at 25 ± 2 °C, 80 ± 10 RH and 14: 10 L: D for 10 days. Dose-response bioassay employed dipping and filter paper methods for both 0.05% Tween 80 and 5% peanut oil formulations. In the dipping bioassay (Tween 80 formulations), *A. flavus* caused significant mortalities ($F_{5, 60} = 5.644$, $p < 0.001$) with LC_{50} of 6.75×10^6 conidia/ml and LT_{50} of 3.3 days as well as *M. anisopliae* ($F_{5, 60} = 6.493$, $p < 0.001$) with LC_{50} of 8.0×10^6 conidia/ml and 3.6 days. However, significant differences in virulence was obtained between *A. flavus* ($M = 44.56$) and *M. anisopliae* ($M = 65.09$) t (10) $= 4.830$, $p < 0.001$, when their mortality rates under this bioassay was compared. Using same formulations for filter paper bioassay, significant mortalities of *E. pallens* were obtained for *A. flavus* ($F_{5, 60} = 5.318$, $p < 0.001$) with an LC_{50} values of 9.36×10^7 conidial/ml and LT_{50} of 5.0 days while *M. anisopliae* ($F_{5, 60} = 8.825$, $p < 0.001$) had an LC_{50} of 6.85×10^6 conidia/ml and LT_{50} of 3.8 days. Comparing their mortality rates, significant difference was obtained between *A. flavus* ($M = 54.55$) and *M. anisopliae* ($M = 71.46$) t (10) $= 5.311$, $p < 0.001$. For dipping bioassay (oil formulations), mortalities were significant for *A. flavus* ($F_{5, 60} = 5.589$, $p < 0.001$) with LC_{50} of 4.75×10^6 conidia/ml and LT_{50} of 3.2 days; and *M. anisopliae* ($F_{5, 59} = 4.314$, $p < 0.002$) had LC_{50} of 2.14×10^6 conidia/ml and LT_{50} of 3.0 days. No difference in virulence was observed between *A.*

flavus (M =60.00) and *M. anisopliae* (M =62.18) and, t (10) =1.284, p > 0.228, when compared. In the filter paper bioassay, mortalities of *E. pallens* were significant; *A. flavus* ($F_{5, 60}$ =4.394, p < 0.002) with LC_{50} of 1.95×10^6 conidia/ml and LT_{50} of 3.0 days and *M. anisopliae* ($F_{5, 60}$ =4.363, p < 0.002) with LC_{50} of 3.92×10^6 conidia/ml and LT_{50} of 3.2 days. No difference in virulence was observed between *A. flavus* (M =52.64) and *M. anisopliae* (M =58.91), and t (10) =1.796, p > 0.103. Hydrolytic enzymes activity for *A. flavus* were 0.587 ± 0.202 U/ml (protease), 0.873 ± 0.028 U/ml (chitinase), 2.513 ± 0.017 U/ml (lipase) while *M. anisopliae* had 0.739 ± 0.155 U/ml (protease), 0.879 ± 0.023 U/ml (chitinase) and 2.592 ± 0.019 U/ml after 4 days of culture at 25 °C. A strong positive (r = 0.999; p < 0.03) correlation was obtained for atoxigenic *A. flavus* while toxigenic strains correlated inversely (r = -0.999; p < 0.03) with *E. pallens* rostrums between the sampling locations. This study demonstrates that the isolates possess potential against *E. pallens* and should be considered ideal candidates for field applications.

Keywords: aflatoxin, biological control: enzyme, entomopathogenic fungus, hemiptera, peanut.

POTENSI PENGAWALAN KEPINDING PEMAKAN BIJI BENIH *Elasmolomus pallens* OLEH FUNGI ENTOMOPATHOGENIK DAN PENILAIAN RISIKONYA BERKAITAN PEMINDAHAN AFLATOXIN

ABSTRAK

Elasmolomus pallens adalah serangga perosak lepas tuai yang menyebabkan hilangnya hasil kacang tanah dan bijan di Afrika dan Asia. Sebagai perancangan dalam mengurangkan penggunaan racun serangga sintetik ke atas alam sekitar, kajian ini menilai potensi kulat entomopatogenik sebagai kawalan biologi *E. pallens* dan penilaian rostrum kepinding berkaitan risiko transmisi aflatoksin. Kepinding dewasa telah direndam dalam kepekatan konidium yang berbeza dari pencilan dan diperhatikan pada 25 ± 2 °C, 80 ± 10 RH dan 14:10 L: D selama 10 hari. Bioasai dos tindak balas respons dijalankan menggunakan kaedah pencelupan dan kertas turas bagi kedua-dua formulasi 0.05% Tween 80 dan 5% minyak kacang tanah. Dalam bioasai pencelupan (formulasi Tween 80), *A. flavus* menyebabkan mortaliti yang ketara ($F_{5, 60} = 5.644$, $p < 0.001$) dengan LC_{50} dari 6.75×10^6 conidia/ml dan LT_{50} dari 3.3 hari serta *M. anisopliae* ($F_{5, 60} = 6.493$, $p < 0.001$) dengan LC50 sebanyak 8.0×10^6 conidia/ml dan 3.6 hari. Walau bagaimanapun, perbezaan ketara dalam kevirulenan diperoleh antara *A. flavus* ($M = 44.56$) dan *M. anisopliae* ($M = 65.09$) $t (10) = 4.830$, $p < 0.001$, apabila kadar kematian mereka di bawah bioasai ini dibandingkan. Dengan menggunakan formulasi yang sama bagi bioasai kertas turas, kematian *E. pallens* yang signifikan diperoleh untuk *A. flavus* ($F_{5, 60} = 5.318$, $p < 0.001$) dengan nilai LC_{50} 9.36×10^7 conidial/ml dan LT_{50} 5.0 hari manakala *M. anisopliae* ($F_{5, 60} = 8.825$, $p < 0.001$) mempunyai LC_{50} sebanyak 6.85×10^6 conidia/ml dan LT_{50} dari 3.8 hari. Dengan membandingkan kadar kematian mereka, perbezaan yang significan diperoleh antara *A. flavus* ($M = 54.55$) dan *M. anisopliae* ($M = 71.46$) $t (10) = 5.311$, $p < 0.001$). Bagi bioasai pencelupan (formulasi minyak), mortaliti adalah signifikan bagi *A.*

flavus ($F_{5, 60}$ = 5.589, $p < 0.001$) dengan LC_{50} daripada 4.75 × 106 conidia/ml dan LT_{50} dari 3.2 hari; dan *M. anisopliae* ($F_{5, 59}$ = 4.314, $p < 0.002$) mempunyai LC_{50} daripada 2.14 × 10^6 conidia/ml dan LT_{50} 3.0 hari. Tiada perbezaan dalam kevirulenan yang diperhatikan antara *A. flavus* (M = 60.00) dan *M. anisopliae* (M = 62.18) dan, t (10) = 1.284, $p > 0.228$, apabila dibandingkan. Dalam bioasai kertas turas, mortaliti *E. pallens* adalah signifikan; *A. flavus* ($F_{5, 60}$ = 4.394, $p < 0.002$) dengan LC_{50} daripada 1.95 × 106 conidia/ml dan LT_{50} 3.0 hari dan *M. anisopliae* ($F_{5, 60}$ = 4.363, $p < 0.002$) dengan LC_{50} dari 3.92 × 106 conidia/ml dan LT_{50} dari 3.2 hari. Tiada perbezaan dalam kevirulenan antara *A. flavus* (M = 52.64) dan *M. anisopliae* (M = 58.91), dan t (10) = 1.796, $p > 0.103$. Aktiviti enzim hidrolisis untuk *A. flavus* adalah 0.587 ± 0.202 U/ml (*protease*), 0.873 ± 0.028 U/ml (*kitinase*), 2.513 ± 0.017 U/ml (*lipase*), manakala bagi *M. anisopliae* adalah 0.739 ± 0.155 U/ml (*protease*), 0.879 ± 0.023 U/ml (kitinase) dan 2.592 ± 0.019 U/ml selepas 4 hari pengkulturan pada 25 ºC. Korelasi positif yang tinggi (r = 0.999; $p < 0.03$) telah diperoleh untuk *A. flavus* tidak bertoksin manakala *strain* bertoksin berkorelasi secara songsang (r = -0.999; $p < 0.03$) dengan rostrum *E. pallens* antara lokasi pensampelan. Kajian ini menunjukkan bahawa pencilan mempunyai potensi terhadap *E. pallens* dan sewajarnya dianggap calon yang unggul untuk aplikasi di lapangan.

Kata kunci: aflatoksin, kawalan biologi: enzim, kulat entomopatogenik, hemiptera, kacang tanah.

ACKNOWLEDGEMENTS

Also, thanks to my labmates and gratitude to one and all who directly or indirectly lent their hands in the course of this work. Thank you all and God bless.

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α	:	Alpha
β	:	Beta
µl	:	Microliter
µM	:	Micromolar
AFB	:	Aflatoxin B
AFG	:	Aflatoxin G
AMP	:	Antimicrobial peptide
ANOVA	:	Analysis of variance
AST	:	Average survival time
AV	:	Ammonium vapor
BCA	:	Biological control agent
BLAST	:	Basic local alignment search tool
CABI	:	Centre for agriculture and bioscience International
CAM	:	Coconut-milk agar medium
CBM	:	Carbohydrate binding domain
CDE	:	Cuticle-degrading enzymes
CFU	:	Colony forming unit
COI	:	Cytochrome oxidase I
DDT	:	Dichlorodiphenyltrichloroethane
DNA	:	Deoxyribonucleic acid
EAO	:	Emulsifiable adjuvant oil
EC	:	Emulsifiable concentrate
EF	:	Elongation factor
EFSA	:	European Food Safety Authority

EPF	:	Entomopathogenic fungi
EU	:	European Union
FAO	:	Food Agricultural Organization
FAOSTAT	:	Food and Agricultural Organization Corporate Statistical Database
FD	:	Fluorescence detection
GH	:	Glycoside hydrolase
GlcNac	:	N-Acetyl-D-Glucosamine
GNBP	:	Gram-negative binding protein
HCC	:	Hepatocellular carcinoma
IARC	:	International Agency for Research on Cancer
IBM	:	International Business Machines
IPC	:	Inter process communication
IPM	:	Integrated pest management
ITS	:	Internal transcribed spacer
LB	:	Luria Bertani
LC	:	Lethal concentration
LD	:	Lethal dose
MTCC	:	Microbial Type Culture Collection
NaClO	:	Sodium hypochlorite
NADH	:	Nicotinamide adenine dinucleotide + hydrogen
OECD	:	Organization for Economic Co-operation and Development
PAMP	:	Pathogen-associated molecular pattern
PCR	:	Polymerase chain reaction
PDA	:	Potato dextrose agar
PDB	:	Potato dextrose agar
PGRP	:	Peptidoglycan recognition protein

PKA	:	Protein kinase A
PKMP	:	Polyketide metabolic pathway
PKS	:	Polyketide synthetases
PO	:	Phenoloxidase
PPO	:	ProPhenoloxidase
PRR	:	Pattern recognition receptor
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
SDB	:	Sabouraud dextrose broth
SPSS	:	Statistical package for the social sciences
SSA	:	Sub-Saharan Africa
TC	:	Terpene cyclase
TEP	:	Thioester-containing protein
UK	:	United Kingdom
ULV	:	Ultra low volume
USA	:	United States of America
USDA	:	United States Department of Agriculture
UV	:	Ultraviolet
VOC	:	Volatile organic compound
WHO	:	World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 Research background

There are significant crop losses worldwide, due to weeds, pathogens and arthropods; causing a barrier towards achieving the quest for global food security and poverty reduction via agriculture. Although the scope and extend of these losses may vary in estimation, crop yield losses due to insect pests in the perspective of food security, may amount to the equivalent of food needed to feed over 1 billion people of the world population (Pretty & Bharucha, 2015). Insect pests are widespread and constitute a global threat to crops produced for the purpose of food, bioenergy and fibers (Valero-Jimenez et al., 2016). Insect pests are key rivals with humans for agricultural resources and their activities are favored by intensive fertilizer applications and crop monocropping in large areas (Oerke & Dehne, 2004). Evolutionary relationship between crop pests and farmers, precede synthetic pesticides for years. Crop losses may be separated on the levels of primary and secondary losses or direct and indirect losses indicating that pest attack on crops can affect both productivity, farmers income, food and feed supplies and the local economies of countries (Popp et al., 2013).

Insects constitute one of the largest group of extant animals with a projected estimate of between 1.5 to 30 million extant species (Freeman & Herron, 2007). Insects form more than two-third of known fauna species in the world and are distributed in diverse environments. They feed on crops, medicinal and forest tress, and weeds. Stored crops are also infested and damaged by insects, affecting their quality and aesthetic values. Insects can be classified as minor or major pests based on the gravity of damages inflicted on crops. Those that can cause between 5-10% losses are considered minor pests while those that can cause damages above 10% are termed major pests (Dhaliwal et al., 2010). Cultivated crop plants especially in large scale mono-cropping are often prone to

infestation and impact due to the destructive tendencies of insects, resulting in yield loss (Cooke, 2006).

Insect damage are often considered as one of the cardinal factors responsible for reduced productivity of most crop species (Pimentel, 2009). Crop damage due to insect attack can occur as pre-harvest or post-harvest losses (Oerke, 2006). Agricultural losses caused by insect pests cannot be exactly estimated in accurate terms since damages caused by insects depend on a host of factors including cultivated plant species, ambient environmental conditions, the socioeconomic status of the farmers, and the applied farming technology. In this perspective, only few governments have developed systematic programs to monitor and evaluate agricultural losses due to insects and other animal pests (Luo et al., 2010). Crop damage by insect pests has been a production problem to farmers of various crop species across the world. Overcoming this menace will require successful interplay of factors that border on the environment, plant species, farmer's educational and economic attainment and the technological inputs used.

Several estimates of global crop losses caused by insect pests have been made in the past decades. Pimentel (1986) reported an estimated 13.0% crop loss due to insect pests in the year 1985. Oerke and Dehne (2004) estimated a 10.1% loss for various cereal grain crops, soybeans, potatoes, sugar beets and cottons caused by insect pests. But recently, Oerke et al. (2012) reported crop losses of between 7.9% and 15.1% as estimated worldwide loss of cereal grain crops, soybean, potato and cotton, even in places where effective control measures are put in place. However, Oliveira et al. (2014) reported an estimated 7.7% average losses of major crops due to insect attacks, resulting in an annual economic loss amounting to about US$ 14.7 billion, affecting the Brazilian economy despite the implementation control measures.

In the USA, it has been estimated that 13% of crop production losses valued at about US$ 33 billion per annum were reportedly due to insect pest attacks (Pimentel & Greiner, 1997)and 9% losses of forest crops valued at about US$ 7 billion per annum (Hall & Moody, 1994) respectively. In the United Kingdom (UK), insect damage to agricultural crops is estimated at US$ 3.2 billion per annum amounting to 10 % crop losses (Oerke et al., 2012) . In Asia, Australia had an estimated US$ 24 billion crop production potential per annum with losses due to insects and mites estimated at 10.7% of the crop production potential of the country (Oerke et al., 2012). Pimentel (2009)in a global review on global insect damage opined that, insects have produced an estimated 14% global crop loss, while plant pathogens have caused 13% crop loss as well as 13% due to weeds. These has led to crop losses with an economic value estimated at US $2,000 billion yearly. However, In Brazil, a decline in crop production capacity has been encountered over the years causing a reduction of almost 25.0 million tons of fibers, foods and biofuels. Of this decline, grains accounted for 12.5 million tons, sugar (4.2 million tons), coffee (0.4 million tons), cassava (0.5 million tons), and ethanol (2.6 million liters) are lost yearly due to insect attacks, causing an estimated economic loss of US$ 12.6 billion. Also reported is the loss of fruits (approximately 3.7 million tons), vegetables (0.5 million tons), natural rubber (95,000 tons), amounting to approximately US$ 2.1 billion lost. However, in comparative terms, extreme losses were noticed per area for apples (US$ 4281/ha), tomatoes (US$ 3806/ha), tobacco (US$ 2729/ha), garlic (US$ 2655/ha), peanut (US$ 1679/ha), natural rubber and grapes (US$ 1004/ha) (Oliveira et al., 2014) respectively.

Currently, the exponential rise in global trade and the increased efficiency and availability of cross border human travels have escalated the trans boarder introduction and establishment of exotic insect species. These insect pest species have successfully

emerged as pests in new areas across the world, causing serious ecological and economic concerns(Pimentel, 2011). World over, insect pests are responsible for most agricultural yield losses encountered by farmers, hence, the need for efficient pest management systems for effective food security and income generation (Zhang et al., 2018). Other than tree crops, forest and medicinal plants, insects also attack oilseed crops either at planting, pre-harvest or during post-harvest and storage.

1.2 Groundnut production

Groundnut (*Arachis hypogaea* L.) is one of the crops of global significance, occupying the fifth position among oilseeds after soybeans (*Glycine max* L.), cotton (*Gossypium hirsutum* L.), rapeseed (*Brassica napus* L.), and sunflower (*Helianthus annuus* L.). Groundnut is an annual legume belonging to the pea family Fabaceae. Its commonly used names include: peanut, goobers, monkey-nut and earthnut. However, for the purpose of this study, the name peanut will be preferred. Peanut is the 13th ranked most significant food crop common to over 100 countries, cutting across the warm temperate, tropical and subtropical regions of the of the world (Nwokolo, 1996; Torres et al., 2014a; Upadhyaya et al., 2012). Its cultivation improves the fertility of soils due to the symbiotic association between the crop and the rhizobia community (Daudi et al., 2018). Cultivation of peanut plant contributes to improving the soil fertility through the leaf droppings, biological nitrogen fixation, and organic remains of the plant. The organic remains of the plant contribute significantly in soil fertility and also in the improvement of the microbial community.

On a global scale, peanut is cultivated on about 22.5 million hectares of land with a total production of about 35.9 metric tons (FAO, 2006) with production reaching approximately 39.9 million tons per year (Torres et al., 2014a). Notable countries

producing groundnuts are China (40.1%), India (16.4%), Nigeria (8.2%), USA (5.9%), and Indonesia (4.1%) (Shuren et al., 1996). It is cultivated worldwide, but major productions come from the developing countries of the world (Umeh et al., 2001b). It has been reported in FAOSTAT (2015), that Africa accounts for approximately 32% of the global peanut production of the year 2015. Peanut cultivation is basically must suitable in warm climates of the world. It serves a great deal in the national income of growing countries. Peanut also contributes significantly to sustaining the soil ecosystem and structure via its symbiotic partners and the organic debris.

The haulms of the plant when harvested towards the dry season are preserved and used as valuable feeds for livestock (Tanzubil, 2016b). Peanut seed is a rich source of oil (48-50%), protein (26-28%), vitamins, minerals and dietary fibers (Daudi et al., 2018). It also contains carbohydrate (10-20 %). Peanut is capable of supplying the protein requirement of numerous families that cannot afford animal proteins (Asare Bediako et al., 2019). The seeds can either be eaten raw, roasted or ground into pastes for making peanut soup, or for oil production. In some locations, both the plant and the seeds can be used for a variety of purposes such as animal feed constitution (seed, green material, straw and oil pressings) and as raw materials for industries (butter, cake, oil and fertilizer). The numerous benefits of the peanut makes it the economic hub of local markets in production areas and also for international trade between countries (Ani et al., 2013). However, other than the production, peanut is constantly faced with the incidences of contamination with aflatoxins, which are classified as mycotoxins of great health significance (Asare Bediako et al., 2019; Torres et al., 2014a). Aflatoxins are secondary metabolites that are synthesized by fungi such as *A. flavus* and *A. parasiticus*. The infection of peanuts by these fungal species is attributed to insect damage to young developing seedlings, drought stressed plant, and elevated soil temperature prior to harvest (Pitt et al., 2015). Infection

of the plant occur in the soil around the pods, whereas drought related stress impact the plant by weakening its natural defense responses against pathogens; due to reduced water activity in the soil, which inhibit other microbes, but encourages the proliferation of *A. flavus* and *A. parasiticus* because they are xerophiles (Torres et al., 2014b).

It is obvious that peanut holds enormous potential on world economy, for its significance in world trade and foreign exchange earnings. Its global demands for consumption and other value added products makes it a crop to protect from damage due to insect attack, drought or disease. However, unfortunately, peanut production is challenged by a devastating seed bug called *E. pallens*, which can infest harvested peanuts on the field, multiply rapidly and within a short time destroy the kernels of the crop right inside the pods. Usually, pods of destroyed peanuts may not be too obvious to detect unless it is broken. Therefore, control measures must be devised to curb the menace of this bug and palliate food supply and safety. And because over the years, chemical insecticides have been applied for insect pest control which has led to cases of resistance among insects, environmental pollution, and health problems; biological control method which known to be environment friendly is seen appropriate to explore with respect to the control of *E. pallens*.

1.3   Insect pests of peanuts

Over the decades, major impediments such as drought, pests and diseases are responsible for the decline in peanuts production in Africa (Lynch, 1990; Umeh et al., 2001a). In West Africa and some parts of Asia, several biotic and abiotic factors including insect pests, aflatoxin contamination, soil borne and foliar diseases, peanut rosette virus, drought and weeds limit peanut production potentials in these regions (Bowen & Mack, 1993; Umeh et al., 2001a; Wightman & Rao, 1993). According to Lynch (1990), the pests

of peanut in Africa and Asia are ranked in their sequence of importance to include: 1) termites, 2) white grubs, 3) thrips (vector of bud necrosis), 4) leafhoppers 5) *A. craccivora* (groundnut rosette vector) and 6) lepidopterous defoliators. However, the groundnut hopper, millipedes and the subterranean ants are considered top pests of peanuts in Southern Africa, West Africa and Southern Asia respectively. But Gadhiya et al. (2014) reported that over 100 insects and mites species are known to be pests of peanuts in production areas. The authors mentioned that, of all the insect pests that attack peanuts, lepidopteron defoliators such as *Helicoverpa armigera* (Hubner) and *Spodoptera litura* (Fabricius) caused serious damage of peanut crops. An estimated 30-40 % yield loss can be inflicted on the crop by *S. litura*. Wightman and Rao (1993) mentioned that peanut crop yield loss due to *S. litura* was observed despite intensive pest management techniques. This was due to the fact that these lepidopteron defoliators displayed some degrees of behavioral tolerance and resistance against various groups of insecticides, making their control somewhat difficult (Gadhiya et al., 2014). The assertion of these authors supports the claim that most insect pests affecting agricultural productions are acquiring remarkable resistance to a host of synthetic pesticides. And considering the impacts and attendant effects of these chemical insecticides, the quest for less deleterious approaches to pest control becomes sacrosanct.

The review of the above mentioned authors, on the insect pests of peanut cannot be considered to be exhaustive because; some of these insects might exist as a pest in one region where peanut is grown, while in another region, it is not considered as a pest. Additionally, insects which are not pests by nature can assume a pest status on alternate food sources when their natural host plants are destroyed. Where this occurs, the insect is classified as a pest in that very region of the world. Such factors may perhaps affect the exhaustive conclusion on the insect pests of peanut. For example, only few authors

reported the Heteroptera *Rhyparochromus littoralis* a serious pest, even when it attacks peanut and sesame, causing significant losses to farmers in the tropics.

The traditional methods of insect pest control in peanuts is dependent on the application of conventional chemical insecticides which has caused resistance among insect population, deleterious effects on the environment and traces of insecticide residues in food and food products (Sharma et al., 2003). But these insects are emerging in resistance to many of the chemical insecticides commonly used for pest management. In this regards, better and ameliorative alternate control measures are better sought than the continued exposure of the environment to these recalcitrant xenobiotics.

1.4   The   seed   bug,   *Elasmolomus   pallens*   (Dallas)   (Hemiptera: Rhyparochromidae)

Elasmolomus pallens commonly called dirt-colored seed bug belongs to the superfamily Lygaeoidea, family Rhyparochromidae, order Hemiptera and the suborder Heteroptera (true bugs). Although the family was previously characterized as a subfamily under the family Lygaeidae, recent studies on the monophyletic capability of the group established the group as a family of its own (Henry, 2009; Weirauch & Schuh, 2011; Yuan et al., 2015). Members of the family are cosmopolitan in nature and are considered the largest of the lygaeoid bugs (Gad, 2013). *E. pallens* has a wider distribution cutting across tropical, subtropical, and parts of the temperate regions of Africa, Asia, and the Pacific Islands. The species has formally been known as *E. sordidus* before *E. pallens* was used to replace the name *E. sordidus* and the original names *Cimex sordidus* Goeze 1778 and Thunberg, 1783 (Tomokuni, 2010) respectively. An important determinant in the dispersal of *E. pallens* is its association with the legume, *Arachis hypogea* L., also called peanuts or groundnuts (Schaefer & Panizzi, 2000a). The bug is a damaging pest of

peanuts in West, Sub-Saharan Africa and parts of Asia. Rehaman et al. (2018) described *E. pallens* as dark brown peanuts trash bug that easily run into hiding at the slightest disturbance, and both adults and nymphs are sap suckers from leaves and buds. But contrary to this report, *E. pallens* is a pest of serious economic significance that cannot just be commonly described as peanut trash bug and sap sucker. This description can only be fitting in locations where the bug does not constitute a nuisance to peanut destruction, either due to improved harvesting and processing techniques, or due to their habituation on alternate host plants. Rao et al. (2010) described *E. pallens* (by the former name *E. sordidus*) as a dark brown bug, about 10 mm long and 2 mm wide. The females lay approximately 100 eggs in an average lifespan of between 30-40 days. Eggs are laid in the soil or on Peanut plant debris on the field or loosely laid on peanut pods and sacks when under storage. Nymphs (first instars) have brightly colored abdomen; which later becomes darker as they mature. Both adults and nymphs attack peanut by perforating the pods with their rostrum causing seed shriveling, increased fatty acids content in the oil, and rancid flavor (Figure 1.1).
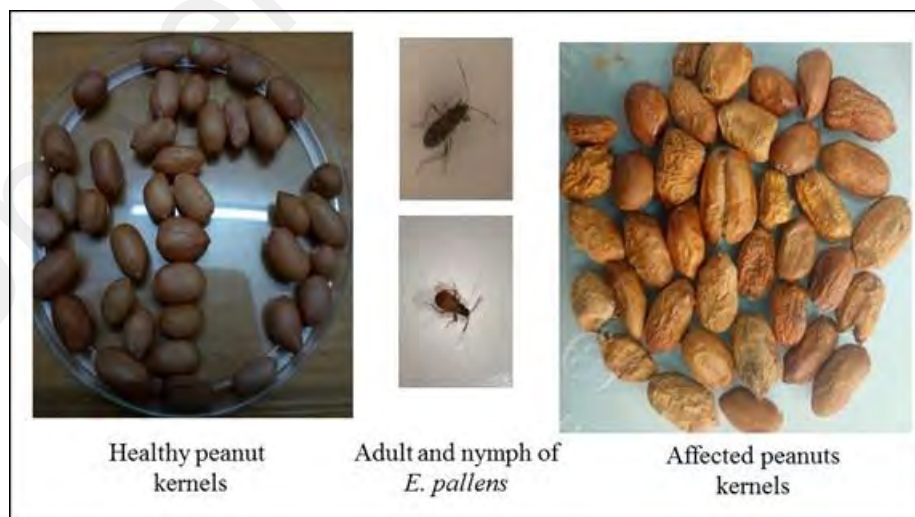
Figure 1.1: Effect of *E. pallens* attack on peanut kernels. Affected seed kernels become shriveled, losing quality and aesthetic value.

In Sub-Saharan African countries where commercial peanut farming is practiced and processing implements are lacking, harvested peanuts are left for weeks on the field to dry before stripping. When this happens, the bugs congregate beneath the heaps and destroy the peanut kernels from intact pods using their rostrum to suck the oil from the kernels. The attacked kernels become soft, oilier and shriveled (Kinati, 2017). Waliyar et al. (2015) reported that subsistence farmers in Mali usually harvest and dry peanuts in the field before stripping and storage. During drying, pods already infected with *A. flavus* are exposed to fluctuating environmental conditions during drying under high moisture condition which may lead to increase in seed infestation by *A. flavus* that has been carried along with the harvested pods. Noteworthy, it is normally during the harvesting and drying periods that *E. pallens* infest and attack the crop, causing losses to farmers. It can also be inferred that the activities of *E. pallens* on peanut could result in both losses and contaminations by aflatoxigenic fungi.

In India (South Asia), *E. pallens* is reportedly found on all peanut growing areas, where eggs from adult females are deposited in the soil, tree trunks or organic debris. Both the nymphal and adult stages of the bug are known to be destructive to peanut during harvest and storage, affecting the oil content, quality and viability of the seeds (Rao et al., 2010). The infestation of *E. pallens* on wheat threshing floor, where they carry off the wheat grains to hiding places has been observed in India. But what nutrient they drive from wheat grains remains doubtful, and the wheat grains carried by the bug was said to be enormous that farmers had to collect them back again in the mornings (Schaefer & Panizzi, 2000a).

E. pallens has also been described as a serious post-harvest pest of sesame in some countries in Africa. High yield loss of sesame due to the bug activity on both field and in

storage (warehouses) has been reported (Berhe et al., 2017). Both adults and nymphs suck oil from the seeds making them become shriveled, bitter and valueless; incurring both physical damage (color change, weight loss and shape) and loss of quality (odor, change in protein content and poor oil yield) (Berhe et al., 2017; Kinati, 2017). Usually, eggs are laid within 2-3 days after mating and hatched within 5-6 days depending on the time laid and the prevailing environmental conditions. *E. pallens* becomes sexually mature within 2-3 weeks of hatching. Being seed sucking bugs, they proliferate more during harvest seasons from summer to autumn. In Nigeria, the bug attacks peanuts by summer (harvest period) and sesame by late autumn when peanut harvest would have been over. Once the harvest of these oilseeds is over, the bug overwinters by penetrating tree trunks to avoid adverse environmental conditions (Unpublished communication). Similarly, Parker et al. (2011) and Assaf et al. (2011b) reported that bugs of the order Hemiptera live from summer to spring hibernating in plant parts before coming out to reproduce under appropriate environmental conditions. However, while in hibernation, they are prone to infection by entomopathogenic and facultative generalist fungi.

In Nigeria, Samaila and Malgwi (2012b) reported the incidences of *Rhyparochromus littoralis* (Hemiptera: Rhyparochromidae) and the severity of its destruction on peanuts during harvest and storage. Their report asserted that both in Nigeria and India, the bug occurs in all peanut growing areas (Malgwi & Onu, 2004; Samaila & Malgwi, 2012b), infesting harvested pods that are left on the field for over one week to dry. Usually, they colonize an area and establish their congregate colony under the peanut haulms to exhibit their destructive activities (Hamdan, 2015; Samaila et al., 2014). The bug feed by perforating the pods using the rostrum (beak) to suck the oil from the kernels. Once the oil is completely sucked, the affected pods become discolored, appearing differently from healthy ones. Severe attacks result in loss of seed viability and the ecstatic value for

consumption. Attacked seeds usually become shriveled with increased free fatty acid content and rancid flavored oil (Hamdan, 2015; Rao et al., 2010; Samaila & Malgwi, 2012b).

Just like *E. pallens*, *R. littoralis* can be found hibernating under peanut plant litters, decaying leaves and feeds on fallen oil seeds. It overwinters on tree trunks or plant litters during summer until spring. By late May, mating between opposite sexes occurs and eggs are laid on the ground and on plant remains and trunks. Mature first-generation adult bugs start appearing in field containing host plants by early August (Samaila & Malgwi, 2012a). Similar in description of the biology of *E. pallens* is the Paleartic species of *R. saturnius* collected from the central California (Henry & Adamski, 1998). The description from the above studies shows some degrees of agreement on the life cycle of the bugs and their common origin. The authors also described that as at the time harvest was over, the bug overwinters to enable continuity of its life cycle. The survival of the bug during overwintering depends on its ability to feed on alternate hosts such as plant sap, fruits and oilseeds. Alternate crops known to be fed upon by the bug during its overwintering period include sesame, cowpea, maize, guinea corn (Henry & Adamski, 1998). Most often, a large proportion of the bug overwinters on the bark of shea tree until another harvest season (Samaila et al., 2018). Because of the close relationship between *E. pallens* and *R. littoralis*, the description of their biology by authors often appeared similar even though they are different.

1.5 Losses of peanut due to insect attack

In Asia, post-harvest losses of peanuts due to insect infestation ranged from 10-25% of the production, resulting in direct losses, while indirect losses due to poor quality of the produce impacts its trade and use (Rao et al., 2010). In India, Dabhade et al. (2012),

reported a post-harvest yield loss of 48.57% in peanut due to insect infestation. In Nigeria, Samaila et al. (2014) described the seed bug *R. littoralis* (which may likely be *E. pallen* being that the bug was not identified based on molecular methods) as one of the major insect pest of peanuts causing colossal post-harvest yield losses of about 68% of peanuts productions per year.

Furthermore, another challenge besides insect pest damage of peanut is aflatoxin contamination. Peanut production in tropical countries has been constrained with the challenge of AF contamination due to the suitability of the weather for the proliferation of toxigenic fungal species. The drought problem, insect menace, and soil temperature are essential determinants of AF prevalence in the tropics. The direct involvement of *E. pallens* in peanut damage is also a warning signal to the propagation of fungal propagules which are capable of secreting this toxic secondary metabolite, which is known to cause fatal aflatoxicosis and cancers.

1.6   Aflatoxins contamination of peanut and effect on health

As developing countries of the world engage in international trade with their trade partners, they must be able to fulfil stringent food safety standards specified for food and food products. Additionally, the concept of globalization has enabled developing countries to acquire expertise that allow them to enact stringent measures towards abating staple food and animal feeds contamination concerns. However, with all these stringent food safety standards put in place, no decrease in aflatoxins contamination has been recorded in food and food products commonly sold in local markets. Most importantly, lack of proper management on and off-farm operations, dangers of consumption of aflatoxins contaminated foods are higher in developing nations compared to developed ones (Waliyar et al., 2015).

Aflatoxins also commonly called mycotoxins are toxic metabolites produced by fungal species that are common contaminants of essential foods and feeds. Their presence in cereals such as peanuts, spices, maize, rice, tree nuts and wheat which are consumed globally, makes them an unavoidable menace (Wang et al., 2018). Most aflatoxins are potent carcinogenic and immunosuppressive agents, nephrotoxins, hepatotoxins, and neurotoxins. Currently, stricter control measures are envisioned worldwide to lessen exposure to aflatoxins. However, the question remains whether the changing environment can be ideal to palliate and control exposure to these toxins (Marroquín-Cardona et al., 2014a). Other than AFs, other mycotoxins are also of great health significance, causing health challenges via contamination of agricultural commodities. But comparing the toxicity of AFs to other mycotoxins such as ochratoxin A, the high toxicity of AFs places them on the radar of priority attention (Wang et al., 2018).

Peanuts are naturally rich in monosaturated fats, proteins and vitamin E. However, there are always safety concerns because of their likely contamination with aflatoxins (Wang et al., 2018). Aflatoxin contamination of peanuts have impacted considerably as a great threat to the health of rural dwellers, and on the economic conditions of peasant farmers who rely on the crop for their welfare and nutrition. For example, in the year 2005, because of the high levels of aflatoxins contamination in Malawian peanuts, 42% of their exported peanuts to European markets were rejected for not meeting food safety standards (Diaz Rios & Jaffee, 2008). There are more pronounced concerns for food safety in Sub-Saharan Africa (SSA) due to aflatoxins contamination by aflatoxigenic *Aspergillus*, compared to temperate zones where such staple food grains are often regarded as aflatoxins infested (Ezekiel et al., 2014). Other food commodities associated with aflatoxin contamination in SSA include oilseeds, legumes, cereals, spices and tree nuts. These commodities constitute largely in the nutrition of people and animals in that

region, thus contamination with aflatoxins can pose serious health threats. Also, the geocarpic nature of peanut exposes the pods to toxigenic fungi that are capable of eliciting metabolites that constitute a threat to health (Waliyar et al., 2015).

The post-harvest infection of grains by *A. flavus* usually lead to increased aflatoxins (AFs) contamination when drying and storage are poorly handled. Usually, post-harvest infection of grains is dependent on fungal infestation of the field, where infected kernels often become potent sources of inoculation on healthy ones. This is propagated all through the storage process, which implies therefore that, fungal infestation of grains from the field are important for mycotoxins formation down the value chain of the product (Battilani et al., 2012). Usually, this is possible for *A. flavus* because of its ability to grow at water activity as low as between 0.73 and 0.85 (Sanchis & Magan, 2004).

Aflatoxins are natural mycotoxins synthesized by certain strains of *A. flavus* and *A. parasiticus*. Most common aflatoxins occurring in peanuts are aflatoxin B_1 (AFB$_1$), aflatoxin B_2 (AFB$_2$), aflatoxin G_1 (AFG$_1$) and aflatoxin G_2 (AFG$_2$). However, the most toxic and frequently encountered is aflatoxin AFB$_1$ (Gong et al., 2016). When food contaminated by aflatoxins is accidentally consumed, the liver metabolizes and transforms the toxin into various kinds of metabolites, including compounds such as AFB$_1$-albumin adducts seen in blood samples and AFB$_1$-N^7-guanine ducts that can be observed in urine samples (Chen et al., 2018; Wang et al., 2018). Cancer studies involving both humans and experimental animals and pool of data from the International Agency for Research on Cancer (IARC, 1993) revealed AFs as group 1 carcinogens that play cardinal role in the development of liver cancers in humans. It has been asserted that 1 ng/kg body weight per day triggers a risk (EFSA, 2007).

Aflatoxin contamination occur worldwide, however, crops in the tropical and subtropical regions of the world are more prone to aflatoxin contamination than in temperate zones (Pitt et al., 2015; Torres et al., 2014b; Waliyar et al., 2015; Wang et al., 2018). Most under-develop countries lie in the tropical zone where the relative humidity and temperatures enhances molds growth on grains and subsequent aflatoxin contamination. Peanut infection by *Aspergillus* spp. and subsequent aflatoxin contamination arises at pre-harvest, during harvest, post-harvest, during storage, processing and transportation down the value chain (Waliyar et al., 2015). Drought stress typical of the tropical climate enhances the menace of infection by *Aspergillus* spp. which is associated with aflatoxin production. Low soil moisture content during drought is responsible for reduced seed moisture, increased threat of insect damage on pods, over maturity and loss of plant vigor (Craufurd et al., 2006). Furthermore, soil dwelling pests such as millipedes, pod borers, white grubs, mites, nematodes and termites have been reported as etiologic agents for the transmission of *Aspergillus* spp. (Umeh et al., 2001b), and because of the geocarpic nature of peanut, the pods are made susceptible to toxigenic molds (Waliyar et al., 2015).

There are reports of the occurrence of aflatoxigenic fungi and aflatoxins contamination in different countries of the world such as United States of America (USDA, 2015; Xue et al., 2003), Brazil (Gonçalez et al., 2008; Nakai et al., 2008), Argentina (Asis et al., 2005; Pildain et al., 2008), Nigeria (Ezekiel et al., 2013; Ezekiel et al., 2012; Odoemelam & Osu, 2009), South Africa and Democratic Republic of Congo (Kamika et al., 2014), Uganda (Kaaya et al., 2006), Malawi (Matumba et al., 2014), Pakistan (Mushtaq et al., 2012), Egypt (Youssef et al., 2008) and Malaysia (Leong et al., 2011). Tropical country like Malaysia, characterized by warm and humid weather conditions provides an ideal condition for the growth and proliferation of *A. flavus* and subsequent aflatoxin

contamination. There are incidences of aflatoxin contamination in commercial peanuts and starch based foods in Malaysia. More also, high levels of contamination in commercial nuts sold in Malaysia has been reported (Leong et al., 2011). In Brazil, aflatoxin contamination in peanut has a long term history. Iha et al. (2016) found 71 out of 100 peanut confection candies in cities of Sao Paulo to be contaminated with aflatoxins. In the same State, (Oliveira et al., 2009) analyzed a total of 942 food samples for contamination with aflatoxin and found contamination be highest for peanut (8.1%).

An estimated 4.5 billion people, with majority from developing nations are at the danger of chronic exposure to foods contaminated with aflatoxins (Shuaib et al., 2010). In Kenya, about 125 people were confirmed death in the year 2005 due to outbreak of aflatoxicosis occasioned by the consumption of locally grown maize (Daniel et al., 2011).

It is a known fact that aflatoxin contamination poses real threat to human population across the world, thus, the need for risk assessment becomes very necessary. Risk assessment based on hazard identification has since been proposed by Wang et al. (2018). Hazard identification has been defined as "the identification of the type and nature of adverse effects that an agent has, an inherent capacity to cause in an organism, system, or sub-population" (IPC, 2004). In other word, hazard identification has been defined as "the identification of biological, chemical and physical agents capable of causing adverse health effects and which may be present in a particular food or group of foods" (FAO/WHO, 2007). It is true that peanut and peanut products are widely consumed across the globe. But one major challenge with the crop is aflatoxin contamination. Therefore, in order to minimize the occurrence of toxigenic fungi and subsequent aflatoxins contamination in peanuts, real assessment of the incidences of these fungi along the production and value chain becomes significant (Martins et al., 2017). Peanuts infested

by *E. pallens* pose a great health risk to consumers because of the potential hazard associated with the feeding habit of the bug. The insertion of the insect beak (rostrum) into peanut kernels via the pods, pose a serious hazard of aflatoxins transmission. Therefore, the risk assessment of *E. pallens* for the transmission of aflatoxins was based on the identification of potential hazard associated with its feeding habits on peanuts. Thus, conducting a risk assessment on the insect rostrum becomes paramount because of its ability to overcome the mechanical barrier (pods) to feed on kernels during harvest and storage. This study in a quest to assay for aflatoxins among the fungal isolates, used the ammonium vapor (AV) test. The coconut milk agar medium (CAM)) was used for the cultural detection of the fungal mycotoxins.

1.7 Chemical control of seed bugs

The use of synthetic insecticides for the control of Hemipteran insects has long been in practice. Because *E. pallens* is a pest of serious economic concerns to the agricultural community and global trade, the need for its control becomes imperative. Therefore, commonly used methods of Hemipteran bugs have been via the use classical biological control and chemical control methods. The classical biological control method involves the application of natural predators such as the bethylid wasp, *Cephalonomia* spp. to parasitize eggs of *E. pallens* whereas the reduviid, *Coranus pallidus* is applied as a predator on both adults and nymphs (Schaefer & Panizzi, 2000a). Other biological control agents used for abating *E. pallens* menace are predators such as lizards, spiders, termites and parasites such as the *Grionini* spp., which causes 40-80% egg parasitism (Terefe et al., 2012). But whether these insect parasites and predators used for the control of *E. pallens* will not assume pest status remains cautionary, likewise the impacts of synthetic chemicals on biodiversity and health. According to Terefe et al. (2012), botanicals such as 10% neem kernel extract and formulated neem oil (Nimex 0.03%) proved successful

at controlling *E. pallens* under storage in airtight containers. Chemical control method such as surface layering and dusting of peanuts stacks with 0.5% lindane and 2% malathion have been found to be effective for the control of the bug (Schaefer & Panizzi, 2000a). Synthetic pyrethroids such as cyfluthrin, deltametrin and bifenthrin insecticides have been applied for the control of *Meccus pallidipennis* (Hemiptera: Reduviidae). For the control of the bug in sesame, conventional chemicals such as Malathion 50% EC and Ethiosulfufan 35% EC are applied at the base of the stalk. Also, dusting of the base of sesame stalks and surrounding soil with carbaryl 85% WP and Ethiolathion 5% dust have been practiced for the control of the bug. Diazone and Fenitrothion are used as foliar spray prior to harvest. While in storage warehouses, Carbosulfan 25% ULV, Ethiosulfan 25% ULV, Malathion 50% EC, Karate 0.8% ULV and Decis 0.5% ULV are known to be effective at doses against *E. pallens* (Terefe et al., 2012). However, these insecticides are not reliable due to the increased rate of bug re-infestation in surroundings after the application of these insecticides, leading to resistance (Flores-Villegas et al., 2016b). In the Kafta-Humera region of Ethiopia, the control of *E. pallens* is totally based on the use of chemical insecticides, which are often less effective at controlling the bug menace, as evidenced from re-infestation after a matter of days (Berhe et al., 2008). The continuous application of synthetic insecticides such as the commonly used organophosphates pose deleterious impacts on both humans and the environment (Senthil-Nathan, 2018). Therefore, the need for a safer environment and better human and animal health via alternative natural pesticides becomes needful. This position was corroborated by Pretty and Bharucha (2015) that alternative pest control methods instead of synthetic pesticides should be adopted that will mitigate pest damage with less cost and minimal negative outcomes.

1.8   Challenges of pest management and global pesticide use

The advent of agricultural production has long been characterized by insect pests which negatively impact crop yields (Deaver et al., 2019). Chemical insecticides have helped elevate the scale of agricultural productions by offering short term protection of crop losses due to insect infestation, but yet, insecticide resistance among insect pests remains on the rise, a threat to different crop species (Huseth et al., 2018). However, so disturbing is the fact that insecticide resistance bears direct effect on the environment and human health and a surge in resistance among vectors of both human and animal pathogens (Reid & McKenzie, 2016).

Pest management is an element of agricultural production with significant economic consequences linked to control options, crop production outcomes, controllled costs, and environmental and societal impacts (Naranjo et al., 2015). The use of pesticides for agricultural pests and disease control has been in practice for decades (Yadav et al., 2015; Zhang et al., 2011), but yet, insects remain the world's biggest threats to cash crops and other important staple crops around the world (Singh & Arya, 2019). Hitherto, the use of chemical insecticides for the control of insect pests remain the convention with the perception that it is the most reliable and beneficial. Haplessly however, the excessive consumption of chemical pesticides is accompanied by negative impacts on the environment, especially in developing countries (Singh & Arya, 2019). Sánchez-Bayo and Wyckhuys (2019) reported synthetic pesticides and fertilizers as some of the major drivers responsible for the global decline of insect population.

Pesticide encompasses a broad range of substances including insecticides, herbicides, fungicides, rodenticides, nematicides, molluscicides, plant growth regulators etc. Organochlorines were successful at controlling vectors and diseases before their

restriction in some technologically advanced countries (Aktar et al., 2009). Pesticides become environmental contaminants of concern when their presence in the environment exceeds their natural threshold, and can also be considered as pollutants where they exert detrimental effects on floras and faunas (Guedes et al., 2016).

With respect to environmental effects, many chemical pesticides have been categorized as xenobiotics which have the tendency to adsorb strappingly to soil particles and remain immobilized for an extended time length. Remedial efforts towards the removal of these immobilized xenobiotics are usually cost intensive and available technologies may not be effective for large scale applications. Also, of great concerns is the drastic effects of these synthetic chemicals on non-target hosts and the disruption of the ecosystem biodiversity. The deleterious effects of these chemicals on non-targeted arthropod population has been reported. Most unfortunately is the fact that insects like the predators and parasitoids that are natural enemies to certain pest species are also usually destroyed via the application of chemical pesticides in the environment (Gill & Garg, 2014). Additionally, the consistent exposure of humans to sub-lethal amounts of these chemicals over a long period of time has been reported to be the leading cause of certain cancers and illnesses involving cardiovascular, renal, respiratory and nervous systems (Germany, 2012; Gill & Garg, 2014).

Currently, there seems to exist a plethora of synthetic pesticides with promising potential for the control of arthropod pests irrespective of their negative consequences. And though pesticides have remarkable global market demands for use in agricultural productions, their environmental impacts, affordability, and economic consequences remain issues of concern. The negative impacts of these synthetic chemicals on biodiversity, food safety, human, animals and aquatic life can be tremendous. Their lack of specificity in killing target species makes all species vulnerable, resulting in actions

that can impact the environment negatively. Therefore, to avert these deleterious effects of synthetic pesticides as enumerated, it is expedient to explore alternatives with less cost of production and that can be easily obtained from the environment and applied to the environment with less effects. Biological control which utilize living organisms instead of synthetic pesticides has been advocated to be promising in this regard.

1.9   Aflatoxins contamination and risk factors

Contamination of food stuff by fungal organisms affects their shelf life and promote several safety concerns in the agricultural and food industries worldwide. Most food stuff are susceptible to microbial infestation, resulting in colossal economic losses. Microorganisms such as the *Aspergillus* sp. are known for high affinity towards grain crops (Tian et al., 2012), oil seeds (peanut, cotton, sunflower, sesame, and soybean), nuts (almond, pistachio, coconut, and walnut), spices (black pepper, coriander, garlic, and turmeric) and milk (Bhatnagar-Mathur et al., 2015). Peanut is one of the crops that is most vulnerable to invasion by *A. flavus*, resulting in aflatoxins contamination (Torres et al., 2014b). *A. flavus* is the most commonly implicated fungus responsible for spoilage and synthesis of aflatoxins, a group of toxic secondary metabolites produced by the fungus and other members such as *A. parasitucus* and *A. nomius*. However, other than just affecting crops, aflatoxins render them unsafe for consumption to humans and animals, thus invoking a threshold boundary of acceptability (Bhatnagar-Mathur et al., 2015). Aflatoxins are the most hazardous forms of these secondary metabolites, possessing both carcinogenic, mutagenic, hepatotoxigenic, teratogenic and immunosuppressive properties which can also impair the functioning of the metabolic system (Peng et al., 2018; Tao et al., 2018; Tian et al., 2014). For instance, for its carcinogenic properties, the aflatoxin B_1 is branded as a Class 1 carcinogen by the International Agency for Research on Cancer for cases of its acute toxicity on human and animal organs such as the liver and kidneys.

AFB$_1$ is considered as highest in potency and effect; followed by AFB$_2$, AFG$_1$ and AFG$_2$ in the toxicity hierarchy (Peng et al., 2018). The digestibility of aflatoxins in animals is difficult and therefore end up in their meats. They are also freeze and heat stable, thus remaining undegraded in foods (Liu & Wu, 2010).

Globally, direct economic effects of aflatoxins on loss of agricultural produce or market worth and healthcare has been tremendous. For example, Africa alone loses more than US$670 million due to compliance to EU safety standards for trans boarder food exports, whereas over US$932 million economic burden has been placed on agriculture worldwide because of fungal metabolite toxins. This often results in a 100% rejection of produce and with resultant economic effects (Bhatnagar-Mathur et al., 2015).

However, biological control is said to be one of the most promising approaches for averting aflatoxins contamination of peanuts in the fields. For instance, aflatoxins incidences in both soil and peanut kernel samples from northern Argentina were found to be reduced upon application of atoxigenic (non-aflatoxin) *A. flavus* strains, which achieved this feat via competitive exclusion of the toxigenic (aflatoxins-producing) strains (Alaniz Zanon et al., 2016). The concept of biological control involves the application of atoxigenic strains to the soil to inhibit the proliferation of native toxigenic strains under conditions that favors aflatoxin production (Abbas et al., 2011). Overtime, the use of atoxigenic *A. flavus* strains for the biological control of toxigenic strains in crops has been investigated. The mechanisms of competitive exclusion due to physical displacement and competition for available nutrients were exploited by the atoxigenic strains against the toxigenic ones. This method has effectively helped in controlling mycotoxins in maize, peanuts and cottonseeds. However, choosing biological control strains remains tasking (Degola et al., 2011). Therefore, conducting aflatoxin screenings

on *A. flavus* isolates is critical to identifying isolates with potential for biological control (Fani et al., 2014) of both toxigenic strains and insects in crop plantations.

Therefore, in order to select from isolates of *A. flavus* strains for the biological control of insects, the need to screen the isolates for their ability to produce aflatoxins becomes important. Isolates of *A. flavus* obtained from the cadavers of *E. pallens* were subjected to aflatoxin screening to enable the selection of atoxigenic strains which can be used for the biological control of the *E. pallens*. The screening of the isolates was necessary to avoid the introduction of toxigenic strains into the environment which may further amplify aflatoxin transmissions. The choice of atoxigenic strains is important to the concept of biological control, because of its dual potential for the control of toxigenic strains and the insect problems. Furthermore, with the view to assessing the potential risk of aflatoxins transmissions by *E. pallens* the rostrums of *E. pallens* were investigation for the association with aflatoxins producing fungu in nature.

1.10   Biological control

Over five decades ago, synthetic chemicals were predominantly employed as the means for pest eradication and management. But the effects of these chemicals on pest resistance and resurgence, surface and underground water contamination, human and animal health, distortion of the ecosystems, altered biodiversity structure and other critical environmental concerns have prompted the quest for finding some more environment friendly strategies for pest control such as the use of biological control agents (BCAs) (Garrido-Jurado et al., 2017; Hussain et al., 2012b). These natural enemies are employed as biological control agents because of their effectiveness in regulating insect pest populations. Several species have been applied as biological control agents of arthropod pests in orchards, glass-house, turf and lawns, ornamentals, range, forestry,

stored products and for the control of vectors and insects of medical and veterinary concerns (Hussain et al., 2016; Kaaya & Hassan, 2000). Considering the negative impacts of synthetic insecticides used in pests control, biological control strategies anchored on the use of naturally occurring entomopathogens emerged as a promising alternative control to synthetic insecticides (Jaber et al., 2016).

Biological control is an environmentally benign approach most commonly used in the control of insect pests. Agricultural crops and products have over the years been under attack by insect pests resulting in colossal losses of fortunes by farmers. These repeated threats of insect pest attacks on agricultural crops and products necessitated the demand for the application of synthetic insecticides for insect control. But because of the deleterious impacts of synthetic insecticides like the organophosphates on both humans and the environment, the search for alternative natural pesticides becomes needful (Senthil-Nathan, 2018) for a safer environment and better human and animal health. Natural pesticides including EPF, viruses, bacteria, nematodes and plant bioactive secondary metabolites are significantly gaining relevance and priority over synthetic insecticides and are being included as integral components of the integrated pest management programs. The virulence of these natural pesticides has been successfully evaluated both under laboratory and field conditions with some positive outcomes. However, the need to further investigate different areas involving naturally occurring pesticides becomes imperative (Senthil-Nathan, 2018). This is imperative because it is likely that there might be increase in the use of EPF in biological control of arthropod pests, due to the advocacy for the withdrawal of synthetic insecticides because of the notable environmental risks, quests for zero pesticides residues on food, legislations

promoting the use of EPF and other biopesticides and the additional concerns of resistance to insecticides by insect pests of socioeconomic significance (Butt et al., 2016).

1.11   Fungi as natural enemies for biological control

Biological control agents possess the advantage of lack of toxicity to pollinators, compatibility to other natural enemies (Glare et al., 2012) while entomopathogens like *M. anisopliae* and *B. bassiana* have been reported to be safer than conventional chemical pesticides and non-pathogenic to animals and humans with just minimal reports of disease (de Garcia et al., 1997; Figueira et al., 2012). In nature, some fungi utilize certain living arthropods by way of resource exploitation for growth, development and reproduction (Roy et al., 2006). However, some of these fungi are natural enemies pathogenic to arthropods while others specialize in exploiting resources from both dead and living plants as well as the soil (saprophytes) (Rohlfs & Churchill, 2011b; St. Leger, 2008). In nature, there are over 1000 fungal species known to kill insects, and are the most studied pathogens of insects (Jaber et al., 2016) in nature.

EPF are significant in their role at regulating insect pest population during epizootics, as they are known for specificity against insect species. These fungi possess characteristic advantage over other biocontrol agents like bacteria, viruses and protozoa as they affect all stages of insect development and as well infect and kill those with specialized chewing mouth parts which often resist infection by bacteria and protozoa (Hussain et al., 2012b; Samuels et al., 2002b; Valero-Jimenez et al., 2016). Several fungal insecticides including *Metarhizium anisopliae*, *Isaria fumosoroseus*, *Beauveria bassiana*, and *Verticillium lecanii* are currently in use for the control of insect pests (Wang, 2012). These fungal insecticides offer comparative advantages of being harmless to humans and animals, cost-effective component of integrated pest management, simultaneous action against multiple

pests and ease of adaptation to prevailing methods of application (https://www.biocontrol.co.za). At present, products formulated from species of *Metarhizium*, *Beauveria*, *Isaria*, and *Laecanicillium* are traded for commercial purposes (Faria & Wraight, 2007; Olombrada et al., 2014). A good example is Mycohit, a formulation of *Hirstulla thomsonii* which showed extensive success against coconut eriophyid mites has been registered and commercialized in India (Sreerama Kumar & Singh, 2009).

1.11.1   Entomopathogenic fungi (EPF)

Fungi are natural pathogens that have the tendency to cause epizootics among population of host insects, possessing numerous beneficial features that place them in pole position as microbial control agents (Lacey et al., 2015b). EPF are widely distributed in the fungal kingdom with majority found in the Deuteromycotina and Zygomycotina. Some possess restricted host range, for example, *Aschersonia aleyrodis* primarily infects only scale insects and whiteflies, while *M. anisopliae* and *B. bassiana* have broad host spectrum with individual isolates becoming more specific (Clarkson & Charnley, 1996). A diversity of molecular means and technologies have been recently used for the reclassification of various species based on phylogeny, as well as linking asexual forms (anamorphs) and sexual forms (teleomorphs) of numerous pathogens in the Phylum Ascomycota (Lacey et al., 2015b). EPF playing key role in arthropod pest control are members of the Fungi Imperfecti, now known as Hypocreales such as *M. anisopliae* (Metschnikoff) (Ascomycota: Hypocreales) *B. bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales). Since early 1980, about 70% of isolates of the above species are used in the formulation of almost 170 commercial myco-insecticides which are mostly

used for the control of coleopteran and hemipteran insect pests in agriculture (van Frankenhuyzen et al., 2016).

EPF constitute the largest group among insect pathogenic microbes. Basically, two fungal groups known to cause infection in insects include fungi that belong to the orders Hypocreales (formerly called Hyphomycetes) and Entomophthorales. However, pathogenicity is not strictly to these few as fungal pathogens from other taxonomic clades are discovered with this ability. It has been established that over 700 species of fungal pathogens from these orders are reported as pathogens of insects (Hussain et al., 2016; Lacey & Kaya, 2007). Fungal entomopathogens confer some major advantages when applied for insect control. Firstly, they function as natural enemies of insects, being able to infect arthropods via the integuments. Secondly, their cultivation and production of infective propagules are relatively easy and cheaper (Hussain et al., 2016).

The potential of EPF for the control of insect pests, dated back to the later part of 19th century when *M. anisopliae* was experimented against the wheat cockchafer *Anisoplia austrica* and *Cleonus punctuventris* (sugar beet curculionid). Several attempts have been made over the past century to use fungi like *B. bassiana*, *M. anisopliae*, *Nomureae rileyi*, *Verticillium lecanii*, *M. anisopliae* and certain entomopthora species for insect pest control (Clarkson & Charnley, 1996). Currently, moderate scale application of fungi for insect pest control is practiced in Brazil, China and Russia. Formulations based on *M. anisopliae*, *V. lecanii* and *B. bassiana* are produced and marketed by a couple of companies in Europe and North America as myco-insecticides under different trade names (Clarkson & Charnley, 1996). For example, a *Metarhizium*-based formulation (Zero QK-S 0,4 DP) from Guatemala has been marketed for many years by Agricola El Sol for application against domestic cockroach pest (Montalva et al., 2016). Some

measures of successes have been made towards investigating and commercializing their application as mycopesticides under field environments (Hussain et al., 2016). Studies have established that numerous species of Hemiptera (Mweke et al., 2018; Santos et al., 2018a; Seye et al., 2014a), Diptera (Amora et al., 2010), Lepidoptera (Karthi et al., 2018), Isoptera (Hussain et al., 2011; Hussain et al., 2010a), Coleoptera (Ansari et al., 2006) are prone to various infection by fungal pathogens. There are sizeable attempts at the formulation of EPF for insect pest control on the global market.

Table 1.1: EPF formulations for commercial purpose.

Fungus	Product and company	Formulation
Metarhizium anisopliae	Bio-path™/Eco Science/OSA	Conidia on medium placed in chambers
M. anisopliae	Biologic Bio 1020/Bayer AG Germany	Granules of mycelia
M. anisopliae	Biogreen/Biocare Tech. Pvt. Ltd/Australia	Conidia grown on grains
Aschersonia aleyrodis	Koppert/Holland	Wettable powder
Beauveria bassiana	Conidia, AgrEvo, Columbia	Suspendible granules
B. bassiana	Naturalis™, Troy-Bioscience, USA	Liquid
B. bassiana	Boverol/Czeck Republic	Wettable powder and dry pellets
B. bassiana	Ostrinil/Natural plant protection/France	Microgranules of mycelia
B. bassiana	Mycontrol-WP/Mycotech.Corp. USA	Wettable powder
B. brongniarti	Engerlingspilz/Andermatt-Biocontrol/Switzerland	Barley kernels impregnated with conidia
B. brongniarti	Betel/Natural plant protection/France	Microgranules of mycelia
Paecilomyces fumosoroseus	Pfr 21/WR Grace USA	Wettable powder
Verticillium lecanii	Vertalec/Koppert/Netherlands	Wettable powder
V. lecanii	Mycotal/Koppert/ Netherlands	Wettable powder

(Hussain et al., 2016)

Over the years, attention on the use of EPF for the control of insect pest populations has been on the rise because they are environmentally safe and serve as innocuous alternative to conventional chemical insecticides; many which have been implicated for toxicity and withdrawn due to their damaging effects on the environment, human, animal and aquatic health (Sonmez et al., 2016). Although, traditionally, EPF have been viewed exclusively as insect pathogens, emerging studies have established the diversities of the ecological niches they occupy (Lacey et al., 2015b). Their role as promising endophytes with mycorrhizal properties of liberating nutrients from infected insects and cadavers (Behie et al., 2015; Jaber & Enkerli, 2016; Jaber & Ownley, 2018), plant disease antagonists, plant growth enhancers and rhizosphere colonizers has been well studied. Other than insect pest control, some of these entomopathogens can serve dual functions of suppressing phytopathogens and parasitic nematodes as well as enhance plant growth (Barelli et al., 2016; Lacey et al., 2015b; Sasan & Bidochka, 2012). The characteristic richness of these fungi presents great potentials for their application for multiple roles.

But as diverse and prolific as insects are among land animals, there exists a plethora of specialized natural pathogens that infect them through different mechanisms. While specialized fungal pathogens possess the ability of adhering and breaching the insect cuticle through enzymatic reactions, the bacterial or viral pathogens must be ingested to trigger infection and death of the host (Pal et al., 2007b). For example, entomopathogens such as *B. bassiana* and *M. anisopliae* have been well characterized with regards to pathogenicity to diverse insect groups and are used as myco-biocontrol agents for the control of agricultural pests worldwide. These fungi are generally known for their wide host range with some clades demonstrating high degree of specificity (Kanzok & Jacobs-Lorena, 2006; Keyser et al., 2014; Zimmermann, 2007). They constitute the largest group of insect pathogens among microorganisms and are diverse in distribution, occurring in

the forest, aquatic and agricultural habitats, and are often exploited as active agents of insect pest control. As natural enemies, they play a vital role of regulating insect population. (Faria & Wraight, 2007; Shin et al., 2014). They are host specific, with minimal risks of attack on non-target pests and other beneficial organisms. They demonstrate a broad spectrum of activity against a variety of insects such as aphids, thrips, and lepidopterous larvae. However, some few species like *Aschersonia aleyrodes* infect basically scale insects and whiteflies while *Aspergillus* and *Fusarium* are known facultative generalist pathogens whereas *Cordyceps* are strictly obligate parasite and are often rarely found (Sandhu et al., 2012b).

The use of EPF for the control of pests of stored-grain products in warehouses have been adjuged to be the most promising option for control (Ak, 2019). They are widely used microbial control agents in storage environments for the control insect pests (Rumbos & Athanassiou, 2017). Broad spectrum species such as *M. anisopliae* and *B. bassiana* have been widely tested on a large number of stored-grain pests (Batta, 2018; Rumbos & Athanassiou, 2017). Because they exist in nature, EPF are known to exhibit minimal environmental impact are generally considered to be environmentally safe with minimal toxicity (Batta, 2016). The use of EPF against stored-grain pest is said to be compatible with food safety and environmental regulation, thus making it an effective alternative to chemical insecticides (Batta & Kavallieratos, 2018).

However, environmental factors such as humidity, temperature and radiation regulate the natural populations of entomopathogens with key effects on their survival and dispersal, thus, posing a serious drawback on the use of these pathogens as bioinsecticides under environmental conditions (Carollo et al., 2010).

1.11.2 Modes of action

EPF infect their target host via adhesion to their cuticular substrata, followed by the synthesis of hydrolytic enzymes which enable the degradation and penetration of the insect cuticle. The insect exoskeleton is made up of two layers, consisting of the epicuticle and the procuticle. The epicuticle which forms the outermost layer of the exoskeleton is a complex structure devoid of chitin, but contains a matrix of phenol-stabilized proteins, non-polar lipids, hydrocarbons, fatty acids and sterols. Most insect species possess long chain hydrocarbons as the major components of their epicuticle, which helps in abating microbial invasion, desiccation, and also serve as recognition molecules or receptor of semiochemicals. However, the procuticle which constitutes a major component of the insect exoskeleton, contains chitin fibrils entrenched in a protein-lipid matrix and quinones. Proteins account for about 70% of the procuticle. But conversely, EPF have developed mechanisms for overcoming these barriers via the instrumentality of hydrolytic enzymes which possess affinity for the cuticular components (Clarkson & Charnley, 1996; Pedrini et al., 2013). In view of their unique characteristics, studies have extensively evaluated these myco-pathogens both as microbial control agents of agricultural and medical insect pests and their potential to produce bioactive metabolites (Boucias et al., 2016; Khan et al., 2012; Roy et al., 2010).

EPF can successfully infect insect pests in different ontogeny stages like the eggs, larvae and pupae, as they need not to be ingested, but can invade the host exoskeleton to cause infection. The insect exoskeleton presents a formidable barrier that must be countered physically because of its properties and functions. Where barriers are breached, fungal invasion occurs between the intersegmental folds, mouthparts or spiracles (Hussain et al., 2016; Sun et al., 2016a). Relatively higher humidity in these areas enhances germination of adsorbed conidia, making the exoskeleton non-sclerotised and

better penetrated (Clarkson & Charnley, 1996; Hajek & St. Leger, 1994). Conidia adhered to the cuticle of potential host could result in compatible (infection) or incompatible (repulsion/resistance) reactions. In compatible reactions which occur between the conidia and the cuticle, conidial recognition and adhesion lead to germination on the insect exoskeleton. Fargues (1984) and Sun et al. (2016a) observed that compartible reaction on the host cuticle results in three successive stages during insect infection: (1) conidial adsorption to the epicuticular surface of the insect; (2) interface consolidation between the pre-germinant propagules and the exoskeleton; (3) germination and proliferation on the epicuticle prior to the penetration of the appressorium. As appressoria are formed, penetration pegs emerge which penetrate the cuticular surface via the effect of mechanical (turgor) pressure and enzymatic means (Sun et al., 2016a). Exocellular mucilage initially thought to facilitate the adhesion of fungal conidia to insect cuticular surface, are hitherto produced by some fungal species during their synthesis of infective structures (Boucias et al., 1991). In *M. anisopliae*, the formation of infective structures and expression of cuticle-degrading enzymes such as proteases, are activated by reduced nutrient levels (Leger, 2008), demonstrating the ability of the fungus to sense environmental changes and host cues to initiate infection.

Metarhizium anisopliae has been used for the control of a wide variety of insect pests, and remains one of the widely used EPF for the formulation of myco-insecticides applied during inundative biocontrols under the integrated pest management system. *M. anisopliae* was first described as *Entomophthora anisopliae* by Metschnikoff, before it was later moved to the current genus *M. anisopliae* by Sorokin (Zimmermann, 2007). Zimmermann (2007), reported that there was originally no teleomorph (sexual reproductive) stage of the genus *Metarhizium*, this led to the classification of the species *M. anisopliae* in the Deuteromycota, Hyphomycetes. Conversely, *Cordyceps taii*

described by Liang et al. (1991) was said to consist of an anarmophic stage which was later called *Metarhizium taii*. However, later, Liu et al. (2001) isolated *Cordyceps brittlebankisoides* which was confirmed afterwards as the teleomorph of *M. anisopliae* var. *majus* (Liu et al., 2002). Thus, today, the genus *Metarhizium* Sorokin are classified as Hypocreales and Ascomycetes (CABI Biosciences, 2007).

Currently, *M. anisopliae* is one of the EPF with known broad spectrum of activities that is applied in the environment for the control of different insect pest species. It has been known to demonstrate pathogenicity against target pests such as grasshoppers and locusts, termites, hemipterans, cockroaches, noctuids, greenhouse pests including thrips or flies, ticks and mosquitoes. Other target insects include the soil inhabiting insects, such as curculionids and different scarab species (Zimmermann, 2007). *M. anisopliae* occurs worldwide, ranging from the tropics to the arctic in soils as well as on insects. Domsch et al. (1980) reported the isolation of *M. anisopliae* from cultivated fields, grassland soils, forest soils, organic remains inside water, swamps, river sediments, mines, birds' roosts, and plant roots. Zimmermann (1986) opined that the *Galleria* bait method is the most widely used method for the isolation of the fungus from soil. But alternatively, there exist some selective media that have been developed for the isolation of the fungus from samples.

Basically, infection by EPF does not require the ingestion of pathogens before infection is initiated in their target hosts. But rather, the adsorbed spores germinate and invade the host directly through the cuticle EPF distinct from other biological control agents (Hussain et al., 2016; Samuels et al., 2002a). This way, fungi can be used for the control of all insects species under appropriate environmental conditions (Sandhu et al., 2012b). In nature, fungi generally constitute the largest insect pathogens causing the

largest expanse of death due to infection (Faria & Wraight, 2007; Pal et al., 2007b). Their high reproductive capabilities, specificity, short generation time and resting stage enable them survive longer outside a living host, making them dependable biological control agents (Chandrashekara, 2007; Kumaraswamy et al., 2014). Usually, the important requirement for the choice of EPF as biocontrol agents is the factor of susceptibility of the insect on one hand and virulence of the fungus on the other (Sandhu et al., 2012b).

1.11.3 Synthesis of extracellular cuticle-degrading hydrolytic enzymes by EPF and their role in virulence.

By nature, insect cuticles are well endowed matrixes of complex polymers including chitin fibrils, lipids, proteins and N-acylcatecholamines which must be countered by invading EPF before infection is established. One major mechanism required to overcome this complex barrier is the secretion of extracellular enzymes that possess the potential to degrade the cuticle and enable the penetration of the pathogen (Dhawan & Joshi, 2017). For insect infection with EPF, overcoming the proteo-chitin complex of the insect cuticle becomes necessary. In this regard, EPF do express various hydrolytic enzyme systems including chitinases, proteases, lipases, catalases, esterases and phospholipase C (Santi et al., 2010a; Wang et al., 2011), as essential virulence determinants required by the pathogens for the penetration of host cuticles (Nunes et al., 2010). Initial events that result in the infection of insect host by EPF is significantly dependent on the activities of these cuticle-degrading hydrolytic enzymes. This enzyme system is exceptional and of great interest as they serve as rudimentary criteria considered during myco-insecticide improvement (Mustafa & Kaur, 2009). For example, *M. anisopliae* has been genetically engineered by way of genetic optimization to overexpress toxic proteases that facilitate rapid killing of hosts (Hu & Leger, 2002). These extracellular hydrolytic enzymes are produced by EPF to degrade the major constituents of the exoskeleton of insects and

enable the penetration of the invading pathogen into the hemolymph. This implies that these enzymes complex is essential for pathogenesis and other vital physiological functions (Cho et al., 2006; Wang & Leger, 2005). Proteases like the virulent Pr1 (Shah et al., 2005) can facilitate conidial germination, and in combination with other enzymes enable the breakdown of the host cuticle for easy access into the hemolymph (Ortiz-Urquiza et al., 2013; Santi et al., 2010a). Comparative studies on EPF genomics revealed the diversity of the genes for encoding chitinases and proteases in EPF than in plants (Gao et al., 2011; Hu et al., 2014; Xiao et al., 2012; Zheng et al., 2012). This genetic endowment has been attributed to the likely adaption of EPF to the vast amount of chitin in the insect exoskeleton, while the expanse of proteases in this regard may reflect their host range potential (Qu & Wang, 2018). It is believed that the production of cuticle-degrading enzymes by EPF which function to disrupt the exoskeleton of insects, places them in greater advantage over other pathogens. Notwithstanding, it is essential to investigate the variety of these enzymes and their activities relevant to pathogenicity (Schrank & Vainstein, 2010b). Determining the presence and activities of these enzymes in EPF isolates used for biological experiments is pivotal, so as to enable the corroboration of existing facts on the infection mechanisms of these fungal pathogens.

1.11.4 Economic value of biological control

Over the years, chemical control has been the method of control of these bugs despite the associated dangers of environmental pollution, resistance and resurgence in insect pest population; and the risk of health problems. Obviously, where these chemicals fail to reach oviposited eggs in the soil, control becomes difficult (Samuels et al., 2002b). However, considering the enormity of additional challenges posed by synthetic pesticides, alternative methods that can moderate arthropod pest damage needed to be applied to curtail the negative impacts associated with synthetic pesticides (Pretty &

Bharucha, 2015). Innovative solutions are required to meet up with the grand challenge of rising global demands for foods and fibers by the rapidly increasing world population. One fundamental factor to alleviating these demands hinges on averting crop yield losses due to insect pests, while preserving the inadequate natural resources and sustaining environmental quality via ecologically and economically all-encompassing pest control systems. Understanding the economic value of biological control will assist in vast measure its usefulness in crop protection and elevate stock between agricultural stakeholders and policy makers that motivate innovations. Approaches of assessing economic values of biological control differ by the scope and scale of the analytical tool and the fundamental questions to be answered.(Naranjo et al., 2015). More importantly, though EPF are known to be slow acting at killing targeted hosts, yet, they are also known to persist when applied in the environment. Where they persist in the environment, they become secondary sources of infection to insect pests of crop plants. Where they function as secondary infection propagules emanating from insect cadavers, there will be less need for farmers to spend money on insecticides because of the epizootic potential of the pathogens. This keeps insect pest population well regulated and the economic conditions of farmers salvaged.

1.11.5 Ecological benefits of biological control

Because of the growing awareness on environmental challenges, concerned government agencies are continually championing the advocacy for the use of eco-friendly technologies towards maintaining environmental sustainability. Biological control and integrated pest management (IPM) practices have resulted in the abatement of chemical pesticide pollution effect and improvement of ecological niches in the environment. It has also served as a panacea to production of healthy crops by reducing the dependence on chemical pesticides like the organophosphates, organochlorines and

the neonicotinoids. Soil health improvement and conservation of beneficial soil dwelling flora and fauna, microbes, insects, reduction in risks of chemical pesticides to human and animal health; and environmental sustainability are cardinal benefits of biological control (Vennila et al., 2018).

1.11.6 Statement of problem

The challenges of food security and safety confronting researchers and the agricultural sector of the world economy is quite enormous. Cultivated crops are under the continuous threat of insect and non-insect pest damage. peanut is one of the crop plants that suffers both pre- and post-harvest destructions by insect pests resulting in losses of fortunes and investments, especially in developing countries with fewer or no mechanized systems of farming. peanut production from cultivation to harvest and storage, ought to be free from insect pest attacks, but that is often not the case as insect pests commonly attack the plant before, during and after harvest. One major post-harvest insect pest of peanut in the tropics, is the seed bug *E. pallens* which belongs to the order Hemiptera. It has been known as a post-harvest pest of peanut in Africa and Asia with sizeable impacts of destruction. Chemical control has been the commonly used method of management of this bug species, as cultural methods fall far less to impede their proliferations. The use of synthetic insecticides for the control of seed bugs is on the rise and most bugs species have acquired resistance against some of these insecticides. Also, these chemicals trigger the associated dangers of environmental pollution and toxicity, resistance and resurgence in insect pest population, and the risk of human and animal health problems. It is noteworthy to mention that other than the damages and economic losses caused by insect pests on crops, control measures adopted for the eradication of insect pests can also trigger indirect economic losses linked to purchase and use of synthetic insecticides, incurred expenses from medical treatment of poisoning from insecticides, and the environmental

contamination problems. However, where these chemicals fail to reach oviposited eggs in the soil, control of these bugs becomes difficult.

But alternatively, the use of synthetic insecticides can be halted by embracing the use of biological control agents such as entomopathogenic microbes. These pathogens are usually obtained from the environment and applied with minimal or no deleterious effects on the environment and its compositions. Over the years, entomopathogenic fungi have been used to control other species of seed bugs that belonged to the order Hemiptera. For instance, EPF have been used to control adult *Diaphoria citri* (Hemiptera: Liviidae), the Chagas vector, *Meccus pallidipennis* (Hemiptera: Reduviidae), the oak lace bug, *Corynthucha arcuate* (Hemiptera: Tingidae), the pea aphid, *Acyrthosiphon pisum* (Hemiptera: Aphididae) and against the eggs of *Blissus antillus* (Hemiptera: Lygaeidae). But surprisingly, there are paucity of reports on the use of EPF for the biological control of *E. pallens* (Hemiptera: Rhyparochromidae) whether under laboratory or field conditions. Even where control measures are developed, it is unlikely that the rise in *E. pallens* population can be effectively controlled in all areas. More also, due to the emerging trend of insecticide resistances among insects, the need for researches into biological control of *E. pallens* becomes imperative. Therefore, in view of the drive for alternative approaches to synthetic insecticides for insect pest control, this study was aimed at evaluating the potential of EPF for the biological control of *E. pallens* under laboratory conditions.

1.11.7 Justification

Growing concerns about the effects of chemical pesticides alongside advances in biotechnology has led to the quest for alternative and ecofriendly insect control measures. These chemical pesticides are costly and can only guarantee transient reliefs, because

insects often evolve mechanisms of resistance to these chemicals. The continuous use of chemical agents has long promoted resistance and cross resistance of insects in the environment, thus posing critical challenges in insect management.

It has basically been established that microbial control agents provide specific targeted actions against specific pests, reducing the risks of other species such as birds and mammals from being destroyed as compared to conventional chemical pesticides. Because the biological control agents come from the natural environment and not synthetic, there are greater tendencies of lessened risks to human health and environmental damages. Microbial control agents (biopesticides) unlike chemical pesticides have very complex mode of action, therefore resistance in pest could not possibly develop. Consequently, microbial control agents can proffer environment friendly solutions to the menace of insect pest than the conventional chemical pesticides which are in most cases recalcitrant. But however, despite it being harmless to humans and environment friendly, biocontrol has not been widely practiced. Biological control of the insect using EPF may in no small measure, proffer an alternative to chemical control.

1.11.8 Objectives

The main aim of this study was to evaluate the potential of EPF for the control of the peanut seed bug, *E.* pallens, via the following objectives:

1. To isolate, identify and characterize via molecular methods fungal isolates from the cadaver and rostrum of *E. pallens* and screen them for the potential for aflatoxin secretion.

2. To conduct a virulence bioassay against *E. pallens* using the fungal isolates obtained from the bug cadavers.

3. To determine the dose-response bioassay for the most virulent isolates (*A. flavus* and *M. anisopliae*) against *E. pallens* and the effect of oil formulation on the virulence of the pathogens against *E. pallens* under laboratory conditions.

4. To assay for the activities of hydrolytic enzymes from the most lethal pathogens among the isolates obtained from the cadavers.

1.11.9 Significance of the research

Biological control is such pivotal for the abatement of pest problems in the agriculture towards achieving and food safety and security. This research will be significant in the following ways:

1. Environmental impact: This research will impact the environment by mitigating the destructive tendencies of conventional chemical pesticides. The advocacy for biological control over the decades is towards a safer environment, being that biological control agents are basically known to be eco-friendly. The prospect of averting land, water pollutions and its attendant

health risks, eutrophication and the maintenance of a balanced ecosystem is feasible through this research.

2. Economic impact: World over, insect pests have been implicated for causing colossal losses to farmers of food crops on both peasant and commercial scales. Recently, most of these insect pests have developed resistance to most of the conventional chemical pesticides. This has affected the economic statuses of farmers where they spent large sums of money on pesticides without corresponding impact but rather derailing their economic status. This research will proffer a cheap and eco-friendly biotechnological palliative toward a reduced economic loss to insect pest control.

3. Food security: For many decades, global food security has been the focus of the Food and Agriculture Organization (FAO). The concept of this research will provide a palliative to food security towards abundance, accessibility and affordability of food.

4. Industrial impact: The scope of the biotechnological industries over the world can be enlarged when the findings and recommendations of this research is adopted. The EPF isolates strains used in this research can help to revolutionize the biopesticides industry.

5. University of Malaya: With this research, University of Malaya will remain hoisted as one of the Universities leading the advocacy for safer environment. The strains used in this research will also impact research, teaching and learning in the University of Malaya.

CHAPTER 2: LITERATURE REVIEW

2.1 Insect pests of groundnuts

In the Sub-Saharan Africa (SSA), peanut infestation and yield loss are attributed to arthropods including millipedes, white grups, nematodes and termites (Brandenburg et al., 2003; Johnson & Gumel, 1981; Sohati & Sithanantham, 1990). Other pests of peanuts that inflict occasional damages include ants, false worms, wireworms and earwigs (Wightman & Rao, 1993). Termites implicated for destroying groundnuts include the *Odontotermes* spp., *Microtermes* spp. and *Macrotermes* spp. While the aforementioned species destroys groundnut pods and seeds; symphilids, white grubs, wire worms damages the roots while scarification of the pods and eating of the haulms are common features of termites damage (Owusu-Akyaw et al., 2005; Ranger Rao & Rameshwar Rao, 2013; Wightman & Rao, 1993). Johnson and Gumel (1981) reported a 40% yield loss in peanut production in Nigeria due to the activity of *Microtermes* spp. In a survey conducted on soil arthropods in peanut crop fields in northern Ghana, Owusu-Akyaw et al. (2005) detailed a compendium of pests including millipedes (Myriapoda: Diplopoda), grubs (Coleptera: Scarabeidae), earwigs (Dermaptera: Forficulidae), symphilids (Myriapoda: Symphyla), wireworm (Coleoptera: Elateridae), mealybugs (Homoptera: Pseudoccoccidae), termites (Isoptera: Termidae) and red ants (Hymenoptera: Formicidae). Also in a preliminary study on the insect pest and disease profile of peanuts in northern Ghana, Tanzubil (2016a) outlined the pests of peanuts to include soil arthropods like millipedes (Myriapoda: Odontopygidae), white grubs (*Lachnosterna* spp.) being preponderant, occupying 70% of farms sampled in the study. Also sampled on the rhizosphere of the crop were termites (*Microtermis* spp., *Odontotermis* spp.), wireworms (*Gonocephalum* spp.) and earwigs (*Forficular* spp., *Anisolabis* spp.). While foliar pests included notable sap suckers like leafhoppers (*Empoasca* spp.), aphids (*Aphis cracivora*) and whiteflies (*Bemisia tabacci*); also, defoliator insects like caterpillars

(*Heliothis* spp., *Spodoptera* spp.), crickets and grasshoppers with sporadic occurrence were observed in all the sampled farms. These findings of Owusu-Akyaw et al. (2005) and Tanzubil (2016a) agrees with earlier reports by other authors on insect pests of peanuts. However, none of the reports indicated the period at which these insects were pests to peanuts; whether at germination, podding or harvest. To better understand the narratives of insect pests of peanuts, the time and season during which an insect becomes a pest should be defined. This is because, most insects mentioned earlier as pests of peanuts are pre-harvest pests, while post-harvest pests like *E. pallens* were rarely mentioned.

In as much as there are pre-harvest pests of peanuts, there also exist post-harvest insect pests that destroy the crop at harvest or storage. Ranga Rao et al. (2010) reported a compendium of economically important post-harvest insect pests of peanuts to include the following (Table 2.1).

Table 2.1: Economically important post-harvest insect pests of peanuts.

Latin name	Common name	Order	Family
Caryedon serratus	Groundnut bruchid	Coleoptera	Chrysomelidae
Tribolium castaneum	Red flour beetle	Coleoptera	Tenebrionidae
Corcyra cephalonica	Rice moth	Lepideoptera	Pyralidae
Elasmolomus sordidus	Pod sucking bug	Hemiptera	Lygaeidae
Abasverus advena	foreign grain beetle	Coleoptera	Silvanidae
Araecerus fasciculatus	Coffee bean beetle	Coleoptera	Anthribidae
Carpophilus dimidiatus	Corn sap beetle	Coleoptera	Nitidulidae
Alphitobius diaperinus	Lesser mealworm	Coleoptera	Tenebrionidae

Attagenus megatoma	Black carpet beetle	Coleoptera	Dermestidae
Cryptolestes pusillus	Flat back beetle	Coleoptera	Laemophloeidae
Dermestes lardarius	Larder beetle	Coleoptera	Dermestidae
Ephestia cautella	Almond moth	Lepidoptera	Pyralidae
Lasioderma serricorne	Cigarette beetle	Coleoptera	Ptinidae
Latheticus oryzae	Red-legged ham beetle	Coleoptera	Tenebrionidae
Liposcelis spp	Booklouse	Psocoptera	Liposcelididae
Necrobia rufipes	Checkered beetle	Coleoptera	Cleridae
Oryzaephilus mercator	Merchant grain beetle	Coleoptera	Silvanidae
Oryzaephilus surianamensis	Saw-toothed grain beetle	Coleoptera	Silvanidae
Plodia interpunctella	Indian meal moth	Lepidoptera	Pyralidae
Sitophilus oryzae	Rice weevil	Coleoptera	Curculionidae
Stegobium paniceum	Drugstore beetle	Coleoptera	Ptinidae
Tenebrio molitor	Yellow mealworm	Coleoptera	Tenebrionidae
Tenebroides mauritanicus	Cadelle	Coleoptera	Trogossitidae
Tribolium confusum	confused flour beetle	Coleoptera	Tenebrionidae
Trogoderma spp	Khapra beetle	Coleoptera	Dermestidae
Trogoderma granarium	Khapra beetle	Coleoptera	Dermestidae
Trogoderma inclusum	Larger cabinet beetle	Coleoptera	Dermestidae
Typhea stercorea	Hairy fungus beetle	Coleoptera	Mycetophagidae

Rao et al. (2010) captured the peanut seed bug by its former name *E. sordidus* and in

the former family Lygaeidae. However, the author described the insect as a post-harvest

insect of economic importance and top among the post-harvest arthropod insect pests enlisted (Table 2.1). But currently, the insect has been reclassified into the family Rhyparochromidae instead and not Lygaedae based on their monophyletic features (Weirauch & Schuh, 2011).

Pods damage by insect attack has been known to result in fungal invasion of the kernels which can result in loss of grain quality, deterioration and development of mycotoxins (Johnson & Gumel, 1981; Tanzubil, 2016a).

2.2   The order Hemiptera

The true bugs (Hemiptera: Heteroptera) are considered the most diverse arthropod taxon, consisting of over 42,000 species from over 5800 genera and 140 individual families (Henry, 2009). The order consists of many members that are economically important as pests of crop plants, disease vectors of animals while some are used as predators in classical biological control systems (Henry, 2009; Schaefer & Panizzi, 2000a). Being referred to as true bugs, they are very diverse and cosmopolitan in distribution. Members of the order include seed bugs, cicadas, bed bugs, shield bugs, assassin bugs, leafhoppers, leaf miners, aphids, thrips and many more. Hemiptera is derived from the Greek words *hemi,* which means half, and *ptera*, which means wings, a characteristic typical of members of the suborder Heteroptera. They possess forewings which vary in structure, as the front half wing is conspicuously thicker and more impervious than the rare half. Hemiptera also possess antennae with few segments numbering about five. They possess few short tarsi that are not more than three in numbers. Other similar insect groups like beetles and cockroaches have much longer antennae and more segmented tarsi than members of the Hemiptera. Other suborders of the Hemiptera include Auchenorryncha, Colleorrhyncha, Heteroptera and Sternorryncha

respectively (https://askabiologist.asu.edu/true-bug-anatomy). Seed bugs and their relatives in the Order Hemiptera possess specialized mouthparts for sucking that are organized into a tube called the rostrum. The piercing and sucking mouthparts of different members of the order Hemiptera show similarity in structures. Three cardinal structures which characterize their mouthparts are: a diminutive triangular labrum, functioning as a shield to the bunch of stylets; the labium, a segmented tubular organ with a musculature complex that are regulated via contraction and shortening of the stylets when inserted into plant parts; and the stylet bundle which is rooted in a groove along the inner walls of the labium. The stylet bundle is characterized by two exterior mandibular stylets that are protective of two inner maxillary stylets (Garzo et al., 2012; Zhao et al., 2010).

2.2.1   The Seed-sucking bugs (Heteroptera)

The seed-sucking insects among Hemiptera (Heteroptera-true bugs) belong to families such as Lygaeidae, Alydidae, Pentatomidae, Rhyparochromidae, Coreidae, Scutelleridae, Corimelaenidae, Rhopalidae and Pyrrhocoridae (Schaefer & Panizzi, 2000a). Majority of heteropterans prefer feeding on immature seeds because they are softer and can be easily penetrated than mature one. Additionally, also, because of the high-water contents of immature seeds. But species from the families Lygaeidae and Pyrrhocoridae have the penchant for feeding on mature seeds. Lygaeidae are generally recognized as "seed bugs", although many species are sapsuckers. During feeding, heteropterans feed on seeds by injecting their stylets on seeds and tissues to invoke damage due to the effect of the penetration and inject saliva that cause the development of tissue necrosis (Panizzi & Parra, 2012).

2.2.2   The family Rhyparochromidae

The family Rhyparochromidae is widely dispersed throughout the world and in the oriental region. According to Gupta and Singh (2014), little or not much work has been

done on the taxonomic grouping of the family since after the initial descriptions by Distant between 1902 to 1918. The Rhyparochromidae as a family was formerly subsumed as a subfamily in the family Lygaeidae until its elevation to the family level by Henry (1997) during the phylogeny-based reclassification of the superfamily Lygaeiodea. The family Rhyparochromidae was defined on the basis of synapomorphy as having sutures between abdominal sterna IV and V fused, being incomplete laterally, and rapt anterolaterally in an arcuate manner. However, these descriptive features are not widespread within the family. For example, the genus *Laryngodus* and few other genera had IV-V sutures in complete and rectilinear fashion. Most species from this family are epigeic and fodder basically on mature seeds of host plants. Noteworthy exclusions include *Gastrodes*s spp., which are mostly arboreal and confined to feeding on seeds contained in cones on coniferous trees. Notable also is *Laryngodus* spp which appear to be strictly arboreal in their mode of feeding with both adults and nymphs found only or close to the cone-like infructescence of diverse monoecious and dioicous *Allocasuarina* spp. (Slater et al., 2009).

While the Rhyparochromids are serious pests of oil seeds in Africa and Asia, Henry (2004) reported the invasive palearctic seed bugs *Rhyparochromus vulgaris* (Schilling) and *Raglius alboacuminatus* (Goeze) as rather nuisance pest invading residential and commercial areas in California and Utah. These bugs were much nuisance to home and business owners rather than being agricultural pests of any significance. Although these bugs did not constitute any threat to agricultural productions, their ability to influence the environment with time makes possible their reversion to constitute agricultural pest problems. Mating between males and females are usually preceded by sexual cues from the male counterpart where the male intromittent organ is inserted into the orifice of the female genitalia. Eggs are laid simply on plant debris or dropped on moist soils. Eggs laid

are usually gray in color when firstly laid before turning brightly red and are hatched between 5-6 days when laid. The eggs hatch into instars which undergo about 4-5 nymphal stages before reaching full maturity.

2.2.3 The bug mouthparts

Insects possess varying mouthpart structures with which they evolved for specialized feeding. Mouthpart types are often characteristic of members of an order, family or genus; making the mouthpart structure an essential component considered for identification, classification, physiological and ecological studies (Gullan & Cranston, 2005). Mouthpart type of Hemiptera are said to be essential for feeding, transmission of phytoplasmas, and also essential for host location (Zhao et al., 2010).

2.2.3.1 The rostrum

The rostrum is a proboscis-like mouthpart structure outspreading from the head and bearing the piercing mouthpart among true bugs. In Heteroptera, the rostrum functions in guiding the piercing stylets, which are retracted into the rostrum when not used. The rostrum is often described as analogous to a blade or beak-like in nature (Brożek et al., 2015). It composed of five segments and a sclerotized groove in which the stylet is anchored and extends back and forth during feeding on plant juices (http://aphid.aphidnet.org/rostrum.php). Many true bugs fold their rostrum beneath their ventral region when it is not in use. In *E. pallens*, the rostrum which protrudes from the head is brown, slender and is folded below the thorax region once it is not in action. The folded rostrum only becomes visible to an observer during the feeding activity of the bug. During feeding, the bug swings the rostrum into position and pierce the plant material to suck up fluids from it. The bug is usually fond of scrubbing the rostrum after feeding using the fore limbs before folding it to position. Feeding on seeds starts when the stylets

on the rostrum is completely inserted pass the shell membranes (of groundnuts) into the kernels during which the insect can remain for a longer period sucking the oil from the kernel.

2.2.3.2 The stylet

The stylet is a set of mouthparts of insects that feed on embedded liquids (plant juices, sap, hemolymph, blood etc.). The stylet is a bundle consisting of mandibles and maxillae which fit together by way of grooves and ridges. The two inner members of the stylet, the maxillae each have two deep grooves that are different to form dual canal system. The functional mouthpart of the Hemiptera is the flexible bundle of the stylet which can penetrate tissues relatively deeper from the body of the insect. During feeding, saliva is released from the stylet through one end while fluid is sucked from the other end. The direction and penetration of the stylet is partly controlled as the penetration of the stylets of some insect species follow an intracellular pattern of probably facilitated by the activity of salivary enzymes (Miles, 1968b).

2.2.3.3 The economic significance of the seed bug, *Elasmolomus pallens*

E. pallens (Dallas) is a seed bug that belongs to the family Rhyparochromidae (Gad, 2013). Earlier studies confined the taxonomy of the bug into the order Hemiptera, family Lygaeidae with distribution known mainly in Nigeria and some few West African countries. The commodity type targeted for attack by this bug is peanut. Other seed bugs earlier classified alongside *E. pallens* into the family Lygaeidae include, *Xylocoris galacticus*, (Fieber), *Xylocoris* spp., *Aphanus* spp., (Fabricius), *Geocoris* spp., and *Aphanus littoralis* Distant (Hagstrum & Subramanyam, 2016). However, Henry (2009) in the recent phylogenetic reanalysis described the Rhyparochromines as a new

monophyletic group. Hence, they are placed in a separate family called the Rhyparochromidae.

The natural food plant of *E. pallens* is peanut or other oil plants that it can feed on such as sunflower (*Helianthus annuus* L.), bean (*Vicia faba* L.) (Miles, 1968a; Wool, 2004) or sesame (Berhe et al., 2017; Kinati, 2017). The bug is a serious post-harvest pest of peanuts, infesting peanut pods left in the field to dry or in granaries. Peanuts and sesame infested by this bug showed shriveled kernels (Suman & Kaur, 2013) with bitter taste and rancid oil. Suman and Kaur (2013) has reported the collection of *E. pallens* from peanut and sesame farms for experimental purposes. According to Berhe et al. (2008), between 5 to 50% of sesame damages in the region of Kafta-Humera sesame field in Ethiopia, was attributed to the destructive activities of *E. pallens*. Both adults and nymphs caused weight loss in harvested sesame pods and kernels by sucking out the oil from it. They also possess the ability of lifting the kernels from the field to their resting places, an activity that results in losses of crop yield and quality (Berhe et al., 2008). The growth stages of *E. pallens* involves the hemimetabolous development from eggs, nymphs and adults with life cycle averaged between 39 and 54 days. Both quantitative and qualitative losses by the bug can reach as high as 100% if left unchecked for an extended period of time (Terefe et al., 2012). The levels of infestation of *E. pallens* vary from year to year depending on climatic factors like rainfall and humidity (Tadesse, 2006). Where high level infestation occurs, crop damages usually significant and occurs in the matter of few weeks. This is because, the bugs the proliferations of the bugs can be massive where infestation is high.

2.3   Molecular identification of Heteropterans

Because of the diversities among the sub order Heteroptera (true bugs), taxonomic classification or identification of members of the group purely on the basis of morphology alone may be tasking and difficult considering the obvious fact that many of them are

pests (Schwartz & Foottit, 1998). More also, identification of immature forms of the bugs using only morphology-based keys are said to be difficult (Park et al., 2011). Park et al. (2011) asserted that DNA barcode can aid the identification of heteropteran groups of arthropods in applied situations by facilitating the ease of identification of cryptic species both in adults and nymphs. According to Hebert et al. (2003), the DNA barcoding approach has been proven to be fast, and accurate at species identification due to its restriction to the analysis of unique, conserved and standardized segment of the organism genome. Previous studies have established that sequence variation in the 5′ terminal of the mitochondrial cytochrome oxidase I (COI) gene is able to provide high species-specific resolution for the identity of various animal groups including moths (Hebert et al., 2003; Janzen et al., 2005), spiders (Barrett & Hebert, 2005), birds (Hebert et al., 2004), springtail (Hogg & Hebert, 2004) and fishes (Ward et al., 2005). Unfortunately however, only a pocket of studies did employ DNA barcoding as a tool for the identification of species of heteroptera (Park et al., 2011). The lack of DNA barcodes and species sequences on genetic databases has on many occasions led to spurious diagnoses among species (Baker et al., 1996), as complete species identification is only possible when the species nucleotide sequences are matched with similar sequences on existing databank (Savolainen et al., 2005). The usefulness of COI at circumscribing novel heteropteran species has been reported by Memon et al. (2006). Recent study by Jung et al. (2011) examined the usefulness of the COI barcode on 139 heteropteran species collected from some parts of East Asian countries (Japan, Korea, Northeastern China and part of Russia) and found unique individual COI barcode sequences except for the genus *Apolygus* (Miridae). This led them to the conclusion that "COI barcodes can reveal new cryptic true bug species and are able to contribute for the exact identification of the true bugs". However, despite the sensitivity of the DNA barcoding at resolving identity among cryptic species, Damgaard (2008) on the contrary, asserted that DNA barcoding of *Gerris*

species from the 3′ end of the COI gene were found to be of limited utility at resolving species variations among the group. Nevertheless, the DNA barcoding of the COI genes remains the most widely used molecular marker for the delineation of arthropods into genus and species respectively.

2.4   Isolation of EPF from infected insect cadavers

Over the decades, EPF have been found to exist as natural regulators of insect population in nature, causing epizootic infections in insect population that lead to death (Hussain et al., 2012a; Lacey et al., 2015a; Sandhu et al., 2012a). They are known natural pathogens infecting insect hosts that can be collected from the field environment either infected or dead and incubated under laboratory conditions to isolate, document, and use the pathogens as biological control agents (Inglis et al., 2001). Their isolation or recovery from infected insect cadavers is most often achieved via direct inoculation of culture media with conidia dislodged from the cadavers (Hajek et al., 2012). Conidial recovery from infected cadavers are is by collecting conidia discharged from surface-sterilized infected insect cadavers under the laminar flow environment to mitigate contamination (Hu et al., 2018).

The isolation of EPF from the insect cadavers has been widely practiced since the surge in interests on EPF as biological agents. Er et al. (2016) demonstrated the isolation of EPF from the cadavers of stored-grains insects, by placing sterilized cadavers in humid chambers (containing damp sterile filter in parafilm-sealed Petri dishes) at $26 \pm 2\ ^{\circ}\text{C}$ with 16:8 LD photoperiod. Jaber and Enkerli (2016) isolated fungi from the cadavers of several insects by suspending the cadavers in 5% Tween 80 followed by vigorous shaking to dislodge attached mycelia and spores from the cadavers. This method allowed for the suspension to be plated on potato dextrose agar (PDA) amended with chloramphenicol.

Some of the isolates obtained from the study were overrepresented, and this included fungi from the genus *Cladosporium*, *Penicillium* (consisting of *P. commune*, *P. digitatum*, *P. frei*) and *Talaromyces* (*T. amestolkiae*) respectively. According to the study, majority of the isolates obtained were saprophytes (depending on organic remains). Two basiodiomycetes, *Fomes fomentarius* and *Wallemia* spp., were isolated from Coleoptera. Also isolated from the cadavers of the insect were *Simplicillium sympodiophorum* and *Engyodontium album*. Other isolates were members of the family Cordycipitaceae including the widely studied *Beauveria*, and *Cordyceps* and the *Metarhizium* from the family Clavicipitaceae. The isolation of *Aspergillus* spp. and *Gliocladium* spp from the cadavers of the rice black bug, *Paraeucosmetus pallicornis* sterilized in different concentrations of alcohol and NaCl solutions has been reported (Sjam et al., 2018). However, a study by Sharma et al. (2018b) isolated fungi from the cadavers of the vine mealy bug, *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) sterilized in 1% NaClO with 3 different rinses in distilled water. EPF isolates obtained from the bug cadaver included *Fusarium solani*, *Sarocladium kiliense*, *Penicillium griseofulvum* and *Purpureocillium lilacinum*. There were also incidences of *Aspergillus sclerotiorum*, *Fusarium oxysporium*, *Penicillium* spp., and *F. fujikuroi*. For isolations on non-selective media, the culture media used for the isolation of fungi were often supplemented with antibiotics such as chloramphenicol, streptomycin or tartaric acid. Furthermore, other isolation from insect cadavers, EPF can be isolated from the soil by the use of the insect-baiting and selective media isolation techniques.

A study by Korosi et al. (2019) utilized the insect baiting method (*Tenebrio molitor*) and the soil dilution method to isolate EPF from the soil. These isolation methods were successful at isolating 33% *Metarhizium* spp. and 26% *Beauveria* spp. from 240 soil samples collected. The two methods were said to be effective for the isolation of EPF. Furthermore, the *Galleria melonella* bait and selective medium methods have been used

by Medo and Cagáň (2011) for the isolation of EPF from soil but with significant differences in the types of isolates obtained between the methods. Several studies have recommended the baiting technique as feasible and to be adopted as a standard method for the isolation of EPF from soil (Ravindran et al., 2016). Kim et al. (2018) corroborated that the insect bait isolation method remains the best alternative to selective medium for the isolation of EPF. This is because, the method allows for the use of insects that are cost-effective to rear, easy to handle and that can be susceptible to a host of EPF. Two good examples of commonly used insects for the recovery of EPF from soil samples are the greater wax moth *G. mellonella* and the mealworm, *T. molitor* as they are easy to rear and handle. Although putative EPF can be adequately isolated via the use of selective medium, the insect bait isolation method contrasts it as a highly sensitive method that aid direct recovery of EPF from soil samples.

In nature, EPF specialize in killing insects by penetrating their cuticles into the haemocoel, unlike other pathogens that must be ingested before infection is initiated (Hussain et al., 2016; Lacey et al., 2015b; Valero-Jimenez et al., 2016). However, Kim et al. (2018), opined that, other than invading the cuticle to initiate infection, EPF can also cause infection when ingested. For example, *T. molitor* in search of food in the soil can ingest conidia of EPF which may invade the hemolymph once the gut is breached. This can result in infection and killing of the host. Consequent upon this later mode of invasion, piercing and sucking insects may not function as suitable baits for the isolation of EPF from the soil.

2.5   Molecular analyses and characterization of fungi

Molecular analytical methods are powerful tools that aid the discovery, identification, characterization or quantification of fungal species. Molecular methods permit direct

detection, identification and analysis of genetic features which are not possible via physiological, morphological and biochemical methods (Inglis et al., 2012). Molecular analysis of fungi follows steps such as homogenization, DNA purification and polymerase chain reaction (PCR) to establish the identity of fungal species.

2.5.1 DNA homogenization

Usually, molecular analysis can be adequately feasible where effective methods of genomic DNA extraction are applied. Even though there are lots of methods for genomic DNA extraction from experimental samples, it remains the pre-requisite to all subsequent analyses. DNA extraction is usually preceded by disruption of the fungal cell via mechanical, enzymatic or chemical means to liberate the cytoplasmic content into required buffers. DNA extraction from actively growing cells is most preferred to older cells because of the chances of reduced or low DNA content in older mycelia that have lost viability. Due to the chitin scaffold in fungi, mechanical disruption and homogenization of the mycelia is required to enable DNA extraction. Different methods of mechanical homogenization including beads beaters, tissue tearors and pestle and mortar have been effectively used (Inglis et al., 2012). The latter method allows for the grinding of the fungal tissues in liquid nitrogen into powders. The liquid nitrogen makes the biomass brittle and easy to grind into powders and minimizes heat buildup during the grinding to protect the DNA from shearing.

2.5.2 Genomic DNA purification

Usually, after the homogenization step is successfully completed, the crude DNA must be purified to remove impurities that may affect its quality and the success of its analysis. Often, when homogenization is completed, fragments of the cell wall, proteins and lipids must be removed for a good quality DNA using techniques like phenol/chloroform extraction, centrifugation, column purification or precipitation with ethanol, polyethylene

glycol, potassium acetate or isopropanol (Schneider et al., 2009). RNA in the crude extract is removed via the addition of RNAse (Lobo et al., 2015).

Upon extraction of genomic DNA, the need for its quality and quantity is usually recommended. This is achieved via the use of the agarose gel electrophoresis with suitable size markers and buffer, followed by staining and visualization of the DNA (Feng et al., 2014). Traditionally, the fluorescing intercalating ethidium bromide dye is used as gel stain for the visualization of DNA, but because it has been known to be carcinogenic, other non-toxic dyes such as SYBR® Green (Molecular Probes, Introgen Life Science) or GelRed® (Biotium Inc.) are now being used. High quality DNA are visible in the gel as single band with higher molecular weight. Most often, DNA quantity is estimated on agarose gel by visualizing electrophoresed genomic DNA relative to a ladder sequence of known concentration. However, alternatively, specialized equipment like fluorometer or spectrophotometer (e.g., Nanodrop) or capillary electrophoresis are used to estimated genomic DNA (Inglis et al., 2012).

2.5.3   Polymerase chain reaction (PCR)

PCR is a powerful molecular tool that is used for the identification and characterization of fungal pathogens. It is applied for the amplification of target sequences of the DNA in a taxon-specific manner (Inglis et al., 2012). PCR can be applied for identity resolution at species level (Johnson & kawchuk, 2005), but differentiation at the subspecies (Castrillo et al., 2003; Destéfano et al., 2004) and the subgenus levels (Schneider et al., 2011) can also be possible (Inglis et al., 2012). Sequence specific regions targeted for PCR amplification are basically unique for each fungal taxon. For this purpose, PCR primers are constructed to hybridize with such specific sequences to facilitate for amplifications that are taxon specific. The sequence regions targeted for amplification in

fungi are the ribosomal RNA gene sequences and flanking sequences (e.g., internal transcribed spacer ITS and intergenic regions) which are good examples of target sequences for amplification with specific primer sets (Inglis et al., 2012).

The Internal Transcribed Spacer (ITS) rDNA region is routinely sequenced for characterization of fungi and for the study of their phylogenetic relationships (White et al., 1990), but this universal molecular marker is challenged not to adequately capture species diversity among fungi (Korosi et al., 2019). In view of the forgoing, molecular markers such as the elongation factor-1 alpha (*tef*) and B locus nuclear intergenic sequence (Bloc) were used to sufficiently characterize *Metarhizium* and *Beauveria* species (Fisher et al., 2011; Korosi et al., 2019). Furthermore, a study by Mitina et al. (2017) has utilized the mitochondrial gene NADH dehydrogenase subunit 1 (*nad1*), elongation factor 1-alfa (*tef*) and β-tubulin gene to identify and characterize different *Lecanicellium* species, where sequencing the *nad1* gene revealed four main halotypes of the *Lecanicellium* species. Another method of molecular identification of EPF is the application of clade-specific primers such as Ma 1763 and Ma 2097 which are situated within the ITS 1 and 2 of the gene cluster of the ribosomal RNA (Schneider et al., 2011). But several studies relied on the use of the ITS1-5.8S-ITS region of the genomic DNA with greater measures of success (Jaber et al., 2016; Lu et al., 2015; Sun et al., 2016a). For example, Er et al. (2016) amplified the ITS1-5.8S-ITS region of the fungal genomic DNA using the primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) for the identification of EPF recovered from dead grain infesting insect. The study of Kim et al. (2018) on "*T. molitor*-mediated entomopathogenic fungal library construction for pest management" also utilized the internal transcribed spacer (ITS1-5.8S-ITS) with the primers ITS1F (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4R (5′-TCCTCCGCTTATTGATATGC-3′).

The polymerase chain reaction (PCR) amplification of fungal ribosomal genes using the ITS1 and ITS4 primer sets was also done for the identification of fungal pathogens isolated from different arthropod cadavers (Jaber & Enkerli, 2016). It has been reported in Amaike and Keller (2011) a comparison that utilized the 18S rDNA and the ITS regions to establish which could best distinguish between *A. flavus* and *A. oryzae*. The finding revealed that ITS sequences can differentiate *A. flavus* from *A. oryzae*, but the 18S rRNA subunit could not differentiate between the two fungi. However, it has been argued in Schneider et al. (2011) that phylogeny in *Metarhizium* clade 1 was less resolved when ITS is used as a molecular tool compared to the translation elongation factor 1-α, suggesting it as the most sensitive and informative genetic marker in species differentiation among the *Metarhizium* clade. The assessment of this gene locus showed numerous single nucleotide polymorphism, distinguishing members of species based on the polymorphisms which made possible the description of *Metarhizium* clade 1. However, Mitina et al. (2017) argued differently by asserting that, to successfully identify species in the genus *Lecanicillium*, a cocktail of complex analyses including ITS-sequence and three mitochondrial genes *nad1*, *nad2* and *nad3* must be adopted comparatively. The study opined that *nad1* was the most informative gene sequence for clarity and identity of the species compared to *nad2* and *nad3* whereas ITS alone did not yield satisfactory information for large number of samples. Besides the *nad1* (mitochondrial gene), the study further suggested the β-tubulin (nuclear gene) and the translation elongation factor (*tef*) 1-α as most informative loci to be amplified for the identification of *Lecanicillium*.

However, considering these arguments, one thing that was common between these studies is the ITS-sequence gene that was considered across different genus for the identification of their clades. There seemed to be more dependence on ITS and the EF 1-

α genes sequences by researchers to resolve identity ambiguities in fungi compared to other techniques mentioned. This therefore means that although there exist other molecular identification techniques for entomopathogenic fungal identification, yet molecular methods based on the ribosomal ITS gene sequence cannot be jettisoned, as it is still considered relevant and reliable. Furthermore, amplified and sequenced DNA have to be processed using the Basic Local Alignment Search Tool to allow for comparisons with existing sequences on the GenBank databse or other databases (Sun et al., 2016a).

2.6   The occurrence and menace of aflatoxins

Aflatoxins obtained their names from the fluorescence (blue or green) that they demonstrate under ultraviolet light at 366 nm. They are categorized into the A and B groups plus the new additions AFM_1 and AFM_2 which were identified from milk of dairy cows that are exposed to groundnut meal contaminated with AFB_1 and AFB_2 There are many existing theories on the significance of aflatoxin production by fungi, but without any clear demonstration regarding these theories. The production of aflatoxins by fungi could likely be a mechanism of response to stress, by way of protecting themselves from damages due to ultraviolet radiation (UV), primary metabolic byproducts, for essential elevation of fungal fitness or provision of protection for reproductive structures such as sclerotia and conidia from predators (Battilani et al., 2012)..

Studies revealed that the polyketide metabolic pathway (PKMP) is the channel through which AFs are synthesized, and the genes responsible are linked to the biosynthetic machineries of *A. flavus* and *A. parasiticus* (Battilani et al., 2012; Marroquín-Cardona et al., 2014a).

In Pakistan, Iqbal et al. (2013) reported aflatoxin contamination in peanuts as 32% in unshelled raw peanuts, 17% shelled raw, 29% roasted unshelled and 36% roasted shelled.

While for peanut products, 25% of peanut butter were contaminated with aflatoxin and 13% found in peanut cookies were found contaminated above the recommended EU safety standards. A study by Waliyar et al. (2015) examined pre-harvest and post-harvest contamination of peanuts from three regions of Mali, and found that peanuts from Mali are heavily contaminated with aflatoxins, and both stages pose a risk to human health. The study recorded 77% aflatoxin contamination for pods form Kayes region, followed by Kolokani (55.6%) and Kita (45.6%) based on mean values for 2009 and 2010 data collected. Representative data on the global occurrence of AFs in peanut and peanut products are presented in Table 2.2.

Table 2.2: Aflatoxin contamination in peanut products in different regions of the world.

Country/Region	Food type (No. samples)	Level of contamination (ng/g)	No. of positive samples (%)	Reference
European Union	Peanuts (8929)	2.69[a]	20	(EFSA, 2007)
São Paulo	Peanut products (23)	2.13[b]	25	(Jager et al., 2013)
South Korea	Raw peanuts (4)	0.2[c]	25	(Chun et al., 2007)
	Roasted peanuts (8)	10.67[c]	50	
	Peanut butter (2)	7.36[c]	100	
Zambia	Raw peanuts (92)	0.43[d]	55.4	(Bumbangi et al., 2016)
China	Peanuts from Henan (1190)	9.43[e]	19.00	(Wu et al., 2016)
	Peanuts from Liaoning (408)	0.55[e]	4.90	
	Peanuts from Guangdong (441)	5.34[e]	14.29	
	Peanuts from Sichuan (455)	18.19[e]	15.60	
Malawi	Local peanut butter (14)	72[f]	100	(Matumba et al., 2014)
	Imported peanut butter (11)	0.7[f]	73	
	Peanut based therapeutics (6)	2.1[f]	100	

Pakistan	Raw unshelled peanuts (22)	6.4[e]	59	(Iqbal et al., 2013)
	Raw shelled peanut (29)	9.6[e]	55	
	Roasted unshelled peanuts (31)	10.4[e]	61	
	Roasted shelled peanuts (19)	12.3[e]	68	
	Peanut cookies (24)	4.6[e]	42	
	Peanut butter (32)	2.4[e]	50	
Yangtze River area (China)	Peanuts (2983)	7.238[g]	31	(Ding et al., 2015)
Haiti	Raw peanuts (21)	<2.0[f]	14	(Schwartzbord & Brown, 2015)
	Peanut butter (Port-au-Prince) (11)	137[f]	91	
	Peanut butter (Cap Haitien) (21)	335[f]	100	
Democratic Republic of Congo	Raw peanuts (60)	229.07[f]	72	(Kamika & Takoy, 2011)
Malaysia	Roasted unshelled peanuts (18)	1.01[h]	N.A	(Leong et al., 2011)
	Roasted shelled peanuts (8)	0.78[h]	N.A	
	Pounded peanuts (19)	5.20[h]	N.A	
	Peanut sauce (4)	2.77[h]	N.A	
	Peanut butter (4)	1.19[h]	N.A	
	Peanut cake (3)	6.03[h]	N.A	
	Peanut soup (2)	1.00[h]	N.A	

N.A: not available; [a] mean of upper bound total aflatoxins content; [b] mean of total aflatoxin content; [c] summation of aflatoxin B_{1-2} and G_{1-2}; [e] geometric means of aflatoxins content; [f] median of total aflatoxins content; [g] mean of upper bound of AFB_1; [h] mean of AFB_1 content.

2.6.1   Toxicity of aflatoxins

One major challenge with peanut production is the incidence of aflatoxin contamination, which occurs as a global phenomenon of great health concerns. Aflatoxins demonstrate a wide range of toxicological effects based on the proportion of doses consumed (Waliyar et al., 2015). Aflatoxins when consumed at higher doses are lethal, causing biological effects on human kidney, myocardia and liver tissues. Aflatoxins cause liver cirrhosis, and are powerful hepatocellular carcinogens even at sub-lethal concentrations (Liu & Wu, 2010; Wild & Turner, 2002). When consumed at higher amounts can result in acute aflatoxicosis. Aflatoxins have also been indicted for growth retardation in children (Gong et al., 2004), and also as potent immune-suppressors (Sahoo & Mukherjee, 2001). Aflatoxicosis primarily affects the liver cells, causing necrosis, carcinomas and cirrhosis alongside other accompanying health issues. Acute signs include nausea and vomiting, abdominal cramps, pulmonary oedema, cerebral oedema, convulsion, coma and death in severe cases (Battilani et al., 2012). Liu and Wu (2010) reported that between 4.6-28.2% of global hepatocellular carcinoma (HCC) cases were due to AFs.

Furthermore, the toxicity of aflatoxin can ensue from the production of "of intracellular reactive oxygen species (ROS) like superoxide anion, hydroxyl radical and hydrogen peroxide during the metabolic processing of AFB_1 by cytochrome P450. High concentrations of ROSs can lead to oxidative stress which can cause cellular damage" (Mahfouz & Sherif, 2015).

2.6.2   Qualitative assay for aflatoxin from fungal isolates

Conducting qualitative aflatoxin assays on toxigenic isolates is critical to identifying isolates with potentials that can be harnessed for biological control. Several cultural

methods for the rapid assay and visual detection of atoxigenic and toxigenic *A. flavus* have been described (Fani et al., 2014). Fani et al. (2014) observed that assay for aflatoxins via the AV test proved effective by giving reliable results compared to the fluorescence detection (FD) and potato dextrose agar (PDA) methods. The study observed high percentages of false negative results ranging from 13 to 15% using the FD in contrast to the 0% incidences obtained for assays on CAM and the 7.2% using PDA. For qualitative detection of aflatoxins production by toxigenic strains, Abdel-Hadi et al. (2011) studied the qualitative fluorescence of aflatoxins on CAM and also a conducive Yeast Extract-Sucrose medium. Their study found an overall 13/18 strains producing AFB_1 and B_2; with only 5 atoxigenic strains respectively. According to the study by Mamo et al. (2018), *A. flavus* strains positive for aflatoxin on CAM appeared pinkish, whereas negative ones appeared colorless.

2.7   EPF as biological control agents

The utilization of biological control agents such as EPF for insect pests control as opposed to chemical pesticides is significant towards improving environmental health and safety (Ahmed & Leather, 1994). They function as vital biotic component for the natural decimation of insect population sizes (Schneider et al., 2011). EPF function as alternatives to conventional chemical insecticides that are used largely for the management of arthropod pests in the ecosystem and form components of the Integrated Pest Management (IPM) systems of insect pest control in the environment (Shah & Pell, 2003). They can infect different insect species at different developmental stages. Sucking insects, forest and soil dwelling insect pests are very much susceptible to infection by EPF, because of their ability to infect the insects via cuticular penetration contrary to control agents such as bacteria, viruses, parasitoids and nematodes that must be ingested to initiate infection (Wang et al., 2010). EPF belonging to the genera *Metarhizium*,

Beauveria, *Isaria* and *Lecanicellium* have been extensively applied for the control of different insect species and several microbial insecticides with different trade names have been developed from species of this genus, and applied for insect pests control in agriculture, forest, livestock, aquatic and urban environments (Keppanan et al., 2019).

EPF kill insect arthropods by causing lethal infection in them through integrated intrinsic and extrinsic factors. They are naturally good regulators of insect and mites populations by epizootics (Hajek et al., 2012; Shahid et al., 2012b; Wang & Leger, 2014). They are usually host specific with less risk to non-target organisms. They possess the advantage of broad spectrum activity against different insect species (Roberts & Humber, 1981), ease of production, the tendency to counter development of resistance in insects, and enhanced specificity against target pests (Ren & Chen, 2012). Although the killing speed of EPF is slow compared to synthetic chemical insecticides, the ability of infected insect host to feed is usually hampered by entomopathogens (Avery et al., 2009; Lezama-Gutierrez et al., 2012; Orduno-Cruz et al., 2015). Other studies reported that the slow killing of target insects by EPF confers the advantage of reduced reproductive fitness and minimal selective pressure for resistance in the target host (Fang et al., 2011; Thomas & Read, 2007). However, species of the genus *Metarizhium* (Hypocreales: Clavicipitaceae) including *M. anisopliae*, *M. acridium*, and *A. flavoviride* are the most diverse and well-studied species (Schneider et al., 2011). They are known to have broad host range and infect insect pests of agronomical significance such as grasshoppers, locusts, termites, beetle larvae, spittlebugs including other hemipterans (Zimmermann, 2007).

2.7.1 *Metarhizium anisopliae*

The genus *Metarhizium* (Hypocreales: Clavicipitaceae) is a soil-borne mitosporic entomopathogens with a cosmopolitan distribution and a wide host range among insects

(Kanzok & Jacobs-Lorena, 2006; Keyser et al., 2014; Zimmermann, 2007). Most species of *Metarhizium* produce greenish conidia on the surfaces of insect cadavers and are referred to as "green muscardine fungus". Several species in this genus are used as biological control agents for the control of insects and vectors of human pathogens (Nishi & Sato, 2017). The potential for the use of *Metarhizium* spp for biological control of insect pests dates back to the early 1888 (Keyser et al., 2014). *M. anisopliae* is a well characterized entomopathogen (Santi et al., 2010a; Santi et al., 2010b; Schrank & Vainstein, 2010a) and has being used extensively as an important biological control agent of many agricultural insect pests, vectors and their larvae (Carollo et al., 2010; de Garcia et al., 1997; de Paula et al., 2008; Lazzarini et al., 2006). In order to lessen the use of chemical insecticides for insect pest control, *M. anisopliae* can be applied as a promising biopesticide for insect control (Santi et al., 2011). *M. anisopliae* is capable of recognizing specific host chemical signals, probably via the presence of epicuticular lipids, where specificity is triggered as a function of these signals (Pedrini et al., 2007). The infection process by *M. anisopliae* occurs when the fungus meets a susceptible host. The conidia adhere to the host integument and germinate under favorable conditions to form a germ tube. Once this occurs, the host integument is breached, and the fungus penetrates the hemocoel through a combination of mechanical pressure and cuticle-degrading enzymes (Ortiz-Urquiza & Keyhani, 2015). Sun et al. (2016a) observed how cuticle-degrading enzymes (proteases, chitinases and collagenases) helped to dissolve the cuticles of *Rhynchophorus ferrugineus* to enable the penetration of the appressoria of *M. anisopliae* into the integument and body cavities down into the hemocoel. The infection process for *M. anisopliae* involves the secretion of hydrolytic enzymes alongside associated molecules. This is because factors such as chemical composition, surface structure, and topology of the insect cuticle affects fungal conidial adsorption with consequent effects on its virulence (Santi et al., 2010c). For instance, Sun et al. (2016b) reported how well

established tissues in the abdomen of *R. ferrugineus* were hydrolyzed and assimilated by the invading hyphae of *M. anisopliae*. Intersegmental membranes and hairs were the target spots for invasion by *M. anisopliae* when invading the larvae of *R. ferrugineus*. This they the authors attributed to the relatively thin structure of the chitin matrix in the intersegmental membrane.

Upon penetration of the hemocoel, the fungus proliferates rapidly by utilizing the available nutrients in the hemocoel to proliferate. Once the nutrients within the host are exhausted, the fungus emerges from the host to the surface of the cadaver either through the broken cuticle or other openings to bloom on the surface of the cadaver, preparatory to secondary infection of susceptible hosts. The white fungal mycelial bloom over the cadaver later turns green characteristic of *M. anisopliae* as a result of conidia formed on the conidiophore (Ferron, 1978; Javar et al., 2015).

M. anisopliae usually produce green rod-shaped spores, both under saprophytic conditions and during its existence on the cadaver of insects when its pathogenic cycle is completed. The conidia being specialized asexual structures of filamentous fungi become involved in asexual reproduction and are dispersed where they persist in the environment to maintain the infection cycle of the fungus. More also, the conidia of *M. anisopliae* play a significant role in host recognition and infection (Carollo et al., 2010; Wang & St Leger, 2007).

A study by Orduno-Cruz et al. (2015) reported *M. anisopliae* as the most virulent entomopathogen against *Diaphorina citri* which produced the highest lethality followed by *I. fumosorosea*; where both caused mortality above 95% while *B. bassiana* and *Hirsutella citriformis* showed lower mortality of below 40%. This shows that *M.*

anisopliae possess the potential for biological control of insects through the inundative approach. Furthermore, Samuels et al. (2002b) observed that isolates of *M. anisopliae* tested against the eggs of *B. antillus* were pathogenic and of high virulence than isolates of *B. bassiana* which produced low infection rate in the eggs. A standard conidial concentration (5×10^6 conidia/ml) of the *M. anisopliae* isolates caused a 100% infection in the eggs when compared to 43.3% infection rate caused by *B. bassiana* isolates at the same concentration.

2.7.2 *Aspergillus flavus*

The first description of the genus *Aspergillus* was done by the mycologist and priest of Florentine, P.A. Micheli. He named the genus based on similarity in structure of the conidiophore to aspergillum, a liturgical tool used for Holy Water sprinkling. There are currently over 200 known species (Amaike & Keller, 2011). *Aspergillus* species is generally ubiquitous in nature, and can grow on plant debris, decaying organic remains and inside animal systems. They can also exist in the soil, aerosols, fresh water and marine environments. Fungi of this genus are also common in outdoor environments such as on the surfaces of buildings, domestic equipment and gadgets; and, in drinking water. Aspergilli have broad spectrum of organic substrate utilization and adapt easily to changes in environmental conditions (Cray et al., 2013). Other than their role of aiding nitrogen recycling from metabolizing decomposing organic substrates, aspergilli produces some hydrophobic conidia which are easily dispersed and transported as aerosols in air. These conidia being stress tolerant, germinate to produce sexual ascospores under appropriate environmental conditions (Stevenson et al., 2014; Wyatt et al., 2015).

Aspergillus flavus was described in 1809 by Link, as an asexual species that yield asexual spores, fruiting bodies and sclerotia (Amaike & Keller, 2011). In agricultural context, *A. flavus* is a significant opportunistic pathogen often seen associating with emerging seeds such as peanuts, cottonseeds and corn. Early contamination of these grains by the fungus results in spoilage and aflatoxin production (Amaike & Keller, 2011). *Aspergillus flavus* is ubiquitous and is known to cause disease in many species of insects, animals, plants and man. Being described as opportunistic pathogens, they rely much on immunocompromised hosts to initiate infection (Amaike & Keller, 2011; Foley et al., 2014). *A. flavus* is a fungal pathogen that is well known to be lethal to humans and animals, other than infesting agricultural grains and its ability to secrete aflatoxins (Gupta & Gopal, 2002). It can cause mild aflatoxicosis which is fatal or chronic aflatoxicosis which develop into cancers (Zorzete et al., 2013). Because of these features of the fungus, researchers are pessimistic in recommending its application as a biological control agent (Gupta & Gopal, 2002). However, many studies have found out that not all strains of *A. flavus* are naturally endowed to produce aflatoxins because, in nature, more than a few strains are atoxigenic and cannot produce aflatoxins (Criseo et al., 2001; Ezekiel et al., 2014; Mamo et al., 2018). *A. flavus* possess certain characteristic features for its virulence and niche selection which include production of secondary metabolite toxins, enzyme production and a complex oxylipin-mediated quorum-like sensing phenomenon. Also significant about *A. flavus* is the promising approach of utilizing atoxigenic strains in field conditions for the competitive exclusion of toxigenic strains (Amaike & Keller, 2011).

The post-harvest infection of grains by *A. flavus* has been observed to lead to elevated levels of aflatoxins where the grains are poorly dried and stored. This arises due to prior colonization of the grains by the fungus on the field (Battilani et al., 2012).

2.7.2.1 The cycle of *A. flavus* infection in crops

Under unfavorable conditions, *A. flavus* survives by overwintering in the form of mycelium or sclerotia in plant residues, which in turn presents as the secondary sources of conidia to propagate infection cycle in new plants. Their population in the soil is influenced by major factors such as temperature and moisture conditions. The infection cycle is categorized into two: colonization of plant residues in the soil and the infection of the crop tissues. Under suitable environmental conditions, the overwintering structures (conidia and sclerotia) germinate and proliferate into mycelia which develop conidiophores which disperse conidia into the air. This secondary inoculum is vectored by insects or dispersed by wind to colonize and infect new growing plant hosts (Battilani et al., 2012). Both insect damage and dispersal by wind are known to be associated with *Aspergillus* infection in crops such as cottonseed, maize, tree nuts and peanuts (Battilani et al., 2012). However, other than the environmental conditions, the infection cycle is affected by factors such as the predominance of conidia in the field, plant host susceptibility, insect population and the cropping system (Bailly et al., 2018).

2.7.2.2 Infection of insects by *A. flavus*

In the tropical and subtropical climates, *A. flavus* is commonly isolated from soils, decaying and forage vegetation, stored grains, seeds and numerous other food product types. They are critical in aiding decomposition and some strains are pathogenic to insect pests (Battilani et al., 2012). However, not all strains of *A. flavus* are toxigenic (producing aflatoxins). For example, strains such as the AF36 is a known atoxigenic strain that is commonly used in fields as biopesticides in cotton to reduce the expanse of toxigenic strains in cotton (Mauro et al., 2015), while some other species have been successfully applied to kill ants (Asma et al., 2017). Over the years, atoxigenic strains of *A. flavus* has been widely used for the biocontrol of aflatoxigenic strains in agricultural plantations.

These strains are able to control the proliferation of the aflatoxigenic strains in plantations via competitive inhibition and bio-exclusion processes (Degola et al., 2011; Gupta & Gopal, 2002; Humphrey, 2017). Similar strains have been reportedly applied in the pistachio orchards during contamination with aflatoxins and other toxic metabolites from fungi; and were found to be valuably effective. Nuts harvested in the orchard thereafter revealed a reduction in aflatoxins, depending on the year of harvest (Doster et al., 2014). Furthermore, the aflasafe project that was aimed at exploring biological means of controlling aflatoxins across the African continent, relied majorly on the use of atoxigenic strains of *A. flavus* to eliminate the toxigenic strains in order to palliate aflatoxins contamination of foods (Asma et al., 2017). This implies that *A. flavus* has the potential to confer a dual advantage when applied as a biological control agent.

A study by Asma et al. (2017) isolated *A. flavus* from the cadaver of almond bark beetle, *Scolytus amygdali* and found it be virulent against both larvae and adults of the insect at a conidial concentration of 4.0×10^7 conidia/ml after 24 hours. After the 7th day of inoculation at a concentration of 5.04×10^7 conidia/ml, the fungus produced a mortality rate of 100% for the larvae and 96% for the adults. The study described *Aspergillus flavus* to possess entomotoxigenic potential and the ability to synthesize numerous secondary metabolites that are potent toxins against insects. Similarly, in a study that tested the insecticidal activity of two isolates of *Aspergillus* sp. and *M. anisopliae* against the mosquito *Culex quinquefasciatus* at a conidial concentration of 2.5×10^8 conidia/ml, Bawin et al. (2016) observed a corrected mortality ranges of 67.5-78.5% for *A. clavatus*,12.5-59.7% for *A. flavus* and 44.0-84.4% for *M. anisopliae* after 72 hours respectively. The corresponding lethal concentrations (LC_{50} and LC_{90}) found in the study were 1.1×10^8 and 3.6×10^8 conidia/ml (*A. clavatus*), 1.8×10^8 and 9.9×10^8 conidia/ml (*A. flavus*), and 1.3×10^8 and 4.0×10^8 conidia/ml (*M. anisopliae*) respectively. From the

corrected mortality and LC values, it can be observed that *A. flavus* demonstrated the virulence against the host compared to other isolates.

Humber (2000) reported that *Aspergillus* spp. are less pathogenic against wild insect populations, but can cause significant mortality in stressed insects population. *A. flavus* has been tested at different conidial concentrations (4×10^2, 4×10^4, 4×10^6, and 4×10^8 conidia/ml) against third and fourth instars of *Spodoptera litura* with remarkable success. The estimated lethal concentration LC_{50} and LC_{90} for the third instar were 8.6×10^4 and 1.6×10^7 conidia/ml while the fourth instar had 2.7×10^5 and 2.3×10^8 conidia/ml respectively. Mortality (percentage) of the insect instars were found to be proportional to the conidial concentration. At the highest conidial concentration (4×10^8 conidia/ml), third instar demonstrated 71% mortality while the fourth instar had 63% mortality rate at the highest conidial concentration (4×10^8 conidia/ml).

Aspergillus sp. remains the least studied among Hyphomycetes isolated from insects for biological control. According to Butt et al. (2016), of the over 750 different species of EPF, studies have focused more on the development of pathogens from the order Hypocreales because of the broad host spectrum and the amenability to mass production. de Faria and Wraight (2007) opined that about 80% of EPF products available in commercial scale are developed from the notable genera *Beauveria* and *Metarhizium*. However, Seye et al. (2009), reported the isolation of *A. clavatus* from the Senegalese locust, *Oedaleus senegalensis* Kraus and showed high virulence against the larvae of *Aedes aegypti* and *Culex quinquefasciatus* producing a 100% mortality after 24 h of treatment with 1.2 mg/ml dry spores. A study by Foley et al. (2014) described *A. flavus* to be pathogenic to laboratory reared honey bee larvae in a dose-response bioassay that aimed to determine the pathogenicity and virulence of the organism. Testing the

susceptibility of the adult honeybees, they were found to be highly susceptible to infection by *A. flavus*. Other *Aspergillus* spp. pathogenic to the larvae were *A. nomius* and *A. phoenicis* to which the adult honeybee adults were also susceptible. Other instances of the lethal effects of *Aspergillus* species different from *A. flavus* have been reported to be successful on some hosts. Jaber et al. (2016) described how *A. ustus* and *A. ruber* killed between 50 and 70% while *A. nomius* and *A. sclerotium* killed *Drosophila* fly subjected to microinjection of the fungal spore suspensions. The study noted that *A. nomius* showed greater lethal effect at killing injected *Drosophila* and *Aedes albopictus* faster than *B. bassiana*.

2.8   Infection process of EPF

The exact mechanisms evolved by EPF to kill arthropod pests remain unclear, although different levels of specificity and virulence have been expressed by different strains. Phylogenetic studies have shown that, although EPF have similar invasive and developmental processes, their virulence against insects often arose independently by convergent evolution (Zheng et al., 2013). Genomic data analysis has shown that EPF vary in vital features of the molecular mechanisms facilitating virulence (Xiao et al., 2012). Generally, both insect hosts and pathogens show much pliability in the co-evolution by acclimatizing to bear each other's armory in tandem with evolving counteroffensive measures (Butt et al., 2016). Usually, EPF pathogenesis involves complex processes that hinge on several factors including CDEs for successful invasion of the exoskeleton of the hosts. Their life cycle is also characterized by the secretion of various components including hydrolytic enzymes, proteins and certain toxins (Keppanan et al., 2017). The key progressive steps in the invasive and developmental procedures of infection by EPF are spore adhesion, germination, differentiation into infection structures,

penetration, proliferation of the hemocoel and emergence via sporulation from the cadavers (Butt et al., 2016; Valero-Jimenez et al., 2016).

2.9   Fungal invasion of the host

The success of entomopathogens at causing lethal infections that regulate natural insect population is a function of factors such as an appropriate environmental condition, virulence of the pathogen and the host response mechanisms. The infection of insect generally occurs through some integrated processes such as conidial adhesion, germination, penetration of the integument, growth and multiplication inside the host, pathogen-host immune response, and death of the host (Pal et al., 2007b). However, infection of arthropod hosts by EPF will also require the secretion of enzymes that alters the physiological structure of the host. This ability confers on the EPF a selective advantage over others. For example, secretion of proteases by *M. anisopliae* during infection is essential for pathogenicity because of the large presence of protein molecules in arthropod cuticle (Santi et al., 2010c).

2.9.1   Spore adhesion and germination

Adhesion of entomopathogenic fungal propagule to insect cuticle can occur via specific and non-specific interactions. The initial contact between the propagule and the insect cuticle occurs randomly through non-specific interactions due to physicochemical factors such as electrostatic, hydrophobic and polar properties of the propagule surface. Specific or induced interactions between the propagule and the host occurs during conidial germination and penetration which are induced by specific molecules present on the cuticle (Jackson et al., 2010). The first step to penetration is the adhesion of the conidia to the insect cuticle (Khan et al., 2016a; Ortiz-Urquiza et al., 2013; Sun et al., 2016a), which occurs via the aid of adhesive proteins and mucilage (Wang & Leger, 2014). And

because mortality of insects exposed to EPF is dose-dependent, it is essential that a substantial amount of conidia adheres to the integument of the host (Butt et al., 2016). Adhesion of fungal propagule to the cuticle of insects can occur in three successive stages: (1) conidial adsorption to the surface of the insect cuticle; (2) consolidation of the interface between the insect epicuticle and the pre-germinant conidia; (3) germination and proliferation on the insect cuticle, resulting in the development of appressoria for penetration into the hemolymph. Successful penetration of the pathogen marks the onset of infection in the host (Sun et al., 2016a). But conversely, certain insect groups (especially social insects) have been reported to elicit microbicidal secretions to cleanse their epicuticle alongside grooming. Plant volatiles sequestered on the insect cuticle also functioned to deter infection in the insect host. An example is the inhibition of *M. anisopliae* infection of the mustard beetle, *Phaedon cochleariae* by the activity of crucifer isothiocyanates which prevented the germination of its conidia (Atsumi & Saito, 2015; Butt et al., 2016). Nonetheless, conidia of virulent EPF strains possess significant levels of the enzyme Pr1 which interact with other synthesized enzymes to breakdown fungistatic compounds secreted on the host cuticle to liberate nutrients that are essential for germination (Ortiz-Urquiza et al., 2013; Santi et al., 2010b). Ortiz-Urquiza et al. (2013) opined that conidial adhesion and germination on host cuticles can be prevented via mechanisms such as chemical barriers inherent to the host and environment derived factors. However, upon successful adhesion, and under appropriate conditions of nutrient availability, conidia germinate on the cuticle to form the appressorium (Anderson et al., 2011; Mishra et al., 2015). Other than adhesion to the insect integument, the conidia must be viable, and the fungal propagule must also be able to infect and exert lethality against the host under the ambient environmental conditions to which the entomopathogen is exposed (Jackson et al., 2010). Under appropriate environmental conditions, virulent strains of EPF are characterized by the rate of germination of their spores. Furthermore,

hosts are better and fast colonized when multiple penetrations occur during conidial germination, leading to a compromised host immune status (Butt et al., 2016).

The insect cuticle is majorly composed of chitin, proteins, associated lipids, and phenolic compounds which function to provide a formidable barrier against invasion by pathogens (Ortiz-Urquiza et al., 2013; Ząbek et al., 2017). It is usually a hostile environment for EPF because conidia are exposed to fluctuating temperature and humidity, harmful UV radiation, microbial antagonists and the host's innate defenses. Notwithstanding, EPF have evolved strategies to overcome biotic and abiotic sources of stress, by evolving genes connected to virulence, conidial cell wall integrity, signal transduction, and osmolyte balance (Ortiz-Urquiza & Keyhani, 2015).

Upon adhesion, germination starts under favorable humidity, temperature and exogenous sources of carbon necessary to stimulate growth. However, the germination of conidia on insect cuticle is highly influenced by the composition of the cuticle, resulting in differences in susceptibility or resistance of the host to the pathogen. Entry of the pathogen into the body cavities of susceptible insect hosts is based on the ability of the growing hyphae to exert mechanical pressure on the cuticle (Wang & Leger, 2005). Naturally, insect hosts possess defenses that are intended to eliminate the pathogen or mitigate the damage exacted on it, while the pathogen responds with immune elusion and utilization of host nutrients. EPF can neutralize the proximate surroundings on the surface of their host integument, and derive some benefits due to the endowed physicochemical properties of the host cuticle and the associated compounds required for the elimination of contending microbes. EPF also possess the ability to reduce trauma that adversely affects both the host and pathogen. They do this through production of blastospores that lack immunogenic properties but are able to enhance quick assimilation of nutrients

within the hemolymph, melanization of the hemolymph, and formation of penetration pegs that alleviate host dehydration (Butt et al., 2016). Nonetheless, diverse mechanisms are used by EPF to bypass the defense system of the insect host (Dubovskiy et al., 2014). But other than the composition of the host cuticle, the immune response of the host is also critical at determining the germination of the adhered conidia on the host cuticle.

2.9.2 Penetration and proliferation of the host insect

The outer layer of the epicuticle of the insect is composed of complex mixture of non-polar hydrocarbons, lipids, esters and fatty acids. The long chain hydrocarbons- alkanes are critical for the prevention of microbial invasion, desiccation and recognition of certain molecules on the waxy cuticle of various insect species (Ortiz-Urquiza et al., 2013). The cuticle as a first line of physical barrier to infection and environmental stress, maintains a harsh physiological environment to infecting pathogens. This inhospitable environment is characterized by low water activity, minimal nutrients and the presence of antimicrobial compounds (Qu & Wang, 2018). However, EPF have evolved strategies of overcoming these barriers by secreting enzymes that degrade cuticular lipids, epoxides, fatty acids, hydrocarbons and other compounds. The alkane component of the insect cuticle is an ideal substrate for cytochrome P450 monooxygenases which facilitate the degradation of insect cuticles (Malhadas et al., 2017; Pedrini et al., 2013). This implies that cytochrome P450 monooxygenase-expressing pathogens will flourish and adequately exploit their target hosts.

Once penetration of the cuticle is successful, the hyphae convert into yeast-like forms called blastospores or hyphal bodies inside the hemocoel (Figure 2.1). This characteristic structure enables the fungus evade recognition by the host immune system while the circulating hemolymph serves as a source of nutrient and a medium for the blastospores to proliferate and colonize the host where they encounter both the cellular and humoral

responses (Khan et al., 2016a). The enzyme trehalase is secreted by the infecting fungus to hydrolyze the carbohydrate trehalose (the most abundant carbohydrate) in the insect hemolymph into glucose units for uptake. More also, virulence can also be mediated by the synthesis and metabolism of other important sugars in addition to trehalose carbohydrate utilization (Valero-Jimenez et al., 2016). Furthermore, the heterogeneity of carbohydrates on the fungal surface plays significant role in non-self-recognition by the host. Blastospores and hyphal bodies formed in the insect hemolymph, possess fewer carbohydrate antigenic epitopes, a mechanism that enables them to evade the host immune complex (Wanchoo et al., 2009). However, the success of the pathogen to kill the infected host remains a function of the integrity of both its innate and specific immunity.

From the moment adhesion occurs, to the death of the host, the pathogen must survive the immune reactions and other external responses like enzymes production, toxins or any antifungal compounds composed in the cuticle. The host defense mechanisms must be overcome either by releasing substances that regulate responses in the host and the effects of blastospores inside the hemocoel. Once the supporting nutrient in the hemolymph is exhausted, the infecting fungus converts into hyphal structures and grow out unto the surface of the host cadaver for conidiation (Valero-Jimenez et al., 2016).
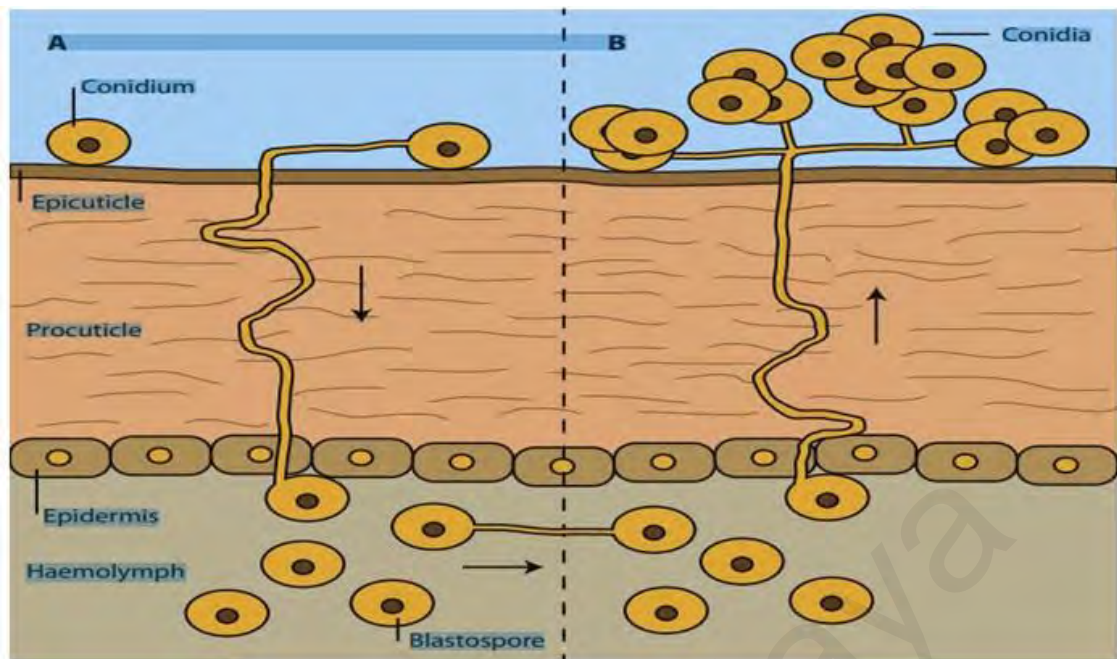
Figure 2.1: The infection cycle of EPF (Valero-Jimenez et al., 2016).

The infection cycle is designated as A and B with seven steps described. Step 1: Adhesion of the fungal spore on the integument of the insect. Step 2: The spores germinate under optimum conditions to form germ tubes. Step 3: The cuticlar structures are degraded through enzymes and mechanical effect to enable penetration. Step 4: The fungus converts into blastospores to exploit nutrients in the host hemocoel. Step 5: The blastospores utilize the available sugars and as well release toxins inside the hemolymph. Step 6: The blastospores suppress the host immune system and release toxins that expedite killing of the host. Step 7: The fungus exits the host through openings on the cuticle to produce spores on the cadaver. The arrow depicts the processes involved in the infection cycle (Valero-Jimenez et al., 2016).

2.9.3   Toxin production

In nature, fungi, bacteria and plants produce numerous varieties of toxins which are mostly proteins targeted against insects. Some of these toxins have been developed biotechnologically for pest control, considering the deleterious effects of conventional agrochemical insecticides on the environment, human health and the fast emergence and resurgence of resistance among insects (Glare et al., 2012; Olombrada et al., 2014).

Toxins are secondary metabolites produced by fungi for adaptive purposes in the environment (Marroquín-Cardona et al., 2014b).

Most hypocrealean EPF secrete large amounts of secondary metabolites that are perceived to form part of the unending evolutionary arm race between fungal pathogens and insects (Wang et al., 2019). Genes responsible for secondary metabolism [(polyketide synthetases (PKSs), non-ribosomal peptide synthetases (NRPSs) and terpenecyclases (TC)] are mostly found assembled in fungal genomes, with their metabolic variations attributed to phenomena such as horizontal gene transfer and gene duplication (Hu et al., 2014; Wang et al., 2019). In *M. robertsii*, some of these metabolites have been identified as serinocyclins and destruxins (Donzelli & Krasnoff, 2016). Destruxins, (the cyclic peptide metabolite) is essentially produced by pathogens to suppress host activity such as antimicrobial peptide (AMP) expression, thus enabling the invading pathogen to withstand the host immune responses (Pal et al., 2007a). Interestingly, the genus *Metarhizium* has developed various biosynthetic pathways for the production of secondary metabolites such as cytochalasins, ovalicins, ergots, diketo-piperazines and resorcyclic acid lactones (Donzelli & Krasnoff, 2016).

Recently, the control of central physiological process as well as communication in microbial community has been linked to the role of secondary metabolites production (Dietrich et al., 2008; Mlot, 2009). The role of gliotoxin as a virulence factor that facilitate systemic mycosis caused by *A. fumigatus* in vertebrates is a good example (Kwon-Chung & Sugui, 2009; Rohlfs & Churchill, 2011b; Spikes et al., 2008). The induction of apoptosis in the hemocytes of insect hosts by destruxin produced by the fungus *M. anisopliae* (Gillespie et al., 2000; Vilcinskas et al., 1997) and the cyclosporine A-facilitated impairment of the host detoxification machinery by *B. bassiana*

(Podsiadlowski et al., 1998) are ways by which fungal secondary metabolites can arrest the immune system functioning of arthropod hosts (Gillespie et al., 2000). However, it can be deduced that not all secondary metabolites of EPF should be considered as virulence factors or toxins. Rather, many may function in more subtle roles of enhancing development, adaptation and survival in diverse environments (Rohlfs & Churchill, 2011a).

2.10   Role of cuticle-degrading enzymes in host infection

Insect pathogenesis by EPF is a multifactorial and complex phenomenon, with specific virulence factors being expressed at different stages of infection and death of host.

Basically speaking, EPF are natural and diverse in occurrence. In nature, some isolates are better endowed with the capacity to kill their host faster than others. The capacity to secrete hydrolytic cuticle-degrading enzymes confers on them the designation of being virulent or nonvirulent. To be successful at eliminating their hosts, they must breach the epicuticle via specific and non-specific events (Mustafa & Kaur, 2009). The synthesis of a cocktail of extracellular hydrolytic cuticle-degrading enzymes including proteases, chitinases, lipases (Santi et al., 2010b), catalase and phospholipase C (Schrank & Vainstein, 2010b) by EPF forms the underlying basis for infection of insects, aiding the degradation of host cuticle and penetration of the host hemocoel (Hussain et al., 2016; Hussain et al., 2010a). Some of these hydrolytic cuticle-degrading enzymes (CDEs) are determinants of virulence for EPF (Nunes et al., 2010) which work in concert to breach the proteo-chitin matrix to enable penetration into the hemolymph and when exiting to the surface of the cadaver for conidiation (Butt et al., 2016). Validations of the significance of these enzymes has been done via gene knockout, overexpression and enzyme-deficient mutants. For example, EPF which are genetically modified to

overexpress chitinases and proteases during infection were found to demonstrate increased virulence compared to the wild type (Zhang et al., 2008).

Mustafa and Kaur (2009) reported how two separate studies on cuticle-degrading enzymes from *B. bassiana* isolates differed in terms of their enzyme production potential. According to the study, strain variation and the methods of cultivation of the isolates were attributed to the marked differences in enzyme activities between the isolates. For example, Kim et al. (2018) observed that enzyme activities in isolates of *M. anisopliiae* were highest when cultivated on Luria Bertani (LB) broth compared with cultivation on Potato dextrose and Sabouraud dextrose broths respectively. The study found that protease, chitinase and lipase activities were highest in *M. anisopliae* than in other isolates. It goes further to establish a relationship between cuticle-degrading enzymes and virulence of EPF against *T. molitor*.

The involvement of CDEs is crucial for the initiation of infection by EPF against a susceptible host. The major hydrolytic CDEs required for pathogenesis are termed virulence determinants, and these include the proteases, chitinases and lipases.

2.10.1 Proteases

Proteases are complexes of hydrolytic CDEs that catalyze the cleavage of the peptide bonds of protein into peptide units and amino acids. They are naturally present in plants and animals because of their cellular role as well as in microorganisms and viruses. Although proteases are broadly found in nature, microbes remain the most ideal source of the enzyme (Mondal et al., 2016). They serve as inevitable determinants of virulence in EPF, catalyzing the degradation of the complex proteins of insect host cuticle (Jarrold et al., 2007). Proteases are also essential for host immune activation and nutrient

acquisition by susceptible hosts (Małagocka et al., 2015). Usually, upon hydrolysis of the insect epicuticle by the lipases, EPF produce a quantum of the serine-protease (Pr1) which function to break down the proteinaceous material of the cuticle. Further degradation of these proteinaceous substrates is catalyzed by the peptidases and exopeptidases to make available nutrients for the infecting fungus (Da Silva et al., 2010). *M. anisopliae* known for broad-spectrum activity is believed to secrete assorted collection of proteases, especially the subtilisins, which function to alter the host cuticle, digest tissues, persist in various niches under saprophytic conditions, and also function to determine virulence of the pathogen and/or host specificity (Santi et al., 2010c). However, two of these proteases including the subtilisin-like serine protease (Pr1) and trypsin-like protease (Pr2) have been most studied; such that, the Pr1 gene has been described to have eleven isoforms including a metallo-protease (Małagocka et al., 2015; Mondal et al., 2016). The role of these subtilisin-like enzymes are well documented in pathogenicity of EPF and have since been categorized as part of the virulence factors (St Leger et al., 1999). But in the views of Hussain et al. (2010b), the conidia bound Pr1 is the most well characterized among proteases of EPF because of its vast role in cuticle invasion. However, in terms of structure, Pr1 contains five cysteines linked by two disulfide bridges while the residual cysteine molecules interact with the catalytic triad consisting of Asp39, Hi69 and Ser224. There has been reports on the activities of the Pr1 and Pr2 proteases among *M. anisopliae*, *B. bassiana*, *Nomurea rileyi*, *M. flavoviride* and *Lecanicillium lecanni* (Liu et al., 2007). These proteolytic enzymes are essential requirements during the onset of cuticle degradation, resulting in the stimulation of the signal relay mechanism when the protein kinase A (PKA) is activated (Fang et al., 2009). Studies have established that the secretion of extracellular proteases are activated in EPF once infection of the host is initiated (Fang et al., 2009; Mustafa & Kaur, 2009). Furthermore, Pr1 proteases have also be described as determinants of virulence in EPF (Castellanos-Moguel et al., 2007; Khan et al., 2016c).

Bye and Charnley (2008) had described how the Pr1 protease and isoforms of *Lecanicillium* spp countered the cuticle of insects by proffering the advantage of regulation of infection process and substrate specificity. On this ground, high numbers of subtilisin isoforms in *M. anisopliae* have been identified based on evolutionary relatedness. These features have endowed EPF with the potential for broad spectrum infectivity across different insect orders (Mondal et al., 2016).

Usually, during infection of a host, penetration of the host hemocoel is usually preceded by enzymatic degradation of the cuticle, for which the serine protease Pr1 constitute most of the cuticle-degrading enzyme complex. This is because, 70% of the host cuticle consisted of proteins. This protein matrix has been known to be considerably degraded by the activity of the Pr1 during infection by EPF (Mondal et al., 2016).

In a study on enzyme assay from isolates of *M. anisopliae* cultivated on Sarbraud dextrose broth at 28 °C for 10 days, Mustafa and Kaur (2009) observed protease activity to be optimum between 6-8 days of incubation. The study found maximum protease activity of 0.96 U/ml for isolate UM5, after 6 days of growth; followed by an activity of 1.52 U/ml for isolates UM1 and UM2 whereas UM13 and AR1 demonstrated protease activities of 0.59 and 0.67 U/ml 8 days after cultivation. A study by Keppanan et al. (2017) investigated the production of proteases by three isolates of *M. anisopliae* (Tk6, Tk29 and Tk37) cultivated for 11 days in casein-containing medium for the expression of higher levels proteolytic enzymes. The study found Tk6 to show highest protease activity (0.83 U/ml) followed by Tk37 (0.64 U/ml) and Tk29 (0.64 U/ml) on the 7th day, followed by a decline in protease activity after the 7th day post-inoculation. In another study that measured the activities of extracellular cuticle-degrading enzymes (CDEs), Dhawan and Joshi (2017) described the variability pattern of CDE secretion from four different strains

of *B. bassiana*, viz: MTCC 2028, MTCC 4495, MTCC 6291, and NBAII-11. The study found maximum mean protease activity observed for the enumerated strains to be 1.22 U/ml (MTCC 2028), 1.26 U/ml (MTCC 4495), 1.22 U/ml (MTCC 6291) and 1.20 U/ml (NBAII-11) after 4 days of cultivation. However, the minimum protease activity observed was 0.91 U/ml from the isolate MTCC 6291. The study also found protease activity to be optimum between 6-8 days of cultivation. In a related study involving *B. bassiana* isolates obtained from *Schistocerca cancellate*, Pelizza et al. (2017) reported a proteolytic activity (1.78 U/ml) from the isolate LPSc 1224 at 4 °C. However, the activity was determined quantitatively by measuring halo-zones around mycelial colony on agar medium supplemented with 0.5% casein. Similarly, a study by Alagesan et al. (2019) on proteases from four different strains of *B. bassiana* found protease activities by the isolates to occur in the range of 0.19 – 0.41 U/ml where strain TP23 had the highest protease activity (0.43 U/ml) while the least activity (0.19 U/ml) was reported for strain TP32. The proteases used in the study was produced in minimal broth medium supplemented with 1% gelatin. Similarly, Castellanos-Moguel et al. (2007) studied the proteolytic activity of extracellular protease of *P. fumosoroseus* and reported the highest activity of 745.7 U/ml for Pr1 protease after 4 days of cultivation in liquid medium. The Pr1 activity reported in this study may be higher compared to others depending on differences in isolates used, growth medium and substrate for determining the enzyme activity. For example, while their study used azocasein for the assay of total protease activity from *P. fumosoroseus*, several other studies relied on the use of casein instead.

It can be observed that the proteolytic enzyme activities reported from all the studies above differed markedly from each other. Factors such as isolate and species type, medium of cultivation, inoculum size, substrate type and environmental changes may be responsible for all these variations. Other likely factors may include human and machine

quantitation errors. Another factor likely to affect the yield and activity of enzymes is whether the isolates are cultured under rotary or static conditions. This factor can affect the yield and activity of enzymes in crude supernatants. The effect of culture medium on the activities of pathogenesis-related hydrolytic enzymes (Pr1 protease, chitinase and lipase) was observed in Kim et al. (2018). The study found highest enzymes production in Luria bertani (LB) broth compared to those observed in Potato dextrose and Sabouraud dextrose broths respectively. The variations in the activities of enzymes produced from these media types was thought to be likely due to slight variations in nutrient compositions of the different culture media. In the study, total protease activity of 15 isolates cultured on LB broth ranged between 0.077 to 3.446 U/ml while protease activity for isolates cultured on PDB medium was between 0.063 to 2.659 U/ml, whereas isolates cultured on SDB medium demonstrated protease activity between 0.054 to 1.816 U/ml respectively.

As efficient as EPF could be, their repeated cultivation on conventional media could cause their attenuation, resulting in limited Pr1 or destruxins which are essential for pathogenicity (Butt et al., 2016). For example, a mutant *M. anisopliae* devoid of dispensable chromosome could produce neither Pr1 nor destruxins and thus become less pathogenic to *T. molitor* (Wang et al., 2002).

2.10.2 Chitinases

Chitin is a natural polymer of β-1,4 N-acetyl glucosamine that is considered one of the most abundant polymers in nature, mostly after cellulose. It constitutes the main structural machinery of fungal cell walls and the exoskeletons of invertebrate animals (Seidl, 2008). Chitin is known to provide structural framework and shape in different organisms while in association with other biomolecules. For instance, a matrix of chitin microfibrils in

combination with proteins, proteoglycans, glycoproteins and diverse sugar molecules are known to form arthropod cuticles, fungal septa and cell walls. In fungal cell wall, chitin occurs in association with complex carbohydrate, while in animals, chitin is found in association with proteins. The fungal cell wall consists of chitin covalently bound to glucans either directly or via peptide bridges. In arthropods, chitin is found in combination with specific proteins that are both covalently and non-covalently bound (Prabu & Natarajan, 2012; Singh & Arya, 2019). Chitinases are hydrolytic enzymes in nature which are capable of inhibiting or degrading chitin in arthropods and fungal cell walls. They function to breakdown chitin polymer via hydrolytic processes to release N, N-diacetylchitobiose (Singh & Arya, 2019). With regards to their mode of action against chitin polymer, chitinases are grouped into endochitinases, which catalyze the random splitting of chitin at internal sites, and exochitinases which liberate N-Acetyl-D-Glucosamine (GlcNAc) monomers or dimers (chitobiosidases) from non-reducing end of the polymer (Adrangi & Faramarzi, 2013; Oyeleye & Normi, 2018; Singh & Arya, 2019; Zhou et al., 2019). However, the release of free N-acetyl-D-glucosamine (GlcNAc) from insoluble chitin polymer via enzymatic breakdown will require a complex of chitinases (Subbanna et al., 2018). In nature, the distribution of chitinase cuts across bacteria, fungi, arthropods, vertebrates and plants. They act in synergy with proteases to breakdown insect cuticle (Mondal et al., 2016) and are involved in the life cycle of EPF (Adams, 2004). Chitinases are known to express physiological functions such as: a) degradation of chitin present in the exoskeletons of dead insects and in fungal cellular walls and use the hydrolyzed products as nutrient source; b) remodeling of the cell walls of fungi during their life cycle, playing major roles during mycelial growth, fusion, branching and autolysis; c) protection against competitor fungi or arthropods within the vicinity of the fungal niche (Adams, 2004; Seidl, 2008). Chitinases are grouped into glycoside hydrolase (GH) 18 and 19 (Mondal et al., 2016; Schrank & Vainstein, 2010b). The GH 28 family

has currently been subdivided into three subgroups including subgroup A (class V) chitinases which possess catalytic domain, but lacks carbohydrate-binding domain (CBM), possessing molecular mass of between 40 to 50 kDa. These group of chitinases majorly possess N-terminal peptide signaling which are bound to the secretory pathway, with few exceptions having intracellular localization. The subgroup B chitinases (class III) also contain N-terminal signal peptide with a size range of 30 to 90 kDa. The subgroup B consists of a cellulose-binding domain that can accommodate large spectrum of polysaccharide that can bind to chitin. The subgroup C consists of chitinases with molecular mass in the range 140 to 170 kDa and have N-terminal signal peptide and other features like the peptidoglycan binding sequence which differentiates them from the rest of the subgroups (Schrank & Vainstein, 2010b). The abundance of subgroup C chitinase is known to strongly vary among different members of fungal species. Although this subgroup differed phylogenetically from subgroups A and B, sequence similarities to subgroup A due to low amino acid sequence points to some progressive catalytic mechanisms. Nevertheless, subgroup C possess a different domain architecture compared to subgroups A and B, suggesting a unique specialization and role of these enzymes in the degradation of chitin substrate (Seidl, 2008).

Chitinases are cuticle-degrading hydrolytic enzymes that are secreted by fungal pathogens. During infection of a host by *M. anisopliae*, chitinases and proteases must work in synergy to degrade the cuticle to enable easy penetration of the invading fungus. Synergism between the enzymes is necessary due to the nature and composition of the cuticle (Santi et al., 2010c).

The virulence of EPF has been linked to their ability to produce extracellular chitinases which are essential components required for the hydrolytic degradation of the insect

exocuticle (Fan et al., 2007). The secretion of chitinases by EPF during infection is essentially to aid the process of penetration of the host cuticle during infection and when the hyphal body undergoes a reorganization inside the host hemocoel into conidiophores that produced conidia on the surface of the host cuticle (Małagocka et al., 2015). According to Mondal et al. (2016), culture medium augmented with insect cuticle, was able to stimulate the production of chitinolytic enzymes from EPF (*M. anisopliae*, *M. flavoviride* and *B. bassiana*) such as endo-chitinases and *N*-acetyl-β-D-glucosaminidases. The chitinase, 1,4- β-chitobiosidases was reported to be produced by *M. flavoviride* when insect cuticle was used to augment the medium of growth. The chitinase enzyme CHIT30 produced by *M. anisopliae* demonstrated the potential for both endo- and exo-chitinase activities which is a key determinant of pathogenicity (da Silva et al., 2005). The production of enzymes that breaks down the integrity of host exoskeleton confers a positive selective advantage on the pathogens (Schrank & Vainstein, 2010b).

A study by Dhawan and Joshi (2017) on the extracellular CDEs of four different strains of *B. bassiana*, viz; MTCC 2028, MTCC 4495, MTCC 6291, and NBAII-11 found their enzymes activity profiles on the fourth day of cultivation to be 0.53 U/ml (MTCC 2028), 0.64 U/ml (MTCC 4495), 0.29 U/ml (MTCC 6291) and 0.57 U/ml (NBAII-11) respectively. However, minimum mean chitinase activity (0.37 U/ml) was recorded in *B. bassiana* MTCC 6291. The mean enzyme activities of these fungal strains were highest on the 6th day of cultivation. In another study involving *B. bassiana* isolated from *Schistocerca cancellate*, Pelizza et al. (2017) reported chitinase activity (2.31 U/ml) from isolate LPSc 1227 at 4 °C. In their study, isolates were grown on potato dextrose agar supplemented with 0.08% Chitin Azure and hydrolytic enzyme activity was measured quantitatively by halo zones developed around the colonies. In another related study involving four strains of *B. bassiana*, Alagesan et al. (2019) reported chitinase activities

of the strains as TP23 (0.48 U/ml), TP32 (0.43 U/ml), KH3 (0.60 U/ml) and KH9 (0.57 U/ml) respectively. The activities of the enzymes were determined using spectroscopic method after reaction of the crude enzymes with 0.5 ml of 1% chitin. The maximum chitinase activity (0.61 U/ml) was obtained on the 7th day of culture. Kim et al. (2018) evaluated chitinase activity from 15 different species of EPF cultivated on different growth medium and found that chitinases produced had highest activities for isolates cultivated on LB medium, with total activity between 0.171 to 3.910 U/ml compared to activities of enzymes cultivated on media like PD and SD broths.

2.10.3 Lipases

Lipases or triacylglycerol acyl hydrolases are serine containing hydrolases with a wide application potential for industrial and physiological purposes. Lipases are responsible for the breakdown of glycerol and long-chain fatty acids and the ester bonds of lipoproteins. Their activities are usually triggered when they adsorb to oil-water interface. The secretion of lipases by non-pathogenic microbes may be for the purpose of absorption of extracellular nutrients, whereas for pathogenic microbes, lipase secretion may be meant to enable the invasion of susceptible hosts (Mondal et al., 2016).

By nature, the insect exoskeleton is hydrophobic and serves as the first line of innate defense against pathogens. It is a polymer of heterogeneous biomolecules including lipids, fatty acids, long chain alkenes and esters. Lipases catalyze the hydrolytic breakdown of ester bonds in lipoproteins, waxes and fats occupying the interior of the host integument (Ali et al., 2009). When lipases are secreted on insect cuticle by EPF, they penetrate the integument and trigger the release of nutrients orchestrated by the hydrolysis of the cuticle. The hydrolytic activities of the lipases is followed the secretion of the Pr1 proteases which breaks down the proteinaceous molecules in the procuticle (Da

Silva et al., 2010).

A study by Kim et al. (2018) on the activities of hydrolytic enzymes from 15 different species of EPF found that total lipase activity for EPF isolates cultivated on LB broth were highest compared to those cultured on other growth media. The total activity found ranged from 0.423 to 2.953 U/ml. Dhawan and Joshi (2017) reported maximum mean lipases activities of 1.36 U/ml for *B. bassiana* MTCC 4495 in a study that measured the extracellular cuticle-degrading enzymes of some groups of the fungal isolates. While minimum mean lipase activity 1.04 U/ml was recorded for the *B. bassiana* NBAII-11. In the study, variability in both the pattern of enzymes secretion and the level of their activities among the isolates was observed. In another study involving *B. bassiana* isolated from *Schistocerca cancellate*, Pelizza et al. (2017) reported lipase activities from three isolates of *B. bassiana* as LPSc 1225 (4.23 U/ml), LPSc 1226 (3.05 U/ml) and LPSc 127 (2.78 U/ml) at 4 °C on agar medium supplemented with Tween 20 as lipid substrate. Hydrolytic enzyme activity was measured quantitatively by halo-zones formed around colonies.

Rocha-Pino et al. (2018) had reported lipase activity (3.6 U/mg protein) for *L. lecanii* grown in medium supplemented with polyurethane foam. In Mondal et al. (2016), the activities of lipase enzymes produced from *M. anisopliae* on different lipid sources were reported as: rice oil (4.46 U/ml), soybean oil (4.26 U/ml), olive oil (4.25 U/ml), sunflower oil (4.23 U/ml), sesame oil (3.51 U/ml) and hydrogenated soybean fat (3.50 U/ml) respectively. This finding obviously shows how enzymes activities can be affected by nutrient sources.

2.11   Host responses to neutralize EPF infection

In the natural sense, arthropods can detect the threat of pathogens in their surroundings, and their survival from infection by EPF depends largely on their defense mechanisms. Baverstock et al. (2009) mentioned that, the capacity of insects to perceive and evade EPF hinges on species and their ontogeny. For example, social insects like termites and ants often nest in soil in dense populations, thus exposing them to infection by EPF. However, these insects possess developed mechanisms that enable them to detect and elude EPF, especially *Metarhizium*. They are also able to detect infected members of the colony via the use of olfactory signals (Yanagawa & Shimizu, 2007). While the termite, *Macrotermes michaelseni*, are able to differentiate between virulent and avirulent strains of EPF, particularly, *M. anisopliae* and *B. bassiana* based on the volatile organic compounds (VOC) profiles produced by the fungi (Mburu et al., 2013). Some behavioral defenses employed against pathogens include self-grooming (Tragust et al., 2013), grooming of nest mates (Ugelvig & Cremer, 2007) and production of antifungal compounds (Bulmer et al., 2009; Tragust et al., 2013). In the presence of EPF, ants are sensitive and capable of altering their behavior in order to evade these pathogens. For instance, workers of *Formica selysi* when exposed to conidia of *M. anisopliae*, tend to increase their frequency of self-grooming, while workers of *Lasius neglectus* manage infected or contaminated workers by enhancing sanitary behavior and brood care. Often in the quest to prevent the spread of pathogens and improve hygiene, some insects undergo conspecific grooming, which are done simultaneously with sanitizers (disinfectants) such as antimicrobial peptides (AMPs), formic acid and protein containing salivary droplets in termites and ants (Tragust et al., 2013). However, in some social insects, antifungal compounds are secreted from the salivary glands or metathoracic glands to guard themselves and nest mates against invading pathogens (Bulmer et al., 2009; Tragust et al., 2013). In termites, secretions from salivary glands have been known

to assume antimicrobial role. For example, termicin and Gram-negative bacteria-binding protein 2 (GNBP2: β 1,3 glucanase) have been reported as components of the salivary secretions from termites (Bulmer et al., 2009). Another behavioral hygiene is observed among the honey bee workers, where larvae infected by the fungus *Ascosphaera apis* are detected among the population by workers via the phenethyl acetate produced by invading pathogens, and larvae are removed from the nest (Swanson et al., 2009). However, in response to infection by EPF, the locust *Schistocerca gregaria* exposed to *M. acridium* do elevate their body temperature via sun bath (basking in the sun) to kill the pathogens (Shah et al., 2010). Whereas in Hemiptera like the pea aphid, *Acyrthosiphon pisum* alarm pheromones are emitted when attacked by pathogens. This alarm pheromone enables the aphid population to retract their stylet and drop off plant surface or move to a new plant to avoid infection by a pathogen. Although in that manner, they might help in the dispersal of the pathogen to new uninfected colonies (Hatano et al., 2012). For *Bemisia tabaci*, pathogens are avoided via a stress response strategy that allows the insect to elevate its capacity to endure infection (Zhang et al., 2014). Internal melanization has also been reported to elevate stress tolerance in some of these insects (Garrido-Jurado et al., 2017).

Defense strategies such as density-dependent prophylaxis and population-level immunity by insects against pathogens possess significant consequences against the use of EPF as biological control agents. This is evident as EPF propagule can cause the elicitation of immune stimulation of target insects rather than death, thus giving rise to the formation of a community that are resistant to infection by EPF (Butt et al., 2016). Under this condition, individual hosts participate more in raising immunity levels under high population densities (Wang et al., 2013). In gregarious locust, susceptibility to infection by *M. anisopliae* is reduced with increase in prophylactic immunity among a population. This concept of prophylactic immunity limits the risks of infection and

epidemics in a population (Qu & Wang, 2018; Wang et al., 2013). For social and gregarious insects, this phenomenon would be expedient for the community structure than in solitary ones.

2.11.1 Innate immune responses against EPF

EPF have developed numerous sophisticated strategies to overcome the immune system of their hosts. However, in response to their invasion, insect hosts revert to behavioral changes, physical barriers and innate defenses to defend themselves against their invasions. The first line of defense for the insect host is the cuticle, which forms a formidable barrier against pathogens. It is a harsh physiological environment, consisting of chemical substances like the AMPs and reactive oxygen species (ROS) which are good inhibitors of fungal growth. More also, the interplay of innate and humoral immunity is critical at preventing the onset of fungal infection of the host (Qu & Wang, 2018). Unlike vertebrates, which are endowed with adaptive immune mechanisms, insects lack antibodies production abilities. Their ability to counter invading pathogens lies majorly on the innate immune complex, which consist mainly of the cellular and humoral mechanisms. Insect hemocytes play vital role as major players in the immune responses against pathogens (Qu & Wang, 2018).

2.11.2 Cellular immunity

Usually, in response to infection by EPF, insect hosts assemble an arsenal of macromolecules and hemocytes such as phenoloxidase and lectins, which are able to wade off, immobilize, and kill EPF. Furthermore, emerging evidence advocates that bioactive components (e.g., antimicrobial peptides and lysozyme) of the host immune system work synergistically to fight infections. Also, functional proteins like apolipophorin III and transferrin are able to perform multifunctional tasks in homeostasis,

metabolism and recognition of pathogens (Butt et al., 2016). Key immune players such as the phenoloxidase and its precursor, prophenoloxidase are used by certain groups of insects against different pathogens (Flores-Villegas et al., 2016a).

Under natural conditions, preventing the loss of the host hemolymph is vital for insect survival. In this respect, hemostatic responses are activated in conjunction with immune defenses when the host cuticle is compromised either by pathogens, predators, herbivores or parasites. In the face of microbial invasion, micro clots are formed that sequester invading microbes in the host hemolymph. When this happens, the enzyme transglutaminase facilitates the anchorage of the invading microbe to the micro clot matrices for it to be killed by antimicrobial peptides (AMPs) and surrounding hemocytes. During microbial invasion of insect hosts, survival of infected insects can be greatly improved during ecdysis, when old cuticle are shed alongside attached conidia that are yet to penetrate the cuticle (Butt et al., 2016). More also, invading pathogens must overcome not only the fungistatic compounds secreted in the epicuticular waxy layer, but also physical factors like cuticle thickness, melanization, mineralization, sclerotization, AMPs, and protease inhibitors (Dubovskiy et al., 2013).

During the event of pathogen's invasion of a target host, the susceptibility of the host to the invading pathogen is largely dependent on the ontogeny (developmental stage) of the host. This is because, newly molted insects are more vulnerable to invasion by pathogens due to their soft and not fully sclerotized cuticle (Liu et al., 2014). Mineralization and sclerotization are major determinants of cuticle hardness, making it intractable to enzyme degradation (Andersen, 2010). However, the thickness of the insect cuticle does not determine resistance to invading pathogens, since EPF are known to have broad spectrum of activity against a wide range of insect hosts, even those with relatively

thick cuticles (Lacey et al., 2015a). Basically, a combination of factors such as cuticle structure, thickness and wax secretions had to interplay to facilitate the susceptibility of the pine scale, *Matsucoccus matsumurae* (Liu et al., 2014). The successful interplay of these factors aid to delay the pathogen invasion of the cuticle, thereby allowing the host time to mobilize the cellular and specific immune defenses to counter the pathogen (Dubovskiy et al., 2013).

2.12   EPF conidial formulation and effects on virulence

Formulation plays a critical role in delivering fungal pathogenic propagules to the environment of interest (Faria & Wraight, 2007), and optimization of the efficacy of myco-insecticides (Alves et al., 1998a). Formulated fungal propagules are mostly compounded as wettable powders, technical concentrates or oil dispersions (de Faria & Wraight, 2007). It has been argued that, for optimal biological efficacy and safety, conidia of EPF must be well formulated. Formulation is simply the mixing of entomopathogenic fungal conidia with non-evaporative ingredients that are essential for stability, viability, efficacy, virulence and protection of the entomopathogen (Burges, 1981).

Water is an essential formulating substance because it is cheap, non-toxic, readily obtainable, and can be easily dispersed with the aid of hydraulic systems. Hydrophilic and lipophilic conidia can be readily suspended in water using simple surfactants. But, numerous studies have revealed that oil-based formulations yield better results than water-based formulations (Agudelo & Falcon, 1983; Bateman, 1997a; Jenkins & Thomas, 1996; Prior et al., 1988). This may be due to the fact that oil-based formulations cause increased adhesion of conidia to the integument of insects, and their spread to inter-segmental locations (membranes) with high humidity than does water-based formulations (Bateman et al., 1993; Prior et al., 1995).

Understanding the mode of interaction between fungal pathogenic propagule, their target insect host and the environment is important during propagule formulations. Because insect cuticle is hydrophobic (repelling water) in nature, interaction with aqueous formulations are usually repelled due to the low surface energy, which must be overcome to allow better interactions. The hydrophobicity of the cuticle is usually countered by the use of suitable surfactants, which reduce the surface tension of the water to facilitate adequate interactions between the fungal propagule and the insect host (Jackson et al., 2010). More also, predictions about the interactions between fungal propagules and their hosts will be reliable when the knowledge of physiochemical properties such as polar, hydrophobic and electrostatic tendencies of their spores is well understood (Holder et al., 2007).

Oils are important in the constitution of superior spray carriers and demonstrate inherent compatibility with lipophilic spores such as found in *Metarhizium* species. Oils are perfect ingredients for ultra-low volume applicators which resist evaporation during application on targets (Bateman, 1997b; Wraight et al., 2001).

Studies have revealed that oil-based formulations of fungal conidia enhance the efficacy of some fungal entomopathogens when compared with the commonly employed water-based formulations. For example, when the effectiveness of oil-based (coconut oil) and water-based (water + 0.01% Tween 80) formulations of *B. bassiana* were evaluated on *Pantorhytes plutus* (adult cocoa weevil), the oil-based formulation had an LD_{50} value 36 times lower than the water-based formulation while the LD_{95} value was 111 times lower than the water-based formulation (Prior et al., 1988). According to Alves et al. (1998a), the addition of 10% adjuvants such as Natur'l and Codacide oils to the water-

based formulation of *M. anisopliae* conidia improved the infectivity of the fungus against *Tenebrio molitor* larvae far above the conventional water + 0.05% Tween 80. Santi et al. (2011), reported a 100% mortality of the cotton stainer bug on the 6th and 7th days after treatment with 10^8 conidia ml^{-1} of *M. anisopliae* formulated in 10% soybean oil, confirming the oil-based formulation as the most effective against both adults and nymphs. A study by Polar et al. (2005a) compared the efficacy of water, oils and emulsifiable adjuvant oils formulation (EAOs) of *M. anisopliae* against *Boophilus microplus* and found that formulation in Coconut oil produced mortalities with average survival time (AST) of 4.6 ± 0.28 days while 10% liquid paraffin had an AST = 4.4 ± 0.15 days compared to water with an AST = 8.4 ± 0.42 days. This indicates that oil-based formulations improved the infectivity and virulence of *M. anisopliae* against *Boophilus microplus*. Also, a study by Rot et al. (2013) reported how *M. brunneum* formulated in mineral oil enhanced on-host mortality of the brown dog tick, *Rhipicephalus sanguineus* compared to unformulated conidia. Mortality in the unformulated conidial suspension was 50.49% while the mineral oil formulated conidial suspension caused a mortality of 70.4%. Furthermore, (Kaaya, 2000) reported how oil formulated *M. anisopliae* induced higher mortalities than aqueous formulation of the fungus against tick species. The study opined that oil formulated conidia in (10^9 conidia/ml) caused 100% mortality in larvae of *Amblyomma variegatum* and *Rhipicephalus appendiculatus*, mortalities in adult varied from 80-90%, whereas the aqueous formulation (10^9 conidia per ml) induced mortalities between 40-50%. This further supports the assertions that oil-based formulations of EPF enhances their virulence against target hosts. It is quite obvious from the above findings that oil demonstrates significant effects on the virulence of EPF applied for biological control purposes compared to water-based formulations.

However, irrespective of the type and method of formulation, insects have developed surface receptor molecules with affinity for conserved molecules on invading pathogens to reveal their identities (Fearon, 1997). The insect receptor-based systems work by identifying molecular motifs such as peptidoglycans, lipopolysaccharides and β (1, 3)-D-glucan (Müller et al., 2008; Wang & Ligoxygakis, 2006). For example, Bulmer et al. (2009) reported that termites express some exceptional β (1, 3)-glucanase enzyme activity in their cuticle, tissues and nests. The two major functions proposed for the β (1, 3)-glucanase in relation to termite immune defense include: (1) to function as sensor for the cleavage and release of pathogen components, which in turn stimulate the defense system of the termite and (2) to facilitate the cleavage and weakening of the cell wall of the invading pathogen, making it vulnerable to the antimicrobial peptides released by the termites.

Fungal formulations may function in reducing stimulated behavioral responses by insects to invading entomopathogens. For example, termites easily activate diverse defensive behavioral responses to fungal entomopathogens to eradicate or curtail the effect on the cluster. This they do via hygiene behaviors such as isolation and removal of diseased workers and sporulating cadavers, grooming of workers (Fefferman et al., 2007; Jackson et al., 2010). However, notwithstanding, EPF have always evolved mechanisms that strive to counter these behavioral modifications of termites and other arthropod pests in their quest to resist infection.

2.13   Laboratory bioassays with EPF

Over the years, there has been no standardized bioassay methods used in the laboratory for entomopathogenic Hypocreales applied for the control of insect pests. This has necessitated the need for the development of a specific bioassay for host-pathogen

interactions. This quest has led to the development of the 'dipping method' which became the most commonly used quantitative bioassay technique for insects that live and feed above the soil surface (Eilenberg et al., 2003). Under this method of bioassay, one or more logarithmic series of conidial suspensions are prepared with known concentrations into which the insects are briefly immersed and removed.

However, in another approach, fungal conidial inocula are prepared using the instrumentality of secondary substrates. The secondary substrate being the host's food source is immersed in conidial suspensions of known concentrations unto which the insects are introduced (Nielsen et al., 2004).

Furthermore, in another approach, insects are exposed to surfaces contaminated with the EPF conidia where they pick up the conidia on their integument, which infect and kill them (Dong et al., 2012a; Nielsen et al., 2004). In a recent publication, García-Munguía et al. (2011) reported that EPF conidia impregnated on filter paper were highly effective at infecting and killing *Aedes aegypti* exposed to the contaminated filter paper. The fungal conidia impregnated were said to be transmitted between the mosquitoes during mating activity. The work of Dong et al. (2012a) supported the assertions that EPF conidial applied on filter paper is highly effective at infecting and killing arthropods during their mating escapade. According to Baverstock et al. (2010), transmission of fungal infection via mating activity is a form of horizontal transmission which is referred to as autodisemination because of its occurrence among individuals of the same species.

In laboratory bioassay studies that determines concentration-response relationship, the term median lethal concentration (LC_{50}) or lethal dose (LD_{50}) are common expressions of virulence of an entomopathogen. The LC_{50} and LC_{90} are conidial concentrations

required to kill 50% and 90% of a test subject population under a given time, whereas LD_{50} specifies the dose required to kill 50% of a given population. The term LC_{50} is basically used with EPF since the methodology permits an estimate of the concentration used and not necessarily the dose received by the test insects (Inglis et al., 2001; Nielsen et al., 2004).

For studies on time-response relationship, the terms median lethal time (LT_{50}) and average survival time (AST) are the commonly used expressions of the time required for killing a given insect population. The period of exposure to a pathogen in a time-dependent bioassay that produce the death of half the test insect population is described as the median lethal time (LT_{50}). The length of the period of exposure directly measures the dose of the pathogen, and an increase in the exposure time leads to an increase in the true dose ratio of the pathogen (Inglis et al., 2012). AST is the average lifetime of test subject population after exposure to a specific pathogen concentration. These terms are mostly used as quantitative expression of the virulence of fungal pathogens. The terms further provide information on what is required to understand the spread of disease among arthropod population and the dynamics of host-pathogen interactions (Inglis et al., 2001; Nielsen et al., 2004).

2.13.1 Concentration-response bioassay

In several concentration-mortality bioassay studies, dead of insects in the treatment is often proportional to increase in conidial concentrations and time of exposure (Karthi et al., 2018; Sevim et al., 2010; Sousa et al., 2013). Usually, as part of selection process in laboratory bioassays, different concentrations of the fungal conidia are used. Conidia harvested from culture plates are most often suspended in surfactants such as Tween 80 (polyoxyethylene sorbitan monooleates) suspension. For example, Seye et al. (2014a)

used five different conidial concentrations (10^3 to 10^7 conidia/ml) of *A. flavus*, *A. clavatus* and *M. anisopliae* suspended in 0.05% Tween 80 during a laboratory bioassay on the aphid, *Acyrthosiphon pisum*. Here the authors observed mortality of the insect to be proportional to the concentrations used and the length of time to which the insects are exposed. The LC_{50} and LC_{90} values reported for the isolates were 1.23×10^3 and 1.34×10^7 conidia/ml for *A. flavus*, 4.95×10^2 and 5.65×10^7 conidia/ml for *A. clavatus*, and 3.67×10^3 and 9.71×10^7 conidia/ml 5 days after exposure of the entomopathogens. Based on the LC values obtained by these researchers, the *Aspergillus* species showed greater virulence against *A. pisum* than *M. anisopliae* which is largely reported to be highly virulent and lethal against arthropod pests.

2.14   Analysis of bioassay data

In bioassays with EPF, the conidial concentration is the only variable considered during bioassays. The response of the experimental subject is presumed to be functionally related to the concentration or dose level, such that as the conidial concentration increases, more experimental subjects respond by dying. The equation of the quantal binary response model with the concentration as a single response variable is given as:

$$Pi = F(a + bxi), \text{where}$$

Pi is the probability of response, x_i is a function of the concentration (logarithm of concentration), *a* defines the intercept of the regression line, *b* is the slope of the regression line, while *F* is the distribution function. The two commonly used functions are the probit and logit functions (Nielsen et al., 2004; Robertson & Preisler, 1992). The commonly used distributions analysis of bioassay data are the normal and the logistic distributions. However, the normal distribution is presumed in probit analysis so that *F* becomes the normal distribution function.

CHAPTER 3: MATERIALS AND METHODS

3.1 Flow chat of the study

The study was designed and implemented according to the flow chart below:

Experimental flow chat
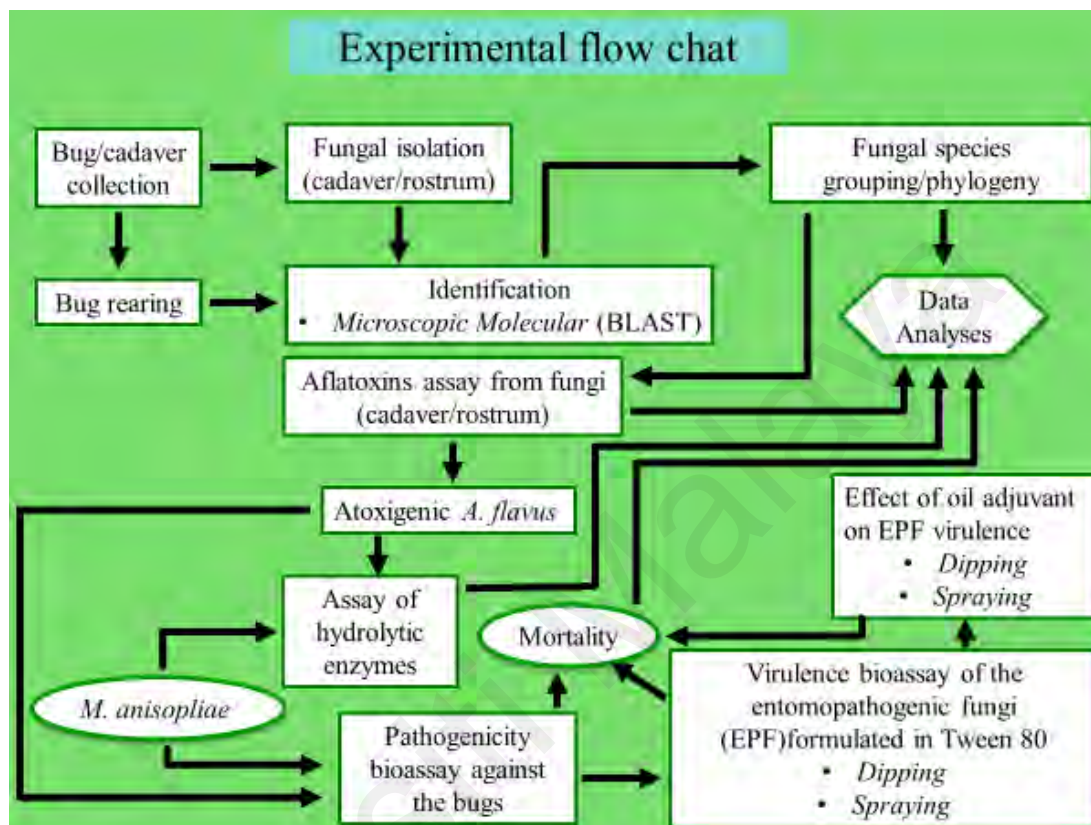
3.2 Bug collection and rearing

Adult bugs were collected from groundnut fields in Menglembu, Perak (4º33ʹ58.29″N and 101º2ʹ53.95″E), Malaysia, from February to April 2017 into plastic containers and transported to the laboratory. The bugs were maintained in the laboratory as in Khosravi et al. (2015). The bugs colonies were reared inside plastic cages (40 × 30 × 20 cm) at 25 ± 2 °C and 70 ± 10% relative humidity (RH) under a 14: 10 h L: D photoperiods. The method was modified by placing moist filter papers in each container to maintain the humidity and cotton wicks soaked in distilled water provided water for the bugs to suck. Fresh groundnuts or sesame seeds were used as diets where groundnuts were not available. The diet used was changed after every 3 days. Gauze materials were placed in

each container as surface for oviposition. Gauze containing oviposited eggs were separated into new containers to hatch at 70 ± 10 (RH). Hatched nymphs were maintained under laboratory conditions until they were mature into adults. Adult bugs, 2-3 weeks, were used for the pathogenicity and bioassay experiments.

3.2.1 Egg laying and biology of the bug

Several copular bugs (n = 30) were used in the study to enable understanding of their biology. One copulated adult female bug each was placed in a plastic cage ($40 \times 30 \times 20$ cm) and cotton gauze placed inside the containers to serve as oviposition surfaces. The bug was maintained under this condition for the period of 16-20 days. To restrict the bug from moving out, a thin layer of Vaseline was rubbed round the edge of the container and covered with two layers of Cheesecloth. After oviposition was completed, adult bugs were transferred into new containers while the eggs where counted for each with the aid of a magnifying hand lens. After counting was completed, cotton balls moistened in sterile distilled water were placed inside the containers to provide the humidity for the eggs to hatch.

3.2.2 Identification of bug species

The identification of the bug species was done via morphological examinations and molecular method. Genomic DNA from the legs of ethanol-preserved specimens were extracted and used for molecular identification (Tembe et al., 2014), while morphological identification was done according to the method described by Katinka (2016) which considered morphological features like the nature of sutures on the head, location of the trichobothria/spiracles, location of the phallus and ovipositor and the pronotum respectively.

3.3 Molecular identification of *E. pallens*

Genomic DNA from the leg sample of ethanol-preserved bug collected from different farms was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. The PCR was done according to the method adopted by Tembe et al. (2014). PCR was conducted in 25 µl volume containing 2 µl of DNA, 12.5 µl of 2×Power TaqMaster Mix, 2µl of 10 µM primers (forward and reverse) and 8.5 µl sterile distilled water. The primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) were used. The amplification conditions were; 1 cycle, 95 °C (5 min), 35 cycles, 94 °C (30 s), 47 °C (30 s), 72 °C (1:30 min), and 1 cycle 72 °C (10 min). PCR products were identified on 1% (w/v) agarose gel electrophoresis containing the gel view stain under UV light. The DNA product obtained was purified and sequenced for comparison with existing species sequences data in GenBank using the Basic Local Alignment Search Tool (NCBI BLAST).

3.4 Isolation of fungi from the cadaver and rostrums of *E. pallens*

Cadaver of *E. pallens* were collected from the field and transported to the laboratory inside sterile centrifuge tubes for further analyses. The method of (Lu et al., 2015), was adopted for the isolation of fungi from the cadavers with minor modifications. To isolate from the rostrum, adult *E. pallens* were briefly cold anesthetized at 4 °C to inactivate them from flying off. Both the cold-anesthetized bugs and the cadavers were surface sterilized separately by dipping in 1% (v/v) sodium hypochlorite (NaOCl) solution for 5 min and rinsed three times in sterile distilled water. The surface sterilized rostrum and cadavers were placed on potato dextrose agar (PDA, Oxoid) plates (95 × 15 mm) amended with 0.5 g/L chloramphenicol and incubated in the dark at 25 °C and relative humidity (RH) ≥ 80% for 5-7 days. Mycelia that developed upon culture were sub-cultured until pure

cultures were obtained and kept on PDA slants at 4 ℃ until required.

The *M. anisopliae* used in this study was obtained from the research laboratory of the Department of Microbiology and Biotechnology, University of Ibadan, Ibadan, Nigeria.

3.4.1   Microscopic observation

The morphology of the isolates was determined using the conventional slide culture technique. Sterile PDA was cut into approximately 1 cm squares and a block each was placed on a sterile glass slide inside Petri plates (90 mm × 15 mm) with sterile filter paper (Whatman 1) placed underneath. Each PDA block was inoculated on the four corners with a fungal colony using sterile needles and a sterile coverslip aseptically placed over it. The filter paper underlay was wetted with distilled water and incubated at 25 ℃ for 3-5 days. The coverslip was gently removed and placed on a glass slide containing a drop of lactophenol cotton blue (LPCB) and examined under the bright field microscope (Woo et al., 2010) (Leica DM500, Leica Microsystems, Wetzlar, Germany) at 40×.

3.5   Molecular identification of fungal isolates

Genomic DNA of the fungal isolates from both the cadavers and rostrum was extracted following the protocol of Genomic DNA Extraction Kit. A fragment of the internal transcribed spacer (ITS) region was amplified using universal primer sets ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCR cycling protocol include initial denaturation at 95 ℃ for 3 min followed by annealing at 50 ℃ for 30 s, extension at 72 ℃ for 30 s, denaturation at 95 ℃ for 30 s, and the final extension at 72 ℃ for 10 min (White et al., 1990). The PCR products were stored at -20 ℃. The size and quality of the PCR products were determined by gel electrophoresis using 1.25% (w/v) agarose gel, stained with gel view stain and visualized

under UV light. All DNA sequences obtained in this experiment were compared with existing species sequences data in GenBank using the Basic Local Alignment Search Tool (NCBI BLAST) (Sun et al., 2016a). Homologous ITS1-5.8S sequences of similar fungal species were selected and aligned with sequences from this study and the phylogenetic relationship generated using the neighbor-joining method of MEGA 7.0.

3.5.1 Phylogenetic analysis of the fungal isolates

The phylogenetic analysis involving the ITS sequence data of the isolates was done by comparing with registered sequences on the GenBank database. ITS sequences were aligned, and unnecessary portions removed before the tree was generated using MEGA 7.0 software, which gave the topology and length of the branches (Kumar et al., 2016). The phylogenetic relationship and the neighbor joining analysis were determined using the Kimura 2-parameter (K2P) model of base substitution in MEGA 7.0 (Tembe et al., 2014).

3.6 In vitro aflatoxins assay from fungal isolates

The qualitative aflatoxin assay enables the detection of fungal isolates with potentials for the synthesis of this secondary metabolite. Strains which are capable of producing aflatoxins on the screening medium usually appear to display certain color appearances depending on reaction with the medium components.

The screening of the rostrum and cadaver isolates for aflatoxin production was done following the ammonium vapour test method adopted by Mamo et al. (2018). *Aspergillus* spp. which are known for aflatoxins production were screened for aflatoxin production on coconut agar medium (CAM) which consisted of commercial coconut milk (from local market) diluted to 40% (v/v) with distilled water and 3 g of agar was added. The medium

was sterilized by autoclaving at 121ºC (Tomy SX-500, high pressure steam sterilizer) before pouring into Petri dishes. A 4 mm agar plug of young culture (3 days old) of each isolate was placed on the center of the medium in a Petri dish and incubated for 3 days at 30 ºC. The cultures plates were inverted and a drop (0.2 ml) of 25% ammonia solution (30% Ammonium Hydroxide solution, analytical reagent, AR; R & M Chemicals) was placed on the lids of the culture plates. The vapour released from the NaOH solution interacted with the fungal culture to cause colour formation. Pink color pigmentation formed due to contact between the vapour and the isolates culture was indicative of aflatoxin production while the absence of colour formation indicated absence of aflatoxin synthesis. The culture plates were recorded as positive or negative with the isolates scored as toxigenic or atoxigenic.

3.7   Fungal conidial harvesting and formulation

The method adopted by Quinelato et al. (2012) was used with some minor modifications. Conidia were harvested from14-day old culture plates by scrapping with a scalpel blade. Harvested conidia were suspended in 20 ml of polyoxyethylene sorbitan monooleate (Tween 80) solution (0.05%, v/v). The conidial suspension was homogenized for 1 min using a vortex mixer. The homogenized conidial suspension was quantified on a hemocytometer and adjusted to a concentration of 10^8 conidia/ml for the pathogenicity bioassay. The conidial concentrations used was calculated following equation 1:

$$C1V1 \; = \; C2V2 \dots\dots\dots\dots\dots\dots. (eq \; 1)$$

$$(where \; C \; = \; concentration \; and \; V \; = \; volume).$$

For concentration-response (dose-response) bioassay, the concentrations $(1 \times 10^8$ conidia/ml, 1×10^7 conidia/ml, 1×10^6 conidia/ml, 1×10^5 conidia/ml, 1×10^4 conidia/ml) were logarithmically prepared using serial dilutions prior to the bioassay.

3.7.1 Conidial viability testing

The conidial viability for all isolates was determined by plating 100 µL of suspension of 10^6 conidia/ml on three replicate Petri dishes containing PDA and incubated at 25 °C under a 14:10 h L: D photoperiod for 16-24 h period. Germination of the conidia was estimated by counting 300 spores/plates (100 spores per area) under a bright field microscope at 40× magnification. The formation of at least half the length of the conidia indicates viability. Also, for comparison, 100 µL of conidia (10^3) was spread onto PDA in culture plates to count the colony forming units (CFUs) (Carrillo et al., 2015).

3.8 Single-dose virulence assessment bioassay of fungal isolates against *E. pallens*

Fungal isolates obtained from cadavers of the bugs and *M. anisopliae* were evaluated for virulence against *E. pallens* according to the method of Orduño-Cruz et al. (2015). Mixed sex adult bugs were cold-anesthetized by placing them briefly at 4 °C before dipping into 1×10^8 mL^{-1} of conidia for 10 s while control groups were treated with 0.05% (*v/v*) Tween 80. For each conidial concentration, five replicate groups of 10 bugs each were used. Treated bugs were placed in plastic containers (65 × 45 mm) containing damp sterile filter paper (Whatman 1). Cotton wool soaked in sterile distilled water was placed in each container to provide water for the insect to suck. Afterwards, the treated bugs and the controls were introduced into plastic containers with perforated lids to prevent the insects from escaping, provide ventilation and maintain the required humidity. Both the treatment and the controls were maintained at 25 ± 2 °C, 80 ± 10% relative humidity (RH) and 14:10 h photoperiod without diet. Mortality was scored after every 24 h for 10 days.

3.9 Multiple-dose virulence bioassay for EPF formulated in Tween 80

This experiment involving multiple conidial concentrations was designed in two fashions to study the effects of EPF. The two designs are the immersion and the wet filter paper methods. Fungal conidia impregnated on the surface of filter paper are said to be transmitted horizontally by insects during their mating activities.

3.9.1 Immersion bioassay

The virulence bioassay of EPF against *E. pallens* was performed following the method of Resquín-Romero et al. (2016). *A. flavus* and *M. anisopliae,* which produced the highest mortality rates, were selected for the multiple-dose virulence bioassay. Five conidial concentrations were prepared via serial dilutions, and the treated bugs and controls were maintained as above (section 3.8). Cadavers were dipped in 1% (*v/v*) sodium hypochlorite (NaOCl) solution for 5 min to surface sterilize and rinsed 3 times in sterile distilled water. The disinfected cadavers were incubated at 25 ± 2 °C on sterile wet filter paper in Petri dishes for 5–7 days to activate conidial growth followed by examination under light microscope.

3.9.2 Wet filter paper bioassay (spraying)

This bioassay utilized filter papers according to the method described by Dong et al. (2012b). Sterile filter papers (65mm in diameter) placed inside plastic containers (65 × 45 mm) were moistened with 1 ml of the conidial suspension for the different concentrations. The method was modified by using moist sterile cotton balls placed inside the containers to provide the required humidity. Cold-anesthetized bugs were introduced into the containers containing the contaminated filter papers. For each conidial concentration, the experiment was replicated five times with each replication having a total of 10 bugs. Cold-anesthetized bugs introduced into plastic containers containing sterile filter moistened with 0.05% Tween 80 served as controls. Mortality was scored

after every 24 h for 10 days. Dead insect cadavers were surface sterilized using 1% (v/v) sodium hypochlorite (NaOCl) solution for 5 min followed by 3 rinses in sterile distilled. Surface sterilized cadavers were incubated at 25 ± 2 °C on sterile wet filter paper in Petri plates for 5-7 days to stimulate fungal growth which was examined microscopically using a light microscope. The experiment was performed thrice, using independent batches of the insect and conidial inoculum each time.

3.10 Concentration-response virulence bioassay (oil-based formulation)

3.10.1 Immersion bioassay

Adult bugs were dipped in various concentrations of fungal isolates conidia formulated in 5% peanut oil to determine the effects of oil on the virulence of the isolates. Control groups were treated with 5% peanut oil solution (conidia-free). A total of five replications each containing 10 insects were carried out for the fungal isolates and the experiment repeated three times for all the different conidial concentrations. Both the treatment and the control were maintained at 14:10 h L: D regimes and mortality data were recorded after every 24 hours for 10 days. Both treated and control bugs were maintained without food for 10 days (to prevent possible introduction of pathogens from food source). Dead bug (immotile, functionless, mummified and brittle) cadavers were surfaced sterilized in 1% NaClO solution for 5 minutes and rinsed three times in sterile distilled water. The surface sterilized cadavers were cultured on damp filter papers in Petri dishes for 5-7 days and later examined using the light microscope at a magnification of 400× to confirm the cause of mortality.

3.10.2 Wet filter paper bioassay

The conidia of the experimental fungal isolates (*A. flavus* and *M. anisopliae*) formulated in 5% peanut oil was applied on filter paper (65 mm in diameter) placed inside

plastic containers (65 × 45 mm) and the insects were introduced. Control groups were introduced into plastic containers that contained filter papers moistened with 5% peanut oil. The experiment was replicated five times with 10 adults each and repeated thrice under laboratory conditions at 14:10 h L: D regimes. Both the treated and control bugs were observed for the period of 10 days without diet, and mortality data taken after every 24 hours. Cadavers were surfaced sterilized in 1% NaClO solution for 5 minutes and rinsed three times in sterile distilled water. The surface sterilized cadavers were cultured on damp filter papers in Petri dishes for 5-7 days and later examined using the light microscope at a magnification of 400× to confirm the cause of mortality.

Mortality data was transformed using equation (1) and was corrected using the Abbott formula equation (2).

$$\text{Percentage of mortality} = \frac{\text{Number of dead bugs}}{\text{Number of bugs used}} \times 100 \dots\dots\dots\dots\dots \text{eqn} \quad (1)$$

$$\text{Corrected percent mortality} = \left(1 - \frac{\text{n in T after treatment}}{\text{n in C after treatment}}\right) \times 100 \dots\dots\dots \text{eqn} \quad (2)$$

where n = number of bugs, T = treatment, and C = control

Dead bug = immotile, functionless, mummified and brittle.

To determine the LC_{50} and LC_{90}, the corrected percent mortality data were subjected to probit analysis using SPSS IBM SPSS Statistics 21.0 software (USA).

3.11   Assay of hydrolytic enzymes from the fungal isolates

The need to assay for the secretion of hydrolytic CDEs from the fungal isolates was essential because of the role of the enzyme in fungal pathogenesis. These enzymes are

believed to be essential for the degradation of the insect host cuticle for ease of penetration by the invading pathogen to initiate infection. They are secreted to hydrolyze the proteo-chitin matrix of the cuticle which serves as a formidable barrier to EPF infection. Considering the significance of these enzymes, the need to examine their production and activity becomes important.

3.11.1 Fungal growth and crude enzyme preparation

Fungal isolates were cultivated according to the method of Kim et al. (2018) with some modifications. Sabouraud dextrose broth (100 ml) inside 100 ml flask was inoculated with 200 µl conidial suspension of 1×10^8 conidia/ml. This conidial concentration was preferred because it was the highest conidial concentration used in the bioassay studies. Inoculated flasks were incubated on a rotary shaker at 28 °C and 150 rpm for 4 days. Cultures were filtered using cheese cloth to remove the mycelia before centrifugation at 13,000 rpm for 10 min at 4 °C. The supernatant being the crude enzyme source was used for the enzyme activity assay.

3.11.2 Spectrophotometric lipase assay

Quantitative lipase activity was assayed using 4-nitrophenyl palmitate as substrate according to the method adopted by Gopinath et al. (2005). A 10 ml isopropanol containing 30 mg 4-nitrophenyl palmitate was mixed with 90 ml of 0.05 M sodium phosphate buffer (pH 8.0) containing 207 mg sodium deoxycholate and 100 mg gum Arabic. A volume of 2.4 ml of the substrate was pre-warmed at 37 °C and mixed with 0.1 ml crude enzyme solution. The mixture was incubated for 15 min at 37 °C and the absorbance determined at 410 nm wavelength against an enzyme-free control. One lipase enzyme unit (U) was defined as the amount of lipase enzyme that released 1 µmol of 4-nitrophenol in milliliters per minute (ml/min). The enzyme assay was carried out in three

replications and the average computed. Standard curve was prepared using different concentrations of the substrate and observed at 410 nm wavelength.

3.11.3   Chitinase assay

3.11.3.1 Colloidal chitin preparation

Preparation of the colloidal chitin substrate was done according to the method adopted by Kang et al. (1999) with minor modification. Analytical grade chitin (20 g) was suspended in 200 ml concentrated HCl with stirring at 40 °C for 3 min. Water (2 L) adjusted to 5 °C was gently added to the chitin suspension to precipitate it as colloidal suspension which was collected by filtering through coarse filter paper. The filtered colloidal suspension was washed in water until the pH dropped to 4.0.

3.11.3.2 Chitinase enzyme assay

The reaction mixture containing 250 µl of 0.5% colloidal chitin, 250 µl of 0.2 M sodium acetate buffer (pH 4) and 500 µl crude enzyme solution was incubated at 37 °C for 2 h before centrifugation. Upon centrifugation, 500 µl from the supernatant was mixed with 100 µl 0f 0.8 M boric acid, and the pH of the mixture was adjusted to 10.2 with KOH. The solution was heated in boiling water for 3 min. Upon cooling, 3 ml of *p*-dimethyl amino benzaldehyde (DMAB) solution (1 g of DMAB dissolved in 100 ml glacial acetic acid containing 1% v/v HCl) was added to the mixture and incubated for 20 min at 37 ºC. Absorbance at 585 (A_{585}) nm was measured against water as a blank. One unit of chitinase activity was defined as the amount of enzyme required to produce sugars equivalent to 1 µmol of *N*-acetylglucosamine per min under the above conditions (Yanai et al., 1992). Standard curve was prepared using different concentrations of the substrate and examined at an absorbance of 585 nm.

3.11.4 Protease assay

Protease activity was measured spectrophotometrically according to the method of Keppanan et al. (2017). The reaction mixture containing 450 µl of 1% (w/v) casein with 50 mM Tris-HCl buffer (pH 8.0) and 50 µl of enzyme solution were incubated at 55 ºC for 20 min. The reaction was brought to a stop be adding 750 µl of trichloroacetic acid (TCA) solution [5% (w/v) TCA, 9% (w/v) Sodium acetate, 9% (v/v) acetic acid] and incubated at room temperature for 30 min before centrifugation at 15000 rpm for 15 min. The absorbance of the reaction supernatant was measured at 280 nm. One enzyme unit was defined as the amount of enzyme that catalyzes the release of 1 µmol of tyrosine per min under the assay conditions. Standard curve was prepared by varying the concentrations of the substrate and the absorbance determined 280 nm wavelength.

3.12 Statistical analyses

Mortality data were corrected using Abbott's formula (Abbott, 1925) and analyzed using Analysis of Variance (ANOVA) and the Least Significant Difference (LSD) test was used to test the differences in mean mortality between the conidial concentration groups of the isolates using SPSS for Windows (Ikram et al., 2016). The lethal concentration (LC_{50} and LC_{90}) values for *A. flavus* and *M. anisopliae* were estimated from the infection-confirmed mortality data by probit analysis. The 95% confidence intervals were calculated for the different treatments where there was no significant difference in their slopes (Finney, 1971). The estimated lethal time (LT_{50} and LC_{90}) against the bugs was determined using probit analysis (Throne et al., 1995). The paired sample t-test statistic was used to test for differences in the virulence of the experimental fungal isolates. Correlation analysis was done to determine the relationship between toxigenic and atoxigenic fungal isolates from the rostrum of *E. pallens* (field-collected and

laboratory) (Ezekiel et al., 2014). For all statistical tests, a significance level of 0.05 was considered.

CHAPTER 4: RESULTS

4.1 Morphological features and biology of *E. pallens*

Morphologically, the bug is dark brown in colour with small head in relation to the body, a long antenna (four segments) and a rostrum (Figure 4.1) positioned on the head. The rostrum is often folded below the abdomen when at rest. It has two compound eyes, and the maxillae and mandibles are fused into a needle-like stylet. Each pair of legs is positioned on a separate thorax and a well-established pronotum. The front wing pairs are positioned on the mesothorax while the metathorax bears the second pairs of wings while the abdomen contains 9 segments. The combined congeneric sequence data obtained after BLAST with the morphological parameters, the bug was confirmed as *E. pallens*.
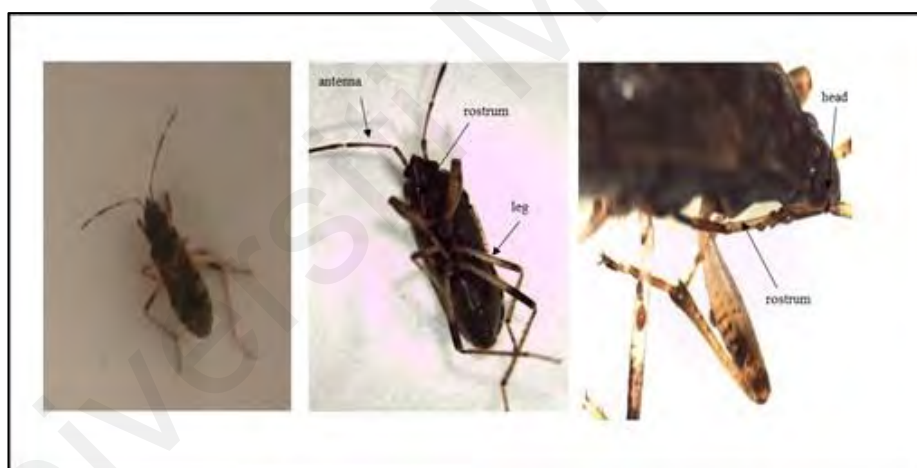
Figure 4.1: The bug, *E. pallens* and some of its key morphological features.

Adults range in sizes from 7-10 mm in length and up 3 mm wide depending on what diets they are fed. Adult females lay eggs that are sausage shaped and pale yellow before maturing into pink colors when incubation is completed (Figure 4.2). This study found that eggs laid by copulated bugs were not different ($F_{29, 60} = 1.122$; $p > 0.05$), where the mean fecundity of female bugs was 113.1 ± 4.82 eggs. The peak of egg laying was attained on the second day after egg laying commenced, with an average oviposition

period of 4.4 ± 0.12 days. The incubation period of the egg was 4.3 ± 0.21 days while nymphal development from the 1st to the 6th instars were 1.76 ± 0.14, 4.6 ± 0.43, 4.1 ± 0.34, 4.0 ± 0.28, 3.9 ± 0.37 and 2.8 ± 0.54 days. The development from egg to adult was 33.2 ± 0.65 days. Adult female and male had a mean longevity of 19.6 ± 0.42 and 9.90 ± 0.41 days respectively. The complete life cycle of the adult ranged from 35.2 to 54 days for male and female respectively. The nymphs and adult feed on seeds using their rostrum. Affected seeds usually become shriveled and the quality lost (Figure 4.2).
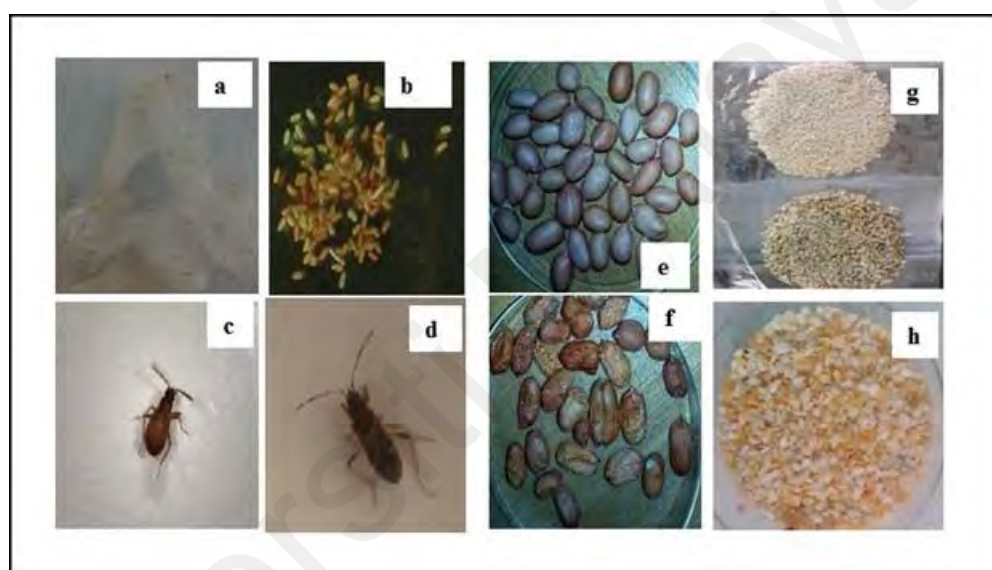
Figure 4.2: Life cycle of the bug and its effect on oil seeds.

Eggs laid on cotton gauze. b) eggs harvested from cotton gauze. c) third instar nymph. d) Adult. e, healthy peanuts kernels. f) damaged and shriveled peanut kernels. g) health and infested sesame seeds; h) sesame seeds fed on by the bugs.

4.2   Molecular analysis of the bug

The identity of the bug was determined via molecular analysis of cytochrome oxidase I (COI). The COI gene sequence was amplified using PCR and sequenced for the molecular identification. The cytochrome oxidase I gene sequences obtained for the bug samples contained sequences of nearly 700 bp for all the samples. The aligned sequence data, after incorporation of GenBank data sequence had a COI sequence data of 749 base pairs (bp) representing members of the family Rhyparochromidae, and genus

Elasmolomus pallens as revealed by congeneric sequences obtained after BLAST.

4.3 Isolation and characterization of fungi from the cadavers and rostrums of *E. pallens*

Fungal isolates were obtained from cadavers sterilized in 1% (v/v) sodium hypochlorite solution for two minutes and then rinsed three times in sterile distilled water before culturing on PDA amended with chloramphenicol (0.05%). A total of 32 fungal strains were isolated from the cadavers of *E. pallens* and morphologically identified into 5 species as *Aspergillus flavus* (11)*, A. niger* (8), *Fusarium proliferatum* (6), *A. tamarii* (4), and *Trichoderma atroviride* (3). The ITS1-5.8S-ITS4 rDNA sequences of representative isolates selected based on colonial growth and conidial viability were sequenced using molecular method. BLAST confirmed the isolates as *Fusarium proliferatum* isolate BAMF2c (F1), *A. tamarii* isolate BAMF2d (F2), *A. flavus* isolate BAMF2a (F3), *Trichoderma atroviride* isolate BAMF2e (F4), and *A. niger* isolate BAMF2b (F5). PCR amplified ITS sequences compared with existing sequence data on the GenBank database gave high level of similarities (Table 4.1).

Table 4.1: Morphological features and identification of fungi isolated from the cadavers of *E. pallens*.

Isolates	Growth morphology	Colony color	Phialides organization	Conidia shape	Probable fungus	Reference	Molecular identity	Identity %	Accession No.
F1	Luxuriant mycelium which becomes folded as culture ages	Peach with purple trace, whitish spots	Simple lateral	Oval, cylindrical, ellipsoidal to macro and microconidia	*Fusarium proliferatum*	(Geiser et al., 2013)	*Fusarium proliferatum*	100	GQ505459.1
F2	Fast growing	Green	Biseriate and radiate	Rough and globose	*Aspergillus tamarii*	(Gardezi, 2006)	*Aspergillus tamarii*	99	LC127424.1
F3	Rapid growth and densely sporulated	Dirty green	Naturally radiate	Classically globose to subglobose	*A. flavus*	(Balogun & Fagade, 2004)	*A. flavus*	100	MF319893.1
F4	Rapid growth, greyish	Glaucous to dark green	Verticillate, solitary, lageniform, and curved	Smooth, dark green, sub globose when fully mature	*Trichoderma atroviride*	(Domsch et al., 1980)	*Trichoderma atroviride*	100	KU896311.1
F5	Rapid growth and conidiation	Black	Biseriate	Coarse, echinulate and globose	*A. niger*	(Balogun & Fagade, 2004)	*A. niger*	100	KY657577.1

The preponderance of the fungal isolates obtained from the cadavers were *Aspergillus flavus* isolate BAMF2a 11 (34.38%) *A. niger* isolate BAMF2b 8 (25%), *Fusarium proliferatum* isolate BAMF2c 6 (18.75%), *A. tamarii* isolate BAMF2d 4 (12.5%) and *Trichoderma atroviride* isolate BAMF2e 3 (9.38%) based on their isolation frequencies (Table 4.2).

Table 4.2: Occurrence and conidial viability of the isolates from the cadaver of *E. pallens*.

Isolate	Name of species	Number of isolates	Mean conidial viability (%) ± S.E	Reports of pathogenicity against insects
F1	*Fusarium proliferatum*	6	94 ± 0.33	(Balogun & Fagade, 2004; NouriAiin et al., 2014; Sharma et al., 2018a)
F2	*A. tamarii*	4	93 ± 0.88	(Gardezi, 2006)
F3	*A. flavus* isolate BAMF2a	11	97 ± 0.88	(Assaf et al., 2011a; Balogun & Fagade, 2004; Karthi et al., 2018; Lee et al., 2015; Scully & Bidochka, 2005)
F4	*Trichoderma atroviride*	3	90 ± 0.33	Mostly used for the competitive exclusion of plant pathogens.
F5	*A. niger*	8	95 ± 0.33	(Balogun & Fagade, 2004; NouriAiin et al., 2014)
	M. anisopliae	-	96 ± 0.33	(Balogun & Fagade, 2004; Flores-Villegas et al., 2016a; Orduño-Cruz et al., 2015; Sönmez et al., 2016)

Fungal isolation was also done from the rostrums of adult bugs by sterilizing the whole insect before gently removing the rostrum using sterile forceps. The fungal isolates obtained from the rostrums were grouped into four genera based om molecular barcoding. These include species from the genus *Aspergillus*, *Talaromyces*, *Annulohypoxylon* and *Rizopus*. Individual species within the genus were *A. flavus*, *A. niger*, *A. oryzae*, *A. tamarii*, *T. verruculosus*, *R. stolonifer*, and *A. stygium* respectively. The ITS sequences of

these isolates were deposited in the Genbank database with appropriate accession numbers assigned to each (Table 4.3).

4.4   Molecular analysis and BLAST comparisons

The molecular characterization of the fungal isolates from both cadavers and rostrums was performed using PCR amplification of the ITS1-5.8S-ITS4 rDNA gene sequences as the molecular marker for the genotyping of the isolates.

The BLAST analysis for isolates obtained from the cadaver showed that the ITS1-5.8S-ITS4 rDNA sequences of isolate F1 (GenBank accession No. GQ505459.1) had 100% similarity with *Fusarium proliferatum*. Fungal isolate F2 (GenBank accession No. LC127424.1) had 99% similarity with *A. tamarii*. Fungal isolate F3 (GenBank accession No. MF319893.1) had 100% similarity index with *A. flavus*. Fungal isolate F4 (GenBank accession No. KU896311.1) was 100% similar with *T. atroviride*. Fingal isolates F5 (GenBank accession No. KY657577.1) showed 100% similarity with *A. niger*. Fungal isolate F6 (GenBank accession No. KP133169.1) showed 99% similarity with *A. stygium* while fungal isolate F7 (GenBank accession No. KU144421.1) had 99% similarity with *Trichoderma* sp. (Table 4.1).

The molecular analysis of the ITS1-5.8S-ITS4 rDNA molecular marker sequences of the fungal isolates obtained from the bug rostrum showed similarities with sequence data on the GenBank database. The isolate FF1, FF2, FF4 and FF6 (GenBank accession No. MH934966, MH934967, MH934974 and MH934971) showed 100% similarity with existing sequences on the database and were identified as *A. flavus*, *A. niger*, *A. oryzae*, and *A. stygium*. However, the isolates FF3 and FF5 (GenBank accession No. MH934972 and MH934969) had 99% similarities and were confirmed as *A. tamarii*, *T. verruculosus* and respectively (Table 4.3). All the isolates were identified based on morphological and molecular features.

Table 4.3: Morphological characteristics and identification of fungi isolated from the rostrums of *E. pallens*.

Isolates	Growth morphology	Colony color	Phialides organization	Conidia shape	Probable fungus	Reference	Molecular identification	GenBank similarity (%)	Accession number
FF1	Rapid growth and densely sporulated	Dirty green	Naturally radiate	Classically globose to subglobose	*A. flavus*	(Balogun & Fagade, 2004)	*A. flavus*	100	MH934966
FF2	Rapid growth and conidiation	Black	Biseriate	Coarse, echinulate and globose	*A. niger*	(Balogun & Fagade, 2004)	*A. niger*	100	MH934967
FF3	Fast growing	Green or Cinnamon	Biseriate and radiate	Rough and globose	*A. tamarii*	(Domsch et al., 1980)	*A. tamarii*	99	MH934972
FF4	Fast growing	Olive green	Phialides bear conidia in chain-like fashion	Globose to subglobose, smooth	*A. oryzae*	(Zhang et al., 2015)	*A. oryzae*	100	MH934974

Table 4.3, continued.

FF5	Moderate growth	Grey with whitish edges	Phialides and metulae branching from conidiophores	Globose to sub-glubose	*Talaromyces verruculosus*	(Chadni et al., 2017)	*Talaromyces verruculosus*	99	MH934969
FF6	Fast growth	Whitish grey before turning blackish at maturity	Conidiophore from substrate	Hyaline, smooth and ellipsoid	*Annulohypox ylon stygium*	(Benjamin et al., 2018)	*Annulohypox ylon stygium*	100	MH934971
FF7	Rapid growth, colonies are cottony, possess rhizoids and aseptate hyphae	White or greyish		Globose, greyish black, flatted at the base, powdery	*R. stolonifera*	(Hernández-Lauzardo et al., 2006)			

4.4.1 Phylogeny of fungal isolates from the cadavers of *E. pallens*

Phylogenetic tree was constructed using the MEGA 7.0 software based on the sequence data of the ITS1-5.8S-ITS4 rDNA region obtained. High similarities between the sequences of fungal isolates and species sequences deposited on the GenBank were obtained (Figure 4.3).
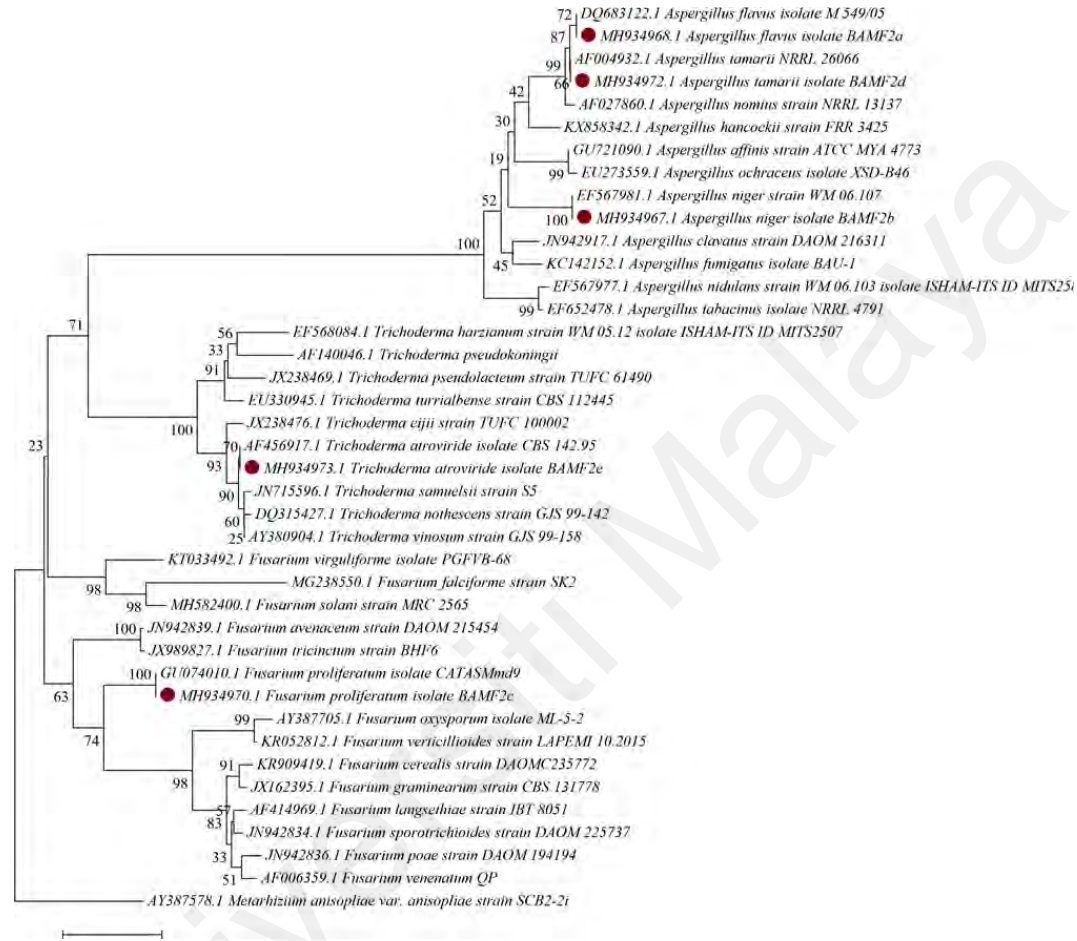
Figure 4.3: The phylogenetic tree of species-representative fungal isolates internal transcribed spacer (ITS1-5.8S-ITS4) gene sequences constructed using neighbor-joining method. The tree shows genetic relationships between isolates obtained from the cadavers of *E. pallens*. Bootstrap values shown by the nodes are based on 1000 replicates. Red dots show isolates used in this study.

4.4.2   Phylogeny of isolates from the rostrums of *E. pallens*

Phylogenetic tree based on the sequence data of the ITS1-5.8S-ITS4 rDNA was constructed using the MEGA 7.0 software to establish the relatedness of the species. High similarities between the isolates and species sequences deposited on the GenBank database were obtained (Figure 4.4). The fungal ITS sequences data were used for the phylogenetic analysis and construction of phylogenetic relationships. The results showed that the isolates obtained from the rostrum were majorly related in the *Aspergillus* section *Flavi* except for the *T. verruculosus* and *A. stygium.*
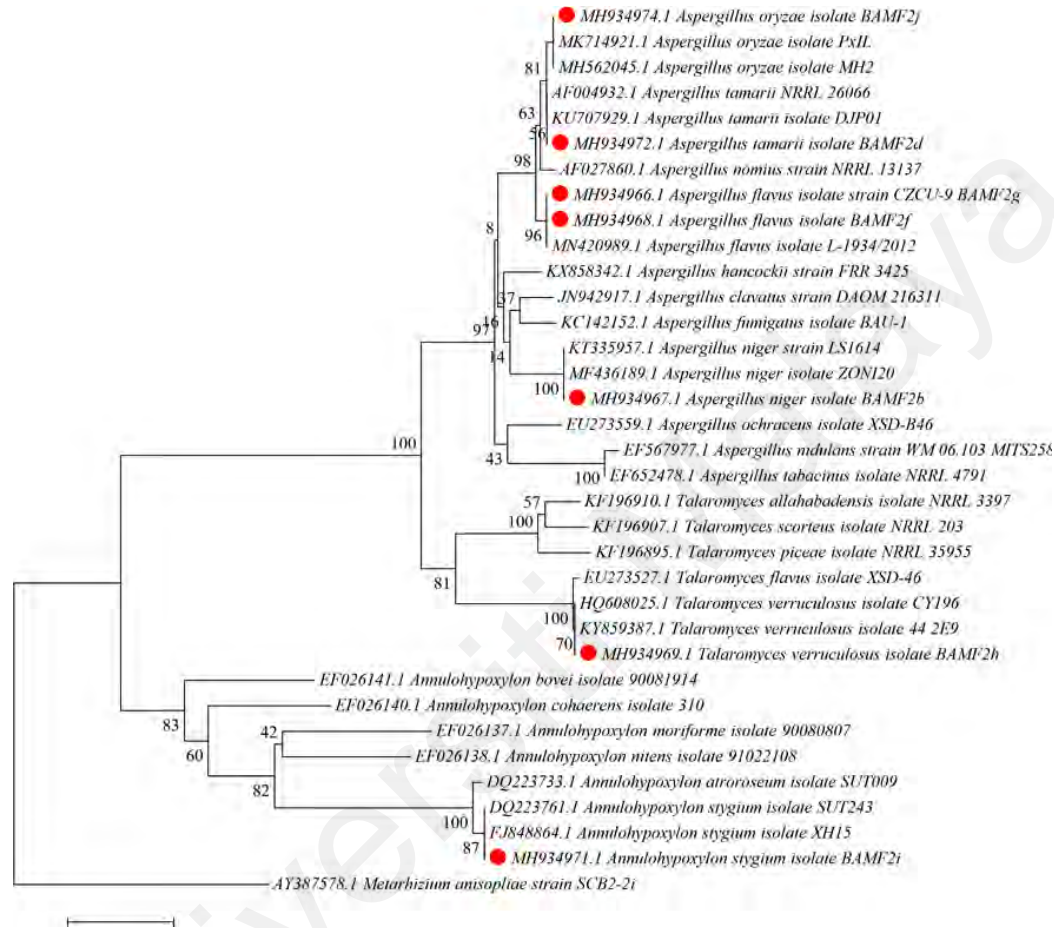
Figure 4.4: The phylogenetic tree of species-representative fungal isolates internal transcribed spacer (ITS1-5.8S-ITS4) gene sequences constructed using neighbor-joining method. The tree shows genetic relationships between isolates obtained from the rostrums of *E. pallens*. Bootstrap values shown by the nodes are based on 1000 replicates. Red dots denote isolates obtained in this study.

4.5   Investigation of *E. pallens* rostrums for the presence of mycobiome

It is believed that insects embody different species of microorganisms both on inside and outside of their extremities. Based on this assertion, we hypothesized that the interaction of *E. pallens* with the environment and their feeding habits on grains may expose them to microbes of potential significance. This hypothesis justifies the need to assess the rostrum for the presence of fungal species with which they are commonly associated. For this therefore, adult *E. pallens* were collected from peanut fields with some reared from eggs and fed with oilseeds under laboratory conditions till maturity.

In this study, a total of 257 rostrums of *E. pallens* collected from 3 different peanut fields were investigated for the presence of fungi (mycobiome) population. From the sample population, a total of 228 (88.72%) of rostrum samples were found to be positive with fungal organisms while 29 (11.28%) showed no fungal growth. The proportion of rostrums found to be associated with mycobiome between the farms were: Menglembu 94 (41.23%), Kundang 86 (37.72%) and Kinta 48 (21%). The fungal genera recovered from the rostrum samples included *Aspergillus*, *Rhizopus*, *Talaromyces* and *Annulohypoxylon* respectively. Species of the genus *Aspergillus* including *A. flavus*, *A. niger*, *A. oryzae* and *A. tamarii* were found to be prevalent in isolation frequencies between the sample locations. Others include *R. stolonifer*, *Talaromyces verruculosus* and *Annulohypoxylon stygium* which showed lower occurrences (Figure 4.5).
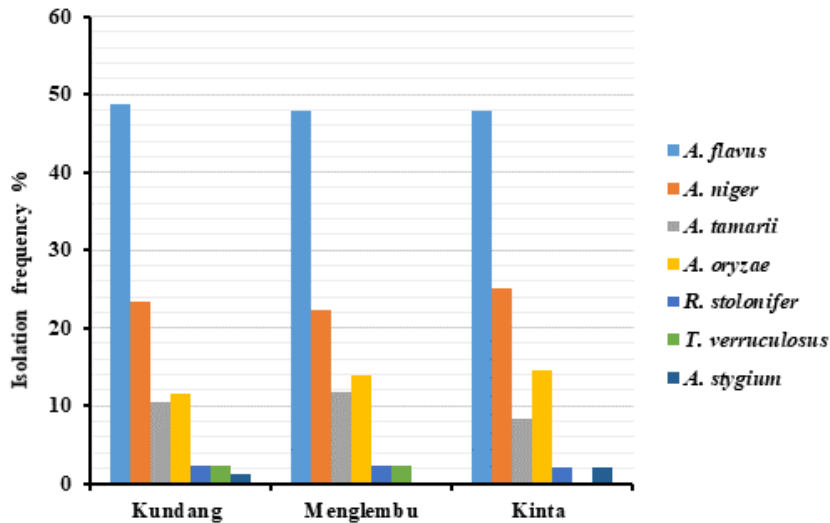
Figure 4.5: Fungal species isolated from the rostrum of *E. pallens* collected from 3 separate groundnut fields.

For the laboratory reared bugs, 170 (82.52%) rostrums were found to produce fungal isolates from the 206 rostrums evaluated. Isolates obtained from the rostrum of this group of bugs were mostly of the genera *Aspergillus* and *Rhizopus* with species including *A. flavus*, *A. niger*, *A. oryzae*, *A. tamarii* and *Rhizopus stolonifera* (Figure 4.6).

From all the rostrum samples investigated, *Aspergillus* species were observed to be the most prevalent fungal isolates (8.33 to 48.84%), *R. stolonifer* (2.08 to 2.33%), *T. verruculosus* (2.33%) and *A. stygium* (2.08%) for all samples. However, most prevalent species among the genus *Aspergillus*, were *A. flavus*, *A. niger*, *A. oryzae* and *A. tamarii* in their order of isolation frequencies. The mean occurrence of *Aspergillus* spp., on the rostrums of *E. pallens* were significantly different ($F_{5,58} = 7.494$; $p < 0.05$) between all the sampling locations.

Aspergillus section *Flavi* still occurred as the most prevalent (12.09 to 33.65%) isolates obtained from the laboratory reared bugs followed by *R. stolonifer* (1.79%) while no incidences of *T. verruculosus* and *A. stygium* were not recorded. The mean isolation of

members of *Aspergillus* section *Flavi* were statistically significant ($p < 0.05$).
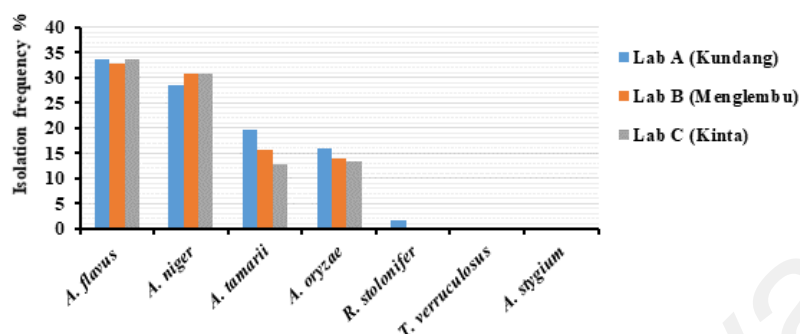
Figure 4.6: Fungal (mycobiome) isolated from the rostrum of *E. pallens* adults collected from different fields and grown from eggs to adult under laboratory conditions.

4.6   In vitro assay for aflatoxin production by *A. flavus*

From all the isolates obtained from both cadavers and rostrums, *Aspergillus* spp was the only species known for aflatoxin production, therefore the isolates were screened on coconut agar medium (CAM) for detection of aflatoxins positive strains. Eleven (11) putative *Aspergillus* spp. strains isolated from the bug cadaver were screened to select a strain that can be applied for the biological control of the *E. pallens* (Figure 4.7). In the in vitro screening, aflatoxigenic (aflatoxin producing) strains appeared pinkish (denoting secretion of aflatoxins) on the culture plates, due to the reaction of the metabolite with ammonium hydroxide vapour, atoxigenic (non-aflatoxin producing) strains appeared colourless (indicating lack of aflatoxin production). Out of the 11 putative isolates of *Aspergillus* spp obtained from the cadaver and screened on CAM, only 3 were aflatoxigenic while the remaining 8 were atoxigenic. *A. flavus* isolate BAMF2a was selected for the bioassay based on it colonial growth, conidial density and viability.
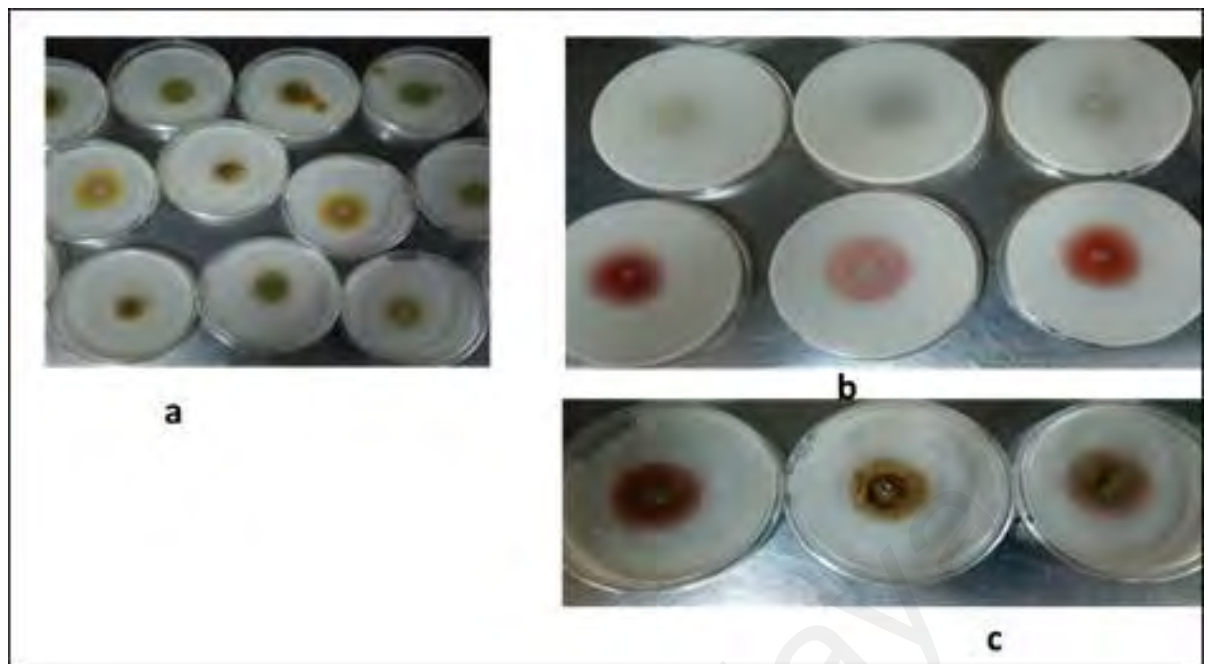
Figure 4.7: In vitro screening of *A. flavus* isolates for aflatoxins production. (a) Growth of *A. flavus* isolates on CAM after 3 days of culture; (b) positive isolates (pinkish) and negative isolates (colorless) from the bottom of the plates; (c) positive isolates view from the top of the plates.

The screening for aflatoxins secretion was also done for isolates obtained from bug rostrum. The prevalence of aflatoxin producing strains isolated from bug rostrum samples from the three farms were Kundang 67.15%, Menglembu 48.89% and Kinta 27.54% respectively. While the aflatoxin producing strains from the laboratory reared bugs were Lab B (13.50%) samples (from Menglembu), Lab A (14.50%) for samples originating from Kundang and Lab C (15.36%) for samples originally from Kinta. However, there was statistically significant difference ($F_{5,58}$ = 5.894; p < 0.05) in the proportion of toxigenic and atoxigenic *A. flavus* strains obtained from samples collected from all the 3 different farms, while no significant difference ($F_{5,58}$ = 6.894; p > 0.05) was observed between toxigenic and atoxigenic *A. flavus* strains from the laboratory samples respectively. A strongly positive (r = 0.999) correlation for atoxigenic strains and strongly negative (r = -0.999) correlation for toxigenic *A. flavus* strains were observed in relation

to the sampling locations. However, isolates from laboratory reared bugs were inversely correlated ($r = -0.999$) with no significant difference ($p < 0.05$) for aflatoxins.
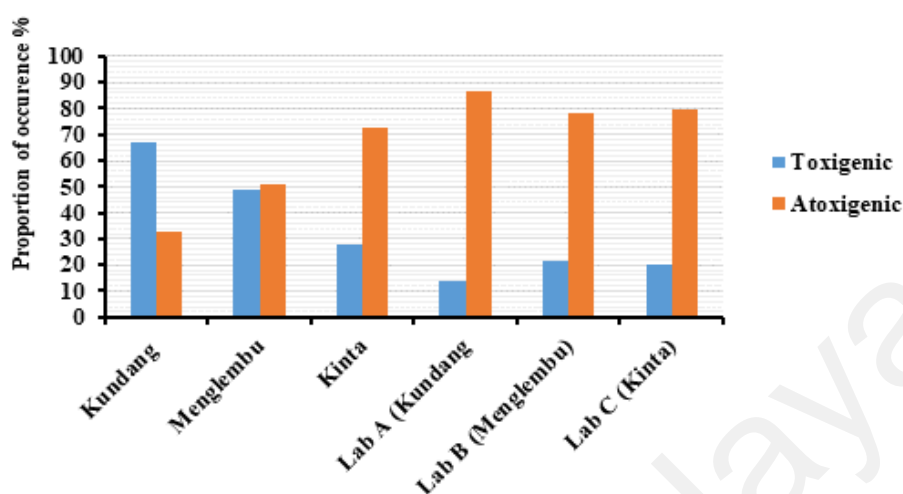
Figure 4.8: *A. flavus* **isolates screened for aflatoxins on CAM**

Isolates of *A. flavus* obtained from the bug cadavers were also screened for aflatoxin secretion. Aflatoxin-negative (atoxigenic) *A. flavus* isolate BAMF2a was selected for pathogenicity and virulence bioassay against *E. pallens* based on colonial growth, conidial density and viability. The *M. anisopliae* used in this study was reportedly isolated in Nigeria, from the cadavers of the grasshopper, *Zonocerus variegatus* (Balogun & Fagade, 2004). For the bioassay experiments, isolates with $\geq 90\%$ conidial viability (Table 4.4) were selected for the in vitro single-dose virulence bioassay against *E. pallens*.

4.7 Fungal conidia viability testing

Bioassays involving EPF are usually preceded by conidial viability testing. Usually, conidial viability is on of the significant virulence characteristics for pathogens. The viability of conidia was determined and compared with the colony forming units (CFU) for all the isolates by converting CFU data to viable conidia ml^{-1}. Isolates with conidial viability > 90% were selected for the bioassay against *E. pallens*. The conidial viability

for both the direct counting and the CFU methods were not different for all the isolates at $p < 0.05$ (Table 4.4).

Table 4.4: Comparison of conidial viability using direct counting under the microscope and the colony forming unit (CFU) methods for all the fungal isolates.

Isolates	Conidial viability (%) ± SE	CFU (%) ± SE	t-test	*P-value*
M. anisopliae	96 ± 0.33	94 ± 0.58	2.65	0.118
Fusarium proliferatum	94 ± 0.33	94 ± 0.33	1.73	0.225
A. flavus isolate BAMF2a	97 ± 0.88	96 ± 0.57	1.25	0.338
T. atroviride	90 ± 0.33	92 ± 1.20	1.89	0.119
A. tamarii	93 ± 0.88	94 ± 0.67	0.66	0.577
A. niger	95 ± 0.33	95 ± 0.33	4.00	0.057

4.8 Single-dose virulence bioassay of fungal isolates against *E. pallens*

Below (Figure 4.9) shows that all the fungal isolates tested in the bioassay showed virulence against *E. pallens*. However, the two most lethal isolates among the pathogens with the highest killing abilities were *A. flavus* isolate BAMF2a and *M. anisopliae*. The fungal isolates produced different mortality rates against *E. pallens* that were significantly different ($F_{6, 70} = 5.758$; $p < 0.05$). It was observed that *M. anisopliae* caused 100% mortality of the bug 7 days after exposure, while *A. flavus* caused 90% cumulative mortality of the bugs 10 days after treatment followed by *F. proliferatum* 68%, *A. niger* 64%, *A. tamarii* 62% and *T. atroviride* 48% compared to the control where mortality never reached 15%. *A. flavus* isolate BAMF2a and *M. anisopliae* produced the highest mortality and were selected for the multiple-dose virulence bioassay to determine the extent of their virulence potential. It was observed that mycosed bugs appeared sluggishly

restless, refused feeding and lose ability to copulate. Mortality of the mycosed bugs were recorded only when they were confirmed death, where the cadaver becomes mummified and brittle in nature.
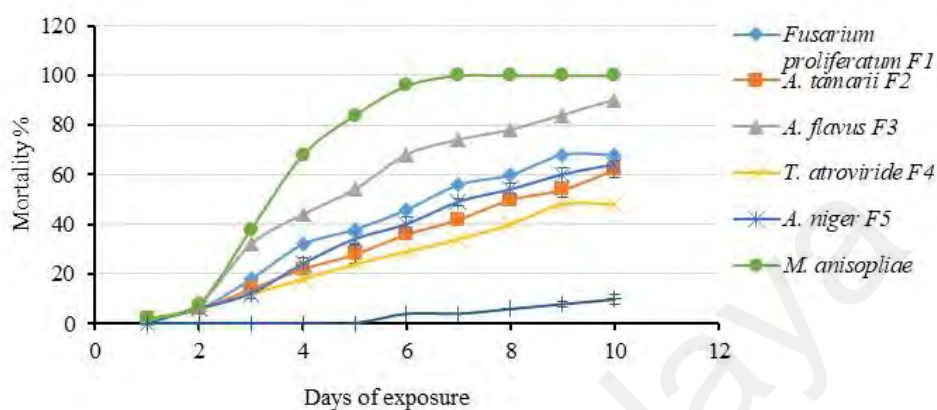
Figure 4.9: Virulence of six isolates of EPF against *E. pallens* treated with 1×10^8 conidia mL^{-1}. Mortality of the bugs was observed after every 24 h for 10 days' post-treatment. Mortalities of *E. pallens* exposed to the fungal isolates. Survival was tested using 10 adult bugs in 5 replications per fungus.

The mean mortality of *E. pallens* exposed to fungal concentration (1×10^8 conidia/ml) in the single-dose virulence bioassay was computed for all the fungal isolates tested. The mean mortality was higher between the 4th and 6th days of exposure to the fungal conidia of all isolates (Table 4.5) compared to the controls. The highest mortality recorded were observed in *A. flavus* and *M. anisopliae* respectively. The mean mortality of the bugs was significantly different between the days of exposure and individual isolates when compared using LSD at $p < 0.05$ (*F. proliferatum*: $F_{5,12} = 25.85$, $p < 0.0001$; *A. tamarii*: $F_{5,12} = 14.30$, $p < 0.0001$; *A. flavus* isolate BAMF2a: $F_{5,12} = 164.80$; $p < 0.0001$; *T. atroviride*: $F_{5,12} = 7.70$, $p < 0.002$; *A. niger*: $F_{5,12} = 30.48$, $p < 0.0001$).

Table 4.5: Mortality of *E. pallens* treated with 10^8 conidial/ml of fungal isolates.

Isolate[a]	[b] % mean mortality ± S. E					
	Day 2	Day 4	Day 6	Day 8	Day 10	Control
F. proliferatum	3.33±0.88	15.67±1.76	8.33±0.67	9.67±1.45	2.67±0.33	3.34±0.15
A. tamarii	2.00±2.08	13.33±1.86	12.33±1.20	6.67±2.67	2.67±0.67	2.33±0.33
A. flavus isolate BAMF2a	3.67±1.76	25.67±0.33	10.00±0.58	1.33±0.88	0.00±0.00	1.67±0.33
T. atroviride	3.67±1.67	10.67±1.33	10.00±1.53	8.00±1.15	5.33±0.88	2.33±0.33
A. niger	2.33±1.20	15.00±2.31	11.00±0.58	6.00±1.53	2.00±1.16	2.67±0.56
M. anisopliae	4.00±0.58	29.33±1.76	16.30±1.86	1.37±0.00	0.00±0.00	3.86±0.78

4.9 Multiple-dose virulence bioassay for EPF conidia formulated in Tween 80

In the multiple-dose virulence bioassay, the virulence of the fungal isolates was investigated using two different methods of formulations (Tween 80 and peanut oil) and applications (dipping in individual conidial concentrations, and spraying filter paper with the formulated conidia). The first method of application is the dipping bioassay consisting of replicate groups of bugs dipped into different cuoncentrations of conidial formulations. The second method was the wet filter paper bioassay where sterile filter papers were wetted inside each container with individual conidial concentrations of the pathogens before the bugs were introduced. Bugs treated under these conditions were observed after every 24 hours for mortality. Mortality data obtained were transformed and subjected to probit analysis to determine the LC_{50}, LC_{90}, LT_{50} and LT_{90} values which are indirect measures of virulence of the entomopathogens.

4.9.1 Dipping bioassay

Adult *E. pallens* were dipped briefly (10 seconds) into individual conidial concentrations (1×10^8, 1×10^7, 1×10^6, 1×10^5 and 1×10^4 conidia/ml) of the pathogens

before introducing them into plastic containers (65×45 mm) containing moist sterile filter papers (65 mm). Bug mortality records were taken after every 24 h. Mortalities of the bug due to *A. flavus* isolate BAMF2a conidial concentrations were significant, $F_{5, 60} = 5.644$, $p < 0.05$ so also for *M. anisopliae* $F_{5, 60} = 6.493$, $p < 0.05$. The result (Figure 4.11) shows that the highest conidial concentration ($1×10^8$/ml) for *M. anisopliae* produced 100% mortality on the 7th day of exposure and 90% for *A. flavus* isolate BAMF2a on the 10th day of bioassay. Here, mortality of the bug increases with increase in conidial concentrations. There were minimal mortalities in the control group compared to the test population exposed to the fungal conidia. Mortality was observed to increase among test population with increase in conidial concentrations. Higher mean mortality values were obtained for higher conidial concentrations (Table 4.6) compared to the control group [Mean mortality values with different lowercase letters are significantly different. (1) *M. anisopliae* ($F = 6.493$; df = 5, 60; $p < 0.05$). (2) *A. flavus* isolate BAMF2a ($F_{5, 60} = 5.644$; $p < 0.05$) using LSD test at 0.05%].

Table 4.6: Mean mortality of *E. pallens* caused by different conidial concentrations of fungal isolates formulated in 0.05% Tween 80.

[a]Conidial concentration	[b] % mortality mean ± S. E	
	M. ansiopliae[1]	*A. flavus*[2]
10^8	$65.09^a ± 11.71$	$44.55^a ± 8.78$
10^7	$58.18^a ± 11.92$	$37.46^{ab} ± 7.25$
10^6	$48.55^{ab} ± 10.78$	$29.64^{bc} ± 6.39$
10^5	$34.91^{bc} ± 7.63$	$25.00^{bc} ± 5.25$
10^4	$28.46^{cd} ± 6.76$	$20.82^{bc} ± 5.25$
Control	$1.09^d ± 0.63$	$2.00^d ± 0.85$
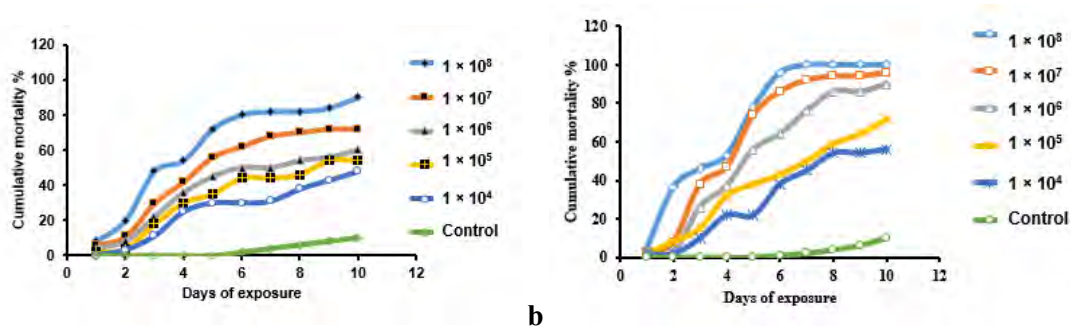
Figure 4.10: Cumulative mortality of *E. pallens* after dipping in different concentrations of fungal conidia formulated in 0.05% Tween 80. (a) *A. flavus* isolate BAMF2a and (b) *M. anisopliae*.

4.9.1.1 Lethal concentrations (LC_{50} and LC_{90}) and lethal time (LT_{50} and LT_{90}) for isolates formulated in Tween 80 for dipping bioassay.

The LC is a measure of the virulence of an isolate by means of the conidial concentrations required to kill the insect test population by half its population over a given time period. Usually, isolates that achieved 50% killing of test population with lower conidial concentration values were considered more virulent than those with higher LC values. While the LT expresses the time (in days) required for a pathogen to eliminate its target host. Lower LT values describe virulent pathogens compared to higher LC values which describe less virulent pathogens depending on the bioassay methods used. From the dose-response bioassay that involved the dipping of *E. pallens* into various conidial concentrations of the isolates, the LC_{50}, LC_{90} and LT_{50}, LT_{90} for the fungal isolates were calculated. For conidia formulated in Tween 80, the median LC_{50} obtained for the two isolates fell in the same range but slightly lower for *A. flavus* isolate BAMF2a compared *M. anisopliae* (Table 4.7). This implies that *A. flavus* isolate BAMF2a performed much better at killing half the population of the test bug than *M. anisopliae*. But conversely, the LC_{90} for *M. anisolpliae* was found to be lower compared to that of *A. flavus* isolate BAMF2a. This could likely be attributed to differences in the mechanism of responses by the fungi and the endowed ability to survive the host immune response. Also, the ability of the fungi to convert into blastopores and proliferate in the hemolymph, their persistence

and how well they exhaust nutrients in the host hemolymph can determine these variations

in the LC values obtained for the isolates.

Table 4.7: Probit analysis outcome for test fungi against *E. pallens* dipped in Tween 80 formulations.

Isolate	LC_{50}	95 % fiducial limits		LC_{90}	95 % fiducial limits	
		Lower	Upper		Lower	Upper
M. anisopliae	8.0×10^6	1.41×10^6	1.2×10^7	6.14×10^8	2.54×10^8	1.76×10^9
A. flavus isolate BAMF2a	6.75×10^6	1.38×10^6	4.11×10^7	4.42×10^9	1.01×10^9	2.57×10^{12}

Usually, the shorter the LT values is for the isolates tested against insects, the higher

the virulence of the isolates. *A. flavus* isolate BAMF2a conidia formulated in Tween 80,

showed the shortest LT_{50} against *E. pallens* compared to *M. anisopliae*; suggesting higher

virulence that resulted in killing 50% of the test insects. But conversely, the LT_{90} was

shorter for *M. anisopliae* than for *A. flavus* (Table 4.8).

Table 4.8: Summary of probit analysis on lethal time (LT) from the dose-response bioassay with *E. pallens* briefly dipped in fungal conidia formulated in Tween 80.

Isolate	LT_{50} (d)	95% fiducial limits		LT_{90} (d)	95% fiducial limits	
		Lower	Upper		Lower	Upper
M. anisopliae	3.6 ± 0.33	1.6 ± 0.43	4.7 ± 0.33	5.6 ± 0.36	1.3 ± 0.32	7.3 ± 0.43
A. flavus isolate BAMF2a	3.3 ± 0.35	1.3 ± 0.53	4.6 ± 0.73	6.2 ± 0.63	5.9 ± 1.23	7.1 ± 1.33

Although the LC_{50} and LT_{50} values for *A. flavus* isolate BAMF2a were lower which

implies greater virulence at killing 50% of the test population, (Table 4.7 and Table 4.8),

M. anisopliae achieved lowest LC_{90} and shortest LT_{90} values compared to that of *A. flavus* isolate BAMF2a. This implies that although the progression of the infection was initially faster for *A. flavus* isolate BAMF2a, yet *M. anisopliae* achieved better LC_{90} mortalities than *A. flavus*. This means that the progression of the infection in the treated insects did increase for *M. anisopliae*, resulting in greater virulence that yielded the killing of 90% of the test population faster than *A. flavus* isolate BAMF2a. Variations in the persistence of the isolates within the host, nutrient exhaustion, and immune depletion of the host may be the factors responsible for these variations.

The virulence of the two isolates used in Tween 80 formulations further compared using the paired sample t-test statistic and were significantly different (10) = 1.284, ($p < 0.05$) between the virulence of *M. anisopliae* ($M = 65.09$, $S = 38.85$) compared to *A. flavus* isolate BAMF2a ($M = 44.56$, $S = 29.12$).

4.9.2   Wet filter paper bioassay

In this bioassay method, mortalities of the bug increased with increase in conidial concentrations for the isolates used. For bugs treated with *M. anisopliae*, mortality reached 100% in 7 days after exposure. The least concentration (1×10^4 conidial/ml) of *M. anisopliae* conidia caused 40% cumulative mortalities of the bug after 10 days of exposure while *A. flavus* isolate BAMF2a produced a cumulative mortality of 92% at the highest concentration (1×10^8 conidial/ml) while the least concentration (1×10^4 conidial/ml) caused a mortality rate of 49% 10 days' post treatment (Figure 4.11).
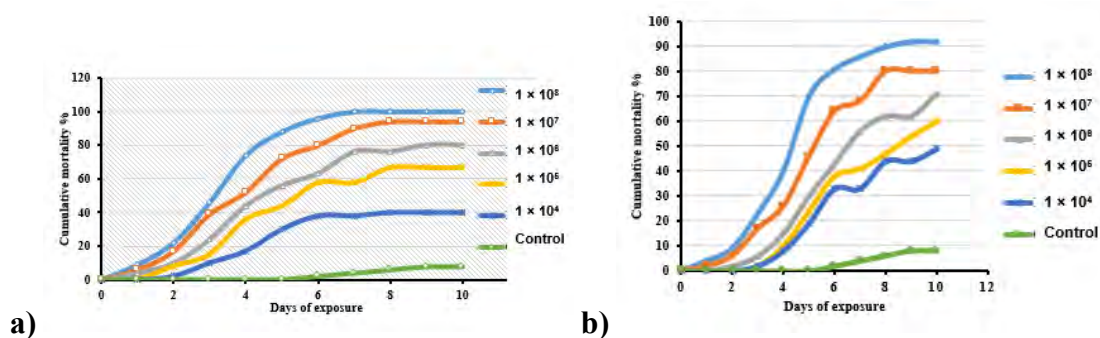
Figure 4.11: Cumulative mortality of *E. pallens* after exposure to fungal conidia formulated in Tween 80 and applied on a filter paper. (a) Virulence of *M. anisopliae* and (b) virulence of *A. flavus* isolate BAMF2a.

The mortality of the bugs caused due to individual fungal isolates were determined using analysis of variance (Table 4.9). Mean % mortality values with different lowercase letters are statistically significant (1) *M. anisopliae* ($F_{5,\,60}$= 8.825; $p < 0.001$) and (2) *A. flavus* isolate BAMF2a ($F_{5,\,60}$= 5.60; $p < 0.001$) using LSD at 0.05%.

Table 4.9: Mean mortality rate of *E. pallens* produced by conidia of fungal isolates formulated in Tween 80 and applied on filter paper.

[a]Conidial concentration	[b]Mean ± S. E	
	M. ansiopliae[1]	*A. flavus* isolate BAMF2a [2]
10^8	71.45[a] ± 10.88	54.56[a] ± 10.92
10^7	59.73[ba] ± 10.38	39.27[ba] ± 10.19
10^6	49.64[bc] ± 8.92	30.56[bc] ± 7.06
10^5	39.18[cbd] ± 7.81	24.56[cb] ± 7.06
10^4	29.27[dc] ± 7.30	20.00[cb] ± 5.76
Control	1.09[e] ± 0.49	1.27[d] ± 0.62

4.9.2.1 Lethal concentrations (LC$_{50}$ and LC$_{90}$) and lethal time (LT$_{50}$ and LT$_{90}$) for formulations in Tween 80 used in Wet filter paper bioassay.

The virulence of the fungal isolates expressed in LC values was determined for the wet filter paper bioassay. Because *E. pallens* is an above ground dwelling insect, filter paper was used as a surface on which fungal conidial suspensions were applied so that the bugs

can pick up the spores on their cuticles by contact. Horizontal transmission of the infection among the bug was observed to be further elevated due to their mating activity, and during feeding, where they mount on the food source during feeding. The result in (Table 4.10) shows that both the LC_{50} and LC_{90} for *A. flavus* isolate BAMF2a was much higher compared to the values obtained for *M. anisopliae*. Under this experimental condition, *M. anisoplae* demonstrated more virulence against the bug than *A. flavus* isolate BAMF2a based on the conidial concentrations required to cause mortalities of the bugs.

Table 4.10: Probit analysis results for fungal conidia formulated in 0.05% Tween 80 applied on filter paper for activity against *E. pallens*.

Isolate	LT_{50} (d)	95% fiducial limits		LT_{90} (d)	95% fiducial limits	
		Lower	Upper		Lower	Upper
M. anisopliae	3.8± 1.33	3.1± 0.36	4.2± 0.83	5.4± 0.73	5.1± 1.43	6.1± 0.33
A.flavus isolate BAMF2a	5.0± 0.83	4.1± 0.45	5.5± 0.97	8.8± 1.13	7.6± 0.33	12.1± 0.33

However, under this experimental condition, *A. flavus* isolate BAMF2a performed poorly based on the LC_{50} and LC_{90} values obtained (Table 4.10). The LT_{50} and LT_{90} obtained were longer days compared to what was obtained for *M. anisopliae* (Table 4.11). This may likely be due to poor interactions between the fungal conidia and the host cuticle or the grooming process that occurs among the bugs, which enable them to wade off conidia adsorbed to their body surface.

Table 4.11: Summary of probit analysis on lethal time (LT) from the filter paper bioassay against *E. pallens*.

Isolate	LC$_{50}$	95% fiducial limits		LC$_{90}$	95% fiducial limits	
		Lower	Upper		Lower	Upper
M. anisopliae	6.85×10^6	1.33×10^6	1.55×10^6	2.57×10^8	1.15×10^8	1.24×10^9
A. flavus isolate BAMF2a	9.36×10^7	1.37×10^7	3.22×10^8	6.50×10^9	4.16×10^9	1.35×10^{12}

To further determine the performance of the two isolates used in the filter paper bioassay against *E. pallens*, their mortality data were compared using the paired samples t-test. This was done to determine whether or not there are differences in virulence between the isolates. The mean mortalities of the bugs compared were significantly different ($p < 0.05$), indicating differences in the virulence potentials of *M. anisopliae* ($M = 71.46$, $S = 35.91$) and *A. flavus* isolate BAMF2a ($M = 54.55$, $S = 36.20$). Where $M = mean$, and $S = standard\ deviation$.

4.10 Effect of oil adjuvant on virulence of the isolates (conidial formulation in peanut oil)

Previous studies (Alves et al., 1998a; Polar et al., 2005b) utilized different non-evaporative oil adjuvants for conidial formulations for application in the biological control of certain insect pests. Peanut oil was rarely used even when it is considered an oil adjuvant for enhancing the virulence of entomopathogens. More also, because *E. pallens* is a pest that exploit peanut oil, testing the virulence of entomopathogens using peanut oil as an adjuvant was worth the study. Here, different concentrations of the fungal isolates conidia were formulated in 5% sterile peanut oil and applied for the bioassay

experiments. For formulations in peanut oil, the dipping and wet filter paper applications were employed to study the lethal effect of the fungal pathogens against *E. pallens*.

4.10.1 Dipping bioassay (formulation in oil)

For the dipping bioassay experiment, the result (Figure 4.12) shows that *M. anisopliae* formulated in oil produced a 100% cumulative mortality of the bugs after 6 days post-treatment. This indicate how the virulence of *M. anisopliae* was enhanced by the oil adjuvant compared to when the isolate was formulated in Tween 80 where 100% mortality was achieved in 7 days. Here, all the conidial concentrations showed increased infectivity compared to formulations in Tween 80. Under this bioassay condition, *E. pallens* mortality reached 100% for the two highest conidial concentrations (1×10^8/ml and 1×10^7 conidia/ml). Mortalities were confirmed by culturing the cadavers on moist sterile filter paper and examined under the light microscope for conidial development.
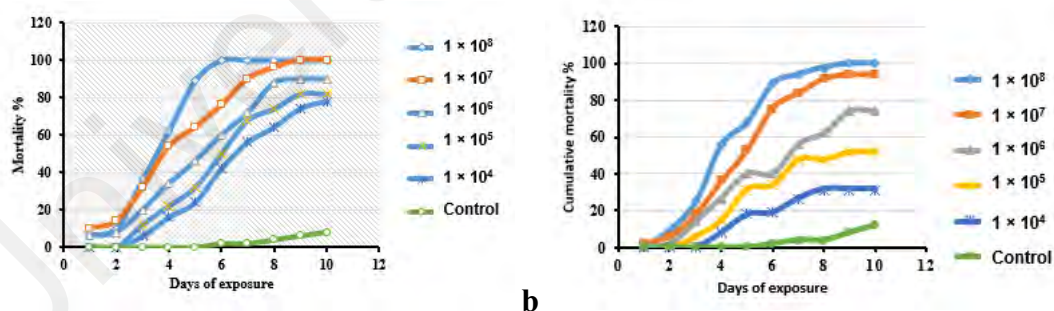
Figure 4.12: Cumulative mortality of *E. pallens* after dipping into different conidial concentrations formulated in oil for the dipping bioassay. (a) Virulence of *M. anisopliae* (b) Virulence of *A. flavus* isolate BAMF2a.

The conidial concentration (1×10^8 conidia/ml) of oil formulated *A. flavus* isolate BAMF2a produced a 100% mortality of *E. pallens* in 9 days, followed by 94% produced by conidia concentration 1×10^7 conidial/ml (Figure 4.12). Under this experimental

condition, conidia formulated showed enhanced virulence by causing a 100% mortality of the bugs in 9 days as against the 10 days observed for formulations in Tween 80. This shows entomopathogenic fungal conidia formulated in adjuvant oil can undoubtedly enhance the virulence of a pathogen.

The bug mortality values recorded for each fungal conidial concentration were subjected to analysis of variance to compare the mean mortality obtained for the bioassay involving the fungal isolates. Higher mean mortality values were obtained for formulations containing higher conidial concentrations. However, low mortalities were observed for the controls (Table 4.12). Mean mortality values with different lowercase letters were significantly different (1) *M. anisopliae* ($F_{5, 59}$ = 4.314, $p < 0.05$) and (2) *A. flavus* isolate BAMF2a ($F_{5, 60}$ = 5.589, $p < 0.05$) using LSD test at 0.05%.

Table 4.12: Mean mortality of *E. pallens* produced by fungal conidia formulated in peanut oil and used for the dipping bioassay.

[a]Conidial concentration	[b] % mortality mean ± S. E	
	M. ansiopliae[1]	*A. flavus* isolate BAMF2a [2]
10^8	62.18[a] ± 12.59	60.00[a] ± 12.06
10^7	57.82[a] ± 11.54	57.09[ab] ± 10.84
10^6	46.73[a] ± 10.65	45.64[ab] ± 10.77
10^5	38.91[ab] ± 10.47	33.09[bc] ± 8.77
10^4	32.73[b] ± 9.36	26.18[cd] ± 8.07
Control	2.20[c] ±0.92	1.09[e] ± 0.63

4.10.2 Lethal concentrations (LC_{50} and LC_{90}) and lethal time (LT_{50} and LT_{90}) for oil formulations used in dipping bioassay.

The virulence of the isolates formulated in oil was estimated from the transformed mortality values using the probit analysis model. The LC_{50} is a measure of the of the concentration of fungal conidia required to kill 50% of the insect test population while LT_{50} measures the virulence of a pathogen by the length of time taken to kill a host. Usually, the lower the LC_{50} and LT_{50}, the greater the virulence of the isolate. The result (Table 4.13) shows that the LC_{50} of *M. anisopliae* was slightly lower than *A. flavus* isolate BAMF2a. However, the LC_{90} for *A. flavus* isolate BAMF2a was higher compared to that of *M. anisopliae*. This depicts greater virulence for *M. anisopliae* both at killing 50 and 90% of the test bug population.

Table 4.13: Probit analysis results the fungal isolates formulated in peanut oil against *E. pallens* in the dipping bioassay.

Isolate	LC_{50}	95% fiducial limits		LC_{90}	95% fiducial limits	
		Lower	Upper		Lower	Upper
M. anisopliae	2.14×10^6	1.97×10^4	9.23×10^6	4.15×10^8	1.60×10^8	1.39×10^9
A. flavus isolate BAMF2a	4.75×10^6	1.38×10^6	4.11×10^7	1.42×10^9	1.01×10^9	2.57×10^{12}

Estimation was also done to determine the LT_{50} and LT_{90} values for fungal isolates formulated in oil. Here, the shortest LT_{50} and LT_{90} were for *M. anisopliae* (Table 4.13). The fungal isolates formulated in oil for the dipping bioassay demonstrated shorter LT values compared to formulations in Tween 80 with similar dipping bioassay. This shows enhanced virulence of the pathogens in oil formulation.

Table 4.14: Summary of probit analysis on lethal time for fungal isolates conidia formulated in peanut oil used for dipping bioassay against *E. pallens*.

Isolate	LT_{50} (days)	95% fiducial limits		LT_{90} (days)	95% fiducial limits	
		Lower	Upper		Lower	Upper
M. anisopliae	3.0± 0.55	1.3± 0.78	4.0± 0.85	5.2± 1.45	5.2± 0.95	7.2± 1.45
A. flavus isolate BAMF2a	3.3± 0.45	1.3± 0.86	4.5± 0.77	5.8± 1.51	6.2± 0.58	7.9± 0.95

To further validate the observed differences in virulence (performance) between the two isolates used for the dipping bioassay, the mean mortalities of the of the bug caused by the fungal isolates (Table 4.12) were compared using the paired sample *t*-test to establish whether there was any difference in virulence between the two isolates. The test statistic (*t*- test) revealed no significant difference ($p > 0.05$) in the killing ability of the fungal isolates. The comparative statistical test result shown as: *A. flavus* isolate BAMF2a ($M = 60.00, S = 40.01$) and *M. anisopliae* ($M = 62.18, S = 41.76$), t (10) =1.284, $p > 0.05$. Where $M = mean$, and $S = standard\ deviation$. This means that under this experimental condition, both *A. flavus* isolate BAMF2a and *M. anisopliae* showed no difference in virulence against *E. pallens*. This therefore supports the claim that conidia formulated in oil often demonstrate enhanced infectivity and virulence.

4.10.3 Wet filter paper bioassay

This experimental bioassay parameter utilized sterile filter papers impregnated with conidia of test isolates at estimated concentrations applied in order to adhere to the cuticles of the bugs. The conidia of the test isolates were formulated in oil as mentioned above before applying unto the filter papers. Adult bugs were then introduced into the plastic container containing the contaminated filter papers to establish contact with the spores on the filter paper.

Under this bioassay condition, all the conidial formulations *A. flavus* isolate BAMF2a showed the capacity to infect the bugs. The highest conidial concentration 1×10^8 conidia/ml caused 100% cumulative mortality 9 days after treatment and 94% cumulative mortality 10 days after treatment for 1×10^7 conidia/ml. However, the least concentration 1×10^4 conidia/ml produced 39% cumulative mortality 10 days after treatment. Mortalities of the bugs caused by the different conidial concentrations were significantly different ($F_{5, 60} = 4.394$; $p < 0.05$). For *M. anisopliae*, the highest conidial concentration 1×10^8 conidia/ml produced 100% mortality 6 days after treatment, while conidial concentration 1×10^7 conidia/ml produced a cumulative mortality of 100% 10 days after treatment. The least cumulative mortality was 46% 10 days after treatment for conidial concentration 1×10^4 conidia/ml. There was significant effect of the formulated conidial concentrations on the mortality of the bugs ($F_{5, 60} = 4.363$; $p < 0.05$) (Figure 4.13). Mortalities in the control were less than 10%. All the cadavers of the bug recovered and cultured on damp filter paper developed mycelia common to the isolates within 5-7 days.
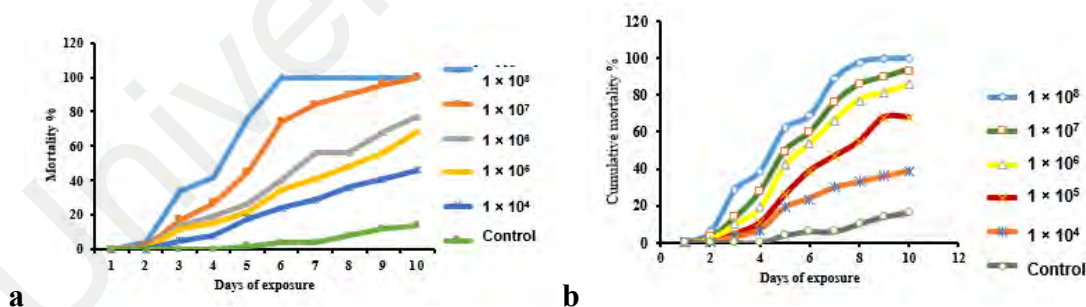
Figure 4.13: Cumulative mortality of *E. pallens* exposed to different concentrations of fungal isolates conidia formulated in oil and applied on sterile filter paper. (a) virulence of *M. anisopliae* and (b) virulence of *A. flavus* isolate BAMF2a.

Under this experimental condition, the mean mortalities of the bug caused by individual conidial concentrations of the isolates were compared to establish the killing potential of the isolates. The mean mortality values were highest for the highest conidial

concentrations (10^8 and 10^7) in all the isolates, compared to the lowest in the control. Mean mortality values with different lowercase letters are significantly different (1) *M. anisopliae* ($F_{5, 60} = 4.363$, $p < 0.05$) and (2) *A. flavus* isolate BAMF2a ($F_{5, 60} = 4.3934$ $p < 0.05$) using the LSD at 0.05%.

Table 4.15: Mean mortality of *E. pallens* produced by fungal conidia formulated in oil and applied on sterile filter paper for the wet filter paper bioassay.

[a]Conidial concentration	[b]Mean ± S. E	
	M. ansiopliae[1]	*A. flavus* isolate BAMF2a [2]
10^8	58.91[a] ± 12.84	52.64[a] ± 11.70
10^7	53.18[a] ± 12.03	45.91[a] ± 10.23
10^6	48.00[ba] ± 10.35	43.46[a] ± 9.77
10^5	35.82[bc] ± 9.28	36.27[ba] ± 8.36
10^4	31.46[cb] ± 8.80	31.09[bc] ± 6.95
Control	2.18[d] ± 0.95	1.09[d] ± 0.63

4.10.4 Lethal concentration (LC$_{50}$ and LC$_{90}$) and lethal time (LT$_{50}$ and LT$_{90}$) for formulations in oil used for the wet filter paper bioassay.

In the wet filter paper bioassay experiment containing oil formulated fungal conidia, the median lethal concentration was determined for *A. flavus* isolate BAMF2a and *M. anisopliae* using the probit analysis model. The LC$_{50}$ of *A. flavus* was lower compared to *M. anisopliae*, (Table 4.16) suggesting that *A. flavus* isolate BAMF2a demonstrated greater virulence against *E. pallens* at killing 50% of the test population under the experimental condition. However, *M. anisopliae* achieved a lower LC$_{90}$ faster than *A. flavus* isolate BAMF2a. This may depend to a large extend on the persistence of the individual test isolates, host immune response and nutrients availability.

Table 4.16: Probit analysis results for fungal conidia formulated in peanut oil and applied on filter paper for infectivity against *E. pallens*.

Isolate	LC_{50}	95% fiducial limits		LC_{90}	95% fiducial limits	
		Lower	Upper		Lower	Upper
M. anisopliae	3.92×10^6	1.86×10^4	7.23×10^6	5.37×10^8	1.78×10^8	1.45×10^9
A. flavus isolate BAMF2a	1.95×10^6	2.17×10^4	1.12×10^7	3.66×10^9	8.56×10^8	11.16×10^9

However, the lethal time for the isolates to achieve 50 and 90% killing effect on the bug was determined for the wet filter paper bioassay. In the result (Table 4.17), *A. flavus* isolate BAMF2a achieved a shorter LT_{50} than *M. anisopliae* suggesting higher virulence at killing 50% of the test population under this experimental condition. However, conversely, *M. anisopliae* achieved a much shorter LT_{90} than *A. flavus* isolate BAMF2a.

Table 4.17: Summary of probit analysis on lethal time from the virulence of fungal isolates conidia formulated in oil and applied on filter paper for activity against *E. pallens*.

Isolate	LT_{50} (days)	95% fiducial limits		LT_{90} (days)	95% fiducial limits	
		Lower	Upper		Lower	Upper
M. anisopliae	3.2 ± 1.55	1.3 ± 0.47	4.2 ± 0.59	5.7 ± 1.25	1.5 ± 0.88	6.3 ± 0.85
A. flavus isolate BAMF2a	3.0 ± 1.43	1.3 ± 0.35	4.1 ± 0.78	6.6 ± 1.44	5.9 ± 0.45	8.1 ± 0.75

Under this experimental bioassay parameter, the mean mortality of the bug caused by these two isolates (Table 4.19 and Table 4.20) were compared to further determine if any differences in virulence exists between the isolates other than just the result of the median lethal concentrations (LC) and the median lethal time (LT) which are the yardsticks for

measuring the virulence of a pathogen. The mean mortality for the isolates were compared using the paired sample t- test. The test statistic revealed no significant difference ($p >$ 0.103) in virulence between *A. flavus* isolate BAMF2a ($M = 52.64$, $S = 38.81$) and, *M. anisopliae* ($M = 58.91$, $S = 42.59$) t (10) = 1.796, $p > 0.05$ under the experimental conditions. Where M = *mean*, and S = *standard deviation*. This therefore means that both fungi formulated in peanut oil and utilized under this experimental condition showed no differences in virulence against *E. pallens*.

4.11   Hydrolytic enzyme assay

The production of hydrolytic enzymes by the fungal pathogens was assayed in the course of this study. This was essential because hydrolytic cuticle-degrading enzymes are known to play important role in the degradation of the host cuticle during fungal pathogenesis. The assay showed a significant difference in the activity of proteases ($F_{7, 62}$ = 14.162; $p < 0.05$) for both *A. flavus* isolate BAMF2a and *M. anisopliae*. However, the activities of chitinases were not different ($F_{5, 48}$ = 1.626; $p < 0.05$) and lipase activities were also not different ($F_{3, 32}$ = 103.976; $p < 0.05$) for both fungal isolates. Enzyme activity (U/ml) values with different lowercase letters are significantly different ($F_{5, 48}$ = 1.626; $p < 0.05$) using the LSD at 0.05%.

Table 4.18: Assay of hydrolytic enzymes from the entomopathogenic fungal isolates.

[a]Total enzyme activity U/ml			
[b]Fungus	Protease	Chitinase	Lipase
M. anisopliae	0.74 ± 0.16^c	0.89 ± 0.12^c	1.59 ± 0.12^d
A. flavus BAMF2a	0.59 ± 0.20^b	0.87 ± 0.03^c	2.51 ± 0.02^d

CHAPTER 5: DISCUSSION

5.1 The morphology and biology of *E. pallens*

The morphological description of *E. pallens* in the perspectives of structures like the head, antenna, thorax, pronotum and abdomen which houses the sternum and trichobothria, are typical of *E. pallens* in the family Rhyparochromidae as similarly reported in Gullan and Cranston (2014) and Katinka (2016). These morphological features used to confirm the molecular identification of *E. pallens* agrees with earlier descriptions by Gullan and Cranston (2014).

The biology of *E. pallens* described in this study, conforms with the findings of Osman et al. (2009) who reported an incubation period of 4.3 ± 0.17 days for the eggs, while a complete development from egg to adult lasted for 33.2 ± 0.52. Additionally, the complete life cycle of the bug agrees with the findings of Berhe et al. (2014) who reported a range of 32-54 days as lifr cycle of the bug. Furthermore, the mean fecundity of the bug agrees with the findings of Tarig (2005) who reported a mean fecundity of 113.1 ± 5.65 eggs with a peak laying period on the second day of an average oviposition period of 4.4 ± 0.07 days.

5.2 Fungal isolation and characterization

Isolation of fungi was done for both the cadavers and rostrums of the bug.

5.2.1 Isolates from the cadavers of *E. pallens*

EPF are known natural pathogens infecting insect hosts that can be collected from the field environment either infected or dead and incubated under laboratory conditions to isolate, document, and use the pathogens as biological control agents (Inglis et al., 2001).

In this study, different species of fungi were isolated from the cadavers of *E. pallens*, some of which have been reported in previous studies. For example, the isolation of *A. flavus* from the cadavers of insects have similarly been reported in Lee et al. (2015) and Assaf et al. (2011a) respectively; whereas the isolation of *Fusarium* spp. was reported in Balogun and Fagade (2004), NouriAiin et al. (2014) and Sharma et al. (2018a). Gardezi (2006) reported the isolation of *A. tamarii* from insect cadaver and testing its pathogenicity against several insect species. *A. flavus* was found to be the most abundant isolate among the fungal isolates identified. Similar finding has been reported in a study on the almond bark beetle, *Scolytus amygdali* (Asma et al., 2017).

5.2.2   Isolation from the rostrum of *E. pallens*

Plant bugs are widely known as damaging pests of a wide variety of plant species. They inflict damages on crop plants by sucking their nutrients via their piercing-sucking mouthparts (Schaefer & Panizzi, 2000b). Like animals, insects live in a world of microbes. They are exposed to various microorganisms on surfaces and in association with food materials. Though few of the microorganisms encountered are pathogenic to the host, however, majority are of no specific importance to the insects and are easily lost during cuticle shedding, poor adhesion or are passed down the gut alongside food (Douglas, 2015).

The mycobiome of *E. pallens* rostrum identified in this study will further the understanding of the relationship complex between these fungi and their insect host, and what potential risks they pose. Fungi belonging to four genera were isolated from the rostrums of *E. pallens* collected from different farm locations, and from adults reared in the laboratory. The isolates characterized included species of *A. flavus*, *A. niger*, *A. oryzae*, *A. tamarii*, *R. stolonifer*, *T. verruculosus* and *A. stygium*. All the isolates obtained

showed consistent occurrence for all the samples investigated except *T. verruculosus* and *A. stygium* which showed sporadic presence between sample locations. *A. flavus* showed preponderance among all the isolates obtained from the rostrums of both field-collected and laboratory reared bug samples. This may be due to common association between the fungus, husks and kernels of peanut (Atayde et al., 2012) during feeding. It has earlier been described as a widely cosmopolitan mold with high affinity for oilseeds and nuts (Amaike & Keller, 2011; Hocking & Pitt, 2012). A number of surveys earlier conducted on the myco-flora of some crop commodities showed *A. flavus* as the most commonly encountered fungal species on oilseeds and nuts, while *A. parasiticus* was rarely found to occur (Fapohunda et al., 2012). Furthermore, the predominance of the *Aspergillus* spp. can be linked to reports that they are associated with the kernels and pods of peanuts of different cultivars (Zorzete et al., 2013) and are considered storage-associated fungi (Opio & Photchanachai, 2018).

However, there were no incidences of isolation of *T. verruculosus* and *A. stygium* from the rostrum samples of laboratory reared *E. pallens*. This suggests that not all fungi isolated from the rostrums of the bug can be described as obligate mycobiome of the rostrum or are grain-associated, considering the absence of these two fungi from the rostrums of laboratory reared bugs. The association of these fungi with *E. pallens* rostrum could be linked to its interactions with the environment. The isolation of *T. amestolkiae* from the red bug Pyrrhocoridae (Hemiptera) has been previously reported (Jaber et al., 2016). Ezekiel et al. (2014) has reported the incidences of *Talaromyces* and *Rhizopus* species from sesame seeds which is one of the target crops for *E. pallens*. Although it is unknown whether *E. pallens* possess any fungivory characteristics, yet, its interactions with organic matter during hibernation and feeding, may be responsible for its rostrum harboring these fungi. The social behavior, niche composition and the spectrum of

substrates to which the bug is exposed both under field and laboratory conditions may be attributed to the fungal presence on their rostrum, and the variations in their distributions. The isolation of *A. stygium* from the rostrum of *E. pallens* may be linked to its feeding activity. Sometimes, the bug feed on leftover peanut pods trapped inside decaying remains of the plant. The presence of this fungus on the bug rostrum, may support the findings of Vasilyeva et al. (2016) who reported unidentified substrate for the southastern Asian species of *Annulohypoxylon*. Recent report by Benjamin et al. (2018) on the isolation of *A. stygium* from decomposing tree trunk suggests that its presence on the rostrum of *E. pallens* may be due to the interaction of the bug with the environment.

The presence of mycobiota on the rostrums of laboratory reared *E. pallens* has also been confirmed in this study. The grain seeds on which the bugs fed under laboratory conditions may be responsible for the presence of grains associated fungi on the rostrums; as they were reared in the laboratory purely on peanuts and sesame. According to Douglas (2009), insects are exposed to various microorganisms on surfaces and in association with food materials. Pitt et al. (2015) asserted that field fungi are commonly associated with the invasion of developing or mature seeds before harvest, while storage fungi contaminate seeds during storage under humid conditions. A good example are *Aspergillus* species, which according to Sellon and Kohn (2014) infect agricultural crops such as corn, peanuts, cotton and tree nuts. These crops are described as highly susceptible to invasion by *A. flavus*, most often during harvest than during transport and storage (Amaike & Keller, 2011).

5.2.3 The phylogeny and ecological proximities of the fungal isolates

Fungi are basically categorized into saprophytes, plant pathogens, myco-parasites, animal pathogens and endophytes based on the diverse nature of their modes of nutrition

(Sharma et al., 2018b). The phylogenetic relationship of the fungal isolates from the cadaver (Figure 4.3 and Figure 4.4) shows very close proximities with regards to their ecological niches. These fungi are generally considered as saprophytes as well as entomopathogens, and their close proximities simply suggests their exclusive biological role regarding these two modes of nutrition. The pathogenicity of the cadaver isolates against *E. pallens* adds to the knowledge of how fungi from the same ecological niche has impacted on the mortality of the bug.

5.2.4   In vitro assessment of *A. flavus* isolates for their ability to produce aflatoxins

It is a generally believed that *A. flavus* is a harmful pathogen to humans and animals, aside its ability to infest agricultural grain products and its ability to produce aflatoxins (Amaike & Keller, 2011; Hedayati et al., 2007; Pasqualotto, 2009). However, because of these traits among some strains of *A. flavus*, some researchers are pessimistic with the view to objecting the recommendations for its use as a biological control agent (Gupta & Gopal, 2002). But contrary to this pessimism, several reports have emphasized and described *A. flavus* as a ubiquitous mould and not all of its strains can produce aflatoxins (Ezekiel et al., 2014; Frisvad et al., 2019; Humphrey, 2017; Mamo et al., 2018). Thus, with proper strain selection, formulation and application technique, the perceived objection, pessimism and paranoia for potential hazards associated with the fungus can be quelled (Gupta & Gopal, 2002). Additionally, atoxigenic *A. flavus* strains are known to be used for the biological control of toxigenic strains in crop plantations via competitive bio-exclusion to prevent aflatoxins contamination in grains (Dorner et al., 1992; Humphrey, 2017). In this study, 8 (73%) of the *A. flavus* strains isolated from the cadavers of *E. pallens* were atoxigenic while 3 (27%) were aflatoxigenic (Figure 4.8). Drummond and Pinnock (1990) found 20% of *A. flavus* isolated from sugarcane mealybugs to be atoxigenic. Similarly, Gupta and Gopal (2002) observed that out of the

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seven (7) *A. flavus* isolates obtained from three insect groups, *Stephanitis typica* (lace bug), *Opisina arenosella*, and *Proutista moesta*; only two of the isolates were toxigenic.

However, proportion of toxigenic to atoxigenic *A. flavus* isolated from the bug rostrum was different from those obtained in the bug cadavers. The abundance of the isolates was linked with the sampling locations. In our study, the proportion of toxigenic *A. flavus* isolates obtained from rostrum samples were 67.15% (Kundang), 48.89% (Menglembu) and 27.54% (Kinta) respectively (Figure 4.8). The result shows that not all strains of *A. flavus* were toxigenic, as some could not produce the toxins. Abdel-Hadi et al. (2011) confirmed that not all wild-type strains of *A. flavus* can produce the secondary metabolite, aflatoxins. A study by Mamo et al. (2018) found 68% of *A. flavus* isolates obtained from different locations in China to be toxigenic. However, greater than 50% of *A. flavus* isolated in Nigeria were found to be atoxigenic (Donner et al., 2010) and in Thailand, 74% of isolated *A. flavus* were found to be atoxigenic (Tran-Dinh et al., 2014). Although toxigenic *A. flavus* isolated from samples collected at Kundang showed higher occurrence, the toxigenic and atoxigenic strains from all the samples occured differently ($p < 0.05$). The observed differences in their frequency of occurrence may reflect adaptation which may lead to different tendencies of vulnerabilities to interactions with toxigenic strains in those locations (Pitt et al., 2015). At Kundang, the bugs were collected while hibernating under decaying groundnut haulms. This might be responsible for the large number of toxigenic *A. flavus* on their rostrums, since, the growth of *Aspergillus* spp. in decaying vegetation has been reported (Meredith & Ulrich, 2013). In this study, a strongly negative ($r = -0.999$) correlations was obtained for atoxigenic and toxigenic *A. flavus* strains from laboratory samples. This could be attributed to the contamination of the diets with which the bugs were fed. This is because contamination of staple foods such as peanuts, maize and rice by aflatoxins producing *A. flavus* as has been widely

described (Kumar et al., 2008; Mamo et al., 2018; Passone et al., 2007). However, we perceive that it is very possible that fungal conidia contained on the rostrum of *E. pallens* could be lost during probing of the peanut shells due to mechanical barriers, abating the fears of aflatoxins contamination.

5.3   Single-dose virulence bioassay against *E. pallens*

The single-dose virulence assessment bioassay shows that all isolates tested were pathogenic against *E. pallens*, although the rate of mortalities differed ($p < 0.0001$). This may be attributed to established facts that sucking, forest and soil-dwelling insect pests are very much susceptible to infection by EPF, because of the vulnerability of their cuticle to conidial adhesion, germination and penetration contrary to control agents such as bacteria, viruses, parasitoids and nematodes that must be ingested to initiate infection (Khan et al., 2012). Their mechanisms of action are said to be due to inherent pathogenicity (the infective capacity of an entomopathogen resulting in disease) and virulence (the degree to which the host tissues are colonized by the pathogen with time) traits, which are gene-specified intrinsic features of EPF. However, these intrinsic features are largely dependent on host immune response, nature of formulations, growth medium composition, abiotic factors, and methods of application used (Santos et al., 2018b).

In the findings of this study, *A. flavus* isolate BAMF2a and *M. anisopliae* showed greater potential for virulence against *E. pallens*. *A. flavus* isolate BAMF2a produced a mortality rate of 90% after 10 days compared to *M. anisopliae* which demonstrated 100% mortality of the bugs 7 days after treatment (**Figure 4.9**). Whereas, other isolates caused lower mortalities. The two most effective entomopathogens were selected for multiple-dose virulence bioassay against the bug based on their lethal effects, which could be

related to the assertion by Ferron (1978), that both fungal species can secrete lipolytic enzymes during infection, which enables them to degrade the proteo-chitin complex. This could most likely explain why the two isolates showed more virulence than the other isolates used. Studies have established that killing of insects by EPF involves some series of successive steps that could lead to the death of the host depending on its ontogenic stage and immune response. These steps include: (1) adhesion of the fungal conidia on the integument of the insect; (2) conidial germination under optimum conditions to form germ tubes;(3) degradation of cuticular structures by hydrolytic enzymes and mechanical effect to enable penetration; (4) conversion of the hyphae into blastospores to exploit nutrients in the host hemocoel; (5) blastospores utilize the available sugars and as well release toxins inside the hemolymph; (6) blastospores suppress the host immune system and release toxins that expedite killing of the host; (7) fungus exits the host through openings on the cuticle to produce spores on the cadaver surface (Khan et al., 2016a; Sun et al., 2016a). Furthermore, Ferron (1978), opined that once total invasion of the cadaver occurs, fungal conidiation on the surface of the mummified insect cadaver is dependent on the ambient environmental relative humidity. The mycelia develop from within the cadavers to the surface to produce conidiophores only when the atmosphere becomes saturated. Otherwise, the mummified cadaver remains dry and brittle. Under this condition, entomogenous agents become preserved in the form of chlamydospores.

5.4   Multiple-dose virulence evaluation of the EPF formulated in Tween 80

Under this virulence bioassay, *A. flavus* isolate BAMF2a induced a 90% cumulative mortalities of *E. pallens* (**Figure 4.9**) in a fashion similar to Seye et al. (2014b) who reported high mortalities of the aphid bug, *Acyrthosiphon pisum* caused by *A. flavus* and *A. clavatus* respectively. However, just like in the single-dose bioassay, *M. anisopliae* hitherto induced a 100% mortality rate against *E. pallens* after 7 days of exposure to the

bug. A similar study by Loureiro and Moino-Jr (2006) observed a 100% cumulative mortality of the aphid (Hemiptera), *Aphis gossypii* and *Myzus persicae* after 7 days of treatment with *M. anisopliae*. Also, a 100% cumulative mortality rate of the aphid, *Aphis craccivora* treated with 10^8 conidial ml^{-1} of *M. anisopliae* was earlier observed in Saranya et al. (2010) after 4 days of exposure to the fungus. Furthermore, Santos et al. (2018b) reported a 100% cumulative mortality of the Hemiptera *Thaumastocoris peregrinus* treated with 10^8 conidia ml^{-1} of *M. anisopliae* after 10 days. The differences between these findings may be attributed to factors such as isolates and strains variations, types of insect species, host immune responses, and the prevailing environmental conditions.

The LC and LT as yardsticks that measure the virulence of a pathogen in terms of conidial concentrations and time length required to kill the test insect population exposed to a given concentration of a pathogen were determined for both pathogens. In the virulence bioassay study against *E. pallens*, *A. flavus* isolate BAMF2a showed slightly lower median lethal concentrations (LC_{50}) and median lethal time (LT_{50}) compared to that of *M. anisopliae*. This demonstrates that *A. flavus* isolate BAMF2a showed more virulence at killing 50% of the test *E. pallens* population faster and at lower conidial concentration than *M. anisopliae*. However, on the contrary, *M. anisopliae* progressed to achieve a lower LC_{90} and LT_{90} compared to *A. flavus* isolate BAMF2a (Table 4.7 and Table 4.8). This could be attributed to the degree of persistence of the pathogens within the host hemocoel, nutrient exhaustion in the host, and the host's immune response to the pathogens over time. The ability of *M. anisopliae* to persist against the host's immune responses, exploit available nutrients and withstand ambient environmental changes would have been responsible for the rise in virulence over *A. flavus* isolate BAMF2a as can be observed in the later stage of the bug infection. Seye et al. (2014b) had earlier

reported higher virulence for *A. flavus* against the aphid, *Acyrthosiphon pisum* (Hemiptera: Aphididae) after 5 days of treatment, producing a lower LC_{50} and LC_{90} values (1.23×10^3 and 1.34×10^7 conidia ml $^{-1}$) compared to *M. anisopliae* (3.67×10^3 and 9.71×10^7 conidia ml^{-1}). Karthi et al. (2018) did report higher virulence for *A. flavus* against third instar and 4th instar nymphs of *Spodoptera litura* respectively. However, the observations in these studies shows that *A. flavus* possess the capacity to perform better in virulence against some arthropod pest than *M. anisopliae*, though its insecticidal activity is rarely reported compared to the widely discussed *M. anisopliae*. But some studies have reported LC_{50} values lower than what obtains in this study. For example, FitzGerald et al. (2016) observed a lower LC_{50} (5.29×10^5 conidia ml^{-1}) for *M. anisopliae* compared to what was found in this study. Also, Ekesi et al. (2000) demonstrated how four different strains of *M. anisopliae* differed in virulence against the aphid, *Aphis craccivora* (Koch) with LC_{50} values ranging between 3.1×10^5 to 7.4×10^6 conidia ml $^{-1}$. But Shah et al. (2005) asserted that fungal species and strains virulence, substrate composition, and culture methods, determine to a large extent whether insecticidal compounds responsible for virulence are produced by conidia of EPF.

The lethal time (LT_{50} and LT_{90}) obtained for both fungi used in this study, agrees with the finding of Mweke et al. (2018) who observed 3.3 to 6.3 days for EPF tested against *Aphis craccivora* (same Hemipteran insect as *E. pallens*). But Saranya et al. (2010) reported an LT_{50} of 5.54 days for *M. anisopliae* against the *Aphis craccivora*, which is longer than obtained in this study. This therefore means that the isolates used in this study showed potential of virulence against *E. pallens*. This may be due to the host type similarity, method of fungal conidial application and the virulence of the strains used.

However, to validate if the mortalities of the bugs were due to infection by the fungal pathogens, the cadavers were cultured and examined for the development of fungal conidia. This is important such that where these fungal agents are applied as myco-pesticides, they must confer the relative advantage of producing spores on the host cadavers to serve as a secondary source of inoculum for continuous propagation, natural hosts infection, reduction of insect menace and costs of applications (Roy & Pell, 2000). Since *E. pallens* attack peanut pods by piercing with their rostrum when they congregate under harvested peanut on the field, secondary infection by EPF which is largely dependent on insect behavior becomes easier due to their susceptibility under such conditions. Treating this bug species with EPF exposes them to infection by the pathogens considering their social interactions and susceptibility of their cuticles under appropriate conditions.

5.5 Multiple-dose virulence bioassay of the EPF formulated in peanut oil

This is the first study that evaluated the virulence of peanut formulated *A. flavus* isolate BAMF2a and *M. anisopliae* conidia against adult *E. pallens* (Hemiptera: Rhyparochromidae). Several studies have demonstrated the pathogenicity of *M. anisopliae* against hemipteran insects and their eggs. For example, *M. anisopliae* has been used to infect the eggs of *Blissus antillus* (Hemiptera: Lygaeidae) (Samuels et al., 2002a), adult *Diaphoria citri* (Hemiptera: Liviidae) (Orduño-Cruz et al., 2015), the Chagas vector, *Meccus pallidipennis* (Hemiptera: Reduviidae) (Flores-Villegas et al., 2016b), and the oak lace bug, *Corynthucha arcuate* (Hemiptera: Tingidae) (Sönmez et al., 2016). In this study, all conidial concentrations of the two fungi showed high virulence evidenced by the mortality rates and positive cadavers confirmed upon culture. The cumulative mortality of the bugs ranged from 46% to 100% for *M. anisopliae* and 39% to 100% for *A. flavus* isolate BAMF2a from the lowest conidial concentration 1×10^4 to 1×10^8

conidia/ml respectively. The highest conidial concentration (1×10^8 conidia/ml) of *A. flavus* isolate BAMF2a produced a mortality rate of 100% at day 9 post treatment, while *M. anisopliae* caused 100% cumulative mortality after 7 days of treatment. The virulence demonstrated by these isolates agrees with Inglis et al. (2001) that virulent entomopathogens may require less propagules, demonstrate high persistence, and possess the ability to adhere to a susceptible host to infect. These features in EPF are essential criteria for the selection of biocontrol agents. Oil-based formulations of biopesticides have over the years been adjudged to enhance the adherence of pathogenic fungal propagules to insect cuticle, promote the spread of the propagules over the insect body, penetration of the insect integument, protection from the effect of ultraviolet (UV) light radiation and enhance infectivity of the propagules even under low moisture (humidity) conditions (Carrillo et al., 2015; Inglis et al., 2002; Polar et al., 2005b). Peng and Xia (2011) reported the increase in the virulence of oil-based formulation of *M. anisopliae* var. *acridium* against grasshoppers and locusts by reducing the dependence on high humidity for germination and maintain the thermos-tolerance and UV tolerance of the fungal conidia. Alves et al. (1998b) used peanut oil formulation to demonstrate the protection of *M. anisopliae* conidia against ultraviolet radiation. Vegetable oil-based formulations compared to water-based formulations have been reported to demonstrate increased virulence against different hosts including *Bemisia tabaci* (Batta, 2003), arid locust, *Schistocerca gregaria* under low humidity (Bateman et al., 1993), and ticks (Camargo et al., 2012; Kaaya & Hassan, 2000; Polar et al., 2005b). Santi et al. (2011) reported formulations containing *M. anisopliae* conidia in 5% and 10% soybean oil as the most virulent against the cotton seed bug *Dysdercus peruvianus*.

Aspergillus species has been reported to kill locust (Balogun & Fagade, 2004) and cause infection among wide range of insects population, but whether or not it is host

specific remains unknown. *A. flavus* tested against *Galleria mellonella* killed 100% of the insect after 48 h when injected with 3×10^3 conidia/ml of the fungus whereas *A. fumigatus* and *A. nidulans* lacked parasitic attributes (Leger et al., 2000).

The germinability of the conidia of *A. flavus* isolate BAMF2a and *M. anisopliae* and the mortality rates of the bugs caused by the two isolates were not different ($p > 0.05$). The formulation of the fungal conidia in peanut oil must have enhanced the virulence of both *A. flavus* isolate BAMF2a and *M. anisopliae* against *E. pallens*. This could mean that, although *Aspergillus* are described as facultative generalists (Sandhu et al., 2012a), their virulence can be enhanced to match promising biological control agents such as *M. anisopliae* when formulated in non-evaporative diluents such as peanut oil. The LC_{50} values obtained in this study for both *A. flavus* and *M. anisopliae* were all within 10^6 conidial/ml indicating no difference in virulence between the fungi. Similar LC_{50} values was obtained by Ihara et al. (2001) for *M. anisopliae* isolate FRM515 that was considered and selected as the most virulent against the brown-winged green bug, *Plautia stali*, and an LT_{50} value of 3.9 days respectively. The LC_{50} obtained also agrees with FitzGerald et al. (2016) who reported similar LC_{50} value for *M. anisopliae* against adult citrus mealybug, *Plannococcus citri*. The LT_{50} of 3.3 days for *A. flavus* isolate BAMF2a and 3.6 days for *M. anisopliae* (Table 4.8) showed no much difference. This may be due to the effect of the oil on the virulence of the isolates, especially *A. flavus* which is often less reported for virulence against arthropods. Balogun and Fagade (2004) reported an LT_{50} of 4 days for *A. niger* and 5 days for *M. anisopliae* against *Zonocerus variegatus*. However, even though the LC_{90} for *M. anisopliae* was found to be 10^8 conidia/ml, and

10^9 conidia/ml for *A. flavus*, there was no significant difference in their virulence against *E. pallens*.

It is important for us to assert that, conidia impregnated on filter paper where effective at killing *E. pallens* for both *A. flavus* isolate BAMF2a and *M. anisopliae* probably due to the adsorption of the conidia on their body surfaces. This finding falls in agreement with the findings of Dong et al. (2012a) and García-Munguía et al. (2011) who reported that EPF conidia applied on filter paper where found to be highly effective at infecting and killing *Aedes aegypti* due to their sexual behavior, which promote horizontal fungal propagule transmission. In our own study, horizontal transmission due to mating activity and feeding habits attributed to the effectiveness and horizontal transmission of filter impregnated EPF conidia among *E. pallens*. This is plausible because, the bug usually mounts their food source during feeding. In that fashion, conidia adhered to their body surface can easily be disseminated on the seeds and can become a contact point for transmission of infection among the bug population.

Furthermore, conidial formulation of the two fungi demonstrated great potential for the control of *E. pallens* under laboratory conditions. The oil-based conidial formulation enhanced the virulence of the fungi respectively, which implies that either of the fungi is capable for use as a biocontrol agent of the bug. The application of atoxigenic *A. flavus* strains for biocontrol of insects in grain crops will no doubt provide dual advantage of insect control and inhibition of toxigenic strains through competitive exclusion. The effectiveness of the formulated entomopathogens at infecting and killing the bugs when applied on the filter paper, mimicking the soil condition makes these fungi potent for field applications with great possibility of success. Exploiting the full potential of the two fungi in inoculative or inundative field applications will undoubtedly improve their commercial

use as biological control agent. Further studies should be conducted to develop and optimize these fungi as biological agents of seed bugs.

5.6   Role of hydrolytic cuticle-degrading enzymes of EPF

The cuticle of insects is usually the primary point where infection by EPF is established. Because it constitute a formidable barrier to intruding pathogens, it is important for this barrier to be overcome for successful pathogenesis by EPF (Keppanan et al., 2017). Previous studies have reported the involvement of extracellular cuticle-degrading enzymes in fungal pathogenesis (Lu et al., 2015; Shahid et al., 2012a). Enzyme secretion by invading EPF involved in the breakdown of constituent polymers of the insect cuticle during pathogenesis aid the penetration of the exoskeleton and release of nutrients that enable fungal growth (Goulet et al., 2005). This makes the degradation of the chitin-rich matrices in the insect cuticle a common ability of EPF (Humber, 2008).

In this study, the isolates expressed appreciable hydrolytic enzyme spectrum that are essential for the degradation of the host proteo-chitin complex. This possibly demonstrates the genetic and biochemical versatility of the isolates; an evidence of their potential to exploit resources that may be available to them. The isolates showed detectable amounts of expressed enzyme, which may be responsible for the degree of virulence exhibited them. Noteworthy, Małagocka et al. (2015) reported the role of hydrolytic extracellular enzymes in the breakdown of host integument and infectivity. Moreover, Khan et al. (2016b) opined that highly virulent clades of EPF secretes quantifiable amounts of extracellular cuticle-degrading enzymes.

Proteases have been considered as essential component of the enzyme system required by EPF for pathogenesis (Mustafa & Kaur, 2009). During infection, EPF produce

significant quantity of proteases that help to breakdown the proteinaceous material into soluble proteins which are further degraded by the peptidases into constituent amino acids which provide nutrient for the pathogen (Dhawan & Joshi, 2017). The isolates evaluated in this study showed maximum protease activities that were significantly different. However, the maximum activities obtained in the study corroborated the findings of Mustafa and Kaur (2009) who reported protease activities in the range of 0.59 to 1.52 U/ml for five different strains of *M. anisopliae* grown on Sabouraud dextrose broth at 28 ºC. Also, same range of proteolytic enzymes activity was reported in Keppanan et al. (2017) from a study involving three strains of *M. ansiopliae*. Nahar et al. (2004) had reported a protease activity of 0.01 U/ml for a strain of *M. anisopliae* cultivated on yeast extract, peptone and glucose (YPG) medium. However, Dhawan and Joshi (2017) reported maximum proteases activity among four strains of *B. bassiana* (MTCC 2028, MTCC 4495, MTCC 6291, and NBAII-11) in the range of 1.20 to 1.26 U/ml. Furthermore, Pelizza et al. (2017), found higher enzyme activity for *B. bassiana* strain LPSc (1.78 U/ml). But on the contrary, Alagesan et al. (2019) found protease activity in a study involving four strains of *B. bassiana* to be in the range of 0.19 to 0.43 U/ml even when the medium was supplemented with 1% gelatin. Though the medium used in this study for the enzyme production was not supplemented with any exogenous substrate, yet, proteases recovered from this medium showed greater activity compared to the medium supplemented with 1% gelatin. Noteworthy, the observable differences in these findings could be attributed to differences in types of fungal species, strains and culture medium used. It is significant to note that the importance of culture medium cannot be overemphasized. For example, The effect of culture medium on the activities of pathogenesis-related hydrolytic enzymes (Pr1 protease, chitinase and lipase) was observed in Kim et al. (2018) to be highest when produced in Luria bertani (LB) broth compared to those observed in Potato dextrose and Sabouraud dextrose broth. The higher

activities in the LB medium was thought to be due to the presence of yeast extract which is likely to provide the vitamins and nutrients required for efficient growth and enzyme yield. In their study, total protease activity of 15 isolates cultured on LB broth ranged from 0.077 to 3.446 U/ml; activity of protease from isolates cultured on PDB medium showed activity of 0.063 to 2.659 U/ml while the protease activity of isolates cultured on SDB medium was between 0.054 to 1.816 U/ml. Considering the role of culture media in enzyme production, fungal strains should be cultured extensively in different growth media in order to fully understand their enzymes production capacities.

Chitinase is one important enzyme required in the enzyme system of EPF to break down the chitin polymer of arthropod cuticle. According to Dhawan and Joshi (2017), there exists a correlation between chitinase activity and virulence in EPF. The chitinase activity obtained for *A. flavus* isolate BAMF2a and *M. anisopliae* were not different. The two isolates showed maximum chitinase activities when assayed. Perinotto et al. (2014) observed marked maximum chitinase activity in the most virulent strain of EPF evaluated on the tick *Rhipicephalus microplus*.

In this study, the maximum chitinase activity recorded was higher than what obtains in Dhawan and Joshi (2017) who found chitinase activities among four strains (MTCC 2028, MTCC 4495, MTCC 6291, and NBAII-11) of *B. bassiana* to range from 0.23 to 0.64 U/ml. Also, the chitinase activities evaluated by Alagesan et al. (2019) which ranged from 0.43 to 0.61 U/ml for four strains of *B. bassiana* were lower compared with what was found in our study. Kim et al. (2018) evaluated chitinase activity from 15 different species of EPF cultivated on different growth medium. Their finding was that chitinase production and their activities were highest for isolates cultivated on LB medium, with total activity ranging from 0.171 to 3.910 U/ml compared to activities of those enzymes

cultivated on media like PD and SD broths. But Pelizza et al. (2017) reported chitinase activity for *B. bassiana* strain LPSc (2.31 U/ml), higher than obtained in this study. In their study, the growth medium (potato dextrose agar) was supplemented with 0.08% Chitin Azure and the enzyme activity was quantitatively measured. These may be responsible for the high chitinase reported for LPSc. However, chitinase from *M. anisopliae* grown on yeast extract, peptone and glucose (YPG) medium for constitutive enzyme production, yielded chitinases with an activity of 0.01 U/ml after 120 h (Nahar et al., 2004).

The mean lipase activities obtained for both *A. flavus* isolate BAMF2a and *M. anisopliae* were not different but were higher compared to the activities of proteases and chitinases. This means that, lipases may be well-thought-out as the first enzyme to be secreted by these isolates during pathogenesis. Similar finding has earlier been confirmed in Dhawan and Joshi (2017). Lipases produced by EPF were involved in preliminary adhesion and penetration of host integument and their extracellular activities were found to be more compared for chitinases and proteases; hence, their consideration as essential enzymes for EPF metabolism (Da Silva et al., 2010). Supakdamrongkul et al. (2010) found that EPF supplemented with purified lipase demonstrated greater virulence against *Spodoptera litura*. Kim et al. (2018) studied the activities of hydrolytic enzymes from 15 different species of EPF and found total lipase activity cultivated on LB broth to be highest compared to those cultured on other growth media. The total activity found ranged from 0.423 to 2.953 U/ml. Dhawan and Joshi (2017) reported maximum lipase activity for *B. bassiana* MTCC 4495 (1.36 U/ml) in a study that determined the extracellular cuticle-degrading enzymes of the fungal isolates, while minimum mean lipase activity recorded for *B. bassiana* NBAII-11 was 1.04 U/ml. Variability in the pattern of enzymes activity among the isolates were observed by the authors. However, in another study

involving *B. bassiana* isolated from *Schistocerca cancellate*, Pelizza et al. (2017) reported lipase activities from three isolates of *B. bassiana* strains LPSc 1225 (4.23 U/ml), LPSc 1226 (3.05 U/ml) and LPSc 127 (2.78 U/ml) that were grown on agar medium supplemented with Tween 20 as lipid substrate and cultured at 4 ℃. But, the hdrolytic enzyme activity was measured quantitively by halo-zones formed around colonies. In Mondal et al. (2016), the activities of lipase enzymes produced by *M. anisopliae* on different lipid sources were reported as: rice oil (4.46 U/ml), soybean oil (4.26 U/ml), olive oil (4.25 U/ml), sunflower oil (4.23 U/ml), sesame oil (3.51 U/ml) and hydrogenated soybean fat (3.50 U/ml) respectively. Nahar et al. (2004) also observed lipase activity of 0.312 U/ml from *M. anisopliae* grown on yeast extract, peptone and glucose (YPG) medium. These findings obviously show the effect of nutrient sources, fungal species type and method of analysis on the nature of enzymes activity. The variation in the enzyme activities and the amount secreted by fungal isolates can also be best correlated with the genetic architecture of the fungal isolates.

The findings of our study strongly suggest that mortalities of *E. pallens* treated with conidia of EPF could be due to the synergistic expanse of hydrolytic enzymes such as proteases, chitinases and lipases. Pelizza et al. (2017) has asserted that isolates that can produce high levels of proteases and chitinases were found to be the most virulent among others; which strongly implies direct correlation between enzymes activity and virulence in EPF.

Although *E. pallens* was susceptible to infection by the isolates evaluated in this study, there are however, trending evidences that long-term protection can be acquired by insects against invading pathogens via their immune system in a process called 'immune priming' or genetic transfer from parents to offspring; otherwise termed as 'trans-

generational immune priming'(Małagocka et al., 2015; Moret, 2006; Roth et al., 2009). Insects with this ability can subsequently develop resistance to EPF (Dubovskiy et al., 2014). Thus, factors like type of fungal species, strains, cultivation temperatures, type of carbon and nitrogen sources used between this study and reports from previous studies may be responsible for the differences in the activities of extracellular hydrolytic enzymes reported for these EPF. Whereas this study utilized *A. flavus* isolate BAMF2a and *M. anisopliae* as EPF, comparisons in their enzymes activities were made with other isolates of closely related species. Furthermore, this present study reports the hydrolytic activities of these enzymes as 'constitutive' enzymes (produced during vegetative growth on medium lacking exogenous substrate) as compared to inducible enzymes previously reported. Therefore, this expanse of factors can be responsible for the observed variations in the reported enzyme activities.

CHAPTER 6: CONCLUSION

This study isolated, identified and characterized fungal isolates from the bug cadaver and rostrum of *E. pallens* via molecular methods; and evaluation of their potential as pathogens of the peanut seed bug, *E. pallens* by immersion in various conidial formulations and exposure of the bugs on conidia-treated filter paper surface. For the different formulations, various conidial concentrations were employed. It was found that *A. flavus* isolate BAMF2a demonstrated greater virulence against *E. pallens* by achieving lower median LC_{50} and LT_{50} values—killing half the population of the test bugs at lower conidial concentrations and reduced exposure time. However, conversely, the LC_{90} and LT_{90} were observed to be lower for *M.* anisopliae. The conidia of *A. flavus* isolate BAMF2a and *M. anisopliae* formulated in peanut oil showed no significant differences in virulence against *E. pallens*. This there shows that oil truly enhances infectivity and virulence of EPF under controlled laboratory environment. Furthermore, oil formulated conidia applied on filter paper showed better infectivity, virulence and mortality of the bug compared to the immersion method. This may be due to enhanced adsorption of the conidia to the insect body surface as they move and unconsciously pick up the conidia on the filter paper. Under this experimental condition, the bugs were unconscious of their predisposition to the infective conidia until the onset of infection. However, conidia formulations used in the immersion method proved less virulent, which may be attributed to the extensive grooming the insects undergo after immersion into the fungal conidial formulations. The ability of the bug to undergo self-grooming by constantly swiping their body is an ideal scenario that perhaps helps to dislodge the conidia already adsorbed to their body surfaces, limiting virulence and mortality especially as observed in the Tween 80 formulations.

It was observed that once the treated bug population becomes infected by the fungal propagules, they become sluggish, restless and lose sexual cues. Death was confirmed only when the bug becomes immotile, functionless, and when the cadaver becomes hard and brittle. When death of the bug occurs, mycelia develop from within the cadavers to the surface to produce conidiospores under saturated atmosphere. Otherwise, the mummified cadaver remains dry and brittle. Additionally, both isolates used in the study expressed appreciable production of major constitutive cuticle-degrading enzymes including proteases, chitinases and lipases which showed remarkable hydrolytic activities. Although *A. flavus* has often been less applied for the biological control of insect pests compared to the widely used *M. anisopliae*, yet, it has demonstrated potential for the biological control of *E. pallens* and should be considered in myco-formulations for field applications.

The study also reported for the first time the mycobiome contained on the rostrum of *E. pallens* collected from the fields and those reared in the laboratory. From the samples investigated, diverse mycobiome species belonging to the genera *Aspergillus*, *Rhizopus*, *Talaromyces* and *Annulohypoxylon* were obtained. The relationship of the bug rostrum with the fungi was not species-specific, involving species that are cosmopolitan, grains-associated and decomposer fungal microbiome respectively. From these mycobiome, strains of *A. flavus* investigated for aflatoxins production consisted of both toxigenic and atoxigenic strains. However, atoxigenic strains had the highest prevalence compared to the toxigenic strains. It was similarly found that strains of *A. flavus* obtained from the bug cadaver consisted of both toxigenic and atoxigenic strains. However, atoxigenic strains were also found to be preponderant. This finding may connote minimal risks of aflatoxins contamination of staple crops grown in those locations. Additionally, the practice of

immediate stripping of the pods after harvest by peanut farmers in the sampling areas could constitute an essential check on aflatoxin contamination of the kernels.

In the light of the foregoing findings, it is imperative that further research on the field evaluation and development of these EPF isolates into biocontrol agents of *E. pallens* and their incorporation into integrated pest management (IPM) systems should be explored. Additionally, whether the mycobiome species associated with the rostrum is beneficial to the host remains imperative for investigation. Furthermore, although this study does not in any way undermine the safety status of peanuts production, further studies on the aflatoxins content of peanuts affected by the bug in comparison with healthy ones is highly recommended.

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