Entomopathogenic fungi as a biological control for the Greater melon fly *Dacus frontalis* (Becker) (Diptera: Tephritidae)

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Abstract

The Greater melon fly *Dacus frontalis* is one of the most economically damaging pests of cucurbit fruits in Africa. The aim of this study was to evaluate entomopathogenic fungi for use against *D. frontalis* and develop better strategies in using these biological agents for integrated fly management.

Laboratory experiments were conducted to evaluate the pathogenicity of some commercial biopesticides based on several strains of entomopathogenic fungi against different life stages of the fly. Met52 Granular biopesticide (MET52) based on *Metarhizium anisopliae* caused the greatest pathogenicity to the adults. Therefore, MET52 was selected for further investigation. Pupal age and an increasing rate of MET52 had no effect on pupal mortality. However, post-emergence mortality increased when the fungus was applied on young pupae. Prior application of MET52 in a granule form caused considerable mortality to larval-pupal stages compared with a drench application. A sublethal effect of MET52 infection, reducing adult reproduction, was found. The fungus was more effective when applied as a granule in soil against emerging adults than as a direct spray against adults. MET52 fungus was able to persist in soil, reducing the adult emergence and subsequent fly population for more than two months after a single application, and also inducing new infections among the fly population. Percentage mortality of untreated females mating with inoculated males was greater than that of untreated males mating with inoculated females. The infection reduced the adult's reproduction resulting in a considerable reduction in pupae recovered in the fungal treatments. Alone or in combination with MET52 neem had a dose-dependent effect on larval mortality and induced various effects on soil stages and adults of the fly. The efficacy of the fungus increased considerably at low concentration of neem showing the shortest LT50. In field cage trials, baited McPhail traps with yeast hydrolysate enzymatic had the greatest number of *D. frontalis.* MET52 gave lower adult mortality than insecticide. In conclusion, soil application of MET52 can be a promising effective control of *D. frontalis*, and can be combined with other control agents providing a possible effective strategy for integrated fly management.

Dedication

To my parents, wife and sons for their unlimited support

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Chapter 1. General introduction

1.1 Background

The provision of sufficient food to meet the increasing human population and the protection of fruit and vegetable harvests are among the pressing challenges faced by the global agricultural organisations and related institutions today. Also, agricultural production has a significant role to play in expanding the economies in many countries in the world (Weinberger and Lumpkin, 2007). Crop yield losses that resulted from numerous insect pests, including fruit flies, have been clearly documented (Oerke and Dehne, 2004; Litsinger, 2009; Dhaliwal *et al.*, 2010). For the last six decades, synthetic pesticides have been excessively used to protect agricultural crops in fields and grain houses from attack by these pests (Kumar *et al.*, 2008). Although many researchers have clearly demonstrated the negative impact of toxic residues and the intensive use of synthetic pesticides on human health (Bolognesi, 2003; Amoah *et al.*, 2006; Ngowi *et al.*, 2007; Mostafalou and Abdollahi, 2013; Fantke and Jolliet, 2015), insect resistance (Vontas *et al.*, 2011; Bass *et al.*, 2014) and the environment (Bues *et al.*, 2004; Badenes-Perez and Shelton, 2006; Wei *et al.*, 2015), many growers still consider pesticide application as a favourite option to protect vegetables and fruit from diseases and pests even with the availability of some alternative control (de Bon *et al.*, 2014). This may be because pesticides are known to offer quicker results. Many insect pests, however, are still considered among the most substantial factors causing losses to global food production (Oerke, 2006; Nicholson, 2007). Therefore, the development of safer insect control tools and strategies is required to limit insect damage and safeguard future food security and the environment. Recently, Billah *et al.* (2015) indicated that further studies on damage losses and the management of fruit flies are required. The potential use of biopesticides as an effective approach for pest management has been previously suggested to be one of the most promising alternatives to pesticides (Nicholson, 2007).

1.2 Cucurbit crops

The Cucurbitaceae are widely cultivated around the world. Cucurbit fruits are among the most important fruit consumed in Africa in general and in Libya in particular. The majority of Libyans consume cucurbit fruits daily, as the fruits are rich in several vitamins and minerals. Many cucurbit species are cultivated throughout the year. For example, cucumber, squash and courgettes are usually planted in open fields from spring to autumn, while they are cultivated under plastic or in protected houses in the winter season.

The cucurbit crops are usually infested by several diseases and insect pests, which reduce the quantity and quality of the crop yield. The pests cause direct damage by reducing and destroying the crop production, or indirect damage by increasing crop protection expenses or by quarantine restrictions causing economic losses to growers and exporting countries. These pests are the most influential factors limiting the yield of the cucurbit species. Pest species belonging to several arthropod groups have been reported to attack cucurbit crops, including the: cotton aphid (*Aphis gossypii*), green peach aphid (*Myzus persicae*), Cotton or tomato whitefly (*Bemisia tabaci*), grasshoppers (*Heteracris littoralis*), onion thrips (*Thrips tabaci*), black melon bug (*Coridius viduatus*), melon ladybird (*Henosepilachna elaterii*), cucurbit leaf fly (*Liriomyza bryoniae*), melon fly (*Bactrocera cucurbitae*), pumpkin fly (*Dacus ciliatus*), black cutworm (*Agrotis ipsilon*), cucumber beetle (*Coridius viduatus*), guava fruit fly (*Anastrepha striata*) and greater melon fly (*Dacus frontalis*) (Jones, 2003; Ellers-Kirk and Fleischer, 2006; White, 2006; Pinto *et al.*, 2008; Foottit and Adler, 2009; Sapkota *et al*., 2010; Manandhar *et al.*, 2009; Goergen *et al.*, 2011; Gameel, 2013).

1.3 Fruit flies

Fruit flies (Diptera: Tephritidae) are one of the most damaging insect pests in the world. Several fruits and vegetables, including cucurbit crops, are attacked by fruit fly species (White and Elson-Harris, 1992; Drew, 2004; White, 2006; Vayssières *et al.*, 2007; Rwomushana *et al.*, 2008; Mwatawala *et al.*, 2010; Goergen *et al.*, 2011; Jose *et al.*, 2013; Cugala *et al.*, 2014; Billah *et al.*, 2015). Numerous studies and reviews have attempted to determine the damage caused by different species of fruit fly (Lux *et al.*, 2003; Dhillon *et al.*, 2005b; Ekesi *et al.*, 2006; Mwatawala *et al.*, 2006; Sapkota *et al*., 2010; Jose *et al.*, 2013; Lu and Ariana, 2013; Redha, 2013; Ali *et al.*, 2014; Amalia *et al.*, 2014; Bhowmik *et al.*, 2014; Cugala *et al.*, 2014; de Oliveira *et al.*, 2014; Sarwar *et al.*, 2014; Billah *et al.*, 2015). For example, in a field study conducted by Ekesi *et al.* (2006), 34% of mango fruits collected from different orchards in Kenya were infested by *Bactrocera invadens*. The melon fruit fly, *B. cucurbitae* has been found to cause average damage of nearly 70% in some species of bitter gourd (Dhillon *et al.*, 2005b). Damage losses of mango and citrus fruits infested with some species of the *Ceratitis* genus have been found to range from 20-30% in Africa (Lux *et al.*, 2003). In a recent study conducted by Sarwar *et al.* (2014), *B. zonata* flies caused approximately 19% infestation of guava fruits. Furthermore, inclusion of some fruit fly species within a quarantine pest lists led to regulations being imposed by fruit and vegetable-importing countries, thereby causing economic losses for exporting countries and reducing international trade (Jose *et al.*, 2013). For example, Ekesi (2010), indicated that *B. invadens* was the reason given for banning the

trade of some plant products, including cucurbit fruits, between some African countries and Africa and the United States. Although the economic importance of fruit flies, including the *Dacus* species, on the fruit and vegetable trade has been well documented, studies related to their ecology, biology and control are limited.

The genus *Dacus* is one of the main groups in the African Dacina fauna (Fletcher, 1987), with 195 described species attacking economically important fruits and vegetables belonging to the Cucurbitaceae, Passifloraceae and Apocynaceae families (White, 2006; White and Goodger, 2009). The genus *Dacus* is indigenous to Africa (Foottit and Adler, 2009) and Asia (Papadopoulos, 2014). It is also found in Australia and the Pacific, with the majority found in Africa (Drew, 2004). *Dacus* is one of the most serious insect pests which damages cucurbit crops and causes economic losses to other fruits and vegetables (Roomi *et al.*, 1993). Some countries that import crop products have placed restrictions to deny the entry of plants products that could be associated with these species. For example, *D. frontalis* with other five *Dacus* species (*D. ciliatus, D. bivitattus, D. lounsburyii, D. punctatifrons and D. vertebratus*) are considered as important quarantine pests in the United States (https://www.law.cornell.edu/cfr/text/7/319.56-48). Strict conditions must be followed before importing some cucurbit fruits from Zambia to prevent the spread of the flies. Also, the Mexican Ministry for Primary Industries has imposed conditions and limitations to the import of plant products from New Zealand (http://www.mpi.govt.nz/mpisearch/). *Dacus frontalis* with another 11 *Dacus* species and several other insect pest species, most of which belong to the fruit flies (*Bactrocera, Ceratitis* and *Rhagoletis*), have been considered as quarantine pests for over a decade (http://www.mpi.govt.nz/mpisearch/).

1.4 The Greater melon fly, *Dacus frontalis* (Becker)

Dacus frontalis is one of the native fruit flies species in Africa (Foottit and Adler, 2009). Information on the ecology, biology and management of *D. frontalis* is very limited, but it is mentioned in broader studies like the taxonomic revision of African Dacina (Dr Ian M. White, personal communications).

1.4.1 *Taxonomy history*

Identification based on morphological characteristics of flies belonging to the genus *Dacus* was not completely precise (Munro, 1948). As *D. frontalis* is morphologically similar to other *Dacus* species the fly was misclassified under different species names. For examples, the fly used to be identified as *D. vertebratus* by Hendel, 1927 and Collart, 1941 cited by Munro

(1948), *D. ciliatus* var. *duplex* (Munro, 1948) and as a synonym of *D. ciliatus* by Munro, 1964 cited by (White, 2006). Then, the fly was classified as an independent species by Munro 1984 cited by Abukhashim *et al.* (2003b) and by White (2006). Currently, *D. frontalis* species can be recognised from *D. celiatus* by a response of the fly males to Cue-lure baited traps (White, 2006). In some more recent studies, investigations have been done to identify some fruit flies including the African genus *Dacus* at species levels by genetic and molecular based techniques. The results indicated that the genetic classification of some members of Tephritidae including the genus *Dacus* was not fully satisfactory suggesting further revisions are required (Virgilio *et al.*, 2009; Frey *et al.*, 2013; Virgilio *et al.*, 2015). In Libya, the fly was initially identified to the rank of a genus *Dacus* and placed under species *D. ciliatus* by the biological control department in the Biotechnology Research Centre. Then, the fly was defined by experts in the British Museum of Natural History and identified as *D. frontalis* (Becker) and recorded under number: 1200/2001 ENQ (Abukhashim *et al.*, 2003b). According to Global Biodiversity Information Facility (http://www.gbif.org/) and True Fruit Flies of the Afrotropical Region (http://projects.bebif.be/fruitfly/index.html), the classification of *Dacus frontalis* is presented in Table 1.

Table 1.1 Scientific classification of *Dacus frontalis.*

Kingdom	Animal
Phylum	Arthropoda
Class	Insecta
Order	Diptera
Family	Tephritidae
Subfamily	Dacinae
Tribe	Dacini
Genus	*Dacus*
Species	*Frontalis*
Scientific name	*Dacus frontalis* Becker, 1922

1.4.2 *Global distribution*

Dacus frontalis is an African native species (Foottit and Adler, 2009) and widely distributed in Palearctic and Afrotropical (Carroll *et al.*, 2006). The fly has been reported in Libya, Sudan, Egypt, Algeria, Angola, Botswana, Cape Verde, Congo, Eritrea, Kenya, Lesotho, Namibia, Tanzania, Zambia, Zimbabwe, Benin, South Africa and some parts of Asia including Saudi Arabia, Yemen and Iraq (Steffens, 1982; Ba-Angood, 1977; Lobo-Lima and Klein-Koch, 1981; Harten and Viereck, 1986; Monteiro Neves and Viereck, 1987; Abukhashim *et al.*, 2003a; White, 2006; Mwatawala *et al.*, 2010; De Meyer *et al.*, 2013). Recently, new records of cucurbit fruits infested with *D. frontalis* have emerged from new regions including the New Valley, Egypt (Gameel, 2013), Al-Baha, Saudi Arabia (El-Hawagry *et al.*, 2013) and four Iraqi provinces (Baghdad, Diyala, Karbala, Amarah, Tuwaitha and Suwaira) (Shawkit *et al.*, 2011; Redha, 2013). More recently, the fly has been recorded in the Central and South Tunisia for the first time (Hafsi *et al.*, 2015). The author also indicated that the fly might recently have entered some countries in the south of Europe. In Libya, the first observation of the fly was in 1992 in Shaabia Marzak farms in the south (Ramadan Abdallah, 2002). Then, the fly spread across the country, but was less abundant in the eastern region (Abukhashim *et al.*, 2003a).

1.4.3 *Economic important and host plants*

Dacus frontalis is one of the economically important fruit flies species which has a negative impact on food security in Africa (Foottit and Adler, 2009). Similar to the tephritid species, direct damage is caused by the larval stage which decreases quality and quantity of the fruit production making the fruits unmarketable. In Cape Verde Islands and Yemen, *Dacus frontalis* attacks all cultivated cucurbits, causing extensive yield losses of up to 100% in the absence of a good control strategy (Steffens, 1982; Ba-Angood, 1977). In Libya, a range of major cucurbit crops are attacked. Results of a survey conducted on cucurbit orchards through the country showed that seven cucurbit species, including *Cucumis sativus, C. melo, C. melo* var. *flexuosus*, *Cucurbita moschata*, *C. pepo, Citrullus lanatus* and *C. colocynthis* are reported as fly hosts (Abukhashim *et al.*, 2003a). The authors also reported *Solanum melonena* (Solanaceae) as a new fly host. These infestations damaged the cucurbit fruits, causing 100% losses of the fruit production and raising concern among growers leading the local authority to propose a national project aimed to study and control the fly in the country. In a recent field experiment conducted by Mwatawala *et al.* (2010), five species of cucurbit cops including *Cucumis dipsaceus*, *C. melo*, *C. sativus*, *Cucurbita moschata* and *Lagenaria*

siceraria was found infested with *D. frontalis*. Recently, Gameel (2013) indicated that *D. frontalis* and other species of *Dacus sp* and *B, zonata* were collected from a field planted with five species of Cucurbitaceae. More recently, the fly was reported attacking neck cucumber fruits, *C. melo* var. *flexuosus* in Tunis (Hafsi *et al.*, 2015).

1.4.4 *Life cycle and infestation symptoms*

Males and females usually mate on rest plants which grow in a cucurbit field (Steffens, 1982). The author observed that *D. frontalis* adults are not found on the fruits during the morning when mostly they are found on the rest plants such as citrus, maize, sunflowers and pigeon pea species which provide protein (from pollen) for the adults. Recently, *D. frontalis* adults have been observed resting on a maize plant growing beside a cucurbit field in Libya in the 2013 season (Figure 1.1.A) (personal observation). More than one adult female could be seen on a single cucurbit fruit during oviposition which usually starts from noon to the early afternoon (personal observations, Figure 1.1.B and (Steffens, 1982). The author reported that the edge of a cucurbit field is more infested than the centre. The fertilised females visit cucurbit hosts laying 7 to 21 eggs daily by inserting the ovipositor under the fruit skin to a depth of 3 mm (Figure 1.2.A) (Abukhashim *et al.*, 2003b). In biology experiments conducted by (Shawkit *et al.*, 2011) *D. frontali*s females laid approximately 22 egg a day. The females prefer to deposit their eggs into young fruits (Steffens, 1982; EL-Sabah and Fetoh, 2010). EL-Sabah and Fetoh (2010), indicated that small squash fruits have more of several nutritional elements such as proteins than medium and large fruits. Damage symptoms and the life cycle of *D. frontalis* are similar to those of other species belonging to the dacine fruit flies. The damage starts with adult female oviposition on different stages and sizes of cucurbit fruits including the fruit setting stage (Figure 1.2.B) (Steffens, 1982; Ba-Angood, 1977). A sticky calcareous material is usually observed produced from oviposition punctures as a result of the infestation. This can be clearly shown when the infested fruits are pressed by the fingers tips (Abukhashim *et al.*, 2003b). Additional infection by microorganisms such as pathogens can occur causing fruit rotting. A pale yellow colour is also visible around the punctures on the infested fruits, which then become brownish yellow (Figure 1.2. B). Depending on environment conditions the eggs (Figure 1.3.B) hatch within 2-4 days into larvae which grow through three larval stages feeding on the fruit flesh and causing the fruit to decompose, rot and often eventually fall from the host plant (Figure 1.2. C.D). The full grown larvae, which have a white milky colour (Figure 1.3.C), bore holes and drop out of the damaged fruit to the ground and burrow into soil where they pupate shortly afterwards at different depths depending on soil type, temperature and moisture. *D. frontalis* pupae are cylindrical in form,

4-5 mm long and 1.5-2 mm in diameters and have a light brown yellow colour (Figure 1.3.D). Pupae develop and emerge to adults (White and Elson-Harris, 1992; Abukhashim *et al.*, 2003b). *D. frontalis* adults are small flies 8-9 mm long, orange to brown, with four yellowish spots located on the thorax and another four at the wing connection. Females usually have a bigger size body than males (Figure 1.3.A) (Carroll *et al.*, 2006).

Development time for the fly life stages (eggs to adult emergence) is climate dependent. So far, no research has been done to study *D. frontalis* generation times under typical conditions in Libya. The fly can be found in the field on the main hosts or rest plants during all the seasons of the year. Dry conditions are usually suitable for the fly to cause high infestation while the fly population declines in the winter season (Steffens, 1982). In a sample collection experiment conducted in Iraq, various infestation levels of the fly were recorded from May to October seasons. The result of the survey revealed that the highest infestation was recorded from August to October reaching approximately 55% on neck cucumber fruits (Shawkit *et al.*, 2011). The fly was also detected between September and December on some vegetables, and in citrus and olive orchards in Tunis (Hafsi *et al.*, 2015).

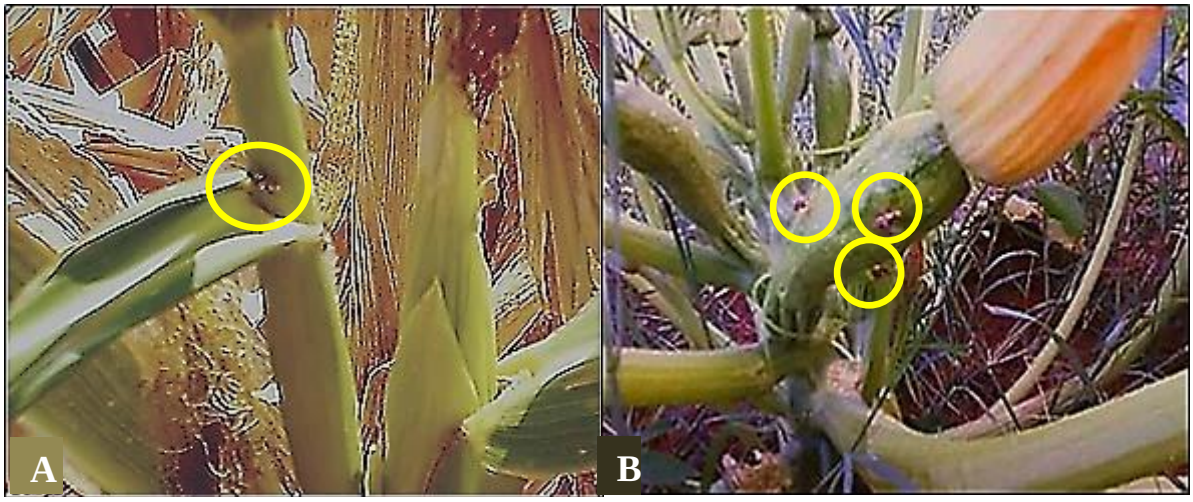


Figure 1.1 *Dacus frontalis* adult resting on maize plant (A) Zucchini attacked by a number of *Dacus frontalis* females (B). The pictures were recently taken in a cucurbit field during field trials in Ben-Ghasheer, Libya, September 2013. (Photographs © were taken by the author. 2013).

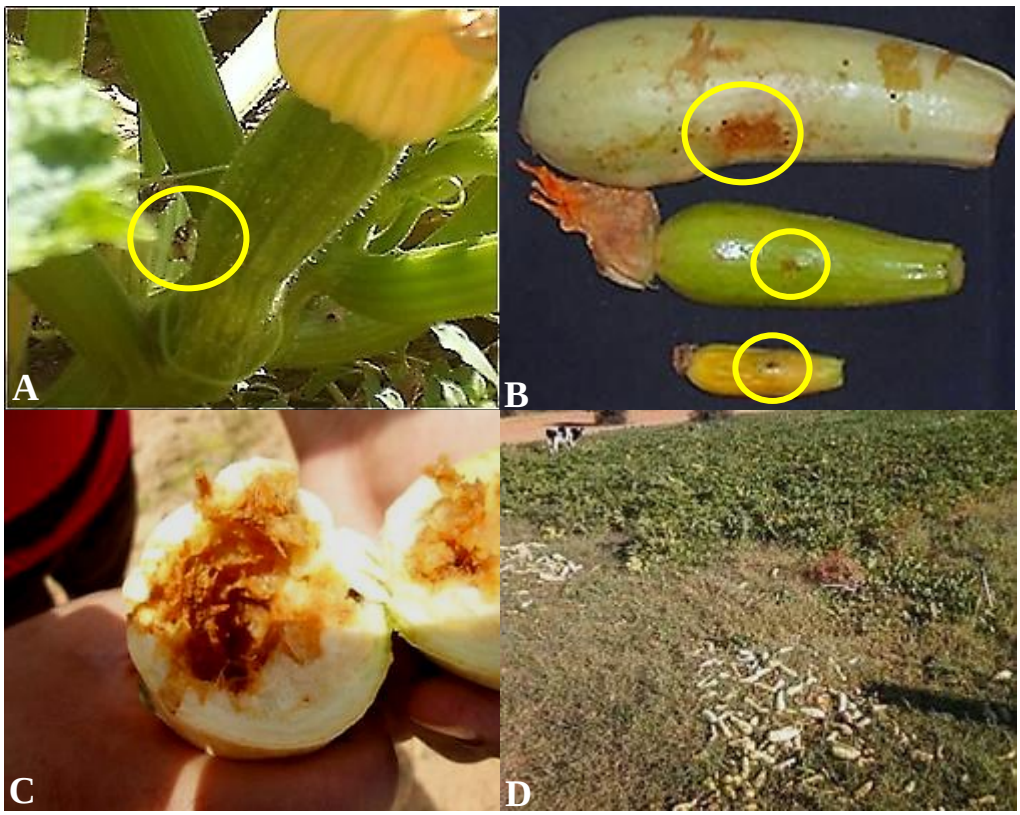


Figure 1.2 A female *Dacus frontalis*, laying eggs into a small zucchini (A) Infestation symptoms on different age stages of cucurbit fruits (B) A cross section of an infested zucchini fruit showing feeding damage by larvae (C) Unmarketable infested zucchini are left on one side of a cucurbit field and offered to agricultural animals (D) The pictures were recently taken in ALmrazeek area in Libya in September 2013. (Photographs © were taken by the author. 2013).

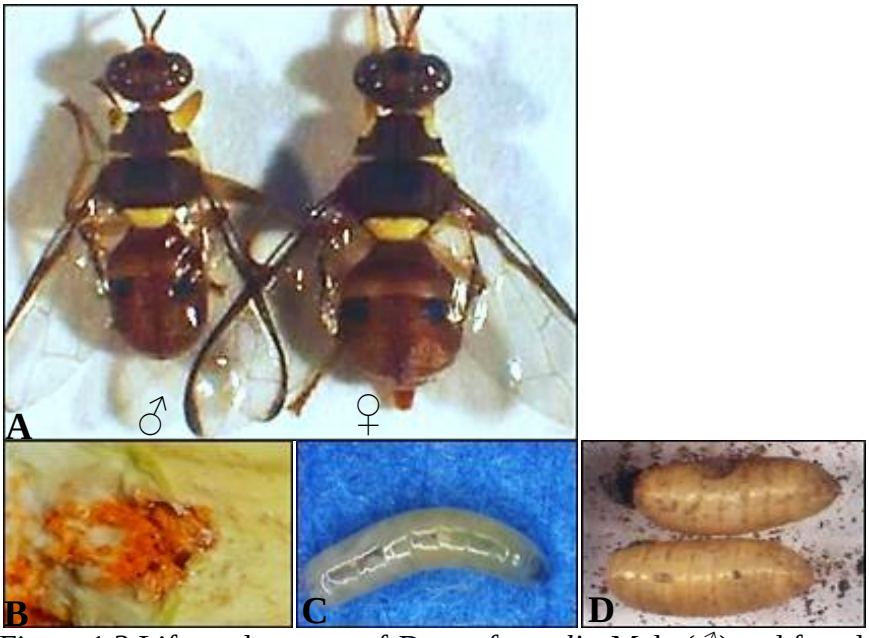


Figure 1.3 Life cycle stages of *Dacus frontalis*. Male (♂) and female (♀) adults (A), Eggs deposited under a cucurbit fruit skin (B), a third instar larva (C) and pupae. (Photographs © were taken by the author).

1.4.5 *Dacus frontalis* ***Control strategy***

To my knowledge, published studies on *D. frontalis* management are very limited. Few procedures have been recommended to reduce the fly population. In old field trials conducted by Ba-Angood (1977), four insecticides (diazinon, fenthion, trichlorphon and fenthion) caused considerable reduction in the fly infestation resulting in greater weight of sweet melon in the treated plots compared to the control. The author recommended three applications of the above insecticides at intervals of one week. In some Sub-Sahara African countries, spraying the edges of the cucurbit crop by insecticides in late afternoon, planting trap plants at the edges of the crop, using bait traps, and uprooting wild host were applied (Steffens, 1982). Also, male annihilation techniques (Hanna *et al.*, 2008) and field sanitation and post-harvest fruit treatment (Ekesi *et al.*, 2010) have been reported to reduce fruit infestation by some African tephritid flies including *D. frontalis*. Quarantine inspections are followed in some importing countries to prevent the entry of serious pests. For example in Zimbabwe, stricter procedures should be followed where cucurbit crops are growing before export to the United States. The cucurbit plants must be grown in special pest-free greenhouses. Baited traps should be placed inside and outside the greenhouses which should be inspected at least once weekly. The area around the cucurbit crop also should be free of any host plants (http://www.gpo.gov/about/).

1.4.6 *Current control in Libya*

Although in the last decade a national project has been proposed for study and control of *D. frontalis* in Libya, a practical control programme has not been established against the pest. Most of the procedures mentioned are seldom applied to protect cucurbit fruits from fly attack. Currently, Libyan farmers still rely mainly on extensive application of several insecticides. Foliar application (Malathion (cholinesterase) and Dursban (chlorpyrifos)) and soil treatment (Delfos (chlorpyrifos ethyl 5%)) are common procedures against larval, pupal and adults stages of *D. frontalis* (personal observation). However, such applications often fail to suppress the fly population, resulting in economic losses. This is probably because the larval stage is protected from pesticide exposure or an insecticide-resistant strain of the fly has arisen recently. The Libyan farmer has little knowledge of using developed control agents and strategies to protect their crops (personal observation). For example, although they are aware of the considerable fly damages they ignore applying even the traditional agricultural methods such as field sanitation. They throw the infested fruits on sides of the field and providing them as a food for their agricultural animals (Figure 1.2.D) instead of the fruits being collected and

correctly disposal. This action might be a reason of increasing damage losses of cucurbit fruits in Libya. Also, some farmers ignore the instructions for using the insecticides. For example, they do not take into account the application time and withdrawal periods of the insecticides used (personal observation). They harvest the contaminated fruits immediately or a few days after the treatment which could create health hazards for consumers.

Although the above control procedures can reduce *D. frontalis* damage, they have limited effectiveness. Also, concerns about using traditional pesticides for insect control programme have increased because of the negative impacts they have on agricultural production, growers, consumers, and the environment. Thus, applying and developing safer effective approaches to control the fly within integrated fly management are required.

To my knowledge, safer effective alternatives to chemical pesticides have not been investigated against *D. frontalis* yet compared to other closely related species of fruit fly.

1.5 Review of integrated fruit fly management

According to Prokopy (2003), integrated pest management (IPM) is: `*a decision-based process involving coordinated use of multiple tactics for optimizing the control of all class of pests (insects, pathogens, weeds and vertebrates) in an ecologically economically sound manner*.` Several control measures for suppressing fruit fly populations have been reviewed (Cini *et al.*, 2012; Billah *et al.*, 2015; Vargas *et al.*, 2015). The authors emphasized the effectiveness of combing use of several control methods against fruit flies. Although the control methods mentioned below can reduce a fly population to low levels, incorporation of more than one control agent together in an integrated fly management programme has been proved to achieve better control with potential advantages of increasing fruit yield and making the environment safer (Cuperus *et al.*, 2004; Cook *et al.*, 2006; Dolinski and Lacey, 2007; Jang *et al.*, 2008; Yan-mei, 2011; Praveen *et al.*, 2012; Halder *et al.*, 2013; Sahayaraj *et al.*, 2013; Haldhar *et al.*, 2014; Ruiu, 2015). For examples, the sterile insect technique (SIT) has been used in combination with other control agents against some species of fruit fly. The Mediterranean fruit fly (*Ceratitis capitata*) was eradicated from Kula, Maui and Hawaii by using SIT together with release of parasitoids (Wong *et al.,* 1992). Jang *et al.* (2008), reported that 90 % of melon fly *B. cucurbitae* was controlled after using bait and trapping systems in incorporation with other control agents. Recently, bait stations based on several attractants have been integrated into sterile insect programmes for many species of fruit flies including the genus *Dacus* showing effectiveness in monitoring and control (Joint, 2007; Jang *et al.*, 2008; Barclay *et al.*, 2014; Suckling *et al.*, 2014).

1.5.1 *Cultural practices*

Cultural methods usually include several traditional procedures which can reduce fly infestation. Some studies and reviews have demonstrated that cultural methods reduce adult emergence rate of different species of fruit flies from soil (Dhillon *et al.*, 2005a; Klungness *et al.*, 2005; Akram *et al.*, 2010; Ekesi *et al.*, 2010; Hasyim and de Kogel, 2013). Fruits infested by fruit flies are one of the potential infection source in the following season which could lead to increasing fly populations in the field (Billah *et al.*, 2015). The authors recommended following field sanitation as a good procedure to reduce fruit fly populations, indicating that infested fruits should be collected from a field, placed into plastic bags and exposed to the sun. In a recent field assessment Hasyim and de Kogel (2013), found that populations of *Bactrocera tau* and the level of damaged fruits were reduced when sanitation procedures were followed in passion fruit orchards. Fruit bagging has been also reported to contribute to reducing fruit infestation by fruit flies (Sarker *et al.*, 2009; Ekesi *et al.*, 2010). Billah *et al.* (2015), showed that covering fruits with different paper materials before fly infestation prevented fruit fly females to depositing eggs into the fruits, increasing fruit quality. Fang and Chang (1987) found that covering bitter gourd fruits with bags after setting reduced *D. cucurbitae* infestation. Furthermore, early harvesting of fruits could reduce fruit damage or even result in infestation- free fruit (Ekesi *et al.*, 2010; Billah *et al.*, 2015; Rojnić *et al.*, 2015).

1.5.2 *Attract and kill technique*

Various lures have been reported to attract fruit flies (Tan *et al.*, 2014). Methyl eugenol, Cue-lure, ketone and trimedlure are widely used as attractants for males of economically important tephritid species (Vargas *et al.*, 2010b; Vargas *et al.*, 2012; Vargas *et al.*, 2014). Methyl eugenol and cue-lure are highly attractive lures to oriental fruit fly, *B. dorsalis*, and melon fly, *B. cucurbitae*, respectively (Fargas *et al.*, 2000). Also, females and males of some fruit flies have been captured in food-baited traps. These attractants provide sugar and protein sources to the adults. Several natural compounds (corn, milk and soy), synthetic lures (ammonium and trimethylamine), bacteria (enterobacteriacea) have been evaluated as food-bait attractants and become commercially available to detect and control many tephritid adults (Epsky *et al.*, 2014). The genus *Dacus* respond to different attractants. For example, *D. celiatus* has been reported to have a response to methyl eugenol but not to Cue-lure (White, 2006). In a field trial conducted by Roomi *et al.* (1993), they found that four species of genus *Dacus* were trapped when the fly males were attracted to extracts of the plant *Ocimum sanctum* and methyl eugenol under field conditions. Published information indicates that *D. frontali*s is

attracted to Cue-lure traps (Hancock, 1985; Carroll *et al*., 2006; Jang *et al.*, 2008; Praveen *et al.*, 2012). In recent field trials conducted by Hafsi *et al.* (2015) both male and female *D. frontalis* were attracted to traps baited with diammonium phosphate during a mass trapping programme for another tephritid species.

The potential of several attractants within integrated tephritid programmes has been investigated by various authors. Bait spray and male annihilation techniques are currently successfully used in some fruit fly control programmes (Barclay *et al.*, 2014; Shelly *et al.*, 2014). Many studies have demonstrated that baiting and male annihilation are effective control procedures either used alone or in combination with other control agents against several species of fruit flies (Hancock *et al.*, 2000; Allwood *et al.*, 2002; Vargas *et al.*, 2003; Stonehouse *et al.*, 2007).

1.5.3 *Sterile insect technique*

Sterile insect technique (SIT) is a species specific (Klassen and Curtis, 2005) and environmentally friendly method to limit populations of different insect pests (Dyck *et al.*, 2005). SIT depends on releasing large numbers of sterile males into infested area where they mate with wild females. This leads the females to produce infertile eggs or not lay eggs. Sterilization can be achieved by irradiation e.g. with x-rays (Robinson, 2005). This method has been successfully used to suppress or eradicate some tephritid fruit fly species around the world (Klassen and Curtis, 2005; Meats *et al.*, 2006). Eradication can be successfully achieved if sterile flies are released in sufficient numbers. It has been reported that reducing the target fly population to low levels before releasing sterilised individuals improves the effectiveness control (Dyck *et al.*, 2005).

1.5.4 *Chemical pesticides*

Synthetic pesticides have been excessively used to protect agricultural crops in fields and grain houses from attack by pests for the last six decades (Kumar *et al.*, 2008). Despite the disadvantages of pesticide applications for the environment, this method is still used alone or in combination with other control agents against numerous fruit flies species. These chemicals have been reported as effective compounds against many species of fruit fly (Shafiq Ansari *et al.*, 2012; Wang *et al.*, 2013; Mahat and Drew, 2015). For example, applying diazinon reduced adult emergence of *C. capitata*, *B. cucurbitae* and *B. dorsalis* considerably when it was drenched on soil (Stark *et al.*, 2013). To limit the negative impact of some insecticides on the environment, Mahat and Drew (2015) evaluated malathion mixed with protein and applied

against *B. tryony* on citrus coursing high mortality. Also, some insecticides have successfully contributed to suppression of populations of some species of Tephritidae when combined with other control agents such as traps and attractants (Joint, 2007) and sterile insect technique (Allwood *et al.*, 2002).

1.5.5 *Biological control*

According to Eilenberg *et al.* (2001), biological control is: *'The use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be.'* The authors described various strategies, including classical biological control, inoculation biological control, inundation biological control and conservation biological control for introducing biocontrol agents within insect control programs.

Recently, introducing insect enemies such as parasites, predators and entomopathogens in agricultural system has been receiving increasing attention. Biological control is an economical method and poses less threat than pesticides to humans and the environment (Rizvi *et al.*, 2009). This has encouraged many researchers to evaluate different biological control agents as alternatives to pesticides against several insect pests including fruit flies (Esser and Lemke, 1995; Rousse *et al.*, 2009).

A. Release of parasites: Hymenopteran parasites have been proved to be good biocontrols against Tephritidae species (Purcell *et al.*, 1997; Ovruski *et al.*, 2000; Aluja *et al.*, 2003; Ovruski *et al.*, 2004; Wang *et al.*, 2004; Bokonon-Ganta *et al.*, 2005; Rendon *et al.*, 2006; García-Medel *et al.*, 2007; Wang *et al.*, 2008; Ero and Clarke, 2012; Bokonon-Ganta *et al.*, 2013; Montoya *et al.*, 2013; Wang *et al.*, 2013; Manoukis *et al.*, 2014; Ali *et al.*, 2015; Poncio *et al.*, 2015). These enemies have the ability to oviposit into a life stage (eggs, larvae or pupae) and completing their larval stages before the host is dead (Rousse *et al.*, 2009). For example, in laboratory bioassays carried out by Mohamed *et al.* (2010), *Fopius arisanus* showed various levels of parasitism to eggs of four species of fruit flies. Recently, *Diachasmimorpha longicaudata* significantly reduced the numbers of larvae of *Anastrepha* spp under natural conditions (Montoya *et al.*, 2013). It has been suggested that release of parasites alone would provide unsatisfactory control for fruit flies including tephritid species (Purcell, 1998; Lux *et al.*, 2003). Therefore, compatible use of some of these biocontrol agents with other control tools has been considered within integrated fly management (Zamek *et al.*, 2012). For example, mass releases of some parasites has successfully contributed to suppression of populations of some species of Tephritidae when combined with other control

agents such as SIT, baits sprays, entomopathogens and mass trapping. (Rousse *et al.*, 2009). In a study conducted by Jang *et al.* (2008), augmentative release of *Psyttalia fletcheri* as a parasite in combination with four other control agents including mass trapping, male annihilation, sanitation, SIT and bait spray were reported to cause approximately 83% reduction of melon fly population.

B. Release of predators: The efficiency of several predators of tephritid species have been reported (Peng and Christian, 2006; Adandonon *et al.*, 2009; Cao *et al.*, 2012; Appiah *et al.*, 2014; Kirkegaard *et al.*, 2014). For example, the ant *Solenopsis geminata* (Hymenoptera: Formicidae) predated up to 25% of *C. capitata* larvae (Eskafi and Kolbe, 1990). Another ant species, a weaver ant, *Oecophylla longinoda*, has been reported as a potential control for larvae of *Ceratitis* spp and *B. invadens* (Van Mele *et al.*, 2007). In recent field trials conducted by El Keroumi *et al.* (2010), four species of ant caused high mortality to larvae of *C. capitata* released on Argan trees (*Argania spinosa*). The authors found that more than 90% of the larvae were predated.

C. Insect pathogens: Insect pathogens are natural microorganisms found in many environments. These organisms can induce a disease in a life stage of their insect host. Pathogens such as nematodes, bacteria and fungi have been shown to be potential biological control agents against fruit fly species and are considered as safe alternatives to chemical pesticides (Dolinski and Lacey, 2007).

1- Entomopathogenic nematodes kill their hosts by introducing bacteria into the host body causing death (Rizvi *et al.*, 2009). *Heterorhabditis* and *Steinernema* species have been reported to cause mortality in some tephritid species (Gazit *et al.*, 2000; Yee and Lacey, 2003; Lin *et al.*, 2004; Kuske *et al.*, 2005; Toledo *et al.*, 2006; Malan and Manrakhan, 2009; Sirjani *et al.*, 2009; Kamali *et al.*, 2013; Langford *et al.*, 2014; Nouh and Hussein, 2014). For example, *Heterorhabditis sp.* and *S. carpocapsae* caused 26%-74% mortality in *C. capitata* larvae under laboratory conditions (Rohde *et al.*, 2013). Larvae and adults of cucurbit fly, *Dacus ciliatus* were also found to be highly susceptible to *S. carpocapsae* under laboratory and greenhouse conditions (Kamali *et al.*, 2013). More recently, pathogenicity investigations conducted by Toledo *et al.* (2014) showed effectiveness of *H. bacteriophora* against *Anastrepha ludens* larvae with high mortality reaching 80%.

2- Entomopathogenic bacteria produce protein toxins or metabolites after being ingested by insects causing death (Rizvi *et al.*, 2009; Ruiu, 2015). Different species of the *Bacillus* genus have been found effective against some economically important tephritid species (Karamanlidou *et al.*, 1991; Robacker *et al.*, 1996; Bel *et al.*, 1997; Sivropoulou *et al.*, 2000; Floris *et al.*, 2007; Molina *et al.*, 2010; Aboussaid *et al.*, 2011; Elleuch *et al.*, 2015). For example, in a survey conducted by Karamanlidou *et al.* (1991), 24 isolates of *B. thuringiensis* caused various mortality levels ranged from 7% to 87% in *Bactrocera oleae* larvae. Some strains of *B. thuringiensis* showed potential toxic effect to *C. capitata* larvae, affecting the size of pupae, emergence rate and fecundity of adults that emerged (Ruiu *et al.*, 2015). In another toxicity assessment of some strains of *B. thuringiensis* against the same fly, 68% and 82% larval and adult mortalities respectively were obtained (Aboussaid *et al.*, 2010). More recently, considerable reduction of *C. capitata* pupae (100%) was found in a primary evaluation of actinibacteria isolates against the fly larvae (Samri *et al.*, 2015).

3- Entomopathogenic fungi have a unique characteristic in that they can cause death to infected insects without being ingested by the host compared to nematode and bacterial pathogens (Lacey and Shapiro-Ilan, 2008). Also, the fungi have a wider range of insect hosts (Lacey and Kaya, 2000). Pathogenicity of several isolates of entomopathogenic fungi to fruit flies species has been previously confirmed (Castillo *et al.*, 2000; Ekesi *et al.*, 2002; Dimbi *et al.*, 2003; Konstantopoulou and Mazomenos, 2005; Mochi *et al.*, 2006; Toledo *et al.*, 2007; Ali *et al.*, 2008; Lezama-GutiÃ©rrez *et al.*, 2009; Daniel and Wyss, 2010; Svedese *et al.*, 2012; Beris *et al.*, 2013; Imoulan and Elmeziane, 2014). These pathogens are effective by killing soil and adult stages of several tephritid species (De La Rosa *et al.*, 2002; Ortu *et al.*, 2009; Beris *et al.*, 2013) or inducing sublethal effects on the hosts (Dimbi *et al.*, 2009; Novelo-Rincón *et al.*, 2009; Sookar and Allymamod, 2014). Dimbi *et al* (2003) found that nearly 87% of *C. capitata* adults were killed 4 days from being exposed to *Metarhizium anisopliae*. Recent results indicated that approximately 30% to 40% of the European cherry fruit fly, *Rhagoletis cerasi* adults were killed five days after being sprayed by two isolates of *M. anisopliae* (Daniel and Wyss, 2009). More recently, efficacy trials of *Isaria fumosorosea B. bassiana* and *M. anisopliae* reached 100% mortality of the peach fruit fly, *Bactrocera zonata* adults when the fungi were applied in different inoculations (Gul *et al.*, 2015). Reviews have indicated that entomopathogenic fungi are considered as an effective control agent within integrated fly management (Ekesi *et al.*, 2007; Daniel and Grunder, 2012). To date, using such pathogens against *D. frontalis* has not been investigated.

1.6 Entomopathogenic fungi

1.6.1 *Overview*

Entomopathogenic fungi are living microorganisms which infect insects causing a disease to the hosts in appropriate conditions. These pathogens have a wide range of hosts and are distributed in different natural habits (Sujeetha and Sahayaraj, 2014). Insect pathogenic fungi are generally safe to the humans and environment (Lacey *et al.*, 2001; Zimmermann, 2007; Hajek *et al.*, 2009). These features have made entomopathogenic fungi one of the more attractive biological control agents (Butt *et al*. 2001; Shah and Pell, 2003) and have prompted many investigations of their pathogencities against a range of economically important insect pests (Contreras *et al.*, 2014; Goble *et al.*, 2014; Kaur *et al.*, 2014; Reddy *et al.*, 2014; Carrillo *et al.*, 2015; Kassab *et al.*, 2015). Details of the advantages and disadvantages of using these microbes are well described by (Khan *et al.*, 2012).

It is estimated that more than700 species belonging to 100 genera of fungi are considered as insect pathogens (Leger and Wang, 2010). Species belonged to *Beauveria, Metarhizium, Lecanicillium* and *Isaria* fungi are commercially produced as biopesticides against several insect pests (Vega *et al.*, 2009). A few of these species are currently commercially produced such as Balence, Mycotrol ES, and PaciHit Rich and used for the management of Dipteran species (Sujeetha and Sahayaraj, 2014). This limited number reflects the difficulty of mass production of some species (Lacey and Kaya, 2000). The most common entomopathogenic fungi belong to the Entomophthorales and Hypocreales orders. Members of Entomophthorales such as *Entomophtora* and *Zoophthora* cause death to the hosts by colonisation (Webster and Weber, 2007). While the genera of Hypocreales such as *Beauveria, Metarhizium, Paecilomyces and Lecanicillium* can release toxins leading death of the host (Arora and Shera, 2014).

1.6.2 *Pathogenicity mechanism and life cycle*

In comparison with other entomopathogens such as nematodes and bacteria, insect pathogenic fungi do not need to be ingested to cause death to their hosts, but penetrate their cuticle (Lacey and Shapiro-Ilan, 2008). Physical and enzymatic mechanisms help the spores penetrate the cuticle (Inglis *et al.*, 2001). Spores have degrading enzymes such as proteases, esterases, lipases and chitinases which modify the host`s cuticle surface and help the spores' attachment before penetration (Khan *et al.*, 2012). Once spores come in contact with insects' cuticle they may germinate and penetrate through the cuticle dependent on environmental

conditions such as moisture (Rizvi *et al.*, 2009) and structure of the host cuticle (Inglis *et al.*, 2001). After penetration, the fungus uses the host body`s nutrients for growth before multiplying rapidly causing starvation and physiological disruption leading to death (Wraight and Inglis, 2000). Then, the fungus emerges throughout the cuticle through the intersegmental regions (Pell *et al.*, 2001) before covering the host cadaver under suitable conditions producing spores (Charnley and Collins, 2007). The spores then spread by direct contact with other hosts or by other factors such as wind causing new infection to susceptible hosts (Lacey and Kaya, 2000).

1.6.3 *Entomopathogenic fungi for insect control*

Entomopathogenic fungi have been considered as a potential biocontrol agent against several insect species (Inglis *et al.*, 2001; Lacey *et al.*, 2001; Shah and Pell, 2003) and as a part of integrated pest management programmes for several others (Lacey and Shapiro-Ilan, 2008; Khan *et al.*, 2012), with limited effect on non-target species (Vestergaard *et al.*, 2003). They do not have effects on some fruit fly parasitoids (Ekesi *et al.*, 2005). Zimmermann (2007), indicated that two species of *Beauveria sp* are considered as safe pathogens with low impact on the environment. Entomopathogenic fungi can be used in agricultural systems with different strategies. Classical control depends on introducing pathogenic fungi permanently into infested areas to suppress the pest in the long term (Hajek *et al.*, 2003). For example, *Entomophaga maimaiga* fungus was introduced to control the gypsy moth, *Lymantria dispar* in eastern North America in 1910-1911. The Japanese isolate caused considerable reduction in gypsy moth populations in USA between 1989 and 1990 (Hajek *et al.*, 1995). While, a inundation strategy is used when the pest`s population should be reduced in a short period (Pell *et al.*, 2001; Ravensberg, 2011). For example, *Metarhizium anisopliae* var. acridum has been used for control locust and related pests in Africa (Shah and Pell, 2003).

1.6.4 *Application strategy*

Choosing an appropriate strategy for applying biopesticides offers better control efficacy against target insects. Different application strategies depend on several factors such as biopesticide formulation, application method, timing, dose and biology and ecology of the target insect species, have been reported to influence the susceptibility of several insects orders to entomopathogenic fungi (Lacey and Shapiro-Ilan, 2008; Rizvi *et al.*, 2009).
Many studies results have shown a potential control of numerous insect orders by spraying different species of entomopathogenic fungi. For example, foliar application of *Beauveria bassiana* strain GHA (Bb-GHA) caused moderate to high rates of infection to Colorado

potato beetle *Leptinotarsa decemlineata* and are suggested to be a valuable tool for an IPM program of the insect (Wraight and Ramos, 2015). Different species of entomopathogenic fungi have also been applied in soil against several insect pests. For example, a granule form of some species and strains of *Metarhizium* fungus has been used as a successful pathogen against black vine weevil larvae, *Otiorhynchus sulcatus* (Moorhouse *et al.*, 1993; Bruck and Donahue, 2007; Ansari and Butt, 2013), Tomato potato psyllid, *Bactericera cockerelli* (Mauchline *et al.*, 2013) and the large pine weevil, *Hylobius abietis* (Williams *et al.*, 2013).

1.6.5 *Factors influencing fungal pathogenicity*

There are various factors related to the insect host, pathogen and environment which have different impacts on the pathogenicity and spread of the disease. Germination, growth, the ability of insect pathogenic fungi to induce a disease and persistence depends on environmental conditions (Wraight and Inglis, 2000). For example, soil moisture, temperature and humidity are known to be important factors influencing survival and persistence of fungal pathogens (Fargues and Luz, 2000; Arthurs and Thomas, 2001; Yeo *et al.*, 2003; Filotas and Hajek, 2004; Fuxa and Richter, 2004; Luz *et al.*, 2004; Lord, 2005; Thompson *et al.*, 2006; Chen *et al.*, 2014). Temperature can influence the speed and rate of the fungal infection (Inglis *et al.*, 2001). Mishra *et al.* (2013), reported that high adult mortality of the housefly was obtained at high humidity (70% to 100% RH) and less than 50% adult mortality at 50% RH when *B. bassiana* was sprayed against the flies. Another study showed different responses of *M. anisopliae* isolates to temperature when larvae of *C. capitata* were released into soil treated with the isolates (Garrido-Jurado *et al.*, 2011c). The authors indicated that insect pathogenic fungi should have a range of temperatures and humidities when they are used for insect control as different species of entomopathogenic fungi have different responses to environmental conditions.

1.6.6 *Combined use with other control agents*

Total suppression of insect pests will not be achieved by a single application of entomopathogenic fungi (Inglis *et al.*, 2001). In order to enhance the fungal efficiency e.g., rapid kill and overall mortality some other control agents could be used in combination with the fungi (Hajek *et al.*, 2009). Different control agents e.g., insecticides, predators, parasitoids, entomopathogens have been successfully evaluated in combination with entomopathogenic fungi against several insect pests. For example, the efficacy of *M. anisopliae* against black vine weevil, *Otiorhynchu. sulcatus* was enhanced when applied in combination with low rates of insect pesticides (Shah *et al.*, 2007). Also, the potential

advantages of combining entomopathogenic fungi with some botanical insecticides have been reported against different insect pests (Santi *et al.*, 2011; Radha *et al.*, 2014). Combinations of neem oil with fungal pathogens have been found to have various impacts on fungal activity inducing additive, synergistic and antagonistic effects on control efficacy (Akbar *et al.*, 2005; Mohan *et al.*, 2007).

1.7 Aims of thesis

The main aims of this project are to evaluate pathogenicity of entomopathogenic fungi against the fly and to develop better strategies for using this approach in integrated fly management. The expected findings may provide an effective biological control agent and a suitable control strategy against *D. frontalis* to safeguard better future food security and the environment.

Objectives

The thesis contains eight themed chapters, including a general introduction and general discussion.

Chapter 2. Potential use of entomopathogenic **fungi as a biological control against the Greater melon fly** *Dacus frontalis* **(Becker) (**Diptera**:** Tephritidae**)**

The efficiency of five entomopathogenic fungi based on several commercial strains was evaluated on larvae, pupae and adult stages of *D. frontalis.* The susceptibility of different life stages of the *D. frontalis* to the entomopathogenic fungi was investigated by using various inoculation methods under laboratory conditions. The most effective formulation, Met52 Granular biopesticide, based on *M. anisopliae* var *anisopliae* strain F52 (MET52), was selected for further investigations. A dose-response of the target pest to MET52 was examined. Effects of formulation, and application time on the efficacy of the fungus were also evaluated. In addition, the comparative susceptibility of two tephritid species, *D. frontalis* and *Ceratitis capitata,* to MET52 infection was assessed.

Chapter 3. Isolation, identification and potential pathogenicity of Aspergillus fungus obtained from a lab culture of *Dacus frontalis* **(Becker)**

This chapter describes attempts to isolate and identify a fungal species found associated with dead pupae collected from a laboratory culture strain of *D. frontalis.* An isolated fungus was subjected to microscopic observations followed with identification based on colonial morphology and microscopic feature.

To confirm the fungus at species level, extraction of DNA, Polymerase Chain Reaction and DNA sequencing were performed.

The potential pathogenicity of the fungus isolated against soil life stages and adults of the fly was determined. Various inoculation methods were used in laboratory conditions to investigate the potential pathogenicity of the fungus against the fly. The fungus and three commercial biopesticides based on several strains of entomopathogenic fungi were compared.

Chapter 4. Influence of soil moisture, humidity, temperature and application method on efficacy of a commercial strain of *Metarhizium anisoplae* against the Greater melon fly *Dacus frontalis* (Becker)

This chapter evaluated the influence of soil moisture, humidity and temperature on efficacy of MET52 against *D. frontalis*. The effects of ten soil moistures (10%- 100% Water Holding Capacity (WHC)), three relative humidity ranges (40%- 50%, 55%- 65% and 75%- 85% RH) and three temperatures (15 ºC, 25 ºC and 35 ºC) were examined under laboratory conditions. Also, the effect of application method was investigated. The fungus was directly sprayed on adults or applied to soil as granules.

Chapter 5. Horizontal transmission and persistence of *Metarhizium anisopliae* in *Dacus frontalis* (Becker) and effect of the fungus infection on fly reproduction

The objectives of this chapter were to investigate the ability of *D. frontalis* adults emerged from MET52-inoculated soil to transfer conidia and induce new infection in untreated adults in different mating combinations. Female reproductive potential in the various mating combinations was also investigated. In addition, persistence of MET52 in soil was determined by assessing infectivity against larval-pupal stages and newly emerged adults.

Chapter 6. Combined use of Met52® Granular biopesticide with two botanical products against *Dacus frontalis* (Becker)

The aims here were to investigate the toxic effects of neem oil and garlic extract on larvae and adults of *D. frontalis.* Seven concentrations of neem oil and garlic extract (5% to 100%) were used separately to assess potential toxic effects on larvae in Petri dish experiments. Also, possible compatibility and toxic effects of four sublethal doses of neem and garlic (2%, 1%, 0.5% and 0.25%) alone and in combinations with MET 52 on larvae, pupae (measured by assessing adult emergence rate) and post-emergence mortality of *D. frontalis* adults were evaluated using soil application.

Chapter 7. Evaluation of Met52® Granular biopesticide for control of the Greater melon fly *Dacus frontalis* (Becker) under semi field conditions

Although successful use of entomopathogenic fungi against insect pests has been proved under controlled conditions, field assessments of these biological agents are limited. Therefore, the effect of MET52 against the target fly under the natural conditions was evaluated. The aims of these experiments were to evaluate efficacy of MET52 in reducing emergence rate and causing mortality post-emergence of *D. frontalis* adult in semi field cages. Also, as there is no specific study published on evaluation of bait traps to attract *D. frontalis* adults, several traps baited with yeast hydrolysate enzymatic were evaluated in attracting and capturing *D. frontalis* adults before starting the main experiments.

Chapter 2. Potential use of entomopathogenic fungi as a biological control against the Greater melon fly *Dacus frontalis* (Becker) (Diptera: Tephritidae)

Abstract

The pathogenicity of five commercial biopesticides based on several strains of entomopathogenic fungi, *Metarhizium anisopliae, Beauveria bassiana* and *Paecilomyces fumosoroseus* against larvae, pupae and adults of the Greater melon fly, *Dacus frontalis* (Becker) was evaluated in soil under laboratory conditions. In the susceptibility test, the results revealed that *D. frontalis* adults are more susceptible to the fungal pathogens than pupae. None of the biopesticides caused mortality of larvae. Met52 Granular biopesticide, based on *M. anisopliae* var *anisopliae* strain F52 (MET52), caused the greatest pathogenicity to the adults ranging from approximately 88 % to100% mortality. Other biopesticides caused very low to moderate adult mortality ranging from approximately 11% to 66%. MET52 was selected for further investigation. Pupal age and increasing rate of MET52 had no effect on pupal mortality. However, MET52 increased mortality of emerging adults by 15% when applied on young pupae. Also, early application of MET52 in a granule form caused a significant reduction in adult emergence compared with a drench and untreated control. The effect of MET52 against *D. frontalis* was influenced by application time with the greatest pathogenicity recorded when treatment occurred two weeks before larvae entered the soil resulting in a 55% reduction in the adult emergence rate. Over all, the present study suggests that early soil application of MET52 offers a promising control for *D. frontalis* by reducing emergence rate and adult flies.

2.1 Introduction

As reviewed in the previous chapter, safer and more effective approaches are required to suppress *Dacus frontalis* damage losses. Biological control is one of the available alternative control strategies to traditional insecticides (Esser and Lemke, 1995). Fungal pathogens are valuable biological agents for controlling some agricultural insect pests (Esser and Lemke, 1995; Butt, 2002; Roy *et al.*, 2010). They are environmentally safe in general (Esser and Lemke, 1995; Wraight and Hajek, 2009). Some fruit flies are susceptible to fungi (Castillo *et al.*, 2000; Ekesi *et al.*, 2002; Dimbi *et al.*, 2003; Konstantopoulou and Mazomenos, 2005; Mochi *et al.*, 2006; Toledo *et al.*, 2007; Ali *et al.*, 2008; Lezama-GutiÃ©rrez *et al.*, 2009; Daniel and Wyss, 2010; Svedese *et al.*, 2012; Beris *et al.*, 2013; Imoulan and Elmeziane, 2014). Various inoculation approaches have been used to determine the pathogenicity of

several entomopathogenic fungi against tephritid flies. Sookar *et al.* (2008), reported that two isolates of *M. anisopliae* (M65 and M235) caused 96% and 98% mortality respectively five days after the treatment when applied by a micropipette to the abdominal surface of peach fruit fly, *Bactrocera zonata* adults. A study by Daniel and Wyss (2009) indicated that species of *M. anisopliae, Isaria fumosorosea* and *B. bassiana* were highly pathogenic when sprayed onto *Rhagoletis cerasi* adults. De la Rosa *et al.* (2002), found low mortalities in larvae and pupae of the Mexican fruit fly, *Anastrepha ludenswere* after they were dipped in suspensions of eight strains of *B. bassianna*. The same study reported a high adult female mortality, ranging from 82% to 100%, after spray treatment of the fungi used. Beris *et al.* (2013), indicated that low mortality, approximately 19% to 24 %, was obtained when *Ceratitis capitata* pupae were dipped in suspensions of three fungi species. However, higher mortality rates were induced after emergence. Soil application of these pathogens has been also suggested as a strategy to reduce emergence rates and induce mortality post-emergence of some fruit flies adults (Ekesi *et al.*, 2005; Garrido-Jurado *et al.*, 2011a). In pathogenicity investigations conducted by Ekesi *et al.* (2002) and Mochi *et al.* (2006) it was found that sand treated with different isolates of *M. anisopliae* had induced up to 100% mortality in the emerging adults of *C. capitata, C. fasciventris* and *C. cosyra*. Recently, good control of larvae of the European cherry fruit fly, *Rhagoletis cerasi* was obtained when the larvae were placed into sand treated with two isolates of *B. bassiana* (Cossentine *et al.*, 2010). More recently, different inoculation methods induced various mortality levels of larval, pupal and adult stages of *B. zonata* to three insect pathogenic fungi (Gul *et al.*, 2015).

Insect pests have different susceptibilities to different strains of entomopathogenic fungi (Butt *et al.*, 1995). Therefore, investigation of pathogenicity is an essential step to select appropriate fungal strains. To date, using such fungal pathogens against *D. frontalis* has not been studied. The aim of this study was to investigate the susceptibility of different life stages of the *D. frontalis* to commercial biopesticides based on different species of entomopathogenic fungi when applied in different methods under control conditions. Also, a dose-response of the target pest to selected pathogens was examined. Effects of formulation and application time on the efficacy of the fungus were also evaluated. This may help to find an effective pathogen and strategy for the fly biological control.

2.2 Materials and Methods

2.2.1 *Insect culture*

A number of *D. frontalis* pupae were obtained from the Biotechnology Research Centre in Libya. A culture was maintained at 25 ºC, 50%-55% relative humidity (RH), and 14:10 hours light to dark (L: D) photoperiod. Adults were kept in transparent perspex cages (25 cm × 25 cm × 25 cm) covered with gauze at one side for ventilation. The cage was supplied with water and artificial diet consisting of 1:3 ratio of Yeast hydrolysate enzymatic (MP Biomedicals, France) and sucrose. Eggs were collected by introducing whole fresh squashes into the cage which were replaced regularly. Larvae were fed on squash in plastic containers (20 cm × 30 cm × 15 cm) filled with sterilised soil (see below) where the full-grown larvae could pupate.

2.2.2 *Bioinsecticides*

Five commercial bioinsecticides were used in this study. The products depend on different strains and isolates of entomopathogenic fungi (Table 2.1). The fungal pathogens were kept at 4 ºC in a refrigerator until used.

Table 2.1 Sources and isolates of entomopathogenic fungi used in the study.

Commercial name	Strain	Supplier	Recommended rate	Concentration
Met52® Granular (MET52)	*Metarhizium anisopliae* var *anisopliae* strain F52	Fargro® Ltd, West Sussex UK	0.5 kg m^{-3} of growing media or 122 Kg ha^{-1} for open ground use	9.0×10^8 Colony Forming Units (CFU) g^{-1}
Bio-Magic	*Metarhizium anisoplae* (Metchnikoff) Sorokin	T.Stanes &Company limited, India	4 Kg ha^{-1} in 500 Litters of water	1×10^8 (CFU) ml^{-1}
Bio-Power	*Beauveria bassiana* (Balsamo) Vuillemin	T.Stanes &Company limited, India	4 Kg ha^{-1} in 500 Litters of water	1×10^8 (CFU) ml^{-1}
Bio-Catch	*Paecilomyces fumosoroseus* (Wize) Brown and Smith	T.Stanes &Company limited, India	4 Kg ha^{-1} in 500 Litters of water	1×10^8 (CFU) ml^{-1}
Naturalis-L®	*Beauveria bassiana* strain ATCC 74040	Belchim Crop Protection	3 litres in 1000 L of water	2.3×10^7 (CFU) ml^{-1}

* Only MET52 and Naturalis-L had information on strain.

2.2.3 *Pupal and adult experiment*

Thirty plastic cups (4 cm height × 4 cm diameter) were filled with 30 g of sterilised sandy clay loam soil (autoclaved at 1.5 bar, 123 °C 25 minutes) (65% sand, 12 silt and 23% clay) obtained from Cockle Park, Morpeth, United Kingdom. Six treatments were prepared as following: Soil was inoculated with 1.5 g of MET52 (9.0×10^8 CFU g^{-1} (manufacture`s estimate)). The fungus was mixed with soil. Then, 20 pupae (2 days old) of *D. frontalis* were placed at 2 cm depth. For other inoculated treatments, 2 ml suspensions of Bio-Power, Bio-Magic and Bio-Catch ($1 \times 10^8$ CFU $ml^{-1}$ (manufacture`s estimate)) and Naturalis-L® (2.3×10^7 CFU ml^{-1} (manufacture`s estimate)) were applied after placing the same number of pupae into soil in cups. Two ml of sterilised distilled water was added to the untreated control and the other treatments. Soil moisture content was maintained at 35% Water Holding Capacity (WHC) daily until adult emergence. The treatment cups were covered with the same size of the cups inverted and perforated at the top for air flow. Cups of each single replicate were sealed together at the sides with parafilm and kept in an incubator at 25 ºC, 60% to70% RH and 14:10 L: D. Five replicates for each treatment were arranged. Nine days later (two to three days before emergence), the cups were transferred to transparent plastic cages 10 cm × 10 cm × 10 cm to assess emerging adult mortality. The cages covered with gauze at one side, were supplied with artificial diet and water as previously described. After emergence the number of emerging flies in the treatments was assessed. The cups were taken out of the cages. Four cages with fifteen emerging adults for each treatment were arranged. The cages were kept in the same conditions described above for the cups. Dead flies were collected daily from the cages and assessed over the period of two weeks. To confirm a fungal infection pupae which failed to produce adults and dead adult flies were individually sterilised with 70% ethanol followed with three rinses in sterile distilled water. The samples were placed into Petri dishes with moist sterile filter papers. The Petri dishes were incubated at 25 ºC in the dark. The insect samples were subjected to microscopic observation every 24 hours for a week to ten days. Pupae and adults covered with fungal mycelium were considered as hosts to fungi only.

2.2.4 *Larval experiment*

Six treatments were prepared and inoculated with fungi following the same process described above. Twenty, third instar larvae of *D. frontalis* were released into the treatments. The cups were kept by following the same process and conditions described above. Five replicates for each treatment were arranged. One week later, pupae were sieved from the soil and examined

under a microscope to determine if any growth of mycelium was apparent. To evaluate emerging adult mortality, all pupae recovered from the treatments were placed into Petri dishes and kept in the same conditions until adult emergence. After ten to eleven days, emergence rates were assessed. Then, 19 adults (zero to one day-old) were placed into adult cages to assess mortality. The cages were kept as previously described for adults in experiment one. Four replicates for each treatment were arranged. Dead flies were collected daily from the cages over the period of 12 days. Fungal infection in the adults was investigated by following the same process described in experiment one.

2.2.5 *Pupal age experiment*

As the result of above experiments indicated that MET52 caused the highest mortality against *D. frontalis* (Figure 2.3), the product was selected for further investigations.

Effect of pupal age (two and eight days old) on susceptibility to MET52 was examined. A number of cups filled with soil inoculated with 1.5 g of MET52 (9.0×10^8 CFU g^{-1}) were prepared as previous described. Four millilitre of sterilised distilled water was added to the untreated control and the other treatments. The cups were kept by following the same process and conditions as described in experiment one. Five replicates with 20 pupae for each treatment were arranged. After emergence the number of dead pupae was assessed. Then, 20 adults (zero to one day-old) were transferred in adult cages, kept as described above, to assess mortality. Four replicates for each treatment were arranged. Dead flies were collected daily from the cages over the period of 12 days. Fungal infection in dead pupae and adults was investigated by following the same process described in experiment one.

2.2.6 *Rate response experiment*

In a similar procedure as previously described in experiment one, MET52 was applied in 30 g soil at different rates (1.5, 1, 0.75, 0.5, 0.25, 0.125 g (9.0×10^8 CFU g^{-1}). Fifteen, two day old pupae of *D. frontalis* were used in each replicate and there were five replicates for each treatment. Four millilitre of sterilised distilled water was added to the untreated control and the other treatments. Then, the cups were kept by following the same process and conditions as described in the experiment one. Ten to eleven days after application, adult emergence was assessed. Then, 12 adults (zero to one day-old) were transferred and kept in adult cages to assess mortality following the same process and conditions previously described. Four replicates for each treatment were arranged. Dead flies were collected daily from the cages

and assessed for 5 to 9 days. Fungal infection in the adults was investigated by following the same process described in experiment one.

2.2.7 *Formulation experiment*

The efficacy of two formulations (Granule and Drench) of *M. anisopliae var anisopliae* strain F52 was evaluated in reducing emergence rate and emerging adult of *D. frontalis*. For a granule treatment, cups filled with 30 g soil were prepared and inoculated with the fungus at 9.0×10^8 CFU g^{-1} as previously described. In the drench application, a suspension of the fungus was prepared in 2 ml water at recommended dose (9.0×10^8 CFU g^{-1}) and drenched into the cups. Both treatments were applied one week prior to release of 20 third instar larvae into the cups. Four millilitre of sterilised distilled water was added to the untreated control and the other treatments. Then, the cups were kept following the same process and conditions described above. Five replicates for each treatment were arranged. After emergence, 15 adults (zero to one day-old) were transferred and kept in adult cages and kept as described above. Four replicates for each treatment were arranged. Dead flies were collected daily from the cages and assessed over the period of two weeks. Fungal infection in dead pupae and adults was investigated by following the same process described above.

2.2.8 *Application time experiment*

The result of the formulation experiment indicated that MET52 caused a high reduction in adult emergence of *D. frontalis* when applied as granule one week prior to releasing larvae in soil (Figure 2.8). Thus, the effect of MET52 applied at different times prior to releasing larvae in the soil on emergence rate and adult mortality were investigated. Cups were filled with 30 g soil inoculated with MET52 at 9.0×10^8 CFU g^{-1} as previously described. The fungus was applied two, four, six, eight and ten weeks prior to 20 third instar larvae being introduced to the treatments. Four millilitre of sterilised distilled water was added to the untreated control and the other treatments. Then, the cups were kept following the same process and conditions described above. Five cups per treatment were used. After emergence, 15 adults (zero to one day old) were transferred into adult cages and kept as previously described. Four replicates for each treatment were arranged. Dead flies were collected daily from the cages and assessed over the period of two weeks. Fungal infection in the adults was investigated by following the same process previously described.

2.2.9 *Pathogenicity of MET52 to another fruit fly species*

The comparative susceptibility of two tephritid species, *D. frontalis* and *Ceratitis capitata,* to MET52 infection was investigated. The effect of MET52 on emergence and post-emergence mortality of the adults of each species were evaluated.

Cups were prepared and inoculated with the fungus at 9.0 x 10^8 CFU g^{-1} as previously described. The pathogen was applied one week prior to release of 20 third instar larvae into the cups. The treatments and control received 3.5 ml of sterilised distilled water. Soil moisture was maintained until adult emergence. Then, the cups were kept following the same process and conditions previously described in Section 2.2.3. Five replicates for each treatment were arranged. After emergence, adult emergence was assessed. Then, 15 adults (zero to one day-old) were transferred and kept in adult cages as described in Section 2.2.3. Four replicates for each treatment were arranged. Dead flies were collected daily from the cages which were monitored over the period of fifteen days. Fungal infection in adults was investigated by following the same process previously described in Section 2.2.3.

2.3 Statistical analysis

Percentages of dead pupae, adult emergence rate and emerging adults were transformed by arcsine and analysed by appropriate Anova test. Then, mean differences among the treatments were compared by Tukey`s test ($P < 0.05$). If data were not normally distributed nonparametric analysis was performed by Kruskal- Wallis (for one factor experiment) or by Scheirer-Ray-Hare test (for two factors). Then, Mann-Whitney was used to compare the differences between the treatments. Probit analysis was used to calculate LT50 and LT90. All the statistical analysis was performed in Minitab 16 Statistical Software.

2.4 Results

2.4.1 *Pupae and adult experiment*

As shown in the Figures 2.1 and 2, mycoses of the fungi applied in soil were observed growing on dead pupae and emerging adults. None of the biopesticides used in the present study caused a significant increase in mortality of *D. frontalis* pupae ($F = 2.43$; $df = 5$; $P > 0.05$). Figure 2.3.A shows MET52 and Bio-Magic biopesticides caused increasing mortality of pupae compared to the other treatments and untreated control giving approximately 22% pupal mortality. After emergence, the mortality of the adults in the fungal treatments ranged from approximately 8% to 88% compared to the untreated control with approximately 3%,

two weeks after emergence (Figure 2.3.B). There was a significant difference in adult mortality between the treatments (F = 74.67; df = 5; $P < 0.002$). MET52 showed the greatest pathogenicity against the fly, inducing approximately 88% mortality (Figure 2.3.B). The mortality of flies treated with MET52 was significantly greater than that of those treated with Bio-Magic and both treatments had significantly greater mortality than other treatments (Figure 2.3.B).

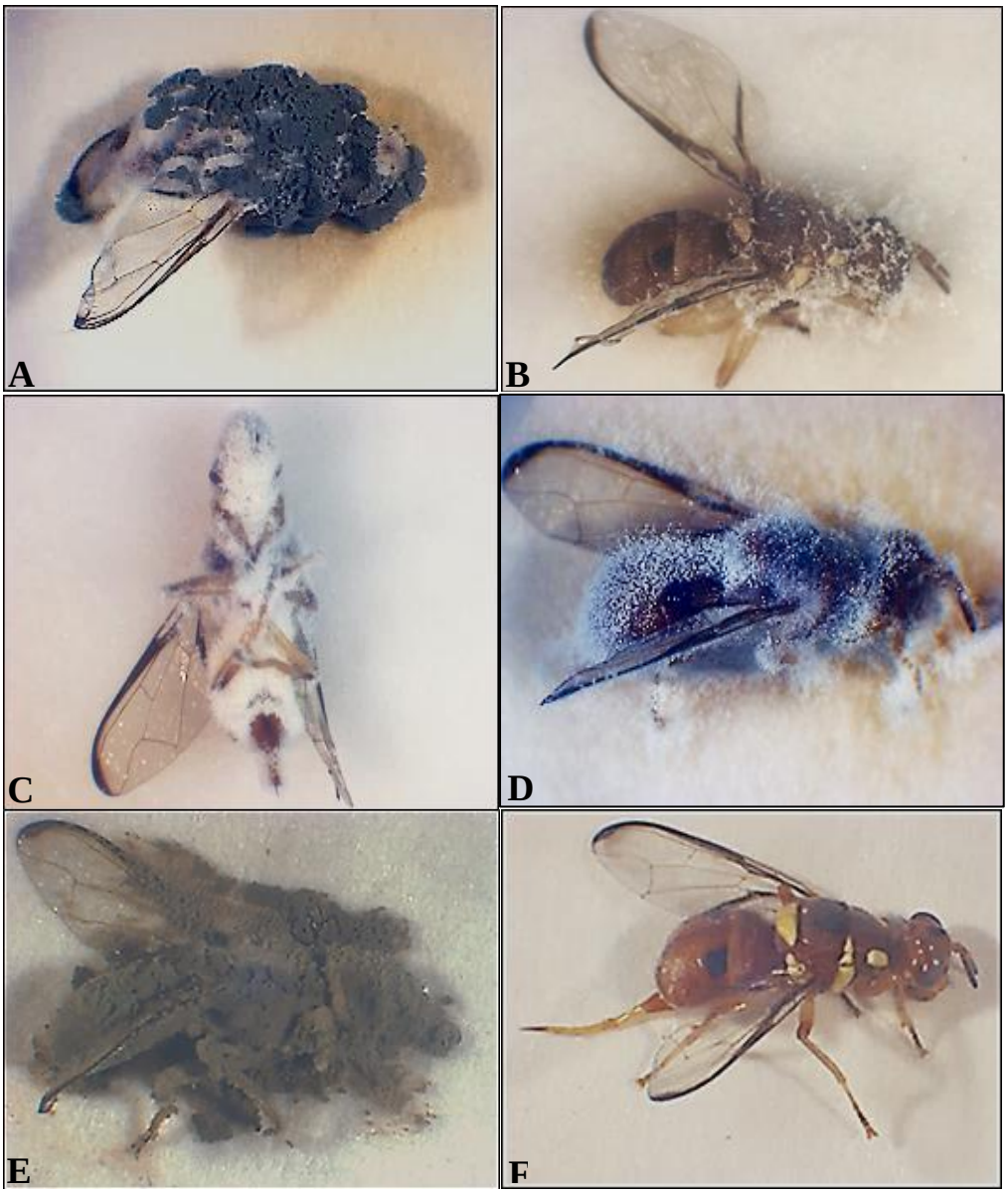


Figure 2.1 *Dacus frontalis* adult infected with *Metarhizium anisoplae* (Metchnikoff) Sorokin (A), *Paecilomyces fumosoroseus* (Wize) Brown and Smith (B), *Beauveria bassiana* (Balsamo) Vuillemin,(C), *Beauveria bassiana* ATCC 74040 (D), *Metarhizium anisopliae* var *anisopliae* strain F52 (E) and normal fly (F) after treated with commercial biopesticides in soil application. (Photographs © were taken by the author. 2013).

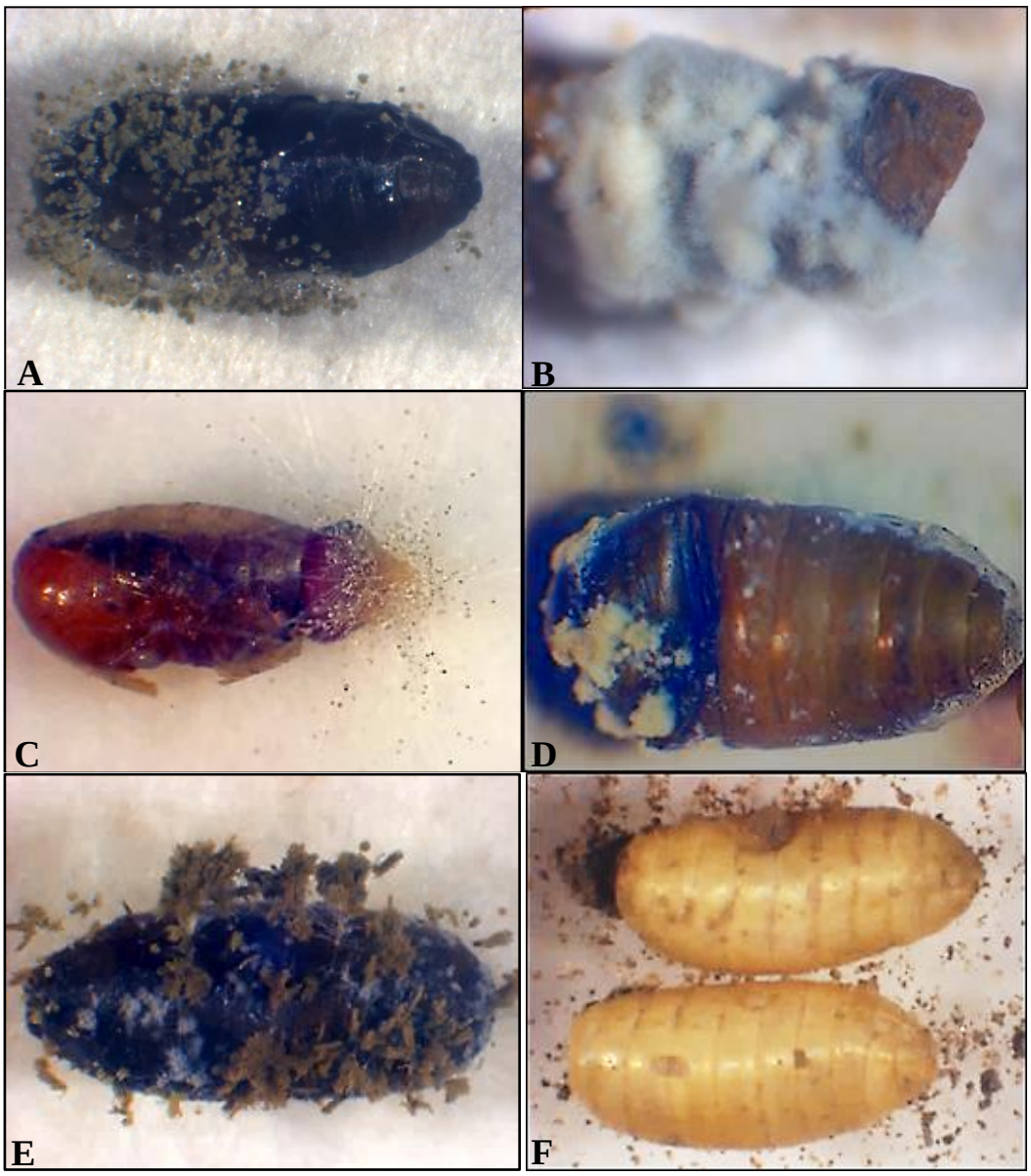


Figure 2.2. *D. frontalis* pupae infected with *Metarhizium anisoplae* (Metchnikoff) Sorokin (A), *Paecilomyces fumosoroseus* (Wize) Brown and Smith (B), *Beauveria bassiana* (Balsamo) Vuillemin,(C), *Beauveria bassiana* ATCC 74040 (D), *Metarhizium anisopliae* var *anisopliae* strain F52 (E) and normal fly (F) after treated with commercial biopesticides in soil application. (Photographs © were taken by the author).

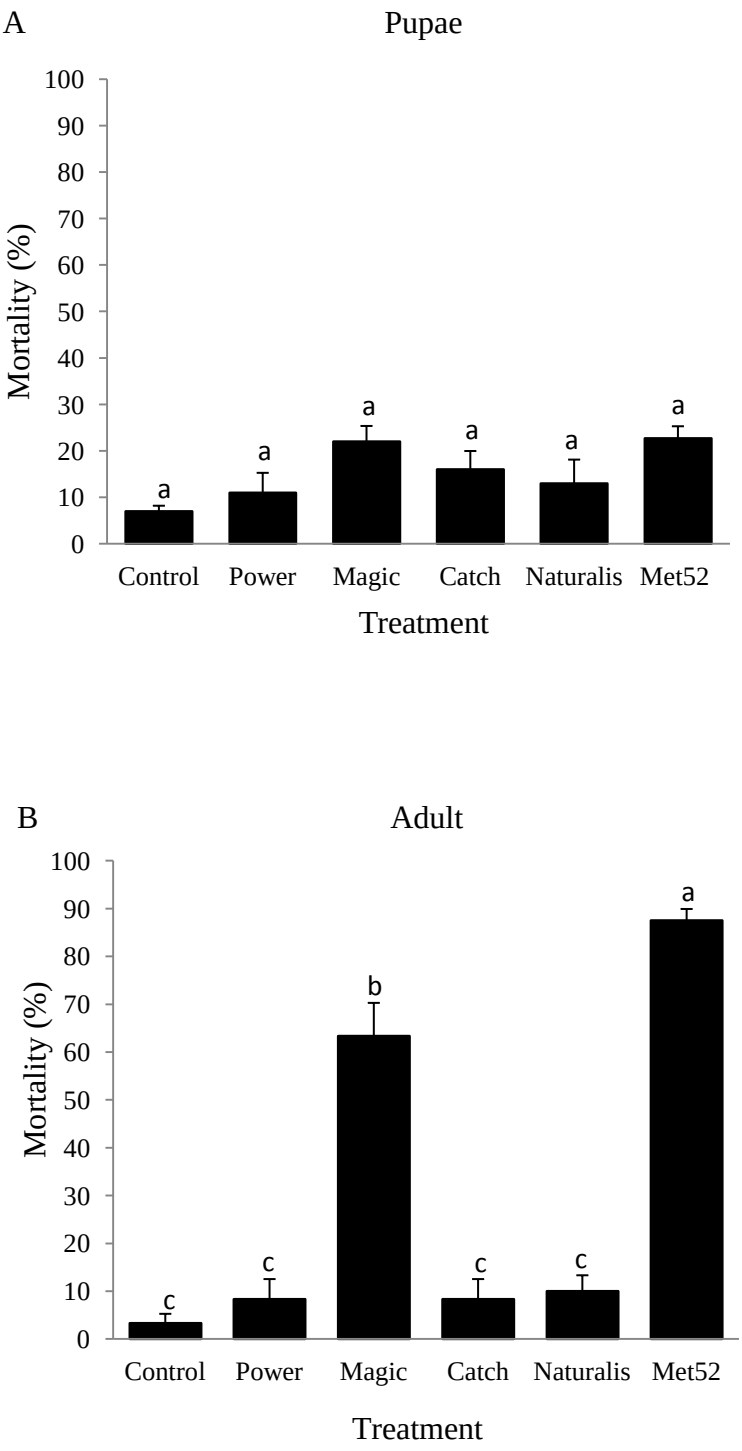


Figure 2.3 Mean (% ±SE) percentage pupal mortality, n = 5 (A) and subsequent adult mortality (two weeks after emergence), n = 4 (B) of *Dacus frontalis* treated with different biopesticides at recommended doses in 30 g of soil. Bars with different letters have significantly different means based on Tukey's HSD test ($P < 0.05$) after Anova.

2.4.2 *Larval experiment*

Larvae of *D. frontalis* pupated normally in all the treatments. The results showed that the larvae were not susceptible to any of the tested strains, with no significant differences in pupal mortality between the treatments and untreated control ($H = 4.57$; $df = 5$; $P > 0.05$). The percentage pupal mortality ranged from 1% to 4% (Table 2.2). Visible mycelium was detected growing around pupae recovered from the soil treated with the MET52 only (Figure 2.4). Twelve days after emergence, a significant mortality was observed in the MET52 cages compared to other the treatments ($H = 17.41$; $df = 5$; $P < 0.005$). The adult mortality was 29% compared to the untreated control with approximately 1% adult mortality. No infected adults emerged from the other fungal treated soil and adult mortality varied from 0% to nearly 3% (Table 2.2).

Table 2.2 Median mortality of pupae and emerging adults (twelve days after emergence) of *Dacus frontalis* following larval treatment with different entomopathogenic fungi at tested doses. n = 5.

Treatment	% Pupal mortality	Median*	% Adult mortality	Median†
Control	1	0	1	1
MET52	4	5	29	29‡
Bio-Magic	4	5	0	0
Bio-Power	3	0	0	0
Naturalis L	4	0	0	0
Bio-Catch	1	0	3	3

*****Kruskal-Wallis: Difference not significant.
†Kruskal-Wallis: Difference is significant.
‡Mann and Whitney test: MET52 treatment showed significant difference compared to the untreated control ($P < 0.05$).

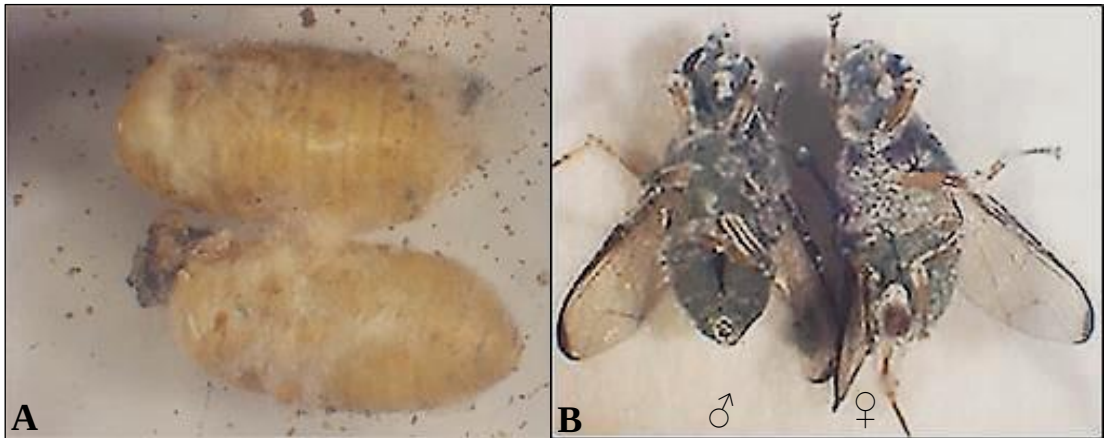


Figure 2.4 *Dacus frontalis* pupae infected with a mycelium of MET52 (A), and infected adult male (♂) and female (♀) emerged from the infected pupae (B). (Photographs © were taken by the author).

2.4.3 *Pupal age experiment*

The age of the pupae did not affect their susceptibility to MET52. No significant difference in mortality was observed between 2 and 8 day-old treated pupae and untreated control ($F = 0.64$; $df = 1$; $P > 0.05$). The pupal mortality ranged from 2% to 15% in the treatments (Figure 2.5). The mortality of adults emerged from 2 and 8 day-old inoculated pupae was significantly higher than the mortality in untreated control over the period of twelve days ($F = 545.68$: $df = 1$; $P < 0.001$). The greatest pathogenicity was found in the 2 day treated pupae treatment, with 100% adult mortality compared to 8 day-old treatment with 85% mortality. There was no significant difference between both the fungal treatments (Figure 2.5). In the untreated control, approximately 1% and 4% adult mortality were found in 2 and 8 day-old treatments respectively.

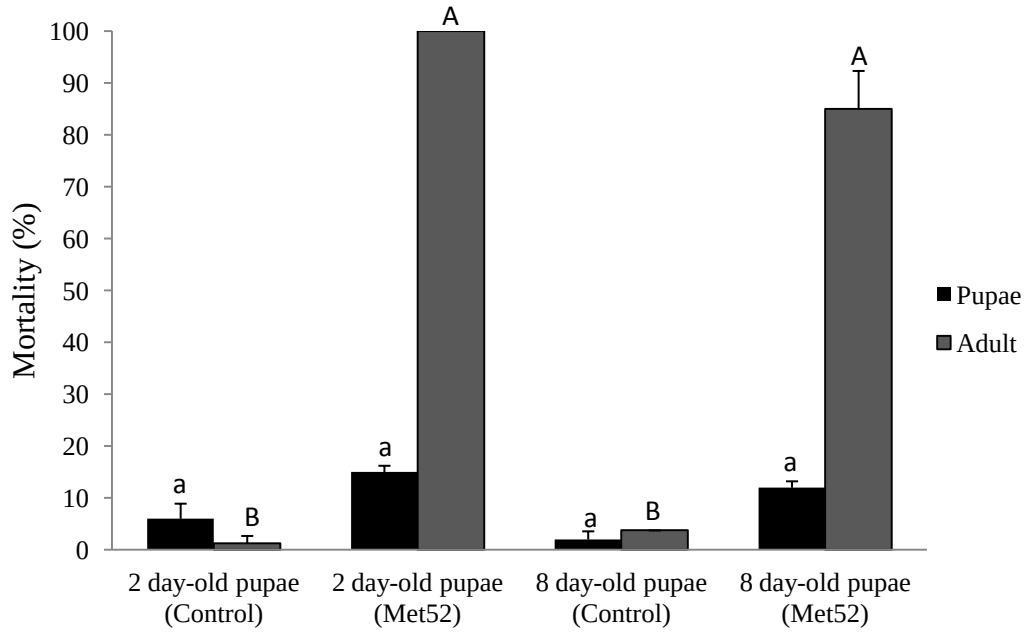


Figure 2.5 Mean (±SE) percentage pupae mortality (n = 5) and subsequent adult mortality, after 12 days (n = 4) of *Dacus frontalis* pupae treated at different ages (2 and 8 day-old) with MET52 at recommended doses. Bars for different life stages with different letters represent significantly different means based on Tukey's HSD test ($P < 0.05$) after Anova.

2.4.4 *Rate response experiment*

Areas of green fungal vegetative growth were visible on the soil surface two weeks after application of MET52 at different rates (Figure 2.6).

There was no significant effect of applying MET52 at rates tested on *D. frontalis* pupae ($F = 1.76$; $df = 6$; $P > 0.05$). The mortality of the pupae ranged from approximately 7% to 20% (Figure 2.7.A). No pupal mortality was recorded in the untreated control. After emergence, the adult mortality increased with the application rate of the fungal pathogen (Figure 2.7.B). In the first five days from emergence, the fungus induced significant mortality in adults from the inoculated soil compared to the untreated control ($F = 5.43$; $df = 6$; $P < 0.01$), with no significant differences between the fungal treatments (Figure 2.7.B). The greatest pathogenicity occurred when the highest rate (1.5 g) of the fungus was applied, giving slightly more than 70% adult mortality compared to the untreated control, in which the adult mortality was 2%. Nine days from emergence, the adult mortality from the fungal treatments had increased significantly compared to the untreated control ($F = 30.4$; $df = 6$; $P < 0.001$), with no significant differences found between the fungal treatments (Figure 2.7.B). The adult

mortality ranged from approximately 79% to 100% in the fungal treatments and 6% in the untreated control. The lethal time to 50% adult mortality (LT50) in the fungal treatments ranged from 5.5 days to 7.7days. The shortest LT50 was found when the pupae had been exposed to 1.5 g of MET52 while approximately 10 days was required to get 90% adult mortality when the pupae were placed into soil treated with the lowest rate (0.125 g) (Table 2. 3).

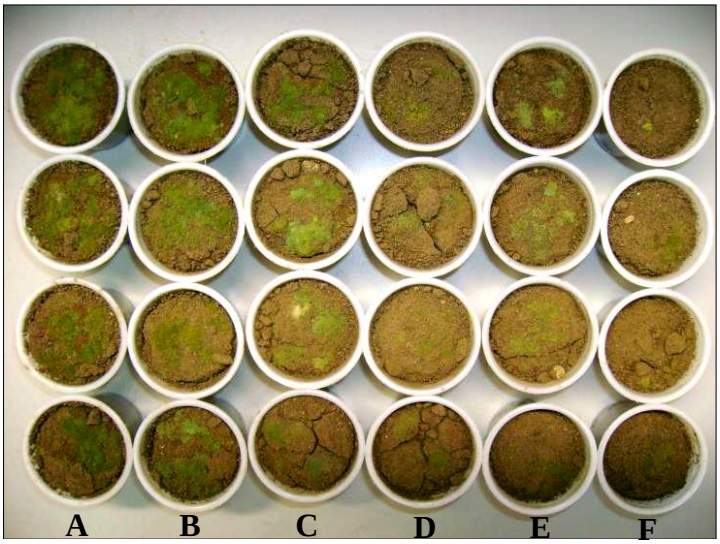


Figure 2.6 Areas of green fungal vegetative growth were visible on the soil surface two weeks after application of MET52 at different rates. A = 1.5 g, B = 1 g, C = 0.750 g, D = 0.50 g, E = 0.25 g and F = 0.125g.

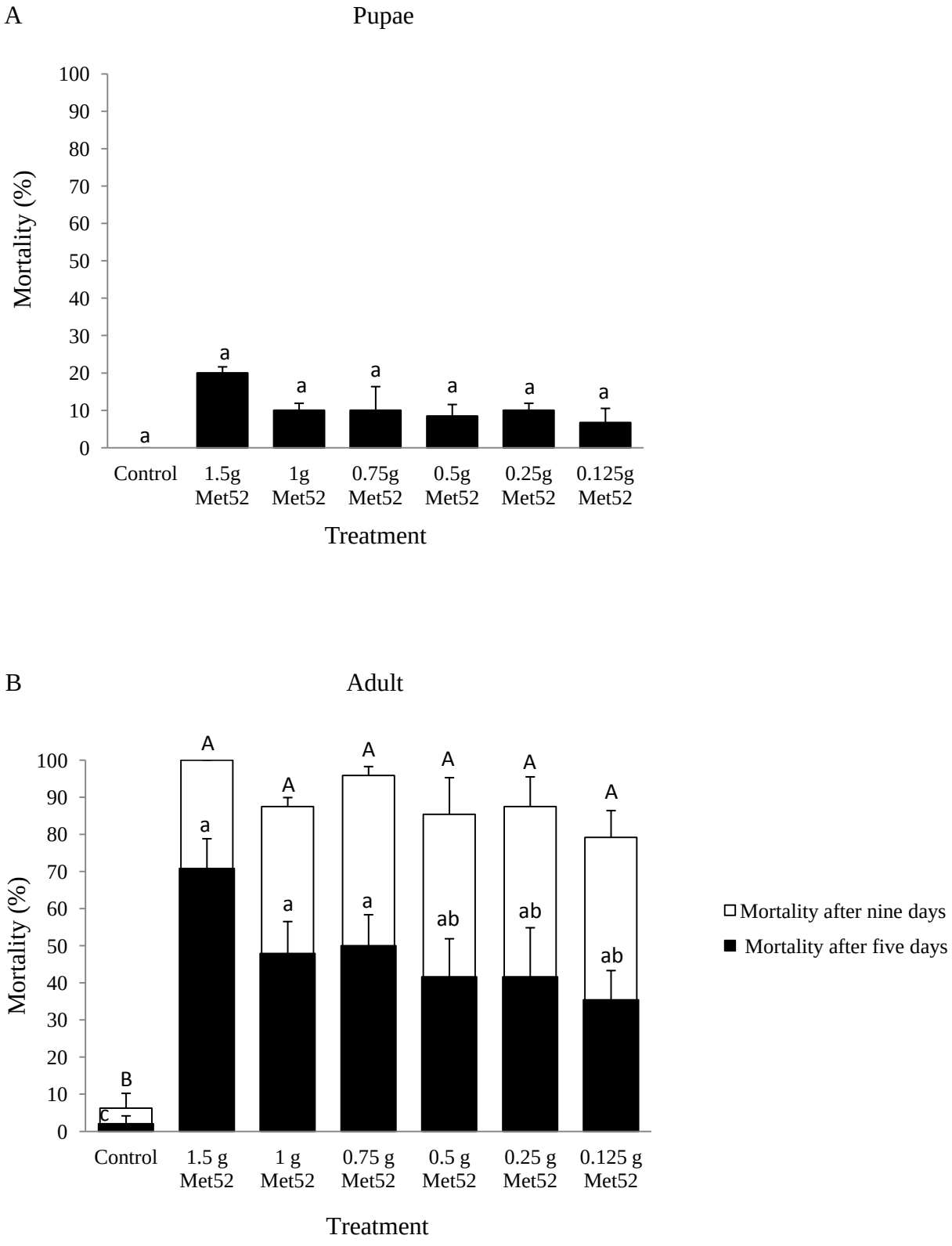


Figure 2.7 Mean (±SE) percentage pupal mortality, n = 5 (A) and subsequent adult mortality (5 and 9 days), n = 4 (B) after *Dacus frontalis* pupae were treated with different rates of MET52 (1.5, 1, 0.75, 0.5, 0.25, 0.152 g) in 30 g of soil. Bars within treatments with different letters represent significantly different means based on Tukey's HSD test ($P < 0.05$) after Anova.

Table 2.3 Mean lethal time (LT50 and LT90) of different rates of MET52 applied against *Dacus frontalis* adult.

Treatment	LT 50 (Days)	LT 90 (Days)
1.5 g	5.5	7.5
1 g	5.8	8.1
0.75 g	6.1	8.2
0.5 g	7.1	9.8
0.25 g	6.7	9.3
0.125 g	7.1	9.9

The values were calculated by Probit analysis.

2.4.5 *Formulation experiment*

The results indicated that applying *M. anisopliae var anisopliae* strain F52 in a granular form caused a significant reduction in adult emergence compared with the drench and untreated treatments ($F = 41.63$; $df = 1$; $P < 0.001$). As can be seen in Figure 2.8.A, the adult emergence reached 52% in the granule treatment compared to the drench treatment with 91% and the untreated control with 97%. In the emerging adult case, both application methods significantly reduced the number of the adults compared to the untreated control ($F = 529$; $df = 1$; $P < 0.001$), The adult mortality was low (35%) when treated with granules compared to the drench treatment (87%) two weeks after emergence, with a significant difference between both treatments (Figure 2.8.B). Only approximately 3% adult mortality was found in untreated control.

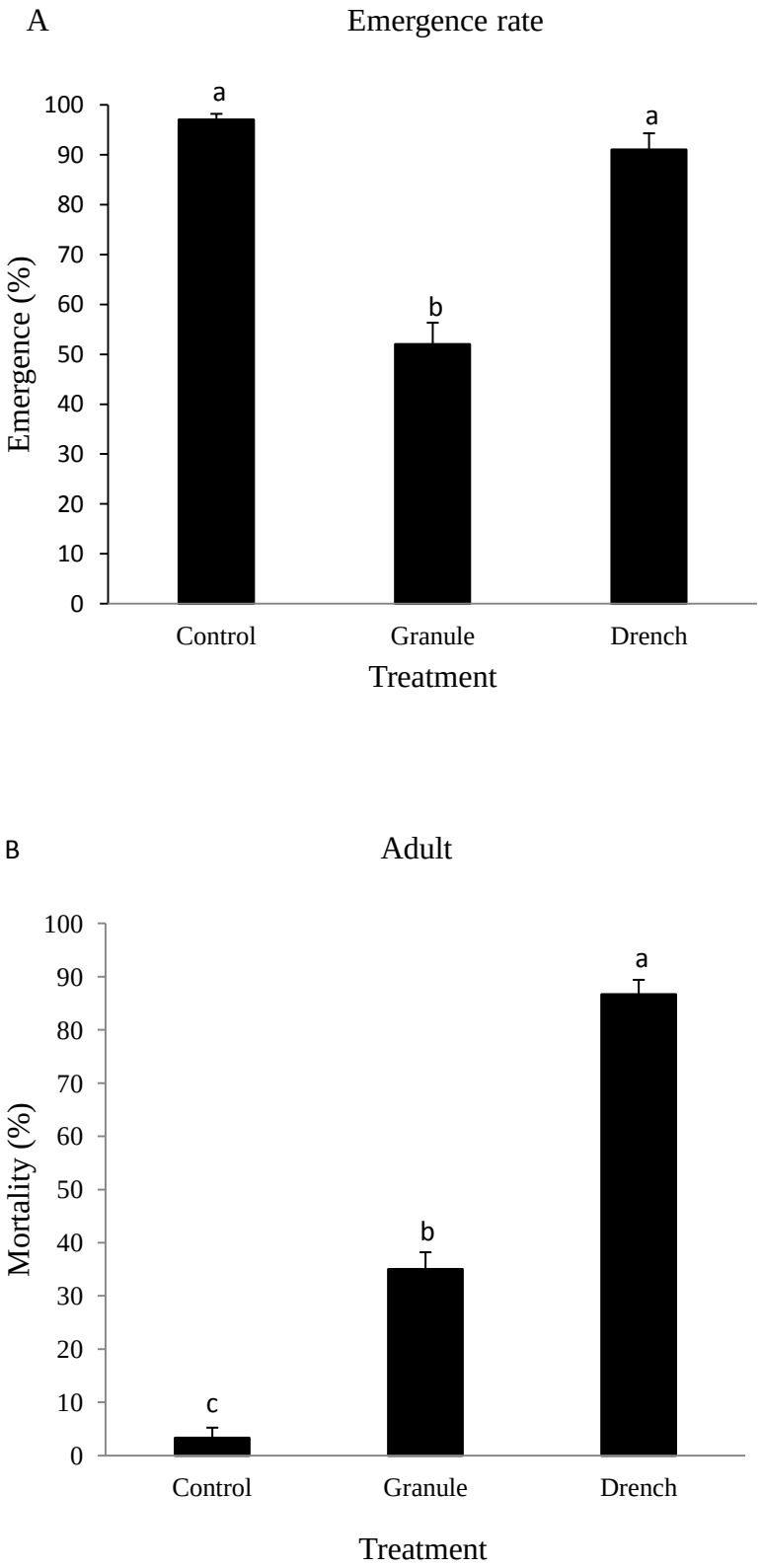
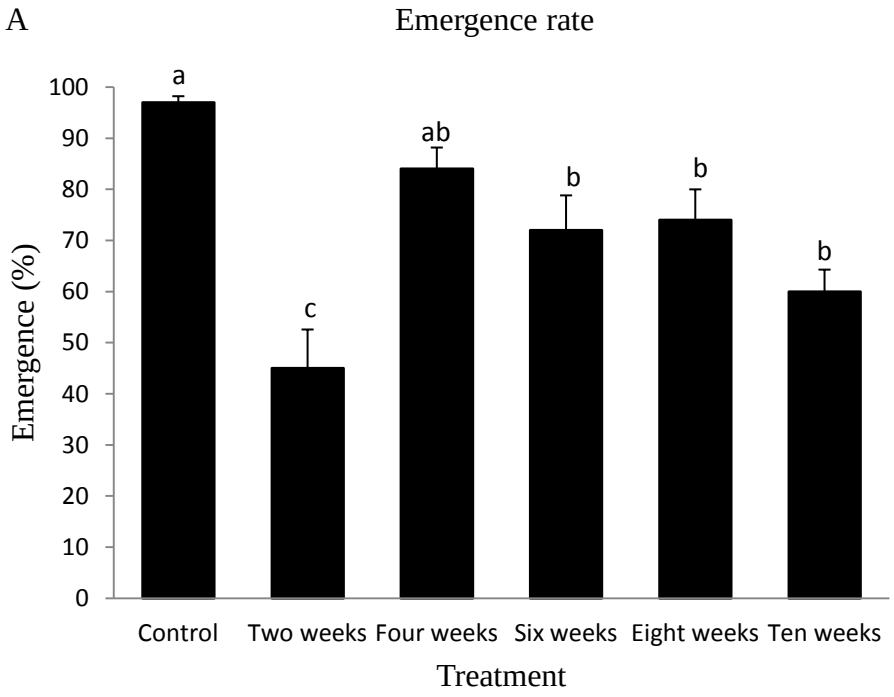


Figure 2. 8 Mean (±SE) percentage emergence rate, n = 5 (A) and subsequent adult mortality (two weeks after emergence) n = 4 (B) after *Dacus frontalis* larvae were released in soil treated with different formulations (Granule and Drench) of *M. anisopliae var anisopliae* strain F52 one week earlier. Bars with different letters represent significantly different means based on Tukey's HSD test ($P < 0.05$) after Anova.

2.4.6 *Application time experiment*

The pathogenicity of MET52 against *D. frontalis* was influenced by application time. The results showed that adult emergence was significantly lower in inoculated soils (with the exception of the four week treatment) than untreated control treatment ($F = 111.44$; $df = 1$; $P < 0.001$). The greatest pathogenicity was recorded in the two week-old treatment with a 55% reduction in the adult emergence rate. The adult emergence reduction in other fungal treatments ranged from 16% to 40%, with significant differences between the treatments (Figure 2.9.A). A high adult emergence (97%) was found in the untreated control. After emergence, the number of dead flies was significantly higher in the inoculated treatments than in the untreated control with an exception for the ten week treatment ($F = 98.02$; $df = 1$; $P < 0.01$), (Figure 2.9.B).The pathogenicity ranged from approximately 32% to 52% adult mortality over the period of two weeks from the emergence. The greatest pathogenicity against the fly was induced when the fungus was applied two weeks from the larvae being released, with no significant difference between the treatments (Figure 2.9.B).The adult mortality in the untreated control was 6.6%.



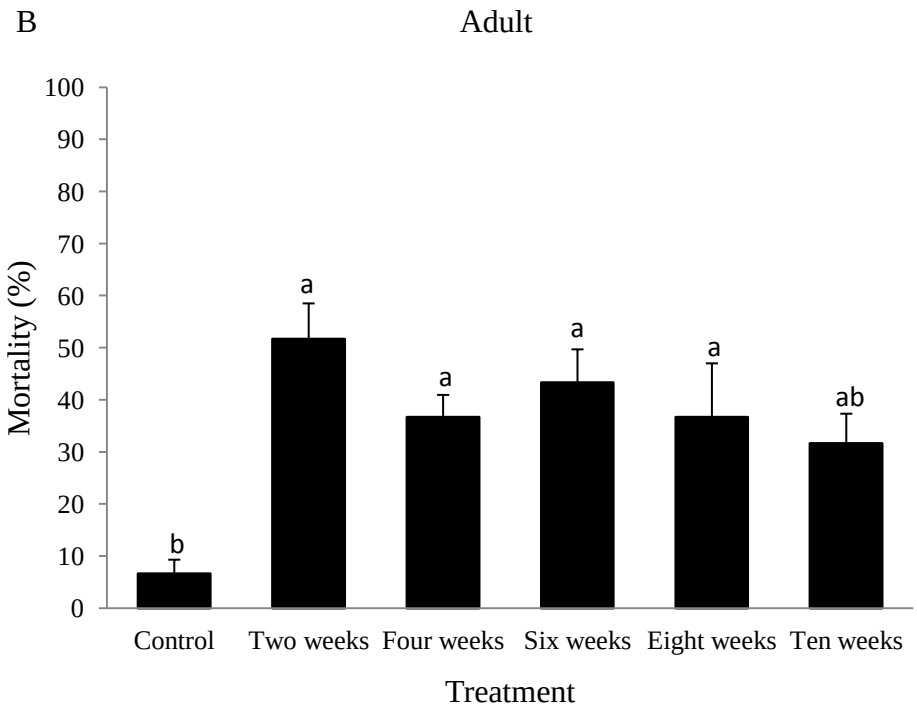


Figure 2.9 Mean (±SE) percentage emergence rate, n = 5 (A) and subsequent adult mortality (two weeks from the adult emergence), n = 4 (B) after *Dacus frontalis* larvae being released in 30 g soil treated with 1.5 g of MET52 at different times. Bars with different letters represent significantly different means based on Tukey's HSD test ($P < 0.05$) after Anova.

2.4.7 *Pathogenicity of MET52 to another fruit fly species*

Larvae of the both species burrowed into the soil in all the treatments. As can be seen in Figure 2.10, the emergence rate in the inoculated treatments was significantly lower at 67% for *D. frontalis* and 75% for *C. capitata* compared to the untreated controls with 95% and 94%, respectively ($F = 64.08$; df = 1; $P < 0.001$), with no significant difference between the species (Figure 2.10). Three days after emergence, an effect of the fungus in killing adults of the both species was observed. Mycelium and green spores of MET52 were observed growing around *C. capitata* adult (Figure 2.11). Adult mortality of the both flies emerged from the fungal treatments was significantly higher than untreated controls five days after emergence ($\chi^2 = 0.9970$; df = 1; $P < 0.005$ (Scheirer-Ray-Hare test)). The highest pathogenicity was induced in *D. frontalis* treatment with approximately 40% adult mortality, and 20% for *C. capitata*. Significant difference was found between the treatments (Table 2.4). Adult mortality was 0% in the untreated controls. Ten days later, the mortality in the fungal treatments significantly increased compared to the untreated controls ($F = 331.00$; df = 1; $P < 0.005$), with no significant difference between the two species (Table 2.4). The mortality was

approximately 82% for *D. frontalis* and 80% for *C. capitata* fifteen days after the emergence. Mortality in the untreated controls was approximately 7% for *D. frontalis* and 10% for *C. capitata.* The LT50 and LT90 are given in Table 2.4. The speed of killing 50% of *D. frontalis* adults from inoculated soil was shorter than *C. capitata* by two days, while approximately 16 days was required to cause 90% adult mortality of the both species.

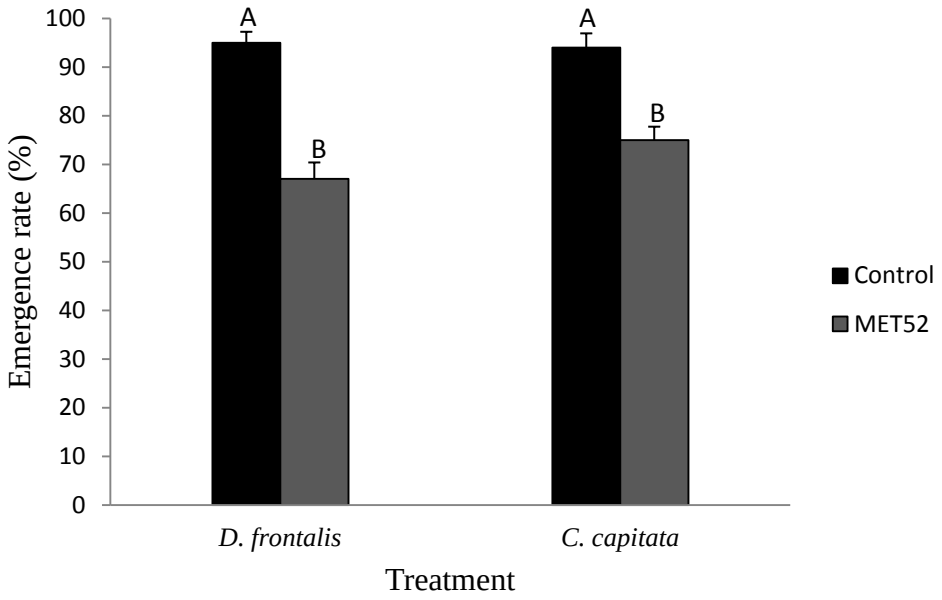


Figure 2.10 Means (% ±SE) percentage emergence rate, (n = 5) of *Dacus frontalis* and *Ceratitis capitata* adults emerging from soil treated with MET52 at 9.0 x 10^8 CFU g^{-1} in 30 g. Means with different letters are significantly different based on Tukey's HSD test ($P < 0.05$) after Anova.

Figure 2.11 *Ceratitis capitata* adult infested with MET52, 48 hours (A) and five days (B) after incubations. (Photographs © were taken by the author).

Table 2.4 Percentage adult mortality post-emergence with mean lethal time (LT50 and LT90), for *Dacus frontalis* and *Ceratitis capitata* adults after larvae were released in 30 g soil treated with 1.5 g of MET52 (9.0×10^8 CFU g^{-1}) at the different times tested. n = 4

Fly species	5 days		15days		LT50	LT90
	% Mortality	Median*	% Mortality	Mean‡	(Days)	(Days)
Treated *D. frontalis*	39.9	36.6A	81.6	76.6a	8	16
Treated *C. capitata*	19.9	19.9B	79.9	79.9a	10	16
Untreated *D. frontalis*	0.0	0.0C	6.6	6.6b	-	-
Untreated *C. capitata*	0.0	0.0C	9.9	9.9b	-	-

*Medians with different letters are significant different (Mann and Whitney test).
‡Means with different letters are significant different based on Tukey's HSD test ($P < 0.05$) after Anova.
The values of LT50 and LT90 were calculated by Probit analysis.

2.5 Discussion

The present investigation compared five different commercial products of entomopathogenic fungi for their efficacy against *D. frontalis* under laboratory condition. This is the first study to demonstrate the susceptibility of *D. frontalis* to entomopathogenic fungi. The susceptibility of other species of fruit fly to entomopathogenic fungi inoculated by different methods have been previously confirmed (Clark *et al.*; De la Rosa *et al.*, 2002; Ekesi *et al.*, 2002; Dimbi *et al.*, 2003; Quesada-Moraga *et al.*, 2006; Sookar *et al.*, 2008; Daniel and Wyss, 2009; Ortiz-Urquiza *et al.*, 2009; Cossentine *et al.*, 2010; Goble *et al.*, 2011; Yousef *et al.*, 2013).

In the adult experiment, MET52 based on *M. anisopliae var anisopliae* strain F52 was the most pathogenic against *D. frontalis* adults. The mortality reached 100% at the dose used. The LT50 ranged from 4 to 5 days through the experiments. This mortality level was reported by other authors (Ekesi *et al.*, 2002; Ekesi *et al.*, 2005; Mochi *et al.*, 2006) who found that approximately 100% mortality in the adults of *C. capitata*, *C. fasciventris* and *C. cosyra* that emerged from sand previously treated with *M. anisopliae*. Also, Sookar *et al.* (2008) reported mortality of *Bactrocera zonata* adult to reach 98% after being treated with *M. anisopliae*. The same study indicated that some strains of *B. bassiana* and *P. fumosoroseus* had caused low pathogenicity and this agrees with the present results which showed that the both fungi caused only 8% adult mortality. In contrast, Daniel and Wyss (2009) and (Cossentine *et al.*, 2010) reported that *B. bassiana* was highly pathogenic to adults of *R. cerasi*. Different target species and different application methods might be the reasons for these different findings. Gul *et al.* (2015), indicated that different inoculation methods induced different susceptibility levels of larval, pupal and adult stages of *B. zonata* to three insect pathogenic fungi.

Larvae in all the treatments did not show susceptibility to the fungi. This was probably because exposure to the pathogens was for a short time (Mochi *et al.*, 2006). Another possible reason for this is that tephritid larvae have soft cuticle lacking any hairs which could limit the numbers of conidia attaching. Similar results were obtained by Mochi *et al.* (2006), who indicated that the E 9 isolate of *M. anisopliae* had no effect on larval stage of *C. capitata*. Also, De la Rosa *et al.* (2002) found that *B. bassianna* caused low mortality against larvae of the Mexican fruit fly, *Anastrepha ludenswere*. Recently, larvae of *R. cerasi* were proved not to be susceptible to entomopathogenic fungi (Daniel and Wyss, 2009).

None of the tested strains caused significant mortality to *D. frontalis* pupae although fungal mycelium was observed growing inside and outside the pupal cuticle (Figure 2.12.A). Also, pupal age and increasing rate of MET52 had no effect on the pupal mortality. This maybe because that pupa has a solid cuticle which might prevent conidia spores to penetrate. However, the fungus induced higher mortality in emerging adults when applied on young pupae. Similar results were obtained by Beris *et al.* (2013), who found that low mortality approximately 19% to 24 % was obtained when *C. capitata* pupae were exposed to three fungi species. However, higher mortality rates were induced after the emergence. Earlier studies by, De la Rosa *et al.* (2002) and Daniel and Wyss (2009) indicated that pupae of *A. ludenswere* and *R. cerasi* were not susceptible to three different fungi species applied to soil nor were there effects on emerging adults, suggesting that the pupal stage is not susceptible to fungal infection. In contrast, Ekesi *et al.* (2002) indicated that different isolates of *M. anisopliae* and *B. bassiana* caused great reduction in adult emergence of three tephritid fruit fly species in Petri dishes experiment. The authors found that the adult emergence decreased when old pupae were used, inducing mortality in emerging adults. Difference in inoculation method and the expected high humidity level in the Petri dish experiment might be the reason for the high mortality.

The application time experiment showed that early application of MET52 reduced adult emergence to 45%. This was probably because of increasing conidia density of MET52 in soil over the experiment time. This may be because the granule form promotes the pathogen to grow, consequently increasing the conidia concentration. Another possible suggestion for this emergence reduction is that the larvae released ingested some conidia spores before entering into pupal stage. The same results was obtained by Ekesi *et al.* (2002), who observed that prophylactic application with *M. anisopliae* was more effective than curative treatment in reducing adult emergence of three species of fruit flies.

The result of the present study also revealed that *M. anisopliae* var *anisoplia*e strain F52 can greatly reduce adult emergence when applied early as granule rather than being mixed in suspension and drenched. While after adult emergence, the mortality was greater by 52% in the drenched treatment. A possible explanation for this might be that adhesion of conidia to emerging adult cuticle in the drenched treatment was greater than in granular treatment. In future investigations, it might be worth to use the fungus in the both formulations tested together for better control. However, the cost should be considered. Our microscopic observations showed that large parts of the emerging adults were obviously covered with greenish dry conidia in granule application (Figure 2.12.B) but not in drenched treatment. Similarly, Ekesi *et al.* (2005) found that a granule form of another strain of *M. anisopliae* was more effective in reducing adult emergence of *C. capitata*, *C. fasciventris* and *C. cosyra* than a suspension and drench treatment.

A granule form of some species and strains of *Metarhizium* fungus has been used as a successful pathogen against other agricultural insect pests. For example, MET52 based on *M. anisopliae var anisopliae* strain F52 has been evaluated against black vine weevil larvae, *Otiorhynchus sulcatus* inducing high mortality (Moorhouse *et al.*, 1993; Bruck and Donahue, 2007; Ansari and Butt, 2013), Tomato potato psyllid, *Bactericera cockerelli* (Hemiptera) (Mauchline *et al.*, 2013) and the large pine weevil, *Hylobius abietis* (Williams *et al.*, 2013), causing 100% mortality for both last species. Another species *M. brunneum* F52 provided 84% to 98% mortality of chilli thrips *Scitothrips dorsalis* when applied as granules (Arthurs *et al.*, 2013).

The soil used in current study is sandy clay loam soil which is different to many in Libya where soil has different texture and characteristics. Very few studies have investigated the effect of soil type on the efficacy of entomopathogenic fungi. In a recent study conducted by Garrido *et al.*, 2011b, soil proprieties have no effects on pathogenicity of *M. anisopliae* EAMa 01/58-Su and *B. bassiana* EABb 01/110-Su against soil stages of *C. capitata*. However, a further investigation with more focus on the type of soil as a factor is therefore required.

In conclusion, *D. frontalis* adults are highly susceptible to some fungal pathogens. The results suggest that applying entomopathogenic fungi as granules to soil could be a promising biological control, reducing adult emergence and causing high mortality to emerging adults. This strategy could provide some benefits for *D. frontalis* control because dead pupae and adults could serve as future infection sources against new fly offspring in soil. Also, soil

provides a good habit for recycling and protecting pathogens which may help increase conidia density and spread in the environment. To get a better understanding of how to maximise the effect of *M. anisopliae* next step is to focus on the effect of abiotic factors such temperature, humidity and soil moisture content on the efficacy of MET52. Also, studying combined use of the product with botanical insecticides could increase larval mortality.

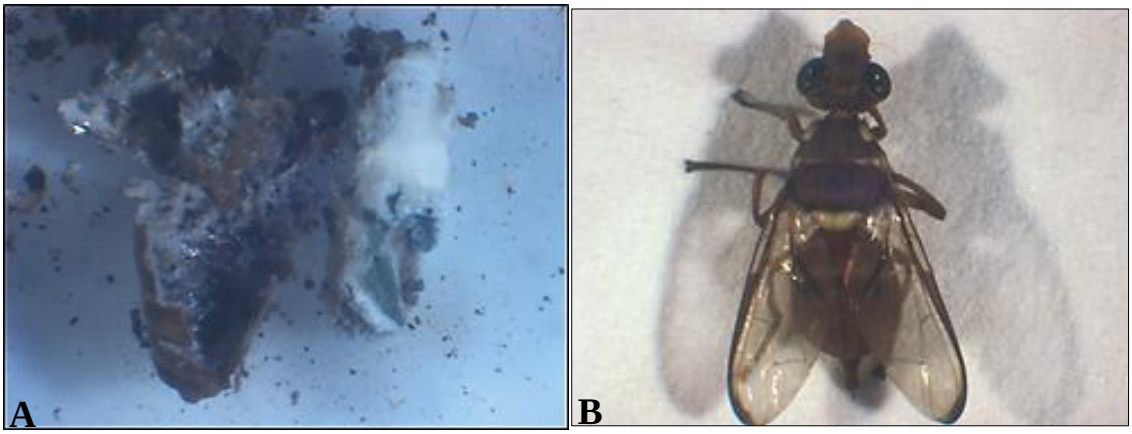


Figure 2.12 Dead pupae of *Dacus frontalis* after being dissected showing growths of white mycelium of MET52 (A) an emerged adult covered with greenish spores of MET52. (Photographs © were taken by the author).

Chapter 3. Isolation, identification and potential pathogenicity of Aspergillus fungus obtained from a lab culture of *Dacus frontalis* (Becker)

Abstract

A fungus, identified as *Aspergillus ochraceus,* was isolated from dead pupae collected from a laboratory strain of *Dacus frontalis*. Various inoculation methods were used in laboratory conditions to investigate the potential pathogenicity of the fungus against the fly. *Aspergillus ochraceus* and three commercial biopesticides based on several strains of entomopathogenic fungi were compared. *Aspergillus ochraceus* showed low mortality against adult flies. The comparative pathogenicity of *A. ochraceus* and *Metarhizium anisopliae* was evaluated against flies by spraying suspensions of the fungi on adults. The *A. ochraceus* treatment resulted in considerably lower mortality than the *M. anisopliae* treatment. *Aspergillus ochraceus* had a dose-dependent effect on adult mortality. The susceptibility of larvae and pupae of the fly to *A. ochraceus* was also investigated. The fungus showed low pathogenicity against pupae only.

3.1 Introduction

Insect pests are associated with different fungal species. Different fungal species have been isolated from several insect pests. Balogun and Fagade (2005), reported that eight fungal species were isolated from locust, *Zonocerus variegates* (Orthoptera). The fungi isolated were identified as *Beauveria bassiana*, *Metarhizium* sp., *Penicillium* sp., *Aspergillus niger* and *Mucor* sp. In another study, six species of fungi including *B. bassiana*, *Nomuraea rileyi*, *Paecilomyces farinosus, P. fumosoroseus, M. anisopliae* and *Aspergillus* sp were isolated from infected silkworm larvae, *Bombyx mori* (Nguyen and Park, 2004*)*. Christias *et al.* (2001), isolated *Alternaria alternate* from dead aphids on cultivated plants, ornamentals and weed. Also, *A. candidus* was isolated from *Bactrocera dorsalis* (Diptera: Tephritidae) (Jiji *et al.*, 2006).

Identification of the isolated entomopathogenic fungi can be achieved by studying colonial morphology and microscopic features of the isolated pathogen such as the size, colour and shape of conidia (Balogun and Fagade, 2005; Anaisie *et al*., 2011). Also, molecular approaches have been used to detect different pathogens in several environments (Elkinton and Burand, 2007). For example, a technique such as using polymerase chain reaction (PCR),

to amplify the internal transcribed sequences (ITS) region in genomic DNA of fungi, helps in definition of molecular characterisation and identifying fungi to species level (Henry *et al*., 2000; Hinrikson *et al*., 2005).

Some of these fungi are considered as common contaminants of food and insect cadavers or pathogenic of plants such as *Fusarium, Aspergillus, Mucor* and *Penicillium* (Balogun and Fagade, 2005; Konstantopoulou and Mazomenos, 2005; Anaisie *et al.*, 2011). It has been reported that some species of these fungi can cause disease to different life stages of several orders of insects. For example, the genus *Aspergillus* has been reported as pathogenic against aphids (Chen *et al.*, 2008), grasshoppers (Balogun and Fagade, 2005; Kumar *et al.*, 2014), mosquitoes (Seye *et al.*, 2009), bugs (da Costa *et al.*, 2003), moths (Anaisie *et al.*, 2011) and some of dipteran species (Castillo *et al.*, 2000).
Published information in terms of the isolation and pathogenicity of species of the genus *Aspergillus* against Tephritid flies is very limited. The first objective of the present study was to isolate and identify a fungal species found associated with dead pupae collected from a laboratory culture strain of *D. frontalis.* The potential pathogenicity of the fungus isolated against soil life stages and adults of the fly was evaluated.

3.2 Materials and Methods

3.2.1 *Fungus detection and isolation*

A total of 5 pupae of *D. frontalis* showing abnormal symptoms were detected and collected from a culture at, Newcastle University. The pupae collected had brown-red to black colour compared to the normal ones which are cream-yellowish. Mycelium and spores were observed growing around the pupae (Figure 3.1.A, B). The pupae were collected in sterile plastic tube and externally sterilised with 70% ethanol and 0.5% sodium hydrochloride for 30 seconds followed with three rinses in sterile distilled water. The samples were placed into Petri dishes with moist sterile filter papers. The Petri dishes were incubated at 28 ºC in the dark. The pupae samples were subjected to microscopic observations every 24 hours. One week later, fungal spores were clearly recognized growing around the pupae. By sterilised loops, mycosed pupae were sub-cultured into sterilised Petri dishes containing potatoes dextrose agar (PDA) under sterile conditions (Figure 3.2.A). The plates were incubated in the same conditions for one to two weeks. The plates were sub-cultured more than once on PDA until pure cultures were established (Figure 3.2.B). Then, pure fungus plates were kept at 4°C and re-cultured regularly on fresh PDA until used for further investigations.

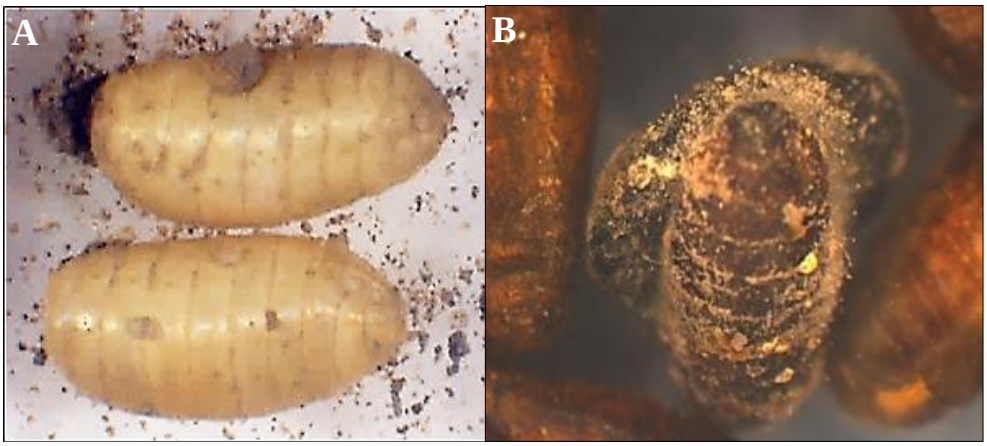


Figure 3.1 A normal *Dacus frontalis* pupae (A) and pupae infested with *Aspergillus sp* (B). (Photographs © were taken by the author).

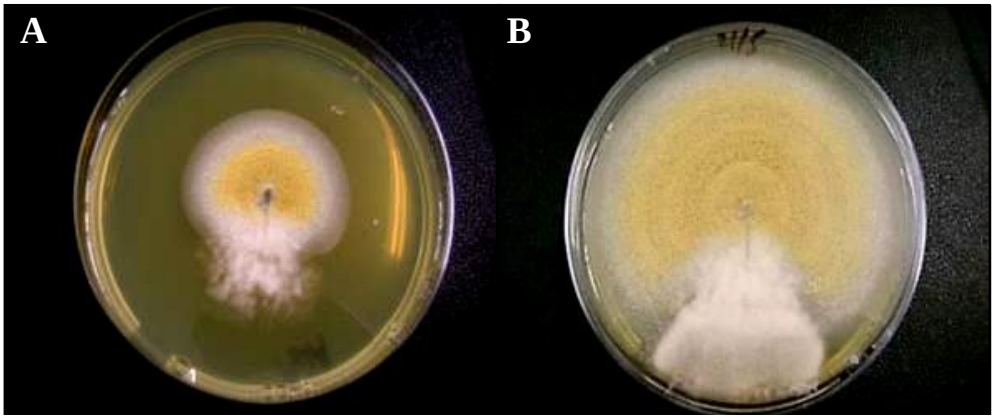


Figure 3.2 An infested pupa was cultured in PDA medium (A) and a pure *Aspergillus sp* cultured on PDA medium (B).

3.2.2 *Primary Identification of a fungal isolate*

A slide of ethylene-blue stained conidiophores picked up from a pure culture of the isolated fungus was subjected to microscopic observations followed with identification based on colonial morphology and microscopic features (Lacey, 1997).

3.2.3 *DNA extraction*

To confirm the fungus at species level, extraction of DNA, Polymerase Chain Reaction (PCR) and DNA sequencing were performed.

A genomic DNA was extracted from a pure two week old *Aspergillus sp* culture by a CTAB-chloroform protocol (Griffiths *et al.*, 2000). 500 µl of CTAB extraction buffer (Hexadecyl

trimethyl ammonium bromide) (Sigma) and phenol/ chloroform/ isoamyl alcohol (25: 24: 1) was added to a micro centrifuge tube (2 ml) containing a fragment of *Aspergillus sp* collected from the fungal culture by a sterilised loop. The fungal mycelium was lysed in fast prep at 5.5/ 30 seconds until well mixed. The sample was cooled on ice for 2 min. At 4 °C, the sample was put in a centrifuge at maximum speed (14000 rpm) for 5 min. By a pipette, the supernatant (top layer containing DNA) was removed and transferred into a clean tube. To remove phenol, equal volume of chloroform/ isoamyl alcohol was added, mixed well by shaking and followed with 5 min centrifugation at maximum speed at 4°C. The upper aqueous layer was transferred in a new tube. To precipitate DNA, two volumes of 30 % of Polyethylene glycol (PEG 600)/ 1.6 M NaCl were added. The sample was incubated for 2 hours at room temperature followed with centrifugation at maximum speed for 10 min. The supernatant was discarded and 500 µl of ice cooled ethanol (70%) was added to wash the pellet. The liquid was carefully poured off. The lid of the tube was opened at room temperature to air dry the pellet before it was suspended in 30 µl of sterile distilled water. Then, 25 µl of RNase (Sigma) and 990 µl of sterile distilled water were added to 10 µl of a genomic DNA. The mixture was incubated for at 37 °C for 30 min. The obtained DNA was stored at - 20 °C.

3.2.4 *Molecular identification*

PCR was performed by PCR Kit Bioline to amplify ITS regions (18s DNA gene) of the fungal isolate. One µl of each of DNA template of the fungus extracted, a pure fungus DNA (*Trichoderma harzianum* (positive control)), and water (negative control) were amplified in a total volume of 24 µl PCR reaction. The PCR mixture contains 0.75 µl of two universal fungal primers (forward primer ITS1 (CTTGGTCATTTAGAGGAAGTAA) and reverse primer ITS4 (TCCTCCGCTTATTGATATGC)), 2.5 µl PCR 10x buffer, 0.85 µl $MgCl_2$, 0.8 µl DNTPs, 17.85 µl dH_2O and 0.5 µl TAQ. The PCR program started with a cycle at 94 °C for 3 min as initial denaturalization followed with 40 cycles consisting of denaturalization at 94 °C, annealing at 49 °C and initial extension at 72 °C for one min. and a cycle at 72 °C for 5 min. for a final extension. Then, 5 µl of the amplified PCR products with 2 µl loading dye, and 2 µl leader 100 bp were loading into 1% agarose gel stained with 3 µl of ethidium bromide and viewed by electrophoresis in TBE buffer (0.5 x) at 100 v running for 30 min. Ten µl of the PCR products and 10 µl of both primers used were sent to Geneius Laboratories Ltd, INEX Business Centre, Newcastle University, UK for purification and sequencing. The sequences obtained were submitted to the NCBI (National Centre for Biotechnology Information) using Blast search to be compared to sequences available in the database.

3.2.5 *Fungi culture*

A commercial product of entomopathogenic fungi, Bio-Magic, based on *Metarhizium anisopliae* (Metchnikoff) Sorokin and *Aspergillus* sp were cultured into PDA media following the same procedure previously mentioned.

3.2.6 *Conidia viability and suspension preparation*

Conidia spores of 20 to 25 days-old PDA cultures of *Aspergillus sp* and *M. anisoplae* respectively were harvested and suspend after adding a sterilised solution of 0.5% Tween 80. The conidia suspensions were gently homogenised by vortex for 60 s. Volumes of 50 µl of both *Aspergillus sp* and *M. anisopliae* conidia suspensions were spread by sterilised loop into Petri dishes contained PDA media. Then, sterilised cover slips were placed immediately on the conidia. The plates were incubated at 25 °C to 28 °C in the dark. The conidia viability was examined after 24 hours. One hundred conidia were examined and counting under a microscope at x 400 magnification to determine germinated conidia spores. The average was calculated from four plates of each fungal strain. The spore concentrations of the both fungi were determined by counting the spores by haemocytometer.

3.2.7 *Pathogenicity test against adults*

The fungus isolated from the infested *D. frontalis* pupae was identified as *Aspergillus ochraceus* (see Section 3.4.2).

Two methods were used to evaluate the pathogenicity of the fungus against *D. frontalis* adults. Firstly, twenty, three day-old adults of *D. frontalis* were dipped for 20 sec into 2 ml suspensions of *A. ochraceus* 5.01×10^7 CFU ml^{-1}, Bio-Power, Bio-Magic and Bio-Catch at 1 x 10^8 CFU ml^{-1}. The same number of adults was dipped in 2 ml of sterile distilled water for control. In another method, two suspensions of pure cultures of *M. anisopliae* (1.21×10^6 CFU ml^{-1}) and *A. ochraceus* (2.32×10^6 CFU ml^{-1}) were prepared. Fifteen, one day-old adults of *D. frontalis* were placed into Petri dishes lined with two pieces of filter paper. The flies were placed on ice for 2 min to be easy to handle. The suspensions of 2 ml of the both fungi were gently shaken by a vortex for 2 min before spraying directly onto the adults by spray bottle (100 ml. Superdrug Stores, UK). The adult flies were sprayed until run-off. The same number of adults was treated with 2 ml of water for the control.

The treated adults of both methods used were transferred into cages and kept by following the same procedure and conditions previously described in Section 2.2.3 (Chapter two). Four

replicates for each treatment were arranged. Dead flies were collected daily from the cages and assessed over the period of fifteen days. Fungal infection in adults was investigated by following the same process previously described in Section 2.2.3 (Chapter two).

3.2.8 *Conidia concentration response*

Four different concentrations (0.95×10^6, 0.95×10^7, 0.95×10^8 and 0.95×10^9 CFU ml^{-1}) of *A. ochraceus* were suspended from 20 to 25 old day cultures. Following the same methodology described above in Section 3.2.7, fifteen adults (one to two days-old) of *D. frontalis* were placed into Petri dishes lined with two pieces of filter paper and sprayed with 1.5 ml of the prepared suspensions. The same number of adults was sprayed with 1.5 ml sterilised distilled water as untreated control. The treated flies were transferred into plastic cages following with the same process and conditions as above. Dead flies were collected daily from the cages and assessed for fifteen days. Fungal infection in the adults was investigated by following the same process previously described in Section 2.2.3 (Chapter two).

3.2.9 *Pathogenicity test against larvae and pupae*

Two methods were used to evaluate the pathogenicity of the fungus against *D. frontalis* larval- pupal stages (measured by calculating adult emergence rate). Firstly, twenty last instar larvae of *D. frontalis* were dipped for 20 seconds in 10 ml suspension of *A. ochraceus* (1.59×10^8 CFU ml^{-1} from 25 day-old culture). Similarly, the same number of larvae was dipped in 10 ml of sterilised distilled. Then, the larvae were released in experimental cups filled with 30 g of sterilised soil moistened with 2 ml sterilised distilled water. The cups were covered with the same size of the cups following the same procedures previously described in Section 2.2.3 (Chapter two). Soil moisture was maintained until adult emergence by adding sterilised distilled water as necessary by weight. Five replicates for each treatment were arranged. The treatments were incubated at 25 ºC and 75%- 80 % RH. Emergence rate was assessed for two weeks.

Secondly, five cups filled with 30 g of sterilised soil were prepared and inoculated with 2ml suspension of *A. ochraceus* at the concentration used by following the similar experimental procedure for soil application experiment described in Section 2.2.4 (Chapter two). Two ml of sterilised distilled water was added to the untreated control. Twenty, third instar larvae of *D. frontalis* were released into the both treatments. The cups were kept by following the same process and conditions previously described. Five replicates for each treatment were arranged. One week later, pupae were sieved from the soil and examined under a microscope to

determine if any growth of mycelium was apparent. Pupae recovered from the cups were placed into Petri dishes and kept in the same conditions until adult emergence. After ten to eleven days, emergence rates were assessed. Fungal infection in the dead pupae was investigated by following the same process previously described in Section 2.2.3 (Chapter two).

3.3 Statistical analysis

Percentages of dead pupae and adults were transformed by arcsine and analysed by appropriate Anova test. Then, mean differences among the treatments were compared by Tukey`s test ($P < 0.05$). In the pathogenicity test against adults, the adult mortality was corrected by Abbot's formula (Abbott, 1925). The formula works by correcting the mortalities relative to the control by the following formula: Corrected mortality % = (1- n in T after treatment/ n in Co after treatment) * 100. Where: n = Insect population, T = treated, Co = control. As the data were not normally distributed after transformation, non-parametric analysis was performed by Kruskal- Wallis. Then, Mann-Whitney was used to compare the differences between the treatments. For the pathogenicity test against larvae and pupae, the results of the methods used were separately analysed by Two-Sample T-Test. Probit analysis was used to calculate LT50 and LT90. All the statistical analysis was performed by Minitab 16 Statistical Software.

3.4 Results

3.4.1 *Primary identification*

The fungus isolate had a yellowish colour on two weeks-old PDA cultures (Figure 3.2.B). Whilst, it appeared pale to brownish on the reverse side. The microscopic observations showed that chains of globose conidia were observed on a swollen and apical vesicle. Vesicle is known to be the typical form for species belonged to the genus *Aspergillus* (Figure 3.3).

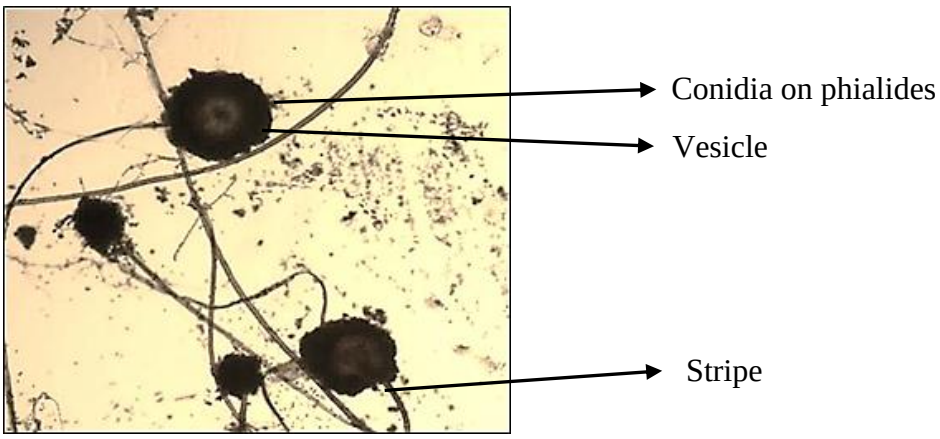


Figure 3.3 Conidia head of *Aspergillus sp.* (Photographs © were taken by the author).

3.4.2 *Molecular identification and sequences analysis*

ITS region of the isolated fungus was amplified with forward primer ITS1 and reverse primer ITS4 producing band of approximately 500 to 550bp for the isolated fungus and 550 to 600 bp for the positive control compared to the negative control (Figure 3.4). In Genbank results, the sequences obtained from the PCR products was analysed against NCBI and characterised as *Aspergillus ochraceus* (Appendix I).

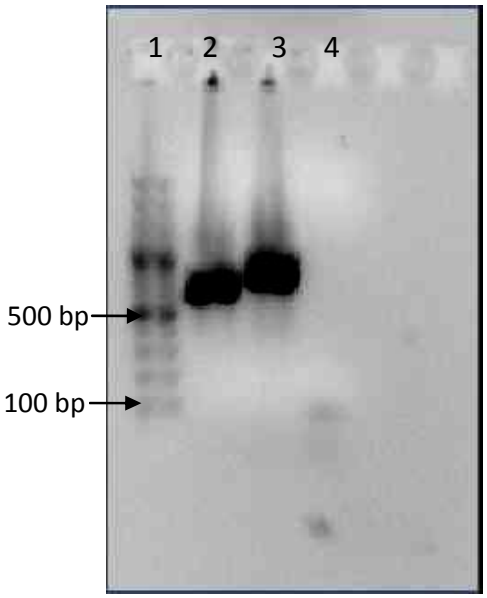


Figure 3.4 Agarose gel electrophoresis of the PCR product of the region ITS of the isolate of *Aspergillus spp* amplified by using ITS1 and ITS4 primers. Lanes represent: 1) Plus ladder (DNA marker, 100 bp), 2) DNA of the fungus tested, 3) A pure fungus DNA (*Trichoderma harzianum*) as positive control and 4) Water as negative control.

3.4.3 *Aspergillus ochraceus* ***pathogenicity test against adults***

In conidial viability tests, percentage germination in all tests ranged from approximately 62% to 75% for *A. ochraceus* and 83% for *M. anisoplae* 48 hours after incubation.

All the fungi used were pathogenic to adults of *D. frontalis*. The mortalities after correction (Abbot's formula) are shown in Table 3.1. A significant mortality was found in the fungal treatments compared to untreated control ($H = 12.72$; $df = 4$; $P < 0.05$ (Kruskal-Wallis test)). *Aspergillus ochraceus* showed the greatest pathogenicity against the fly, inducing approximately 25% mortality. The mortality of the adults in other fungal treatments ranged from approximately 5% to 21%, two weeks after application, with significant differences found between the treatments (Table 3.1).

Table 3.1 Median mortality, after 14 days of adults of *Dacus frontalis* treated with different entomopathogenic fungi at tested doses (corrected mortalities are shown). n = 4.

Treatment	% Adult mortality	Median
Bio-Power	5.57	5.88bc
Bio-Magic	21.13	23.53ab
Bio-Catch	18.34	20.59ab
A. ochraceus	25.54	30.49ab

Medians that do not share a letter are significantly different (Mann-Whitney test).

In the spray method, *M. anisopliae* and *A. ochraceus* showed pathogenicity against the adult flies (Figure 3.5). Growth of mycelium of the fungi tested on the dead adults was observed 24 hours after the incubation. The results showed that both fungi caused significant mortality to *D. frontalis* adult compared to the control ($F = 141.56$ $df = 2$; $P < 0.001$). *M. anisopliae* caused the highest adult mortality (100%) compared to approximately 20% in *A. ochraceus* treatment two weeks after application. A significant difference was found between the fungal treatments (Figure 3.6). In the untreated control, approximately 3% mortality was found.

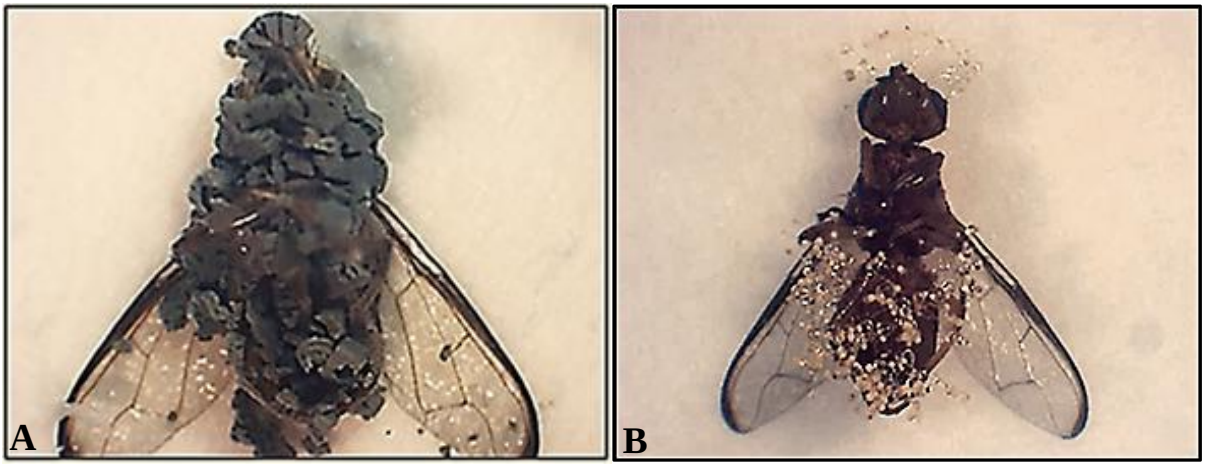


Figure 3.5 *Dacus frontalis* adult infected with *Metarhizium anisoplae* (A) and *Aspergillus ochraceus* (B). (Photographs © were taken by the author).

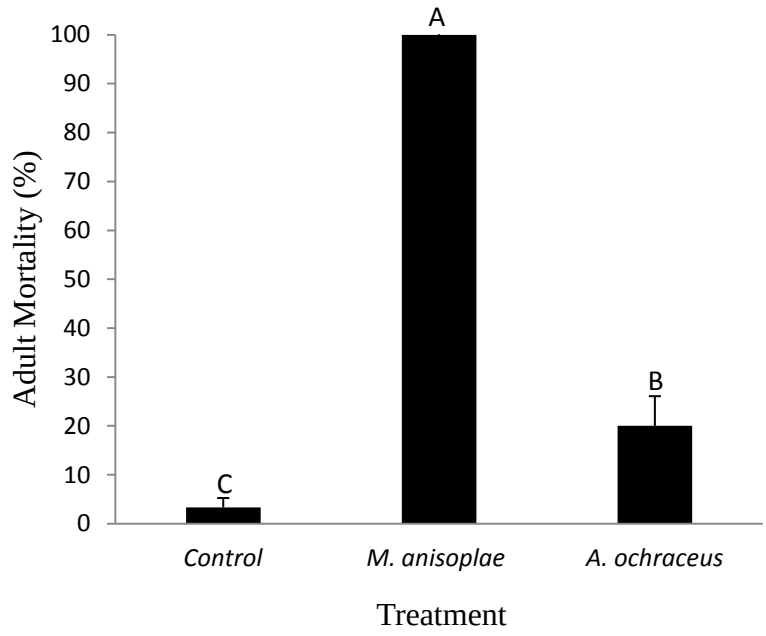


Figure 3.6 Mean (% ±SE) percentage subsequent adult mortality (two weeks after application), n = 4 of *Dacus frontalis* sprayed with *M. anisopliae* and *A. ochraceus*. Bars with different letters represent significantly different means based on Tukey's HSD test ($P < 0.05$) after Anova.

3.4.4 *Conidia concentration response*

As can be seen in the Figure 3.7, the effectiveness of *A. ochraceus* on *D. frontalis* adults is concentration-dependent. The adult mortality increased with increasing the concentration of *A. ochraceus* fifteen days after application. A significant difference was found between the fungal treatments and untreated control ($F = 24.13$; $df = 4$; $P < 0.001$). The greatest

pathogenicity occurred when the highest concentration (0.95×10^9 CFU ml^{-1}) of *A. ochraceus* was applied, giving 66% adult mortality compared to the untreated control with approximately 7%. The adult mortality ranged from 20% to 55% with significant differences in the other fungal treatments (Figure 3.7). Percentage mortality of flies emerging from the treatments increased with time and varied between the treatments (Figure 3.8). The results of LT50 and LT90 of flies emerged from soil inoculated with *A. ochraceus* at different concentrations are given in the Table 3.2. In the fungal treatments, the LT50 value ranged from approximately 11 days to 21 days. The shortest LT50 was found in the highest concentration tested, whereas approximately 32 days was required to get 90% adult mortality in the lowest concentration tested.

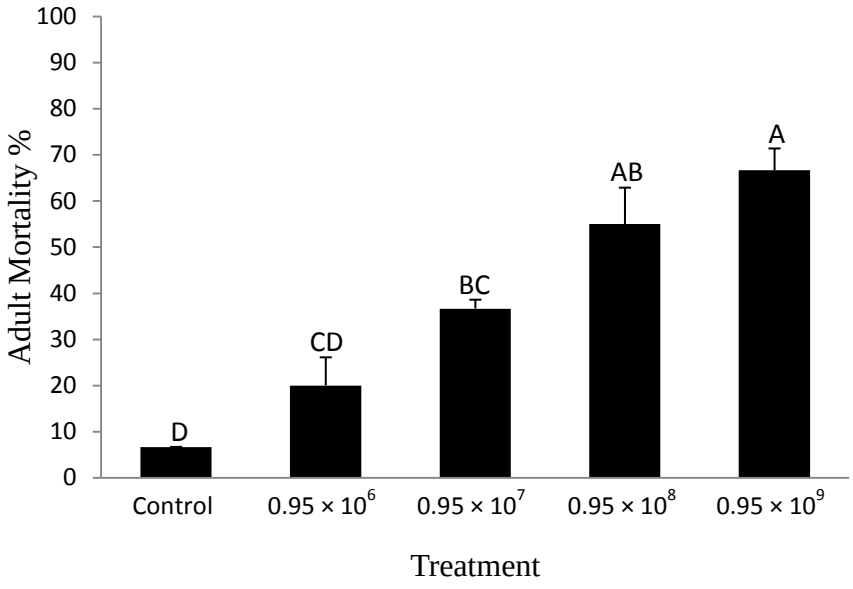


Figure 3.7 Means (% ±SE) mortality percentage, after 15 days of *Dacus frontalis* adults treated with different concentrations of *A. ochraceus*. Bars that do not share a letter represent significantly different means based on Tukey's HSD test ($P < 0.05$) after Anova. n = 4.

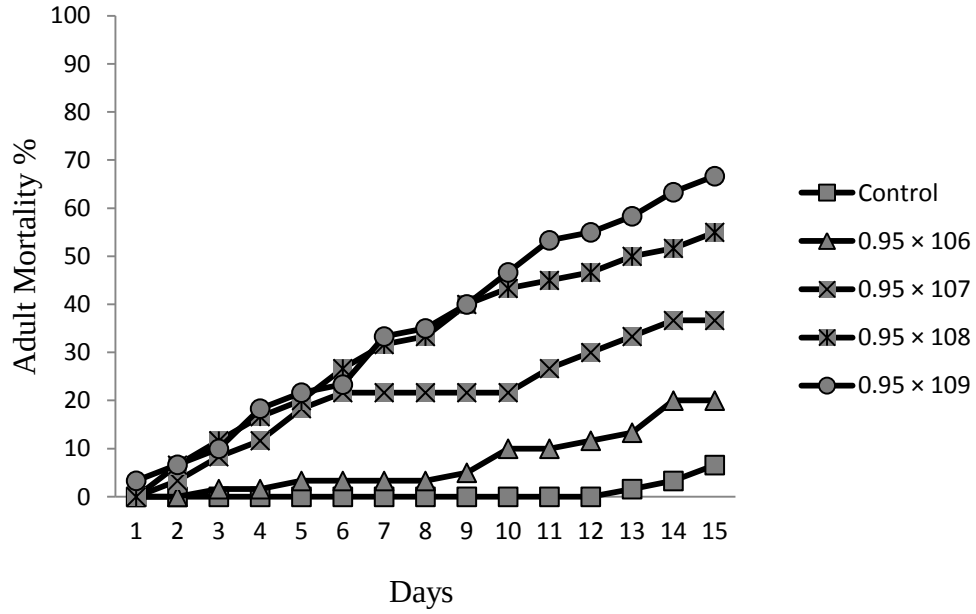


Figure 3.8 Time-mortality response of *Dacus frontalis* adults treated with *Aspergillus ochraceus* at different concentrations over the period of fifteen days from emergence.

Table 3.2 Mean lethal time (LT_{50} and LT_{90}) of different concentrations of *A. ochraceus* applied against *Dacus frontalis* adult.

Concentration (CFU ml^{-1})	LT50	LT90
0.95×10^6	21.33	31.56
0.95×10^7	17.48	31.38
0.95×10^8	12.46	23.10
0.95×10^9	11.05	19.96

LT_{50} and LT_{90} were estimated by Probit analysis.

3.4.5 *Aspergillus ochraceus* ***pathogenicity against larvae and pupae***

Some of the recovered pupae were dead and affected by *A. ochraceus* (Figure 3.9). The results showed that the fungal treated larvae of *D. frontalis* did not cause a significant effect on adult emergence compared to untreated control (T = 1.63; df = 4; $P > 0.05$ (Two-Sample T-Test)) for dip application and (T = 0.00; df = 8; $P > 0.05$ (Two-Sample T-Test)) for soil application. As can be seen in the Figure 3.10.A and B, the emergence rates in the fungal treatment and untreated control ranged from 98% to 100%.

Figure 3.9 *Dacus frontalis* pupae infected with *A. ochraceus* two weeks after incubation. (Photographs © were taken by the author).

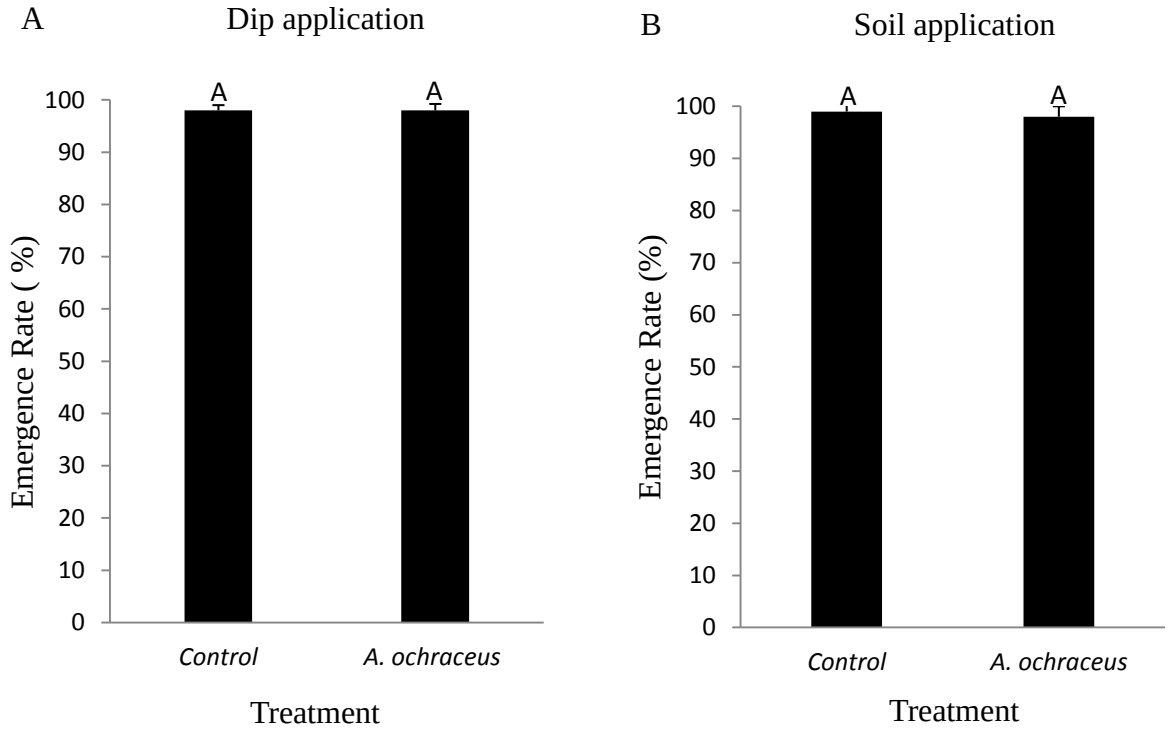


Figure 3.10 Means (% ±SE) Adult emergence percentage of *D. frontalis* when larvae treated with *A. ochraceus* by dip application (A) and soil application (B). Bars with similar letters do not represent significantly different means based on Two-Sample t-test ($P < 0.05$). n = 5.

3.5 Discussion

This is the first report of isolation of a fungus from *D. frontalis* pupae.

In the current study, the fungus was isolated from pupae collected from a laboratory strain and identified as *A. ochraceus*. The *Aspergillus* fungus is considered as a facultative generalist pathogen (Sujeetha and Sahayaraj, 2014). The fungus was found to have a low to moderate pathogenicity to adults causing 66% mortality with a weak growth of mycelium. The estimates of LT50 and LT90 calculated from probit analysis in Table 3.2 are in excess of the normal lifespan of the adult insects in control conditions (about 21 days). The isolation and pathogenicity of *A. ochraceus* has been previously reported against *Ceratitis capitata* adults by (Castillo *et al.*, 2000), who found in a different inoculation method that the fungus caused less than 20% and 40% mortality 6 and 10 days respectively after treatment with 1×10^6 conidia/ ml. Sales *et al.* (2002), reported that other species of the genus *Aspergillus* including, *A. niger*, *A. flavus* were isolated from the housefly adults, *Musca domestica*. *A. candidus* isolated from *B. dorsalis* has been found to cause high mortality to *B. cucurbitae* (Jiji *et al.*, 2006*). A. flavus* and *A. tamarri* isolated from adults of *B. cucurbitae* have been found to cause high mortality to adults but low to the soil stages (Yang *et al.*, 2015). Other biopesticides used in the present study caused very low adult mortality ranging from approximately 5% to 21%, which agree with the results obtained from different inoculation method in Section 2.4.1 (Chapter 2). However, Bio-Magic based on *M. anisopliae* (Metchnikoff) Sorokin caused higher mortality (100%) when was cultured on PDA media, suspended in water at lower concentration (1.21×10^6 CFU ml^{-1}) and applied by dip method against the adult flies compared to the same strain when applied in a commercial formulation at 1×10^8 CFU ml^{-1} in soil application causing approximately 60% adult mortality.

The results showed that pupae had low susceptibility to *A. ochraceus*, whereas larvae had no susceptibility to the fungal infection. Results of another study revealed that several species of *Aspergillus* fungus (*A. ochraceus, A. kanagawaensis* and *A. sulphurous*) were effective against larvae of two mosquito species causing at least 80% mortality (Lage de Moraes *et al.*, 2001). In another study, *A. ochraceus* and two other *Aspergillus* species have been reported to cause less than 80% mortality of larvae mosquito (Powell *et al.*, 1994). This is probably because of different insect species used compared to the insect used in the present study.

Isolation and pathogenicity of other species of the genus *Aspergillus* have been previously reported against other insect pests (Balogun and Fagade, 2005; Seye *et al.*, 2009; Anaisie *et al.*, 2011; Kumar *et al.*, 2014). However, some of these species such as *A. niger* were

suggested not to be strictly entomopathogenic fungi to *Z. variegates* but opportunistic microorganism (Balogun and Fagade, 2005).

In a primary test, the fungus was also pathogenic to Medfly adults, *C. capitata* (Figure 3.11). The results were not presented in the current study.

Figure 3.11 *Ceratitis capitata* female infected with *A. ochraceus.* (Photographs © were taken by the author).

On the other hand, although *A. ochraceus* is pathogenic to several insect pests, the fungus has been reported to cause infection to humans (Ravelo *et al.*, 2011; Reponen *et al.*, 2012) and to animals (Ghibaudo and Peano, 2010).

In conclusion, the results of this study indicated that *A. ochraceus* isolated from a laboratory strain of *D. frontalis* could be a potential biological control agent against the fly. However, the negative impact of using the fungus to human and animals should be considerable. Further investigations related to culture, pathogenicity, sublethal effect and a safe use in the environment should be studied.

Chapter 4. Influence of soil moisture, humidity, temperature and application method on efficacy of a commercial strain of *Metarhizium anisoplae* against the Greater melon fly *Dacus frontalis* (Becker)

Abstract

The effects of ten soil moistures (10%- 100% Water Holding Capacity (WHC)), three relative humidity ranges (40%- 50%, 55%- 65% and 75%- 85% RH) and three temperatures (15 ºC, 25 ºC and 35 ºC) on the pathogenicity of Met52® Granular bioinsecticide (MET52) to *Dacus frontalis* were investigated under laboratory conditions. Moisture of soils treated with MET52 did not affect the adult emergence rate of *D. frontalis*, but significantly affected post-emergence mortality. The greatest post-emergence mortality (93%) was observed in 70% WHC treatment which had the shortest LT50 at 5 days. In the humidity test, 75%- 85% RH reduced the emergence rate and increased post-emergence mortality. MET52 was effective at all temperatures used. The highest post-emergence mortality was obtained at 25 ºC, showing the shortest LT50. MET52 was more effective when applied as a granule in soil against pupae (85% mortality) than as direct spray against adults (37% mortality) when assessed five days after the emergence. Mortality by both application methods increased up to 98% twelve days after application. The results obtained in this study indicated that efficacy of *M. anisoplae* granules against *D. frontalis* is influenced by environmental conditions.

4.1 Introduction

The susceptibility of *D. frontalis* to entomopathogenic fungi was demonstrated in the previous Chapters (2 and 3), suggesting that pathogenic fungi have the potential to effectively control the fly. Met52® Granular bioinsecticide (MET52) based on a commercial strain of *Metarhizium anisopliae* was found to be highly effective against *D. frontalis* adults. However, soil moisture, temperature and humidity are known to be important factors influencing survival and persistence of fungal pathogens (Fargues and Luz, 2000; Arthurs and Thomas, 2001; Yeo *et al.*, 2003; Filotas and Hajek, 2004; Fuxa and Richter, 2004; Luz *et al.*, 2004; Lord, 2005; Bruck and Donahue, 2006; Thompson *et al.*, 2006; Chen *et al.*, 2014). For example, *M. anisopliae* (F52) was able to infect the black vine weevil larvae in different locations across the Willamette Valley, Oregon, USA over two growing season (Bruck and Donahue, 2007). Few studies have investigated the impact of soil moisture, relative humidity and temperature on the efficacy of fungal pathogens against fruit flies. In previous studies tephritid adults showed varied susceptibility to different isolates of *M. anisopliae* under a

wide ranges of temperatures (Dimbi *et al.*, 2004). Ekesi *et al.* (2003), indicated that soil temperature and moisture content can affect efficacy of the pathogen to *Ceratitis capitata*, *C. fasciventris*, *C. rosa* and *C. cosyra.* Garrido-Jurado *et al.* (2011c), found that isolates of *M. anisopliae* and *Beauveria bassiana* when used against *C. capitata* pupae have different responses to temperature and soil moisture. Also, recent research has shown the influence of temperature and humidity on *B. bassiana* efficacy when the fungus was used against housefly larvae (*Musca domestica*) (Mishra *et al.*, 2013). In addition, the influence of some abiotic factors to other pathogens has been reported. In a recent study by Rohde *et al.* (2010), indicated that the virulence of entomopathogenic nematodes is influenced by temperature and moisture. The authors found that inactivity of *Steinernema carpocapsae* against *C. capitata* larvae caused highest mortality at high moisture content. In a recent laboratory study Gul *et al.* (2015), found that inoculation of *B. zonata* adults by spraying suspensions of three different entomopathogenic fungal species gave higher mortality than those that treated by oral application. The same study indicated that the reduction in adult emergence was greater when the pest was treated in the larval stage rather than in the pupal stage.

To date, no studies have investigated the influence of environmental conditions on the efficiency of fungal pathogens against *D. frontalis*. This study, therefore, evaluates the effect of soil moisture content, humidity, temperature and application method on efficacy of MET52 based on *M. anisopliae* against *D. frontalis* under laboratory conditions.

4.2 Materials and Methods

4.2.1 *Moisture content experiment*

The effects of ten different soil moisture contents on the effect of MET52 on adult emergence and post-emergence mortality of adult *D. frontalis* were examined. Following similar experimental procedures described in Section 2.2.3 (Chapter two), plastic cups (4 cm high × 4 cm diameter) were filled with 30 g of sterilised soil. The soil was mixed with MET52 at 9.0 x 10^8 CFU g^{-1}. Eighteen pupae (1- 2 days old) of *D. frontalis* were gently placed at 2 cm depth in each cup. Then, the target soil moisture contents were prepared for the treatments and untreated control by adding 0.6, 1.2, 1.8, 2.4, 3, 3.6, 4.2, 4.8, 5.4 and 6 ml sterilised distilled water to get 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% Water Holding Capacity (WHC (the amount of water that a given soil can hold for crop use)) (Appendix III). The treatment cups were covered with cups of the same size inverted and perforated at the top for air flow. Cups of each single replicate were sealed together at the sides with parafilm and kept in an incubator at 25 ºC, 75% to 85% RH and 14:10 L:D. The soil moisture contents

were maintained by weight the cups daily until adult emergence. Four replicates for each treatment were arranged. Nine days later (two to three days before emergence), the cups were transferred to transparent plastic cages 10 cm × 10 cm × 10 cm to assess emerging adult mortality. The cages covered with gauze at one side, were supplied with artificial diet and water as previously described for insect culture. The number of emerging flies in the treatments was assessed. The cups were taken out of the cages. Four cages containing twelve emerging adults (one day-old) for each treatment were arranged to assess post-emergence mortality. The cages were kept in the same conditions described above for the cups. Dead flies were collected daily from the cages and assessed over the period of twelve days. To confirm fungal infection dead adult flies were individually sterilised with 70% ethanol followed with three rinses in sterile distilled water. The samples were placed into Petri dishes with moist sterile filter papers and incubated at 25 ºC in the dark. The insect samples were subjected to microscopic observation over a week. Only adults covered with fungal mycelium were considered as hosts to fungus.

4.2.2 *Humidity experiment*

The influence of three levels of relative humidity (40%- 50%, 55%- 65% and 75%- 85% RH) on the effect of MET52 in reducing emergence rate and causing post-emergence mortality of *D. frontalis* adults were evaluated. Cups filled with 30 g soil were prepared and inoculated with the fungus at 9.0 x 10^8 CFU g^{-1} as previously described. The pathogen was applied one week prior to release of 15 third instar larvae into the cups. The treatments and control received 1.2 ml of sterilised distilled water. Following the same process described above, soil moisture was maintained at 10% to 20% WHC. The cups were maintained in three incubators at the humidity levels tested, 25 °C and 14:10 L:D. The humidity was regulated by controlling the supply of free water within the incubator and monitored daily. Five cups for each treatment were arranged. The number of emerging flies in the treatments was assessed. Four cages containing twelve emerging adults (one day-old) for each treatment were arranged to assess post-emergence mortality. The cages were kept in the same tested conditions. Dead flies were collected daily from the cages and assessed over the period of two weeks. Fungal infection in dead adults was investigated by following the same process previously described.

4.2.3 *Temperature experiment*

The influence of three temperatures (35 ºC, 25 ºC and 15 ºC) on post-emergence mortality of *D. frontalis* adults caused by MET52 was assessed. Twenty larvae were released into cups filled with soil treated with MET52 at 9.0 x 10^8 CFU g^{-1} prepared as previously described. The treatments and control received 1.2 ml of sterilised distilled water. Soil moisture was maintained at 10% to 20% WHC until adult emergence. Then, the cups were kept following the same process described above and incubated at the tested temperatures, 75%- 85% RH and 14:10 L:D. Five replicates for each treatment were arranged. The number of emerging flies in the treatments was assessed. After emergence, 15 adults (one day-old) were transferred and kept in adult cages and kept in the same tested conditions. Four replicates for each treatment were arranged. Dead flies were collected daily from the cages and assessed over the period of twelve days. Fungal infection in dead adults was investigated by following the same process previously described.

4.2.4 *Application method experiment*

This experiment aimed to compare efficacy of MET52 based on *M. anisopliae var anisopliae* strain F52 when applied using different methods against *D. frontalis* adults. The fungus was directly sprayed on adults or applied to soil as granules. In the case of soil application, one hundred, two day old pupae were placed in soil treated with MET52 at 9.0 x 10^8 CFU g^{-1} in 4 cups (25 pupae for each) filled with soil prepared and kept as previously described. Four ml of sterilised distilled water was added to the untreated control and the fungus treatment. Following the same process described above, soil moisture was maintained at 65% to 70% WHC. After emergence, 15 adults were transferred into cages and kept following the same procedure and condition described in experiment one. In the spray application, a suspension of MET52 was prepared at 9.0 x 10^8 CFU g^{-1}. Following the similar experimental procedure described in Section 3.2.7 (Chapter three), fifteen adults (one to two days old) were placed into Petri dishes lined with two pieces of filter paper. The flies were placed on ice for 2 min to be easy to handle. A suspension of 2 ml of MET52 was gently shaken by a vortex for 2 min before spraying directly onto the adults by spray bottle (100 ml. Superdrug Stores, UK). The adult flies were sprayed until run-off. The same number of adults was treated with 2 ml of water for the control. The treated adults were transferred into cages and kept following the same procedure and condition described above. Four cages per each treatment were arranged. Dead flies were collected daily from the cages and assessed for 5 to 12 days. Fungal infection in the adults was investigated by following the same process previously described.

4.3 Statistical analysis

Percentages of emergence rate and post-emergence mortality of *D. frontalis* adults were transformed by arcsine and analysed by Two-Way Anova test. Then, mean differences among the treatments were separated by Tukey`s test ($P < 0.05$). Percentages of adult emergence and adult mortality were corrected using Abbott`s formula where is necessary (Abbott, 1925). If data were not normally distributed non-parametric analysis was performed using the Scheirer-Ray-Hare test (for two factors). Then, Mann-Whitney was used to compare the differences between the treatments. Probit analysis was used to calculate LT50 and LT90. All the statistical analysis was performed in Minitab 16 Statistical Software.

4.4 Results

4.4.1 *Moisture content experiment*

As shown in the Table 4.1, the soil moisture contents used did not affect the adult emergence rate of *D. frontalis* compared to the untreated control ($\chi^2 = 0{,}999$; df = 1; $P > 0.05$ (Scheirer-Ray-Hare test)). The lowest adult emergence (91%) was found in inoculated soil at 90% WHC. The emergence ranged from approximately 93% to 100% in other treatments (Table 4.1). After emergence, an effect of soil moisture content on the efficacy of the fungus in killing adults was observed. Four days after emergence, adult mortality in the fungal treatments was significantly higher than untreated control at all the moisture contents tested with the exception of the 10% treatment ($\chi^2 = 1$; df = 1; $P < 0.05$ (Scheirer-Ray-Hare test)). The highest pathogenicity was induced in 70% treatment with median adult mortality of approximately 51%. Significant differences were found between the treatments (Table 4.1). A low adult mortality (0%) was found in the untreated control. The effect of the fungus against adults had increased eight days later. Adult mortality was significantly higher in all fungal treatments than untreated control (which had approximately 1% to 8% adult mortality) ($F = 2440.27$; df = 1; $P < 0.05$). The highest mortality was observed in inoculated soil at 70% WHC with approximately 93% adult mortality. However, increasing soil moisture contents to higher levels at 80%, 90% and 100% WHC significantly reduced the effect of the fungus on the adults (Figure 4.1), causing approximately 72%, 50% and 54% mortality, respectively. Significant differences were observed between the treatments (Figure 4.1). The results of lethal time to kill 50% (LT50) and 90% (LT90) of flies are given in Table 2. LT50 in the fungal treatments ranged from approximately 5 days to 10 days. The shortest LT50 was found when pupae were exposed to the fungus at 70% WHC. The LT90 was greater when soil

moisture content increased above 70% WHC. The longest LT90 at approximately 20 days was found in adults emerging from inoculated soil at 90% WHC treatment.

Table 4.1 Percentage emergence rate (data was corrected by Abbott`s formula) and median post-emergence mortality (uncorrected data are shown) after four days, of *Dacus frontalis* pupae treated with MET52 at 9.0 x 10^8 CFU g^{-1} in soil moisture contents (10% to 100% WHC). n = 4.

Treatment		Water Holding Capacity (%)									
		10	20	30	40	50	60	70	80	90	100
MET52	Emergence rate (%)	100	94.8	92.7	97.9	100	100	97.9	100	91.5	100
MET52	*Median adult mortality	2.7 C	21.6 b	19.2 b	17.6 b	22.8 b	26.3 b	51.3 a	28.4 ab	26.6 b	15.4 b
Control	*Median adult mortality	0 c	0 c	0 c	0 c	0 c	0 c	0 c	0 c	0 c	0 c

*Medians with different letters are significant different (Mann and Whitney test).

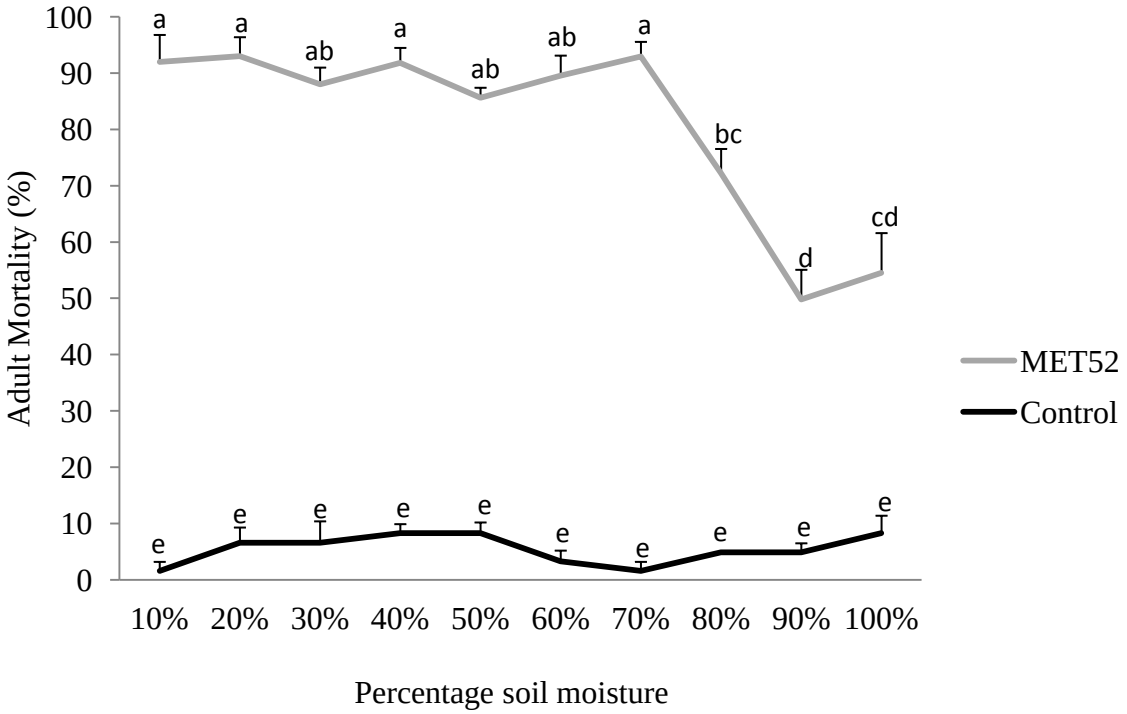


Figure 4.1 Mean (% ±SE) percentage post-emergence adult mortality, after 12 days of *Dacus frontalis* treated with MET52 at 9.0 x 10^8 CFU g^{-1} in soil with different moisture content (10% to 100% Water Holding Capacity). Different letters indicate significantly different means based on Tukey's HSD test ($P < 0.05$) after Anova. n = 4.

Table 4.2 Mean lethal time (LT50 and LT90) in days of MET52 applied against *Dacus frontalis* adult at different soil moisture contents (10% to 100% WHC). n = 4.

WHC	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%
LT 50	7.2	5.9	6.5	6.4	6.6	6.0	5.4	7.5	10.3	9.7
LT 90	10.3	9.5	11.1	10.5	11.4	10.0	10.8	13.4	19.5	17.1

The values were calculated by Probit analysis.

4.4.2 *Humidity experiment*

In general, the humidity levels used in this study had an effect on MET52 efficacy on emergence rate and post-emergence adult mortality of *D. frontalis*.

In the case of the emergence rate, the greatest reduction in the adult emergence was found in MET52 treated soil at high relative humidity (75%- 85% RH) with approximately 36% reduction in adult emergence. At lower humidities, the emergence reduction decreased to approximately 25% at 55- 65% RH, and 23% at 40- 50% RH. The emergence at the highest relative humidity was significantly lower in the treated soil than untreated control ($F = 38.48$; $df = 1$; $P < 0.05$). No significant differences were observed between all other treatments (Figure 4.2.A). In the untreated controls, the adult emergence rates ranged from approximately 95% to 100%. After the emergence, the mortality of *D. frontalis* adults from the treated soil at all levels of the humidity used was significantly higher than the untreated control two weeks from the emergence ($F = 94.07$; $df = 1$; $P < 0.001$). The greatest adult mortality was observed at the highest humidity inducing approximately 80% mortality. A lower mortality was found in the other fungal treatments at medium and low humidity which induced equal pathogenicity with approximately 31%. Significant differences were found between the fungal treatments (Figure 4.2.B). The adult mortality in the untreated control ranged from approximately 2% to 6%.

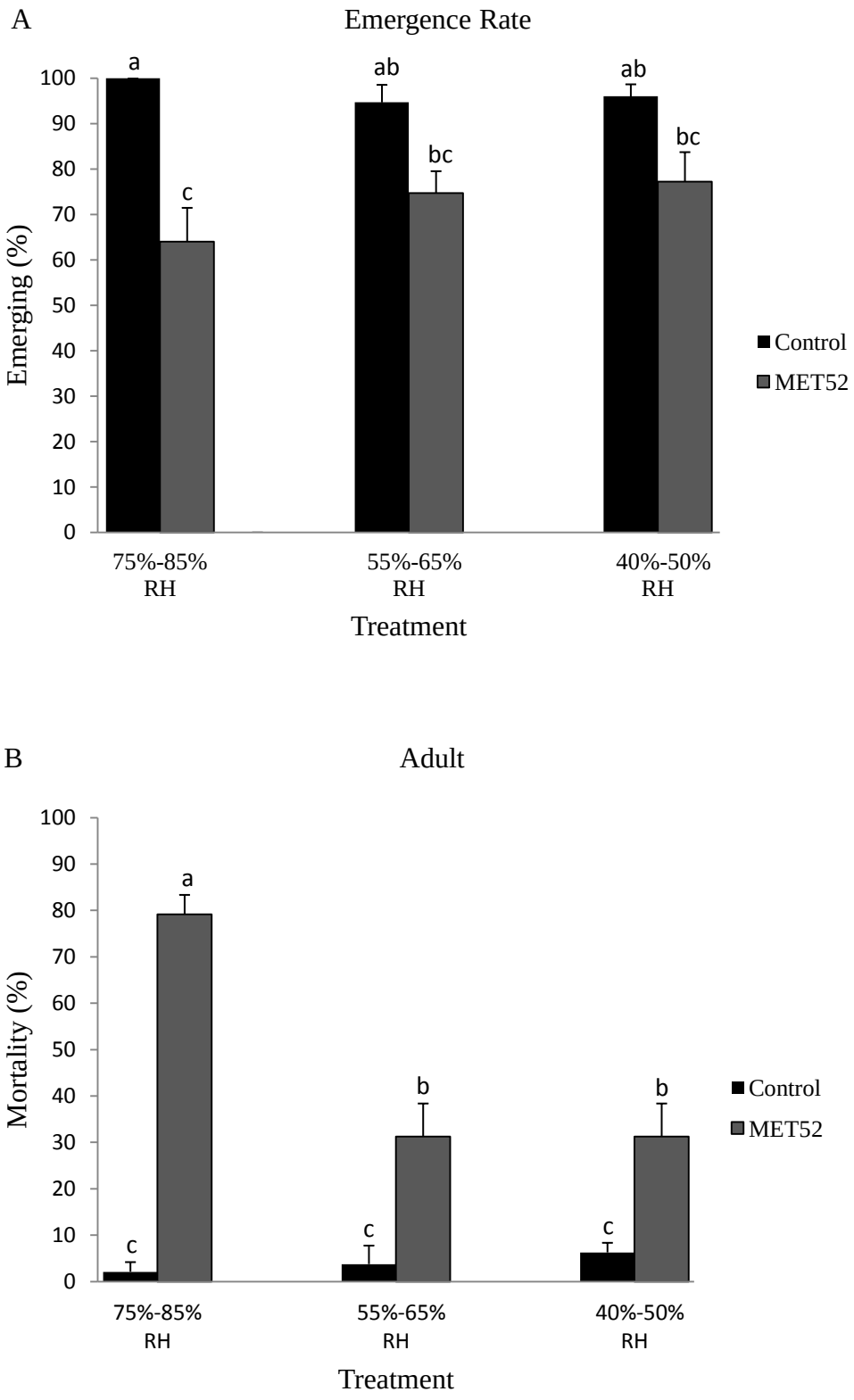


Figure 4. 2 Means (% ±SE) percentage emergence rate, n = 5 (A) and subsequent adult mortality after 14 days (B), n = 4 of *Dacus frontalis* emerging from soil treated with MET52 at 9.0 x 10^8 CFU g^{-1} in 30 g at different humidity levels. Means that do not share a letter are significantly different based on Tukey's HSD test ($P < 0.05$) after Anova.

4.4.3 *Temperature experiment*

Percentage mortality of flies emerging from inoculated soil was strongly affected by temperature (Figure 4.3). Five days after emergence, the mortality of *D. frontalis* adults from the treated soil at all temperatures used was significantly higher than the untreated control (χ^2 = 0.99999; df = 1; $P < 0.001$(Scheirer-Ray-Hare test)). The greatest adult mortality (66%) was observed at 35 ºC. At 25 ºC and 15 ºC the mortalities were 50% and 25% respectively. Significant differences were found between the three temperatures (Table 4.3). Nine days after emergence, 100% mortality of adults from inoculated soil and approximately 21% mortality from untreated soil were observed in 35 ºC treatment. The mortalities after correction (Abbot's formula) are shown in Table 4.3. The mortality of flies from inoculated soil was significantly higher than flies from untreated soil (χ^2 = 0.999987; df = 1; $P <$ 0.001(Scheirer-Ray-Hare test)). No significant differences were found between the three temperatures (Table 4.3). The effect of the fungus on the flies increased to produce a significant difference compared to the untreated control at 25 ºC and 15 ºC twelve days after emergence (χ^2 = 0.999; df= 1; $P < 0.001$) causing approximately 100% and 82.5% adult mortality respectively. No significant difference was found between the two treatments (Table 4.3). The LT50 and LT90 are given in Table 4.3. The speed of killing 50% of adults from inoculated soil increased with temperature (Figure 4.3). The LT50 in the fungal treatments ranged from 4 days (35 ºC) to approximately 8 days (15 ºC). In the 25 ºC treatment approximately 9 days was required to cause 90% adult mortality.

Table 4.3 Mean lethal time (LT50 and LT90) and median mortality of *Dacus frontalis* adults emerging from soil treated with MET52 at temperatures of 15 ºC, 25 ºC and 35 ºC. Mortality data were corrected by Abbot's formula. n = 4.

Treatment	Temperature	LT50	LT90	*Median mortality		
	°C	(Days)	(Days)	after 5 days	after 9 days	after 12 days
MET52	35	4	13	66a	50a	-
MET52	25	5	9	50a	64a	100a
MET52	15	8	14	25b	46.5a	80a

*Scheirer Ray Hare test: Differences are significant between the treatments and untreated control. Medians of each column with different letters are significant different (Mann and Whitney test). The values of LT50 and LT90 were calculated by Probit analysis.

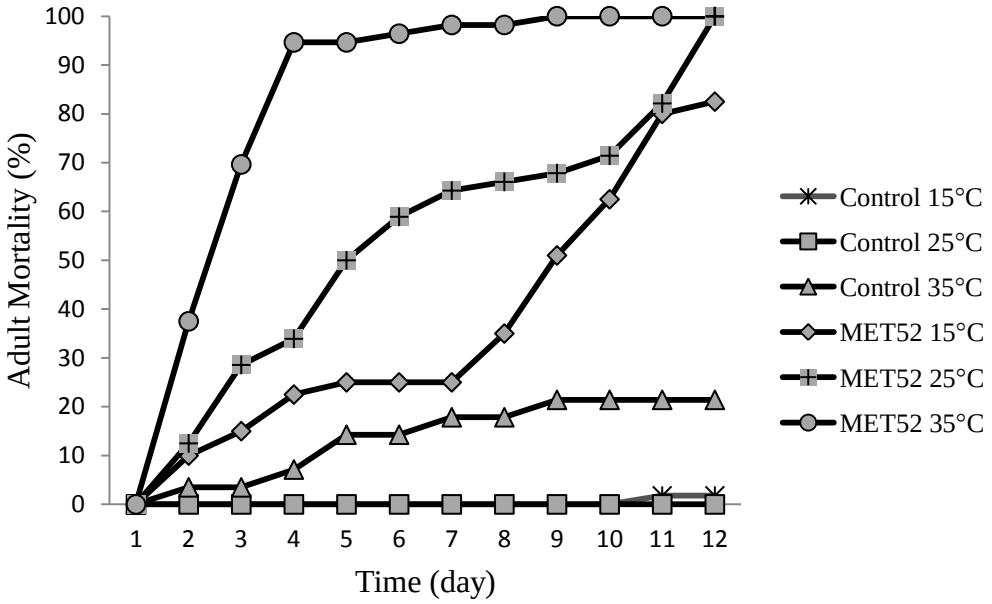


Figure 4.3 Time-mortality response of *Dacus frontalis* adults treated with MET52 at different temperatures over the period of twelve days from emergence (uncorrected mortalities are shown).

4.4.4 *Application method experiment*

Metarhizium anisopliae var anisopliae strain F52 showed significant effects on *D. frontalis* adults when applied either by granules on soil or by direct spray onto adults compared to the untreated control over the period of twelve days from treatment ($F = 237.33$; df = 1; $P < 0.001$). The pathogenicity of adults was significantly ($P < 0.05$) greater following the soil treatment (85%) rather than the spray (37%) (assessed five days after spray application) (Table 4.4). One week later, the adult mortality had increased further in the fungus treatments compared to the untreated control ($\chi^2 = 0.99$; df = 1; $P < 0.001$(Scheirer-Ray-Hare test)), causing 100% adult mortality in spray treatment and approximately 98% in soil application. There was no significant difference between the two approaches (Table 4.4). The LT50 and LT90 are given in Table 4.4. The fungus was more virulent when applied in soil inducing 50% adult mortality after approximately 4 days. While nearly 6 days was required to cause 50% adult mortality when the flies were directly sprayed with a fungus suspension.

Table 4.4 Mean lethal time (LT50 and LT90), mean and median mortality of *Dacus frontalis* adults treated with *M. anisopliae var anisopliae* strain F52 in different approaches. n = 4.

Treatment	Application approach	LT50 (Days)	LT90 (Days)	Adult mortality 5 days (means)*	12 days (Median)†
M. anisopliae	Granule in soil	4.1	7	85a	97.5A
M. anisopliae	Spray	5.9	8	36.3b	100A
Control	Granule in soil	-	-	1.3c	0B
Control	Spray	-	-	1.3c	2.5B

*Means that do not share the same letter are significantly different. Tukey's HSD test ($P < 0.05$) after Anova.
†Scheirer-Ray-Hare test: Differences are significant between the treatments and untreated control.
Medians with different letters are significant different. Mann and Whitney test ($P < 0.05$).
LT50 and LT90 were estimated by Probit analysis.

4.5 Discussion

The results of the present study demonstrate that soil moisture, humidity and temperature influence the virulence of *M. anisopliae* var. *anisopliae* strain F52 to *D. frontalis*.
The impact of these factors on pathogenicity of entomopathogenic fungi have been previously shown against other dipteran species *C. capitata, C. fasciventris, C. rosa* and *C. cosyra* (Ekesi *et al.*, 2003; Dimbi *et al.*, 2004), *C. capitata* (Quesada-Moraga *et al.*, 2006; Garrido-Jurado *et al.*, 2011c) and house fly, *Musca domestica* (Mishra *et al.*, 2013).

The current results showed that increasing soil moisture level did not enhance the pathogenicity of MET52 in reducing emergence rate. The emergence was high at all the tested soil moistures; however, increasing the moisture to high levels increased the post-emergence mortality of *D. frontalis* adults in the fungal treatments. This is the first report that shows the effect of entomopathogenic fungi on dipteran adults increasing with soil moisture and then sharply decreasing at highest moisture levels. This maybe because of insufficient oxygen for the fungi at high moisture levels (Li and Holdom, 1993), which could affected the fungal survival in the soil (Ekesi *et al.*, 2003). Also, this suggests that the MET52 spores may have been washed to a lower level in the soil at the high moisture level. The manufacturer's manual for the MET52 product indicates that the fungus efficacy can be reduced in extreme moisture conditions (http://www.fargro.co.uk/).

The present results show that the moisture level of soils treated with MET52 did not affect the adult emergence rate of *D. frontalis*. In a recent study conducted by Quesda-Moraga *et al.* (2006) the authors found that increasing the soil moisture did not produce higher pupal mortality when *M. anisopliae* (EAM 01/58Su) was applied against *C. capitata* pupae. In contrast, Ekesi *et al.* (2003) found that pupae of four tephritid fruit fly *C. capitata, C. fasciventris, C. rosa* and *C. cosyra* treated by a different methodology with other isolates of *M. anisopliae* in sandy loam soil showed high mortality with increasing moisture. Also, a study by Garrido-Jurado *et al.* (2011c) showed *M. anisopliae* caused high mortality to *C. capitata* pupae in soils with a high moisture content.

In the humidity test, the fungus reduced the adult emergence of *D. frontalis* at the highest humidity used (75%- 85% RH) and caused mortality post-emergence to adults at all the tested humidity levels, but this decreased at lower humidity (45%-65% RH). The results are similar to those of Mishra *et al.* (2013), who reported that approximately 72% to 100% adult mortality of the housefly was obtained at high humidity (70% to 100% RH) and less than 50% adult mortality at 50% RH when *B. bassiana* was sprayed against the flies. The same study indicated that 50% larval mortality was obtained at 75% RH and reduced at lower humidity.

The present results revealed that MET52 caused mortality to *D. frontalis* adults at all tested temperatures which clearly influenced the efficacy of the fungus 5 days from emergence. The mortality was low (25%) in 15 ºC treatment. However, the impact was not significantly different between the three temperatures after 9 days suggesting that the fungus granules can be effective at a range of temperature. This agrees with the manufacturer's manual for MET52 which indicates that the fungus granules can work effectively at a temperature range of 15 ºC to 30 ºC (http://www.fargro.co.uk/). Similarly, Dimbi (2004) reported that the effects of six isolates of *M. anisopliae* (ICIPE 18, ICIPE 20, ICIPE 32, ICIPE 40, ICIPE 41 and ICIPE 62) against adults of *C. capitata, C. fasciventris,* and *C. cosyra* varied with temperature ranged from 15°C to 35 °C. The same study indicated that applying *M. anisopliae* at 25 °C-35 °C had induced greater mortality than at lower temperature. The current results showed that *M. anisopliae* fungus virulence induced a maximum mortality of 100% at 25 °C after 12 days. Also, 100% adult mortality of house flies, *M. domestica* (Diptera: Muscidae) and Queensland fruit flies, *Bactrocera tryoni*) was obtained when the flies were treated with *M. anisopliae* isolate (FI-369) at 25 °C and 30 °C (Carswell and Spooner-Hart, 1998).

A recent study shows different responses of *M. anisopliae* isolates (EAMa 01/58-Su and EAMa 01/158-Su) to temperature when larvae of *C. capitata* were released into sandy-loam

soil treated with the isolates (Garrido-Jurado *et al.*, 2011c). Also, *M. anisopliae* has been reported to cause different mortalities (98%, 93%, 87% and 49%) to Black vine weevil larvae *Otiohynchus sulcatus* at 25 °C, 20 °C, 15 °C and 10 °C respectively (Moorhouse *et al.*, 1994). In other field trials against the same weevil, MET52 caused high larvae mortality when applied at 20 °C but lower mortality at lower temperature (Ansari and Butt, 2013). The influence of a range of temperatures (15 °C to 45 °C) on *B. bassiana* efficacy was reported when the fungus was sprayed on housefly adults, *M. domestica* (Mishra *et al.*, 2013). The percentage of adult mortality was approximately 20%, 72% and 66% at 15 °C, 25 °C and 35 °C at 75% RH respectively.

In the current study it was difficult to design an experiment with more than one factor due the large number of replicates that would have been required. Further investigations therefore are required to get a good reflection of abiotic factors on the pathogenicity of MET52 against the fly. This may enhance our understanding of the impacts of the interaction between temperature, relative humidity and soil moisture on the virulence of the fungus.

The results of the current study indicated that the application methods used for the fungus influence the adult mortality after 5 days. Efficacy of *M. anisopliae* when applied as a granule in soil was higher than as a direct spray against *D. frontalis* adults. Adult mortality exceeded 85% five days after the emergence while approximately 37% adult mortality was found in the spray treatment. The efficiency of the inoculation methods used here has not been compared together in a specific study. However, the adult mortality levels obtained are reported by other authors. In results obtained by Dimbi *et al.* (2003), nearly 87% of *C. capitata* adults were killed 4 days from being exposed to dry conidia of *M. anisopliae* applied by different methodology. While, other recent results indicated that approximately 30% to 40% of *R. cerasi* adults were killed five days after being sprayed by two isolates of *M anisopliae* (Daniel and Wyss, 2009).

Overall, optimal conditions to obtain the maximum control of *D. frontalis* are when MET52 granules are applied early in soil at 25 °C, 75% to 85% RH at 70% soil moisture. These findings suggested that the abiotic factors evaluated have impact on efficacy of MET52 in reducing emergence rate and adults of *D. frontalis*. Further studies, including field trials to examine the complex abiotic interactions affecting inactivity, survival and persistence of MET52 are needed to provide a better understanding of the role of entomopathogenic fungi in *D. frontalis* management.

Chapter 5. Horizontal transmission and persistence of *Metarhizium anisopliae* in *Dacus frontalis* (Becker) and effect of the fungus infection on fly reproduction

Abstract

The ability of *Dacus frontalis* flies emerged from soil inoculated with Met52 Granular biopesticide, based on a pathogenic fungus, *Metarhizium anisopliae,* var *anisoplia*e, to induce new infection in untreated flies was investigated. Contaminated adults were able to transmit the fungus conidia to untreated individuals of the opposite sex, resulting in above 30% mortality of females and 15% of males. The impact of MET52 on adult reproductive success in different mating combinations was assessed. The numbers of progeny pupating was affected by the treatments. The lowest pupal number was produced when inoculated males were paired with untreated females resulting in a nearly 89% reduction. The persistence of MET52 was assessed in terms of infectivity against larval-pupal stages (measured by calculating adult emergence rate) and adults of *D. frontalis* under laboratory conditions. The fungus reduced emergence rate and caused mortality in newly emerging adults even two months after a single application.

Overall, MET52 fungus was able to persist in soil, reducing the adult emergence and subsequent fly population for more than two months after a single application, and also inducing new infections among the fly population reducing the adult's reproduction.

5.1 Introduction

The efficacy of entomopathogenic fungi is influenced by the interaction between the fungus and the target insect which is affected by the transmission of fungi. Fungal pathogens can change or alter infested insect's behaviour (Dimbi *et al.*, 2009; Novelo-Rincón *et al.*, 2009). Horizontal transmission of entomopathogenic fungi through mating or physical contacts has been previously reported in several insect pests (Kaaya and Okech, 1990; Furlong and Pell, 2001; Kreutz *et al.*, 2004; Brooks and Wall, 2005; Toledo *et al.*, 2007; Beris *et al.*, 2013; Maniania *et al.*, 2013; Svedese *et al.*, 2013). However, such a strategy has not been investigated against *Dacus* species.

Different inoculation methods have been investigated for fungus transmission among fruit flies under laboratory and field conditions. Autodissemination devices are usually provided with attractants and insects become contaminated with the fungus before escaping;

subsequently the inoculated insects disseminate the pathogen spores among the target species population (Dimbi *et al.*, 2003; Quesada-Moraga *et al.*, 2006; Ekesi *et al.*, 2007; Quesada-Moraga *et al.*, 2008; San Andres *et al.*, 2014). Direct application of dry or wet conidia on target flies can induce new infections during mating or other contacts with non-infected individuals (Almeida *et al.*, 2009; Daniel and Wyss, 2010; Dimbi *et al.*, 2013). Recently, using sterile males to transfer fungus spores among wild populations has been also assessed (Toledo *et al.*, 2007; Novelo-Rincón *et al.*, 2009; Flores *et al.*, 2013; San Andres *et al.*, 2014; Sookar *et al.*, 2014).

A granular formulation of some entomopathogenic fungi has been evaluated against larvae, pupae and emerging adults of some tephritid species (Ekesi *et al.*, 2003; Ekesi *et al.*, 2005; Garrido-Jurado *et al.*, 2011b; Garrido-Jurado *et al.*, 2011c). However, to my knowledge, the potential for entomopathogenic fungi when applied in soil to disseminate as conidia from infected emerging flies to uninfected individuals has not been investigated. In the previous experiments in the current study, applying MET52 granules, based on a pathogenic fungus, *Metarhizium anisopliae,* var *anisoplia*e strain F52, in soil was demonstrated to cause high pathogenicity against *D. frontalis* (Chapter 2).

Fungal infection has been reported to have effects on mating behaviour of some dipteran species. Dimbi *et al.* (2009), reported that there were delays in male calling and mating competitiveness when adults of *Ceratitis capitata, C. cosyra* and *C. fasciventris* were inoculated by *M. anisopliae* isolate ICIPE 62 as the flies started to groom after the fungus infection. This behaviour may have a negative impact on copulation which would affect the efficiency of horizontal transmission and female reproductive success. Quesada-Moraga *et al.* (2006), demonstrated that *M. anisopliae* has reproductive impact on the German cockroach. Also, a reduction in fecundity and fertility can result when *C. capitata* females are inoculated by *M. anisopliae* and *Beauveria bassiana* (Quesada-Moraga *et al.*, 2006).

Persistence is an important factor which contributes to the successful use of fungal pathogens against insect pests which spend all or some of their life stages in soil. Persistence of *M. anisopliae* with different formulations of conidia in soil has been reported to be effective against some African tephritid fruit flies (Ekesi *et al.*, 2005). The authors indicated that granular formulation of the fungus was able to reduce emergence rate and cause mortality to newly emerging adults of *C. capitata, C. cosyra* and *C. fasciventris* over one year in a field cage experiment. In another study, soil application of granules containing *M. anisopliae* isolate ICIPE reduced populations of *Bactrocera invadens* in mango orchards during 2006/

2007 seasons (Ekesi *et al.*, 2011). Garrido-Jurado *et al.* (2011a), indicated that a suspension of *M. anisopliae* isolate EAMa 01/58-Su can persist in soil to a level which protects olive crops from being infested by the olive fly *B. oleae* for 56 days.

The objectives of this chapter were to investigate the ability of *D. frontalis* adults emerged from MET52-inoculated soil to transfer conidia and induce new infection in untreated adults in different mating combinations under laboratory conditions. Female reproductive potential in the various mating combinations was also investigated. In addition, persistence of MET52 in soil was determined by assessing infectivity against larval-pupal stages and newly emerged adults.

5.2 Materials and Methods

5.2.1 *Persistence experiment*

In this experiment, assessment of MET52 persistence was measured by examining the ability of the fungus to reduce emergence rate and induce mortality-post emergence of *D. frontalis* adults under laboratory conditions.

Following similar experimental procedures described in Section 2.2.3 (Chapter two), 15 plastic cups were filled with 30 g of sterilised soil. The soil was inoculated with 0.25 g of MET52 (9.0×10^8 CFU g^{-1}). The fungus was mixed with soil and applied 7, 42 or 70 days prior to third instar larvae being released into the cups. The same amount of soil was prepared for the control. All the cups received 3.6 ml of sterilised distilled water to enable pupation. Then, twenty larvae of *D. frontalis* were released into the cups. The cups were covered with the same size of the cup following the same procedures described in Chapter 2. Soil moisture was maintained at 65% to 75% until adult emergence by adding sterilised distilled water as necessary by weight. Then, the cups were kept at 25 ºC, 60% to70% RH and 14: 10 L: D. Five replicates for each treatment were arranged. Ten to eleven days after application, adult emergence was assessed. Then, 15 adults (one day-old) were transferred and kept in adult cages to assess mortality following the same process and conditions previously described. Four replicates for each treatment were arranged. Dead flies were collected daily from the cages and assessed over two weeks. Fungal infection in the adults was investigated by following the same process previously described in Section 2.2.3 (Chapter two).

5.2.2 *Fly to fly transmission experiment*

This experiment was conducted to investigate whether MET52-inoculated adults of *D. frontalis* have the ability to transfer conidia to and induce pathogen infection in untreated flies of the opposite sex.

Ten cups filled with sterilised soil inoculated with 1.5 g (9.0×10^8 CFU g^{-1}) of MET52 were prepared by following the similar experimental procedure previously described. Two hundred pupae (one day-old) of *D. frontalis* were gently placed at 2 cm depth into the cups. The same number of pupae was used for control. The treatments and control received 0.6 ml of sterilised distilled water. Soil moisture was maintained at 10% WHC. The treatments were kept at 25 ºC, 75%- 80% RH and 14: 10 L: D. On the day of emergence, the flies were put into Petri dishes and placed on ice for 2 min to be handled. Adults of each sex were separated under a dissecting microscope. Three treatments were arranged each with four groups of 16 one day-old *D. frontalis* adults (8 males and 8 females) as following: 1) untreated male × untreated female as control; 2) Inoculated male × untreated female; 3) Inoculated female × untreated male. The flies were transferred and kept in adult cages as previously described. Four replicates for each treatment were arranged. Dead flies were collected daily from the cages over the period of two weeks. Fungal infection in the adults was investigated by following the same process previously described.

5.2.3 *Effects of the fungus infection on adult reproduction*

To determine the impact of MET52 infection on fly reproduction of *D. frontalis,* all the experimental mating combinations previous used in fly to fly transmission experiment were used. Organic fresh squash were used for oviposition. The fruits were sterilised in 70% ethanol followed with rinsing three times in sterilised distilled water. The fruits were cut to into equal sized pieces, placed into Petri dishes and offered to the adults when mating behaviour was observed (4 days from the emergence). The fruits were replaced daily until 9 days from emergence and kept into small plastic containers (15 cm × 10 cm × 15 cm) filled with sterilised soil for pupation. The containers were covered with fine gauze and maintained at room temperature. Due to some of the fruits samples being quite decomposed it was difficult to count the precise numbers of eggs deposited by females in all the treatments. For that, pupae were recovered after two oviposition intervals (5 to 6 and 8 to 9 days from emergence). Pupae numbers were counted twelve days post the infestation to give a sufficient time for all larvae to complete their feeding in the fruits and move into the soil. The fruits

were also examined to make sure there were no larvae being left. The number of dead females was considered during the analysis.

5.2.4 *Sublethal effect of MET52 infection on adult reproduction*

Based on the results presented in Section 2.4.4 (Chapter two), high and low rates of MET52 (1.5g and 0.125g/ 9.0 x 10^8 CFU g^{-1}) which induced approximately 71% and 35% adult mortality respectively five days after adult emergence were selected to investigate their impact on adult reproduction of *D. frontalis*. The effect of fly sex on the susceptibility to the fungus infection at the rates tested was also evaluated.

In a similar procedure as previously described in Section 2.2.6 (Chapter two), cups filled with sterilised soil had MET52 applied at two rates: 1.5 g and 0.125 g (9.0 x 10^8 CFU g^{-1}). Sterilised distilled water (0.6 ml) was added to the untreated control and the other treatments. Twenty larvae of *D. frontalis* were added to each replicate and there were five replicates for each treatment. After adult emergence, 10 males and 10 females (one day-old) were transferred and kept in adult cages to assess mortality following the same process and conditions previously described. Dead flies were collected daily from the cages which were assessed over 11 days. Four replicates for each treatment were arranged. Whole fresh baby courgettes, externally sterilised, were offered to the adults for oviposition from the eighth to eleventh days after emergence. Courgettes were replaced with fresh once daily and kept in plastic containers as previously described. Pupae were recovered after two oviposition intervals (8 to 9 and 10 to 11 days from emergence). The number of dead females was considered during the analysis.

5.3 Statistical analysis

Percentages of emergence rate and mortality post-emergence of adults were transformed by arcsine and analysed by two-way Anova test. Then, mean differences among the treatments were compared by Tukey`s test ($P < 0.05$). If data were not normally distributed nonparametric analysis was performed by Scheirer-Ray-Hare test. Then, Mann-Whitney was used to compare the differences between the treatments. Two-Sample t-test was performed to compare mean mortality of untreated males and females after being infected by MET52-inoculated adults of the opposite sex. Probit analysis was used to calculate LT50 and LT90. All the statistical analysis was performed by Minitab 16 Statistical Software.

5.4 Results

5.4.1 *Persistence Bioassay*

MET52 persisted in soil over the period of 70 days and was able to reduce the emergence rate and cause post-emergence mortality of *D. frontalis* adults during the experimental period. As can be seen in the Table 5.1, a significant reduction in the adult emergence was observed in the fungal treatments compared to the untreated control ($\chi^2 = 0.999685$; df = 1; $P <$ 0.001(Scheirer-Ray-Hare test)), with no significant differences found between the fungal treatments. The greatest pathogenicity against larval-pupal stage was found in the seven days-old treatment reducing the emergence to 86%. Higher emergence rates ranging from 88% to 97% were found in the other treatments (Table 5.1). In the case of adults, the mortality in all the fungal treatments was significantly higher than the untreated control over the period of two weeks after emergence ($\chi^2 = 0.9999$; df = 1; $P < 0.001$(Scheirer-Ray-Hare test)), with significant differences found between the treatments (Table 5.1) The greatest pathogenicity against adults was found in the 42 days-old treatment inducing approximately 78% mortality. Lower adult mortality ranging from nearly 37% to 50% was found in the 7 and 70 days-old treatments respectively. In the untreated control, approximately 13% adult mortality was found. Percentage mortality of flies emerging from the treatments increased with time and varied between the treatments (Figure 5.1). The results of LT50 and LT90 of flies emerged from soil inoculated with MET52 at different times are given in Table 5.1. In the fungal treatments, the LT50 value ranged from approximately 8 days to 15 days. The shortest LT50 was found in the 42 days-old treatment, whereas approximately 25 days was required to get 90% adult mortality in the 7 days-old treatment (Table 5.1).

Table 5.1 Emergence rate, and adult mortality post-emergence (two weeks after emergence) together with mean lethal time (LT50 and LT90), for *Dacus frontalis* adults after larvae were released in 30 g soil treated with 0.25 g of MET52 (9.0×10^8 CFU g^{-1}) at the different times tested.

Treatment	Emergence (%)	Median	Adult mortality **(%)**	Median	LT50 (Days)	LT90 (Days)
7 days	86	85b	36.66	36.66B	14.98	25.54
42 days	88	90b	78.33	83.33A	8.29	14.18
70 days	92	90b	44.99	43.33B	13.47	22.29
Control	97	95a	13.30	13.30C	-	-

Medians that do not share a common letter are significantly different (Mann-Whitney test).
LT50 and LT90 were estimated by Probit analysis.

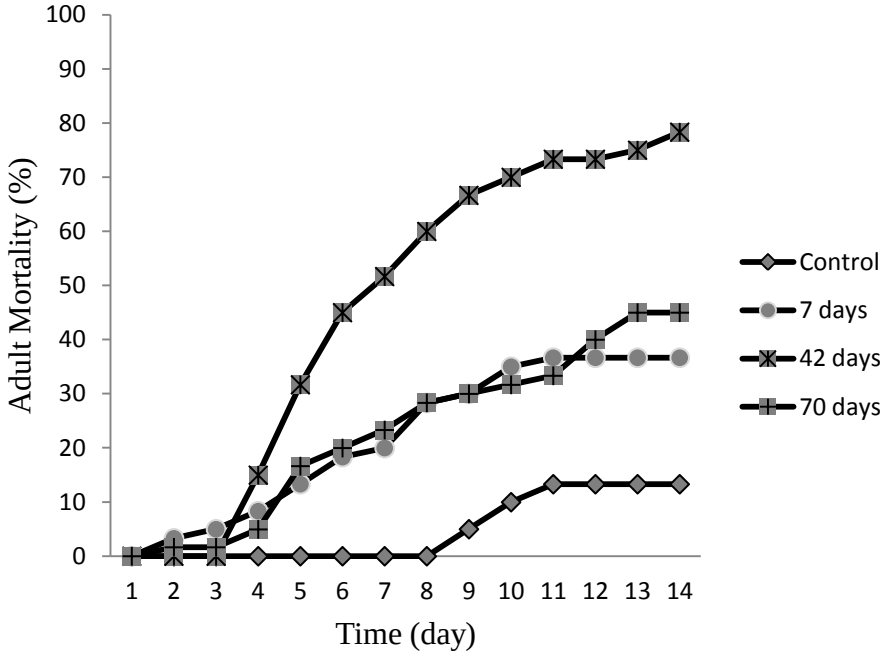


Figure 5.1 Time-mortality response of *Dacus frontalis* adults emerged from soil treated with MET52 at different times.

5.4.2 *Fly to fly transmission*

Mating contacts between males and females were observed in some experimental cages starting from the fourth day and recorded for nine days after adult emergence. It was clearly observed that males mounted the females. The inoculated adults of both sexes were able to cause mortality to the untreated flies in treated cages. As can be seen in Figure 5.2, adult mortality in the fungal treatments were significantly higher than untreated control over the period of two weeks ($F = 140.01$; $df = 1$; $P < 0.001$). The mortality in the fungal treatments ranged from approximately 48% to 62%, with a significant difference between the two treatments (Figure 5.2).

Percentage mortality of untreated females (ca, 31%) coupled with inoculated males was greater than that of untreated males (ca, 17%) coupled with inoculated females two weeks after the treatment (Figure 5.3), and showed very low percentage with presence of mycelium 10% and 0% respectively. There was no significant difference between the two treatments ($t = 1.39$; $df = 5$; $P > 0.05$ (Two-Sample t-test))

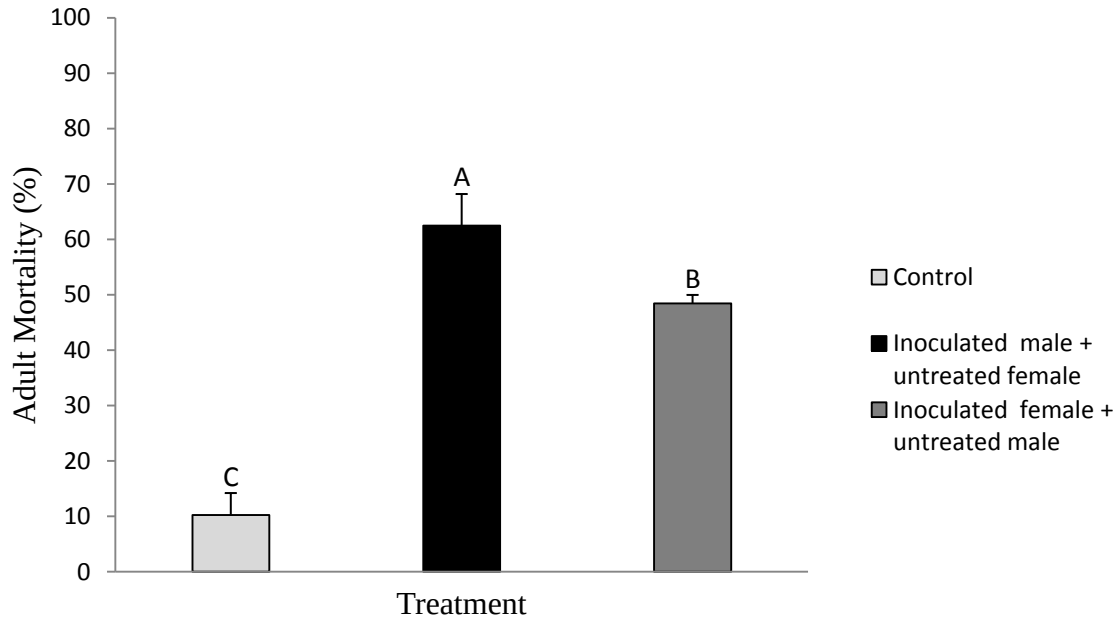


Figure 5.2 Mean (% ±SE) percentage adult mortality, after two weeks of MET52 inoculated *Dacus frontalis* adults paired with untreated adults of the opposite sex. Bars with different letters have significantly different means based on Tukey's HSD test ($P < 0.05$) after Anova. n = 4.

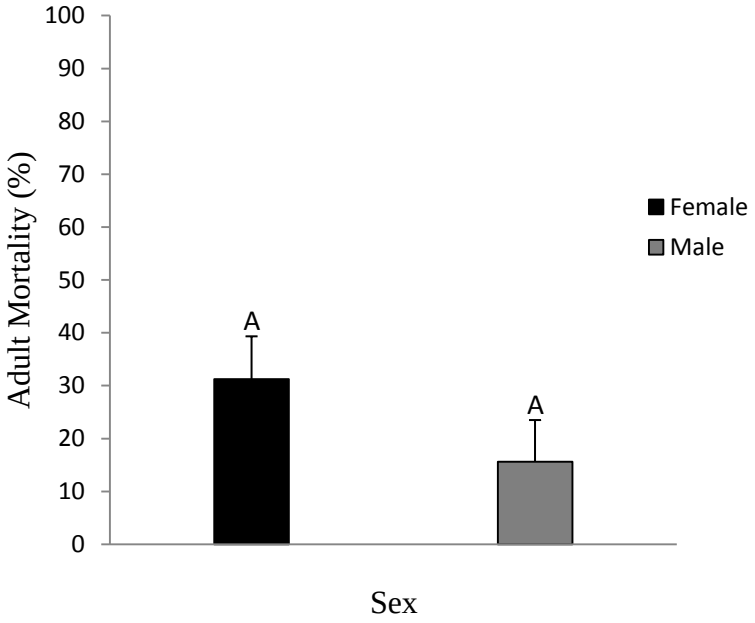


Figure 5.3 Mean (% ±SE) percentage adult mortality, after two weeks of untreated *Dacus frontalis* adults paired with MET52 inoculated adults of the opposite sex. Bars with the same letters are not significantly different based on Two-Sample t-test ($P < 0.05$). n = 4.

5.4.3 *Effects of the fungus infection on adults reproduction*

The results of the reproductive impact of MET52 on *D. frontalis* adults are summarised in Table 5.2. There was no significant effect of the fungus on the number of pupae recovered from inoculated flies 6 days after the emergence compared to untreated control ($F = 0.95$; $df = 1$; $P > 0.05$), with the pupal yield ranging from 3 to 4/ female/ day in all treatments. However, there was a significant reduction in pupae recovered in the fungal treatments compared to untreated control 9 days after emergence ($F = 53.58$; $df = 1$; $P < 0.005$). The greatest reduction (88.71%) was found when inoculated males were paired with untreated females, producing approximately one pupa/ female/ day compared to 57.69% pupae reduction with approximately 3 pupae/ female observed when inoculated females were paired with untreated males. A higher pupa number was found in untreated control with approximately 8 pupae/ female/ day.

Table 5.2 Impact of MET52 on reproductive output of *Dacus frontalis* adults paired with untreated adults of the opposite sex.
Means in the same column that do not share a letter are significantly different. Tukey's HSD test ($P < 0.05$) after Anova. n = 4.

	Oviposition interval									
	Pupae recovered from first and second day of oviposition (5 and 6 days after emergence)					Pupae recovered from fourth and fifth day of oviposition (8 and 9 days after emergence)				
Treatment	% male mortality	% female mortality	Male: Female	Pupae/ F/ day	% reduction*	% male mortality	% female mortality	Male: Female	Pupae/ F/ day	% reduction*
Untreated male × untreated female	0	0	1: 1	4.2a		0	3.12	1.03: 1	7.80A	
Inoculated male × untreated female	28.12	0	1: 1.3	3.7a	11.90	75	12.50	1: 3.5	0.88B	88.71
Untreated male × inoculated female	3.12	40.62	1.4: 1	3.0a	28.57	6.25	68.75	3: 1	3.30B	57.69

* % reduction = control value – MET52 treatment value/ control value × 100.

5.4.4 *Sublethal effect of MET52 infection on adult reproduction*

The results of pupal production in the oviposition intervals tested were analysed separately for each interval alone (8 to 9 and 10 to 11 days from emergence) and when assessed together (8 to 11 days from emergence). Overall, total pupal production of *D. frontalis* adults obtained from the oviposition intervals tested together was affected by the treatment.

The results of the reproductive impact of the MET52 on *D. frontalis* adults are summarised in Table 5.3 and 4. There was significant effect of the fungus on the number of pupae recovered from inoculated flies 9 days after the emergence compared to untreated control (χ^2 = 0.999685; df = 1; $P < 0.005$ (Scheirer-Ray-Hare test)). The greatest reduction (92.9%) was found in 1.5 g MET52 treatment, producing approximately one pupa/ female/ day compared to 77.8% pupae reduction with approximately 3 pupae/ female/ day obtained from adults inoculated with 0.125 g MET52. A higher pupa number was found in untreated control with approximately 8 pupae/ female/ day. There was no significant effect of the fungus on the number of pupae recovered from inoculated flies compared to untreated control 11 days after emergence (χ^2 = 0.439593; df = 1; $P > 0.05$ (Scheirer-Ray-Hare test)), with the pupal yield ranging from 330 to 425 in all treatments (Table 5.3). For the total pupal production (both oviposition intervals together), shown in Table 5.4, there was a significant reduction in the total pupae recovered in the fungal treatments compared to untreated control over the period tested 8 to 11 days after the emergence (F = 18.35; df = 1; $P < 0.005$). The greatest reduction (60.23%) was found in 1.5 g MET52 treatment, producing approximately 367 pupae compared to 41.38% pupae reduction with approximately 541 pupae observed in 0.125 g MET52. A higher pupa number was found in untreated control with approximately 923 pupae.

The adult mortality and effect of sex on the susceptibility to the fungus infection, are shown in Figure 5.4, there was significant effect of the MET52 on *D. frontalis* adult mortality compared to the control (F = 91.19; df = 1; $P < 0.001$). The mortality of the adults ranged from approximately 5% to 72% with no significant differences found between the fungal treatments (Figure 5.4). Mycelium of the fungus was observed growing on male and female 24 hours after incubation (Figure 5.5). The fly sex did not affect susceptibility to the fungus infection 11 days after emergence (F = 0.65; df = 1; $P > 0.05$).

Table 5.3 Impact of MET52 applied at different doses on reproductive output of *Dacus frontalis* adults.

	Pupae recovered 8 and 9 days after emergence				Pupae recovered 10 and 11 days after emergence			
Treatment	Pupae out put		Pupae/ F/ day (Median)	% reduction*	Pupae out put		Pupae/ F/ day (Median)	% reduction*
	Number	Median			Number	Median		
Control	523	127.5a	7.9a		400	69.5A	4.3A	
1.5 g MET52	37	10.5b	1.1b	92.9	330	63.0A	16.5A	17.5
0.125 g MET52	116	26.5b	2.7ab	77.8	425	99.5A	21.8A	

Medians in the same column that do not share a letter are significantly different. (Mann-Whitney test).
* % reduction = control value – MET52 treatment value/ control value × 100.

Table 5.4 Effect of MET52 on reproduction of *Dacus frontalis* adults over the oviposition intervals tested (8 days to 11 days after the emergence).

Treatment	Means of pupae/ female/ day	Pupae output		% pupal reduction*
		Number	Mean	
Control	6.9A	923	233a	
MET52 (1.5 g)	8.1A	367	91.7b	60.23
MET52 (0.25 g)	13.6A	541	135.2b	41.38

Data in the same column that do not share the same letter are not significantly different. Tukey's HSD test ($P < 0.05$) after Anova. n = 4.
* % reduction = control value – MET52 treatment value/ control value × 100.

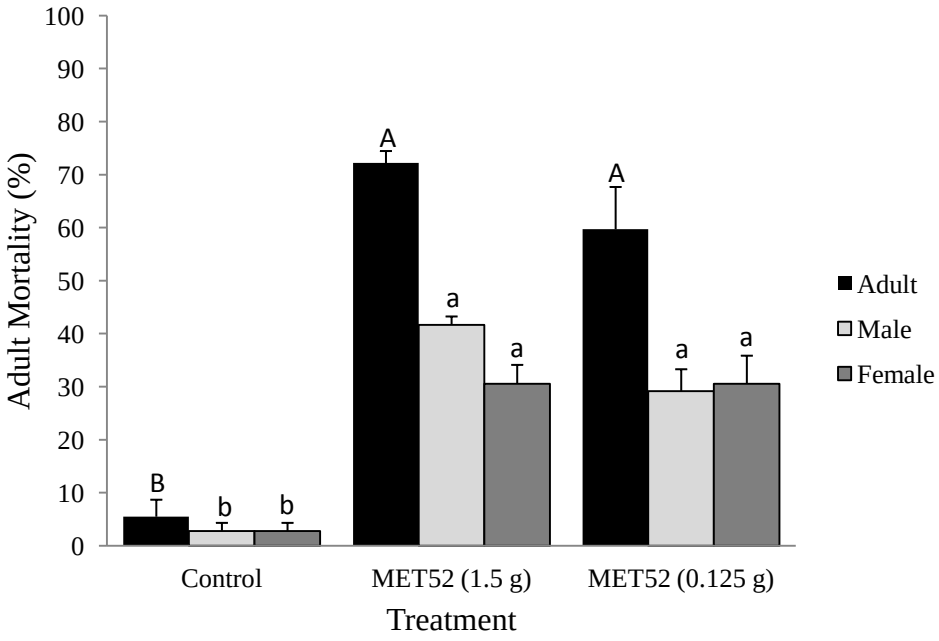


Figure 5.4 Mean (% ±SE) percentage adult, male and female mortality, after eleven days of *Dacus frontalis* larvae being treated with different rates of MET52 (1.5 and 0.125 g) in 30 g of soil. Bars within treatments with different letters (upper case letters for adults and lower case letters for males and females) represent significantly different means based on different Tukey's HSD tests ($P < 0.05$) after Anova. n = 4.

Figure 5.5 *Dacus frontalis* male (♂) and female (♀) infested with MET52.

5.5 Discussion

The present study investigated the ability of MET52-contaminated adults of *D. frontalis* to induce a fungus disease in a healthy fly population. To my knowledge, the ability of newly emerging flies, which are inoculated by entomopathogenic fungi in soil, to transmit the fungal infection to non-infected individuals has not been previously demonstrated. Recently, horizontal conidia transmission of various strains of entomopathogenic fungi to some fruit fly species has been confirmed by different approaches (Toledo *et al.*, 2007; Quesada-Moraga *et al.*, 2008; Dimbi *et al.*, 2013; Sookar and Allymamod, 2014). It was difficult to compare the results obtained in the current study with other published works because of the differences in the inoculation method and insect species used in the current study.

The contaminated flies in the present study started to die 4 days after the treatments, which confirms the results previously obtained in the current work, while the untreated adults of the opposite sex started to die 3 to 4 days (seventh day from the emergence) after mating with the contaminated flies. This result suggests that the fly to fly transmission of the fungus conidia did not occur in the first few days but during mating. Also, this confirms the ability of the contaminated adults to induce new infection in healthy flies, resulting in more mortality in the fly population.

In the current study, low mortality of untreated females (approximately 31%) and males (approximately 16%) mated with inoculated adults of the opposite sex was found. Low mortality ranges of the tsetse fly, *Glossina morsitans* were found by Kaaya and Okech (1990)

when males sprayed with a suspension of *M. anisopliae* or *B. bassiana* caused 13% and 20% mortality of untreated females respectively two weeks from combination treatments. Beris *et al.* (2013), also obtained low mortality with17% and 21% mortality of untreated female and male respectively resulted when the opposite sex of *C. capitata* treated with suspension of *M. anisopliae*-TMB04. In contrast, a higher range of mortality (85% to 100%) was induced in a study conducted by Toledo *et al.* (2007), who reported a high mortality (approximately 99%) of untreated females of *Anastrepha ludens* after being paired with males inoculated with two commercial products, based on *B. bassiana*. In another laboratory assessment, Quesada-Moraga *et al.* (2008) found males of *C. capitata,* inoculated with dry or wet conidia of *M. anisopliae* strain 01/58-Su caused 90% and 70% mortality to untreated females respectively. The authors also reported higher mortality of healthy females than males when they were paired with inoculated individuals of the opposite sex. In the present results, conidia transmission was greater when MET52-inoculated males coupled with untreated females, inducing higher mortality, than when inoculated females coupled with untreated males. The reason for this may be related to the body position of the tephritid adults during a mating contact (Quesada-Moraga *et al.*, 2008). The mortalities reported by Toledo *et al.* (2007) and Quesada-Moraga *et al.* (2008) were higher than in the present study. This might be because of the different experimental protocols and insect species used. For examples, treatment time, fungus strain, insect behaviour and inoculation methods are known to have different impacts on the effectiveness of transmission. Almeida *et al.* (2009) and Dimbi *et al.* (2013) indicated that behaviours such as aggregation of males and homosexual mating in some tephritid species may increase conidia transmission among a fly population.

MET52 infection showed an effect on *D. frontalis* female reproduction by reducing the number of pupae recovered from inoculated flies in both combination treatments, resulting in a reduction in pupae ranging from 58% to 89%. Also, in the sublethal effect experiment, the total effect of MET52 (1.5g and 0.125g/ 9.0 x 10^8 CFU g^{-1}) infection on *D. frontalis* was shown on the fly`s behaviour and reproduction 11 days from treatments. Pupal number reductions and a delay in oviposition period were detected in MET52 treatments. The results suggest that the fungal infection had an effect on mating behaviour causing a delay in female oviposition time and also had an effect on the fly progeny. A reduction in numbers of pupae as a measure of reproductive effect of entomopathogenic fungi on fruit flies has not been previously investigated. While, effect of fungal infection on fecundity and fertility of females of some fruit flies have been confirmed. In the current study, as larvae were fed on a natural diet (squash), because of the lack of an artificial diet for larval stage of the tested fly, it was

difficult to count the precise numbers of eggs deposited by females in all the treatments. Therefore, the direct effects of MET52 infection on fertility, fecundity and ovary development were not investigated. This should be taken in account when the current results are compared with other findings. In a study conducted by Castillo *et al.* (2000), fecundity reduction in a range of 40-65% in females of *C. capitata* inoculated with different species of entomopathogenic fungi was produced, Fewer eggs were produced by flies of *Bactrocera cucurbitae* infected with *M. anisopliae* compared to untreated individuals (Sookar and Allymamod, 2014). Various fecundity reductions ranged between 37%-82% of three species of fruit flies female inoculated with *M. anisopliae* were obtained when paired with an equal number of healthy males (Dimbi *et al.*, 2013). There is no published information on the effect of fungal infection on *D. frontalis* mating behaviour. Meadow *et al.* (2000), suggested that *B. bassiana* infection caused weakness to females of the Cabbage Root Fly, *Delia radicum* L. Interestingly, although the ratio of males to females was 1: 3.5 in treated male and untreated female combination, the number of pupae recovered per female was considerable lower than in untreated male and treated female combination with ratio of 3: 1 male to female. A possible explanation for this might be that the treated males might have less sexual ability than healthy males. Sexual competiveness assessments conducted by Novelo-Rincón *et al.* (2009) showed that mating frequency between a fungus- treated male and untreated female of *Anastrepha ludens* was considerably lower than when untreated males paired with untreated females. Hence, inducing new infections among the fly population and reducing the adult's reproductive output could increase the efficacy of MET52 by reducing the progeny. Development of an artificial diet for the fly will allow more accurate determination of fertility and fecundity by counting the eggs deposited by females. This may give more clarity related to the effect of fungus infection on the fly`s reproduction.

In the persistence experiment, the results confirmed the ability of MET52 granules to persist in soil. MET52 reduce emergence and caused mortality in newly emerging adults of *D. frontalis* even two months after a single application. Percentage mortality of the emerged flies increased with time after emergence and varied between the treatments. The shortest LT50 was found in the 42 days-old treatment. Ekesi *et al.* (2005), found using a different experimental procedure that *M. anisopliae* granules were effective in supressing adult emergence of three species of the *Ceratitis* genus ranging from 37% to 54% even nearly two years after treatment. Persistence of some species of the *Metarhizium* fungus and other fungal species has been investigated against other insect pests. In a potting media experiment, *M. anisopliae* (F52) was able to infect 50-60% of the black vine weevil larvae over two growing

season (Bruck and Donahue, 2007). Coombes *et al.* (2013), reported that *Thaumatotibia leucotreta* larvae were being infected with two isolates of *M. anisopliae sensu lato* and *B. bassiana sensu lato* six months after soil treatment.

In conclusion, the ability of *D. frontalis* adults emerged from MET52-inoculated soil to transfer conidia and induce new infection in untreated adults was demonstrated for the first time. The findings support potential entomopathogenic fungi as a valuable biological control candidate for the fly. Therefore, in future investigations, it might be useful to evaluate the potential for conidia transmission of MET52 by an autodissemination strategy which has previously proved useful for some fruit flies (Ekesi *et al.*, 2007; Toledo *et al.*, 2007; Quesada-Moraga *et al.*, 2008).Also, studying the possibility of transferring the fungus conidia through subsequent generations may give more insight into its potential effects. A further study could assess the long-term effects of entomopathogenic fungi on the fly population under Libyan environment conditions.

Chapter 6. Combined use of Met52® Granular biopesticide with two botanical products against *Dacus frontalis* (Becker)

Abstract

The combined use of MET52 biopesticide, based on a pathogenic fungus, *Metarhizium anisopliae*, var *anisopliae* strain F52, with two botanical products was investigated to achieve greater control against larval-pupal stages (measured by calculating adult emergence rate) and adults of *Dacus frontalis* under laboratory conditions. In an initial step, seven concentrations of neem oil and garlic extract (5% to 100%) were used separately to assess potential toxic effects on larvae in Petri dish experiments. The results showed that only neem had a dose-dependent effect on larval mortality, which reached 100% when it was used at high concentrations. In combinations experiment, insect third instar larvae were exposed to sublethal doses of neem and garlic applied alone or in combination one week after application of MET52 in soil. Percentage reduction in emergence rates and mortality post-emergence of *D. frontalis* adults varied among the treatments. Mortality in the larval-pupal stage when MET52 was combined with 2% neem was higher by 10% than when the fungus was applied alone. In the case of adults, combination effects ranged from antagonistic to additive effects on adult mortality. The combination of MET52 with a concentration of 2% neem showed an antagonistic effect on adult mortality, whereas the efficacy of the fungus increased significantly at a lower concentration of neem (0.25%), showing the shortest LT50. Overall, MET52 was an effective biological control agent against *D. frontalis* when used alone and when combined with low doses of neem oil providing a possible strategy for integrated fly management.

6.1 Introduction

There is an increasing use of botanical insecticides in integrated pest management strategies. Currently, neem is one of the commercial natural insecticides available in several formulations (Isman, 2006). Use of neem as an alternative to chemical pesticides has been evaluated previously for management of several pests (Isman, 2008; Akhtar and Isman, 2013). Neem is safer than synthetic pesticides to humans, beneficial insects and the environment, whilst acting by feeding deterrence, anti-feeding, anti-oviposition and growth regulation against exposed insects (Schmutterer, 1990; Khattak *et al.*, 2009) In a recent study conducted by Silva *et al.* (2011), neem seed cake has been shown to have toxic effects on the larval-pupal stage of *Ceratitis capitata* and there was an indication that neem delays pupal development.

The potential advantages of combining entomopathogenic fungi with botanical insecticides have been evaluated against some insect pests (Santi *et al.*, 2011; Radha *et al.*, 2014). Combinations of neem with fungal pathogens have been found to have various impacts on the fungal activity inducing additive, synergistic and antagonistic effects on control efficacy (Akbar *et al.*, 2005; Mohan *et al.*, 2007). Applying neem in combination with *B. bassiana* fungus has compatible effects against sweet potato whitefly, *Bemisia tabaci* (Islam *et al.*, 2011). In more a recent study, Halder *et al.* (2013) investigated the compatibility of neem oil with three different species of entomopathogenic fungi against five vegetable sucking pests with various responses.

In previous experiments in the present study, larvae of *D. frontalis* were not susceptible to entomopathogenic fungi. However, a considerable reduction in adult emergence was obtained when MET52, based on *M. anisopliae*, was applied early against larvae in soil, giving a low to moderate reduction in the emergence ranging from 20% to 55%. The results suggested that the full developed larvae were not exposed to conidia spores for sufficient time as they shortly moult to the pupal stage which might prevent the fungal infection. The early application of the fungus at different times (week to two months before larvae being released) also showed inconsistent results leading to poor control of newly emerging adults (Chapter 2 and 5). At present, few studies have shown neem to enhance the efficacy of entomopathogenic fungi, especially *M. anisopliae* against insect pests. To my knowledge, the effects of such botanical extracts in combination with entomopathogenic fungi have not been investigated against fruit flies, including *D. frontalis* yet.

The aims of this chapter were to investigate the toxic effects of neem oil and garlic extract on larvae and adults of *D. frontalis*, and to investigate potential interactions of sublethal doses of the botanicals with MET52 against the fly.

6.2 Materials and Methods

6.2.1 *Botanicals resource and preparation*

Neem oil and garlic extract were obtained from Neem Biotech Ltd, UK. Seven concentrations (5%, 10%, 20%, 40%, 60%, 80% and 100%) of each product were used. To obtain these concentrations, 0.25, 0.5, 1, 2, 3, 4 and 5ml of each product were diluted with water to make 5 ml. The dilutions were mixed on a shaker for 60 seconds.

6.2.2 *Botanical extracts experiment*

Twenty, third instar larvae of *D. frontalis* were immersed in 10ml tubes containing 2ml of the concentrations of neem or garlic for 15 to 20 seconds. Sterile distilled water was used as untreated control. Then, the treated larvae were placed gently in Petri dishes (10 cm × 1.5 cm) lined with two sterilised filter papers. The Petri dishes were kept at 25 ºC. Four replicates per each treatment were arranged. One week later, the samples were examined under a microscope and larval mortality was assessed.

6.2.3 *Combined application experiment*

In this test, possible compatibility and toxic effects of four sublethal doses of neem and garlic alone and in combinations with MET 52 on larvae, pupae (measured by assessing adult emergence rate) and post-emergence mortality of *D. frontalis* adults were evaluated using soil application. Following the similar experimental procedure described in Section 2.2.3 (Chapter two), 30 g of sterilised soil was inoculated was with 1.5 g of MET52 (9.0×10^8 CFU g^{-1}) in plastic cups. The fungus was mixed with soil and applied one week prior to third instar larvae being released into the cups. Eighteen treatments were prepared as follows: 1) nine single treatments, including MET52, and four concentrations (2%, 1%, 0.5% and 0.25%) of each of neem and garlic; 2) eight combined treatments of MET52 with all the above concentrations of neem and garlic separately and 3) soil treated with sterilised distilled water as control. To obtain these concentrations, 0.4, 0.2, 0.1 and 0.05 ml of the botanicals were diluted in 20 ml sterilised distilled water. The dilutions were shaken as described above. All the cups received 3.6 ml of sterilised distilled water to enable pupation, and then 2 ml of each treatment solution were added to the cups. Twenty larvae of *D. frontalis* were then released onto the soil surface. The treatment cups were covered with inverted cups of the same size following the same procedures described in Chapter 2. Soil moisture was maintained at 50% to 60% until adult emergence by adding sterilised distilled water as necessary by weight. The treatments were kept at 25 ºC, 75%- 80% RH and 14: 10 L: D. Five replicates for each treatment were arranged. Adult emergence was assessed eleven to fourteen days after application to determine larval and pupal mortality. Then, 15 adults (one day-old) were transferred and kept in adult cages to assess mortality following the same process and conditions previously described. Four replicates for each treatment were arranged. Dead flies were collected daily from the cages over the period of two weeks. Fungal infection in the adults was investigated by following the same process previously described in Section 2.2.3 (Chapter two).

6.3 *Statistical analysis*

Percentages of dead larvae, emergence rate and post-emergence mortality of adults were transformed by arcsine and analysed by Anova test. Then, mean differences among the treatments were compared by Tukey`s test ($P < 0.05$). If data were not normally distributed nonparametric analysis was performed by Scheirer-Ray-Hare test. Then, Mann-Whitney was used to compare the differences between the treatments. Combination effects of MET52 with the botanical products tested was determined by χ^2 test (Morales-Rodriguez and Peck, 2009). The expected mortality of the interactions was calculated by the formula $ME = M_M + M_B (1-M_B/100)$ (Morales-Rodriguez and Peck, 2009). M_M and M_B are the percentages of the observed mortality induced by the MET52 and botanicals alone, respectively. Results values obtained from the χ^2 test were compared to the χ^2 table at 1 degree of freedom (df), using the formula $\chi^2 = (M_{MB}-M_E)^2/M_E$, where M_{MB} is the observed mortality for the MET52-botanical product combination. The effect between the two agents is considered to be synergistic or antagonistic if the χ^2 value exceeded the χ^2 table value. A positive value of $M_{MB}-M_E$ means synergistic interaction while the negative value is considered as antagonistic. Additive effect between the fungus-botanicals was suspected when the χ^2 value did not exceed the χ^2 table value (3.841). Probit analysis was used to calculate LT50 and LT90. All the statistical analysis was performed in Minitab 16 Statistical Software.

6.4 Results

6.4.1 *Botanical extracts experiment*

The results showed that larvae of *D. frontalis* were affected when treated with neem compared to no effects in other treatments. The neem treated larvae struggled to move normally, turned firstly yellowish and then brownish to blackish, then died within 10 to 15 minutes of treatment (before pupating) (Figure 6.1). In the other treatments, the larvae pupated normally. As can be seen in Table 6.1, applying neem at concentrations of 20% to 100% gave significant mortality of the larvae compared with other treatments ($\chi^2 = 1$; df = 2; $P < 0.005$ (Scheirer-Ray-Hare test)), with significant differences found between the neem treatments. The greatest larval mortality (100%) was observed in concentrations of 60% to 100% of neem, whereas lower concentrations of neem (5% and 10%) had no significant effect on the larvae. In the garlic treatment and untreated control, no mortality of larvae was found.

Figure 6.1 Dead larvae of *Dacus frontalis* treated with neem.

Table 6.1 Medians mortality of *Dacus frontalis* larvae treated with different concentrations of neem and garlic. n = 4.

Treatment	Larvae mortality (median)
Neem 100%	100a
Neem 80%	100a
Neem 60%	100a
Neem 40%	79b
Neem 20%	42b
Neem 10%	3.8c
Neem 5%	0.0c
Garlic 100%	0.0c
Garlic 80%	0.0c
Garlic 60%	0.0c
Garlic 40%	0.0c
Garlic 20%	0.0c
Garlic 10%	0.0c
Garlic 5%	0.0c
Control	0.0c

Medians that do not share a letter are significantly different (Mann-Whitney test).

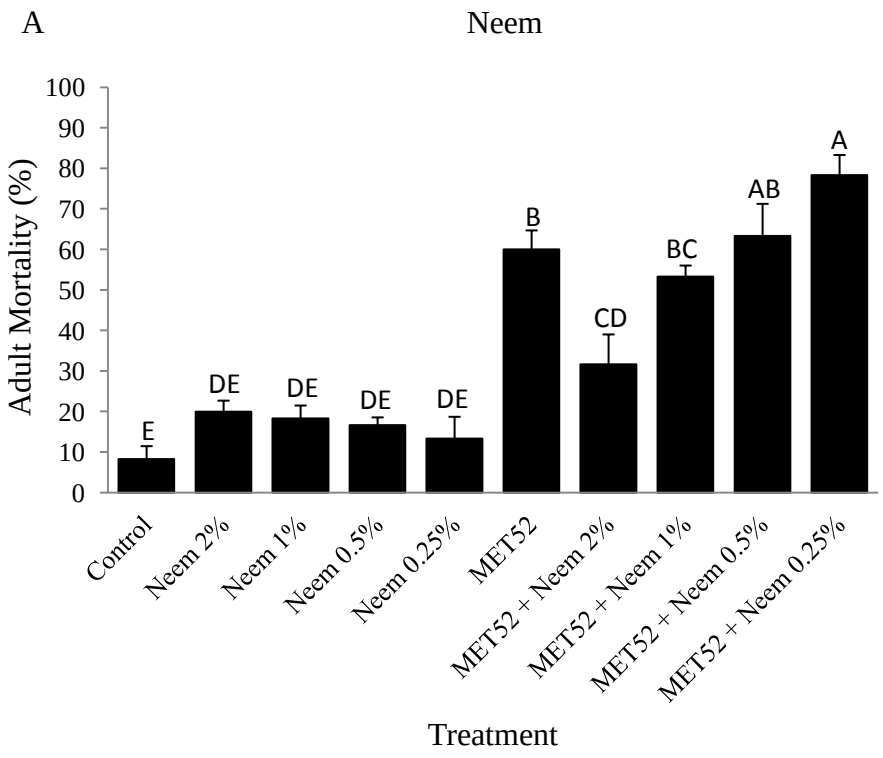
6.4.2 *Combined application experiment*

Larvae burrowed into the soil in all the treatments. Adults emerged from the treatments 11 days after the application. Percentage reduction in emergence rates and post-emergence mortality of *D. frontalis* adults were affected by the treatments (Table 6.2 and Figure 6.2). The results showed that treatments based on fungus alone or in combination with either of the botanical products had significant effects on the emergence rates compared to the control (χ^2 = 1; df = 5; $P < 0.005$ (Scheirer-Ray-Hare test)). There were no significant differences between the fungal treatments (Table 6.2). The lowest emergence rate (57%) was found when MET52 was combined with the highest concentration tested of neem (2%). High adult emergences ranging from 89% to 94% were found in the single neem and garlic treatments (Table 6.2). Only additive effects on larvae and pupae were observed in all the combination treatments (Table 6.3). In the case of adults, the mortality in all the fungal treatments was significantly higher than other treatments (F = 81.20; df = 5; $P < 0.001$), with significant differences found between the treatments (Figure 6.2). In the combination treatments, the highest mortality occurred when MET52 applied with 0.25% of neem and 0.5% of garlic separately giving approximately 78% and 72% respectively compared with approximately 60% and 8% in the single fungus treatment and control, respectively. Applying neem at the highest tested concentration (2%) had a significant effect on the MET52 efficacy reducing the mortality to approximately 32%, whereas when neem was combined at 0.25% the efficacy increased causing a significant mortality (Figure 6.2.A). As can be seen in Table 6.4, antagonistic and additive effects on mortality post-emergence were observed in the combinations treatments. For neem combinations, one antagonistic effect was detected when neem was combined at 2% and additive effects for the other lower concentrations. For garlic combinations, only additive effects on adult mortality were observed. In the single neem and garlic treatments, the adult mortality was low ranging from approximately 5% to 20%, which did not differ significantly from the control (Figure 6.2). Percentage mortality of flies emerging from the combination treatments increased with time (Figure 6.3). The results of lethal time to kill 50% (LT50) and 90% (LT90) of flies inoculated with MET52 alone or in combination treatments with neem and garlic are given in Table 6.5. LT50 in the combination treatments ranged from approximately 8 days to 16 days for neem and 10 to 11 for garlic compared to MET52 treatment alone with fewer than 11 days. The shortest LT50 and LT90 were found when the fungus was combined with the lowest concentration tested of neem (0.25%). The longest LT50 and LT90 at approximately 16 and 27 days respectively were observed in adults emerging from soil inoculated with the fungus when combined with 2% of neem.

Table 6.2 Median adult emergence of *Dacus frontalis* larvae treated by MET52 alone and in combination with different concentrations of neem and garlic separately. n = 5.

Treatment	Emergence rate (median)
Control	93a
Neem 2%	89a
Neem 1%	90a
Neem 0.5%	94a
Neem 0.25%	92a
Garlic 2%	95a
Garlic 1%	91a
Garlic 0.5%	93a
Garlic 0.25%	93a
MET52	67b
MET52 + Neem 2%	57b
MET52 + Neem 1%	63b
MET52 + Neem 0.5%	70b
MET52 + Neem 0.25%	69b
MET52 + Garlic 2%	71b
MET52 + Garlic 1%	67b
MET52 + Garlic 0.5%	70b
MET52 + Garlic 0.25%	65b

Medians that do not share a letter are significantly different (Mann-Whitney test).



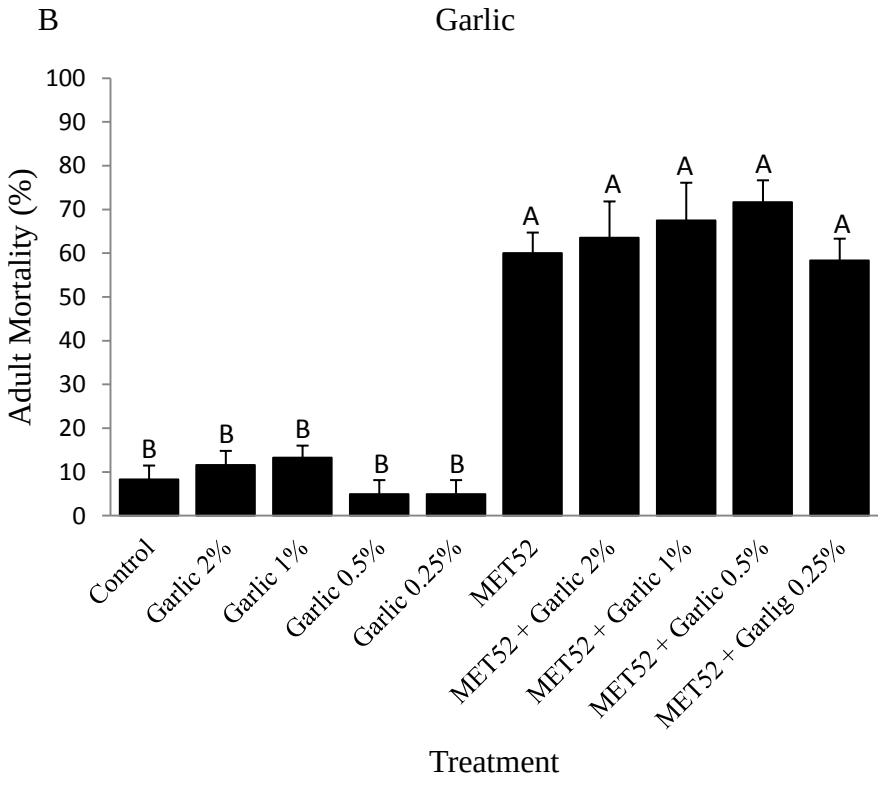


Figure 6.2 Mean (% ±SE) percentage mortality two weeks from emergence, n = 4 of *Dacus frontalis* adults after larvae were treated with MET52 alone and in combination with different concentrations of neem (A) and garlic (B). Means that do not share a letter are significantly different (Anova test).

Table 6.3 Combination effects between entomopathogenic fungus (MET52) with different concentrations of botanicals (neem and garlic) for control of *Dacus frontalis* larvae and pupae.

Botanical product	Mortality (%)		χ^2	Combination effect
	Observed [a]	Expected [b]		
Neem (2%)	43	40.37	0.17[c]	Additive
Neem (1%)	37	39.7	0.18	Additive
Neem (0.5%)	30	37.02	1.33	Additive
Neem (0.25%)	31	38.36	1.41	Additive
Garlic (2%)	29	36.35	1.49	Additive
Garlic (1%)	33	39.03	0.93	Additive
Garlic (0.5%)	30	37.69	1.57	Additive
Garlic (0.25%)	35	39.51	0.51	Additive

[a] Observed larval and pupal mortality caused by MET52 combined with one of the botanicals tested. The mortality is measured by assessing adult emergence rate 11 to 14 days after the treatment.
[b] Expected mortality = sum of efficacy of MET52 and one of the botanicals applied separately.
[c] Combination effect was determine by Chi Square analysis (χ^2). Additive effect was detected in treatments of which χ^2 value did not exceed the table χ^2 (3.841) at df = 1 and $P = 0.05$, and the mortality in combined treatment is greater than the MET52, neem or garlic treatments alone.

Table 6.4 Combination effects between entomopathogenic fungus (MET52) with different concentrations of botanicals (neem and garlic) for control of *Dacus frontalis* adult.

Botanical product	Mortality (%)		χ^2	Combination effect
	Observed [a]	Expected [b]		
Neem (2%)	31.66	67.98	19.4[c]	Antagonistic
Neem (1%)	53.33	67.31	2.90	Additive
Neem (0.5%)	63.33	66.64	0.16	Additive
Neem (0.25%)	78.32	65.30	2.59	Additive
Garlic (2%)	63.50	64.63	0.02	Additive
Garlic (1%)	67.50	65.30	0.07	Additive
Garlic (0.5%)	71.66	61.96	1.52	Additive
Garlic (0.25%)	58.32	61.96	0.21	Additive

[a] Observed adult mortality caused by MET52 in combinations with one of the botanicals.
[b] Expected mortality = sum of efficacy of MET52 and one of the botanicals tested.
[c] Combination effect was determine by χ^2 test. χ^2 exceeds the table χ^2 (3.841), with df = 1 and $P = 0.05$, this means synergic or antagonistic effect, otherwise additive effect was determined.

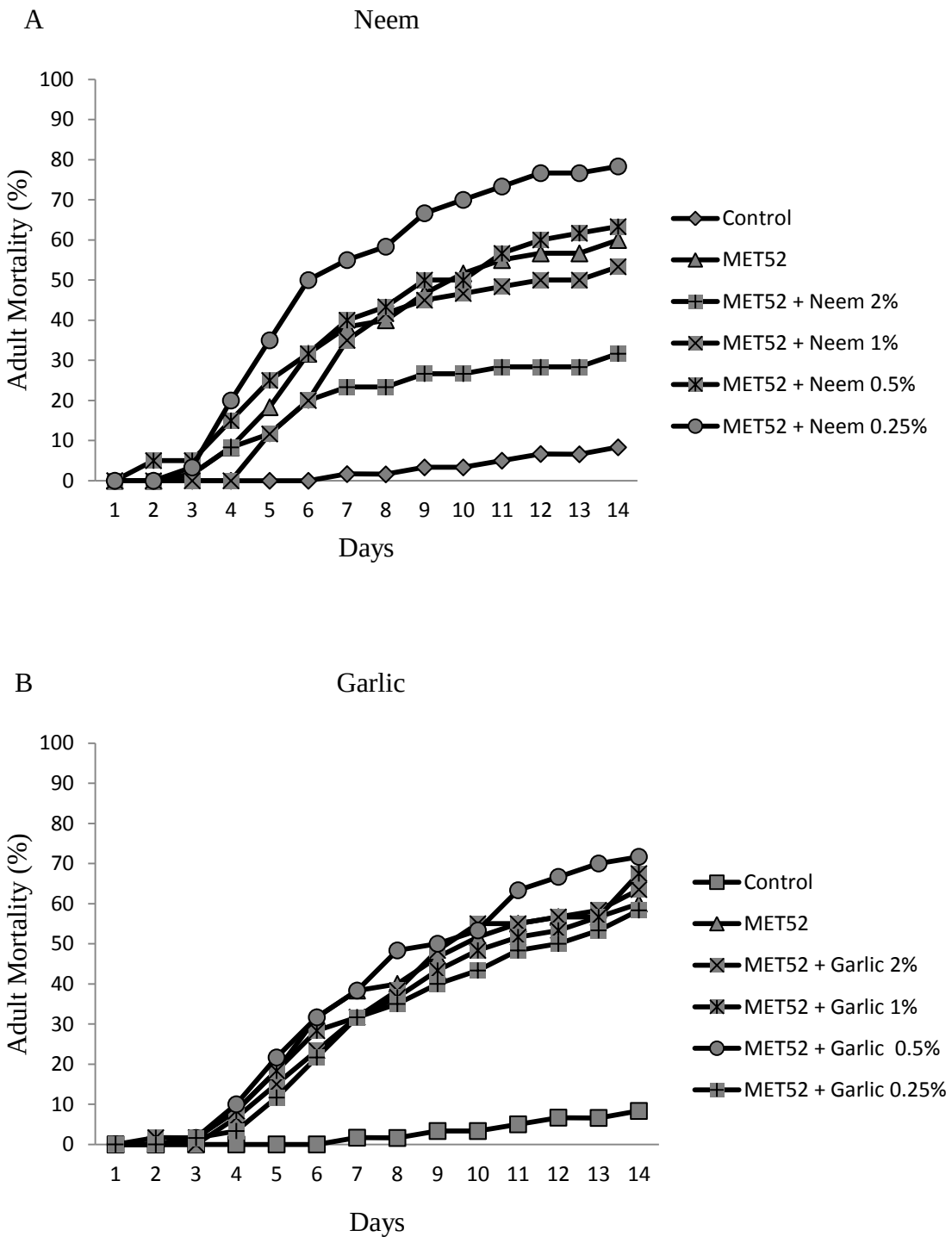


Figure 6.3 Time-mortality response of *Dacus frontalis* adults emerging from soil treated with MET52 alone or in combination with various concentrations of neem oil (A) and garlic (B) separately over the period of two weeks emergence.

Table 6.5 Mean lethal time (LT50 and LT90) in days for *Dacus frontalis* adults emerging from soil treated with MET52 alone or in combination with various concentrations of neem oil or garlic n = 4.

Treatment	MET52	Neem				Garlic			
		2%	1%	0.5%	0.25%	2%	1%	0.5%	0.25%
LT 50	10.64	16.14	11.44	10.17	8.02	10.60	10.83	9.62	11.49
LT 90	17.89	27.34	18.42	17.96	14.13	17.0	17.92	15.84	18.45

The values were calculated by Probit analysis.

6.5 Discussion

To date, there is no other research that has evaluated the effects of botanicals alone or in combination with entomopathogenic fungi on *D. frontalis*. This is the first report confirming that neem oil is toxic to *D. frontalis* larvae and increases mortality in adults contaminated with entomopathogenic fungi.

In the present study, effects of neem oil and garlic extract were firstly determined on larvae. The results showed that neem oil, but not garlic prevented development of larvae into the pupal stage. The larval mortality increased up to 100% with increasing neem oil concentration. While the garlic extract applied even at high rates had no toxic effect on larvae, pupae and adult of *D. frontalis.* Toxic effects of different formulations of neem have been previously reported against immature and mature stages of several important insect pests (Renden *et al.*, 1998; Khan *et al.*, 2007; Silva *et al.*, 2011; Alvarenga *et al.*, 2012; Tomé *et al.*, 2013). For examples, Renden *et al.* (1998), found that emergence rate of the western cherry fruit fly, *Rhagoletis cerasi* was reduced when larvae were exposed to a neem based insecticide added to artificial diet. Also, high mortality was reported when two commercial neem products (Neemazal-T/S® and Greeneem® oil) were tested against young and older instars of the cedar leaf moth, *Acleris undulana* under laboratory and field conditions (Erler *et al.*, 2010). In contrast, Khattak *et al.* (2009), in a different experiment procedure, found that emergence rate of *B. cucurbitae* was not affected when melon fruit was treated with either with neem oil or neem seed extract before the fruits were infested by the fly. Also, a low mortality was found when adults of the South American fruit fly, *Anastrepha fraterculus* were exposed to different concentrations of commercial neem products (Efrom *et al.*, 2011). The mechanism of toxicity of neem was not investigated in the present study. Neem has been reported to contain hormone analogues (ecdysteroids) which have effects on development in

immature insects (Isman, 2006). Very few studies have investigated the role of garlic for insect control. In a study conducted by Bahar *et al*., (2007), garlic caused low aphid mortality under laboratory and field conditions. The author described the effect as unexpected indicating that garlic extract has strong pungent odour. Repellent and oviposition deterrent effects of garlic-based products have been also reported against numerous insects (Sarwar, 2012; Thakur and Gupta, 2013; Bharadwaj *et al*., 2015). In contrast, Prowse *et al*., (2006) reported that garlic juice was toxic to adults of dipteran insects, *Delia radicum* and *Musca domestica*. Different target species and different application methods might be the reasons for these different findings. Hence, it seems that garlic effectiveness against insects depends on the manufacture`s product sources and methodology. Therefore, further studies with different strategies of applying several garlic products against the fly are needed.

Evaluations of the effects of the combined use of insect fungal pathogens with botanical extracts on fruit flies are none. The potential use of neem oil with MET52 against *D. frontalis* larvae and adults was confirmed in the current study for the first time. Tukey test and Morales Rodriguez method were used in the combined application experiment. The both approaches were used to evaluate effects of single and combinations of treatments, however, determination of the type of interactions and strength of synergistic effects can be analysed by Morales Rodriguez method but not by using Tukey test by which the difference among treatments can be compared and whether the differences are significant or none. Combination of neem oil and garlic extracts with *M. anisopliae* produced additive effects against larvae, pupae and adult stages. The emergence rate of the fly was reduced by 10% by combining fungus with neem oil (2%) compared to the treatment with the fungus alone. Combining neem at a low concentration (0.25%) with MET52, significantly increased mortality in adults by 18% compared to MET52 treatment alone, decreasing the time to mortality compared to those obtained from the single treatments of both agents. This result may be explained by the fact that neem decreases feeding behavior of adults (Schmutterer, 1990; Su, 1999). Another possible explanation for these results is that neem oil is known to assist adhesion and germination of fungal spores on insect cuticle (Prior *et al.*, 1988), consequently, causing weakness and increasing the insect's susceptibility to the fungal infection.

Very few studies have investigated the effect of neem oil on the *Metarhizium* genus and never against tephritid flies. In a recent study conducted by Halder *et al.* (2013), combinations of neem oil with three different species of insect pathogenic fungi against some species of vegetable sucking pests showed varying results inducing a lower LT50 than the fungal treatments applied alone. Shah *et al*., 2008, reported that neem seed cake increased the overall

control of the black wine weevil by inducing 95% larvae mortality when used in combination with *M. anisoplae* strain V275 in pots experiment. *Metarhizium anisoplae* var *anisoplae* strain F52, used in the current study, has been previously shown to cause various interaction effects on the white grubs *Amphimallon majale* and *Popillia japonica* larvae when applied in combination with two neonicotinoid insecticides (Morales-Rodriguez and Peck, 2009). In the current study, the mortality of the fly was increased by 10% by combining fungus with garlic (0.5%) compared to the treatment with the fungus alone. This result may be explained because the garlic extract tested was not oily like the neem oil tested so it did not enhance the germination of MET52 conidia, facilitate adhesion of conidia on the fly cuticle nor has pest growth regulating action.

In the present study, an antagonistic effect on adult mortality was only observed in a combined treatment of neem at 2% with MET52. In this treatment, the visible green vegetative growth of the fungus on the soil surface was obviously lower or non-existent compared to other combined treatments. This maybe because the neem oil had a negative effect on spore germination and reduced or inhibited the fungus, resulting in a reduction of 47% of emerging adult mortality compared to the lower concentrations applied in the other treatments (Figure 6.2.A). There are a few studies that have shown inhibitory effects of botanicals, including neem oil, on growth and pathogenicity of entomopathogenic fungi (Hirose *et al.*, 2001; Akbar *et al.*, 2005; Mohanty *et al.*, 2008; Asi *et al.*, 2010; Sahayaraj *et al.*, 2013). For examples, spore number and germination of *M. anisoplae* were significantly reduced when the fungus was combined with neem oil in an *in vitro* experiment (Hirose *et al.*, 2001). In another *in vitro* assessment, Haroon *et al.* (2011) found that growth and the number of spores of *M. acridum* declined with increasing concentration of neem oil indicating that a neem concentration of 2% and higher is toxic to the fungus in a culture medium, whereas at lower concentrations neem oil was compatible with the fungus. The authors in the same study also reported the toxicity of neem oil alone on nymphs of the tree locust, *Anacridium melanorhodon* causing 23% mortality under field conditions. The results of the current study also showed a low toxic effect of neem oil on adults that emerged from the neem control (2%). This effect could be also a result of the feeding deterrence of neem on adults which caused a death to the adults (Su, 1999). There was no evidence of any synergistic effects in the current experiment. The explanations of this result might be related to the fixed concentration of the fungus, the application method and time on both agents used. The results obtained in the current study indicate that neem oil had varied toxic effects on larvae and adults of *D. frontalis* and enhanced MET52 efficiency. The sublethal effects (such as on egg

oviposition, fecundity and fertility) of different neem products have been also been reported from several species of fruit fly (Chen *et al.*, 1996; Singh, 2003; Khan *et al.*, 2007; Silva *et al.*, 2015) which encourages using such botanicals as potential control agents to suppress the progeny of *D. frontalis* in integrated fly management programmes.

In conclusion, the findings of this study suggest that neem can offer considerable reductions in *D. frontalis* populations when used in combination with fungi for integrated fly management strategies. Despite these promising results, further investigations should be done to evaluate the effect of combining applications of neem with MET52 on the persistence of the fungus. To fully understand the role of neem oil in enhancing the fungus efficacy further combinations of neem with sublethal concentrations of the fungus need to be investigated. This may provide positive synergies and reduce the costs of control.

Chapter 7. Evaluation of Met52® Granular biopesticide for control of the Greater melon fly *Dacus frontalis* (Becker) under semi field conditions

Abstract

Field trials with soil application of Met52® Granular biopesticide (MET52) were conducted to control the soil stages and adults of the Greater melon fly *Dacus frontalis*. In a first step, attractiveness of three traps and food bait was evaluated under field condition. Yellow traps performed much better than McPhail and Delta traps. Baited McPhail traps with yeast hydrolysate enzymatic had the greatest number of captured *D. frontalis* over one week. In a second step, pathogenicity of MET52 against larval-pupal stage (measured by calculating adult emergence rate) and mortality post-emergence of *D. frontalis* adult was investigated in field application trials. The results showed that percentage reduction in emergence rate and mortality post-emergence of adults varied among the treatments. Mortalities in the larval-pupal stage and the adults that emerged in insecticide treatments were higher than from the fungal treatment. In conclusion, soil application of MET52 can be an effective control of *D. frontalis*. The results provided evidence of the efficiency of trapping as a potential strategy which could be used in integrated fly management.

7. 1 Introduction

Entomopathogenic fungi have been evaluated with different application methods, formulation and strategies, giving effective control of several insect pests not only under controlled condition, but also under field and greenhouse conditions (Hajek *et al.*, 2006; Shah *et al.*, 2007; Ansari *et al.*, 2010; Haukeland and Lola‐Luz, 2010; Mauchline *et al.*, 2013).

Granule forms of some species and strains of *Metarhizium* fungus have been used as potential biological control agents under field conditions against several insect pests. For example, MET52 based on *M. anisopliae var anisopliae* strain F52 has been evaluated against black vine weevil larvae, *Otiorhynchus sulcatus* inducing high mortality (Moorhouse *et al.*, 1993; Bruck and Donahue, 2007; Ansari and Butt, 2013). F52 has also been assessed against Tomato potato psyllid, *Bactericera cockerelli* (Mauchline *et al.*, 2013) and the large pine weevil, *Hylobius abietis* (Williams *et al.*, 2013), causing 100% mortality for both species. Another species, *M. brunneum* provided 84% to 98% mortality of chilli thrips *Scitothrips dorsalis* when applied as granules (Arthurs *et al.*, 2013). The same strain was investigated in the field with foliar application causing high mortality to first and second instar larvae of pear psylla, *Cacopsylla pyri* L. (Erler *et al.*, 2014). Also, foliar application of another commercial

product (Naturalis L), based on *Beauveria bassiana* ATCC74040, was shown to be effective in causing fungal infection of tephritid adults under field conditions (Daniel and Wyss, 2010). The authors found that the number of cherry fruits infested with the European cherry fruit fly, *Rhagoletis cerasi* was significantly reduced by 65% following treatment.

In previous laboratory experiments the pathogenicity of various insect pathogenic fungi to different life stages of *D. frontalis* was investigated in sandy clay loam soil (Chapter 2, 3, 4, 5 and 6). The results revealed that emerging adult flies were more susceptible to fungal infection than the soil stages. The experiments demonstrated the efficacy of the commercial product Met52® Granular biopesticide (MET52), which is based on *Metarhizium anisoplae* var *anisoplae* strain F52, against the fly in early soil application causing considerable reduction in adult emergence, inducing a high adult mortality. The fungus was more effective when applied as a granule to soil than as a direct spray against adults. Also, conditions of 25 ºC, with 75%-85% humidity and 70% moisture were optimal for MET52 to induce high mortality rates in the flies. However, conditions in the field such as temperature, relative humidity and soil moisture content are usually inconsistent and different from those in laboratory. This could influence the efficacy of the pathogen applied. Therefore, evaluation of the efficacy of field application of entomopathogenic fungi was necessary.

Ekesi *et al.* (2007), indicated that field studies on the efficacy of insect fungal pathogens against fruit flies are limited although their important role has been demonstrated in integrated fly management. Currently, there is no published field work investigating entomopathogenic fungi against *D. frontalis*. The aim of these experiments was to evaluate efficacy of MET52 in reducing emergence rate and causing mortality post-emergence of *D. frontalis* adult under field condition. As there is no specific study published on evaluation of bait traps to attract *D. frontalis* adults, several traps baited with yeast hydrolysate enzymatic (YHE) were evaluated in attracting and capturing *D. frontalis* adults before starting the main experiments.

7.2 Material and methods

7.2.1 *Experiments site*

Field cage experiments were carried out in Almrazeek area in Ben Ghasheer Town 35 km from Tripoli, Libya. The town is a famous agricultural area for growing some species of Cucurbitaceae family. Squash species are usually planted throughout the year either in open field or under plastic tunnels in the winter. The town is one of the most important areas

supplying several vegetable crops to the local market in Tripoli. Soil in this area is generally characterised as sandy clay.

7.2.2 *Infested fruits collection and insect culture*

Infested squash fruits with *D. frontalis* were collected from a heavily infested farm located in Almrazeek area in early August 2013 (Figure 7.1.A, B). The fruits were immediately taken to a biocontrol pest laboratory in the Biotechnology Research Centre and placed on plastic trays 50 cm × 20 cm filled with soil collected from the same site for pupation. The trays were kept at 27 ºC to 28 ºC, 40% to 50% Relative Humidity (RH) and 12:12 hour Light: Dark (L: D). One week to ten days later, pupae were collected by sieving the soil and placed into a transparent plastic cage 25 cm × 15 cm × 15 cm (Almahari shop), covered with gauze at one side (Figure 7.1.C). The cage contained water and artificial diet consisted of 1: 3 ratio of yeast hydrolysate enzymatic (MP Biomedicals, France) and sucrose. The fly culture was kept in the above laboratory conditions. After seven days, whole fresh squashes were introduced into the cage to receive the eggs. The fruits were replaced daily and placed on the plastic trays as above where the full-grown larvae could pupate. Larvae were fed on fresh squashes in plastic containers (35 cm × 25 cm × 15 cm) filled with soil where the full-grown larvae could pupate.

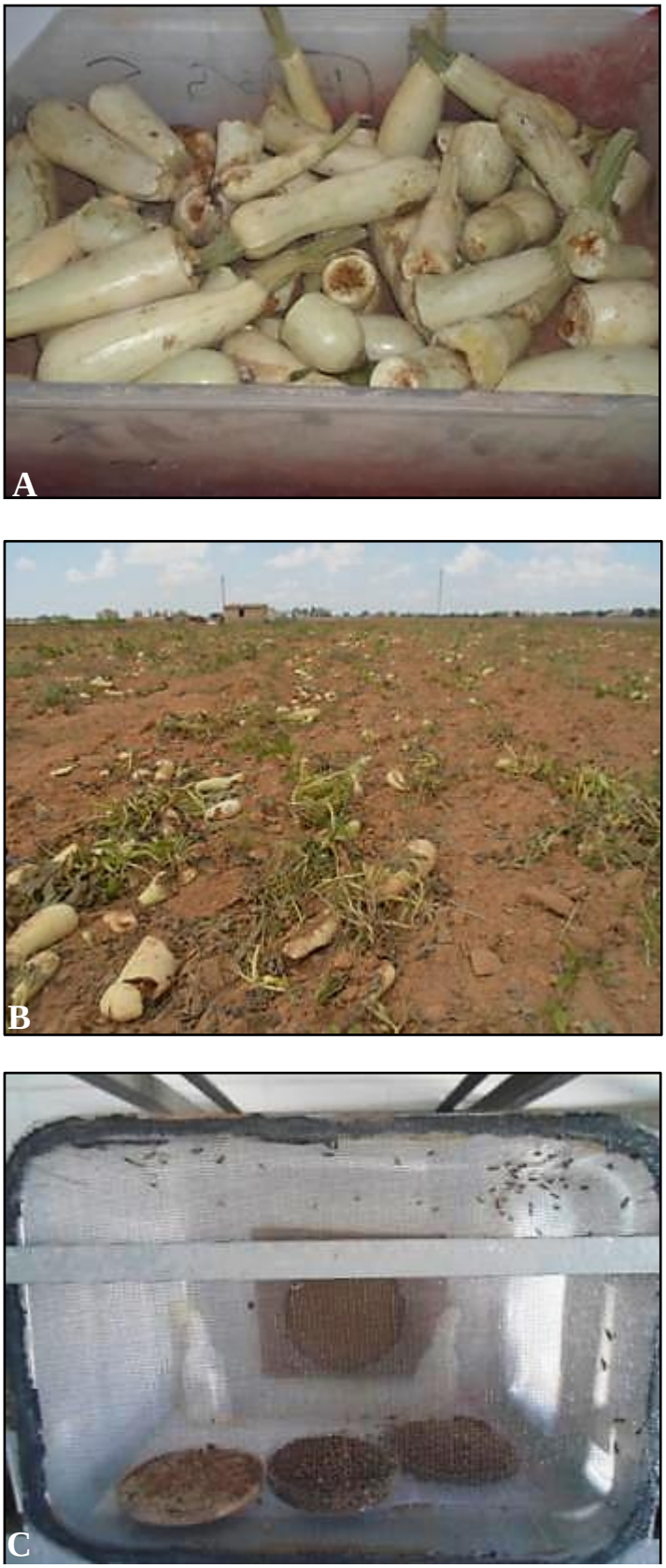


Figure 7.1 Infested squash fruits with *Dacus frontalis* (A) a heavily infested squash field located in Almrazeek area in Ben Ghasheer, Libya (B) and a lab culture of *D. frontalis* (C).

7.2.3 *Trapping and bait tests*

The aim of this test was to evaluate the efficacy of insect traps and food bait in attracting and capturing *D. frontalis* adults. The results of these tests were used later to assess the effect of MET52 on emergence rate and survival of emerging adults under field conditions.

Two tests were conducted in a (approximately) 2000 m^2 field planted with a two months-old squash crop. Some infested squash fruits were observed left on the sides of the field (Figure 7. 2). Other vegetable crops such as corn, pepper, cabbage and olive trees were growing around the cucurbit field. The experiments were done in September 2013. For the trap test, three commercial traps; McPhail filled with 250 ml water (UK), Delta traps (Cooper Company, UK) and yellow sticky traps (Russell IPM, UK) were used. For trap and bait experiment, McPhail and Delta traps were used. The treatments consisted of: 1) McPhail traps baited with 50 g YHE, 2) Delta traps baited with 50 g YHE, 3) McPhail traps filled with 250 ml water and delta traps without food were used as controls. Five replicates per treatment were used organised in a randomised block design with a distance of 30 m between the traps. The traps were hung on wooden supports 90 cm high near the plants. The trap experiment ran for four days after which time the treatments were inspected. The trap/bait experiment lasted a week and the treatments were inspected every 48 hours. The traps were supplied with the bait and refilled with water or completely renewed when necessary. All the captured insects were removed from the traps, examined and recorded.

7.2.4 *Field and cage preparation*

The experiments were conducted in a field of approximately 200 m × 30 m. Traditional agricultural procedures such as removing previous crop residues and soil tillage were applied before planting. The soil was fertilized by Super phosphate and Urea. Squash seeds (Syngenta Seeds B.V, Enkhuizen, Netherlands) were planted in lines in early August 2013 and irrigated when needed by a traditional spray system over the period of the experiment. Wooden cages (100 cm high × 70 cm diameter) were set up and completely covered by a wire mesh with a mesh size of 1.2 mm (Figure 7.3). The cages were sunken 10 cm into the soil and contained a single plant. A small opening was made in one side of the cage for applying the treatments, collecting the samples and for recording the results during the experiment period.

Figure 7.2 Infested squash fruits left on the sides of the experimental field located in Almrazeek area in Ben Ghasheer Town.

Figure 7.3 Experimental cages in a field located in Almrazeek area in Ben Ghasheer Town.

7.2.5 *Pre-emergence treatment*

The treatments were arranged in randomised complete block design with four replicates per each. The treatments consisted of: (1) Untreated control; (2) MET52 granules applied at 5 g / 10 cm^2 and mixed with soil; (3) Delfos 5G (chlorpyrifos ethyl 5%) (Spain) applied at 1 g / 10 cm^2 and mixed with soil. The treatments were applied 40 days after planting the squash seeds. A large number of third instar larvae of *D. frontalis* were carefully collected from the culture in small tubes and taken immediately to the experiment site. Twenty larvae were released into

each treatment. The larvae which failed to burrow into the soil were replaced. The cages were irrigated regularly (every two to three days). A McPhail trap baited with mixture of 50 g YHE and 250 ml water and also a yellow trap were hung at the upper middle side of each cage to attract and capture the emerging flies (Figure 7.4). The traps were introduced into the cages eleven days from the application. Three days after observing adult emergence, the traps were taken out the cages. The flies captured were collected into small plastic tubes and examined in the laboratory. The captured flies were counted to assess the effect of the treatments on the fly emergence rate.

Figure 7.4 McPhail traps baited and yellow traps were hung into the experimental cages.

7. 2.6 *Post-emergence treatment*

The same treatments in the previous experiment were used, but replacing Delfos as the chemical pesticide treatment with Dursban 48% (chlorpyrifos) (Riva Ltd, France). Whereas Delfos was applied as a pre-emergence treatment, Dursban was applied post-emergence. The treatments were organised in randomised block design. Seven replicates were arranged. Twenty larvae were released into each treatment. A week after adult emergence, Dursban was sprayed for fixed period at the recommended dose (100 ml/ 100 l water) against adults in the pesticide treatment. However, the flies in all cages had disappeared after Dursban application a day later. This is probably because of a technical error (the insecticide was sprayed against adults in the pesticide treatment without protecting the control and fungal cages from the insecticide effect). Therefore, this experiment was repeated following above procedure with some modifications. The trial was conducted in another field of approximately 60 m × 20 m located in Ben Ashour area in Tripoli. The land used to be a farm for growing some

vegetables. Trees such as orange, pomegranate, lemon and several ornamental plants were growing around the experimental site. Wooden cages (100 cm high × 70 cm diameter) were set up without fertilization or planting. The treatments were applied in early November 2013. Here, larvae were not available so twenty 10 days-old pupae of *D. frontalis* were placed into the treated soil at approximately 10 cm depth. The soil was moistened by adding 3 l of tap water to each. The cages were supplied with artificial diet placed on the top of the cages. Two days later, emerging adults were observed in the cages. A week after the emergence all the cages, with exception of insecticide cages, were covered by plastic bags for few minutes to avoid being affected by insecticide application. Then, Dursban was sprayed for a fixed period at the recommended dose (100 ml/100 l water) against adults in the pesticide treatment. McPhail and yellow traps were placed into the cages three days after the Dursban application. Three days later, the traps were removed. The captured flies were collected from the traps and counted to assess the adult mortality.

7. 3 Statistical analyses

No statistical analysis was performed for the trap test because of the small sample size obtained. Performance evaluation of the insect traps and food bait in attracting and capturing *D. frontalis* adults was measured by calculating the number of the flies collected from traps before being analysed by an appropriate Anova test. Percentages of larval-pupal stage mortality and mortality post-emergence (measured by calculating captured adult flies) were transformed by arcsine before an Anova test was performed. Mean differences among the treatments were compared by Tukey`s test ($P < 0.05$). Percentage mortality of adults which emerged experiments was analysed before and after being corrected by Abbott`s formula (Abbott, 1925). All the statistical analysis was performed in Minitab 16 Statistical Software.

7.4 Results

7.4.1 *Trapping and bait tests*

For the trap test, total captured flies are presented in Table 7.1. Yellow traps captured more *D. frontalis* adults compared to other traps. A single adult was detected in the Delta traps and no flies were found in McPhail traps. The captured adults were all females (Table 7.1). For the trap and bait test, particular trap and bait combinations had significant effects on the number of flies captured over the period of one week ($F = 18.53$; $df = 1$; $P < 0.001$) for trap and ($F = 19.16$; $df = 1$; $P < 0.001$) for bait. Also, a significant interaction between the traps and baits was found ($F = 18.43$; $df = 1$; $P < 0.001$). The largest number of *D. frontalis* adults (834) was found in McPhail baited traps. In Delta trap treatments, the number of flies captured was

larger in baited traps (12 flies) than unbaited ones with no significant difference between both traps (Table 7.2). Ratios of 8: 1 and 5: 1female to male were found in baited McPhail and Delta traps, respectively. The captured males were detected in baited traps only. Two males were recorded for Delta and 93 males in McPhail traps.

Table 7.1 Efficacy of traps in attracting and capturing *D. frontalis* adults over four days.

Treatment	N. flies captured	Means	Females %	Males %
Delta trap	1	0.2	100	0
McPhail trap	0	0.0	0	0
Yellow trap	12	2.4	100	0

Table 7.2 Efficacy of trap/ bait in attracting and capturing *D. frontalis* adults over one week. Values with different letters have significantly different means based on Tukey's HSD test ($P < 0.05$) after Anova.

Treatment	N. flies captured	Means	Females %	Males %	Female: male
Delta control	2	0.2b	100	0	2: 0
Delta baited with YHE*	12	1.8b	75	25	5: 1
McPhail control	0	0.0b	0	0	0: 0
McPhail baited with YHE*	834	166a	89	11	8: 1

* YHE = Yeast hydrolysate enzymatic

7.4.2 *Pre-emergence treatment*

Temperature (°C) and relative humidity (RH) readings during the experiment period are presented in Appendix II- Figure 1. The temperature and humidity averages were 25.5 °C and 76% RH respectively. Rain occurred three times during the experimental period. The rains were neither heavy nor recorded. As can be seen from the results in Figure 7.5 insecticide treatment with Delfos significantly reduced adult emergence of *D. frontalis* compared to the

MET52 and untreated control treatments (F = 32.90; df = 2; $P < 0.005$). The greatest larval-pupal mortality was recorded in the insecticide treatment with a 100% reduction in adult emergence. In the fungal treatment, the adult emergence reduction was lower indicating 63.7% larval-pupal mortality, with no significant difference in adult emergence rate with the untreated control (Figure 7.5). A lower adult emergence reduction (60%) was found in the untreated control.

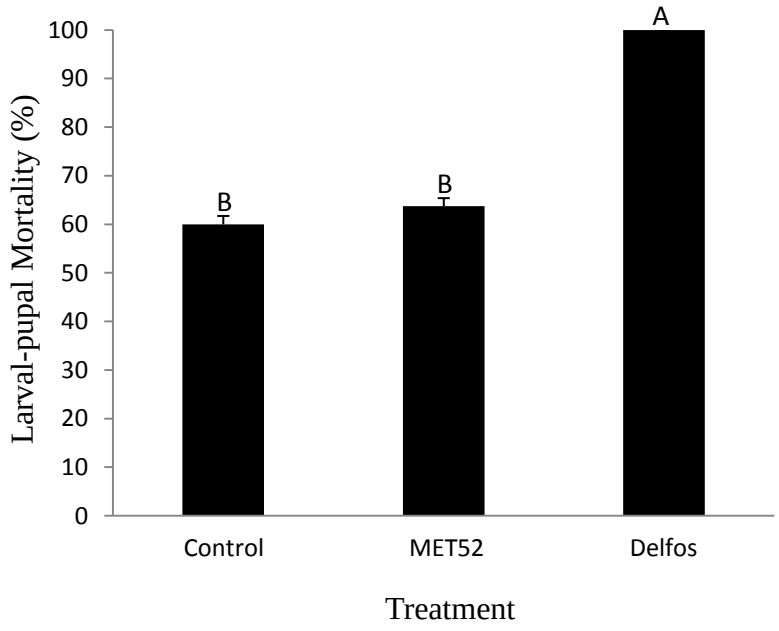


Figure 7.5 Mean (±SE) percentage larval-pupal mortality of *Dacus frontalis* treated with MET52 and Delfos at recommended doses, n = 4. Values with different letters have significantly different means based on Tukey's HSD test ($P < 0.05$) after Anova.

7.4.3 *Post-emergence treatment*

Temperature (°C) and relative humidity (RH) readings during the experiment period are presented in appendix II- Figure 2. The temperature and humidity averages were 21.3°C and 71.9 % RH respectively. Strong wind and heavy rain was recorded two times one week after the adult emergence. In this trial, three dead flies were observed and collected from the fungal cages. One out of three of the flies collected showed mycelium after incubation at room temperature and dark. As can be seen from Figure 7.6, Dursban treatment significantly reduced the population of adult *D. frontalis* compared to other treatments (F = 43.50; df = 2; $P < 0.001$). The greatest adult mortality was observed in the insecticide treatment with 100%.

In MET52 and control treatments, the adult mortality (before correction by Abbott` s formula) was lower reaching approximately 86% and 65% respectively, with significant differences found between the treatments (Figure 7.6.). After adult mortality was corrected by Abbot's formula, the mortality in the fungal treatment reached approximately 41% (Figure 7.7), which was significantly different from the insecticide treatment ($F = 43.50$; $df = 2$; $P < 0.001$). Ants were observed in the fungal cages two days from the application and before adult emergence (Figure 7.8). The ants were also observed into all the experimental cages a few days after adult emergence until the end of the experiment.

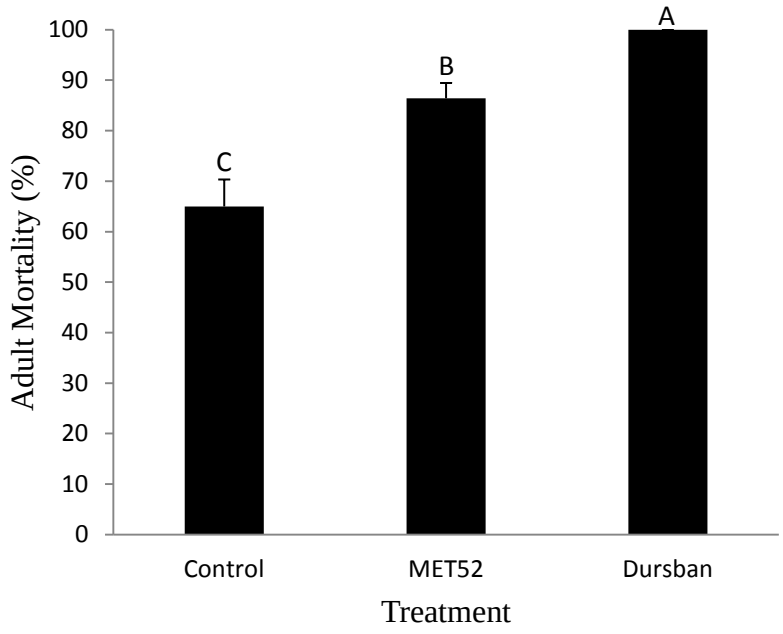


Figure 7.6 Mean (±SE) percentage adult mortality of *Dacus frontalis* treated with MET52 and Dursban at recommended doses (uncorrected data are shown), n = 7. Bars with different letters have significantly different means based on Tukey's HSD test ($P < 0.05$) after Anova.

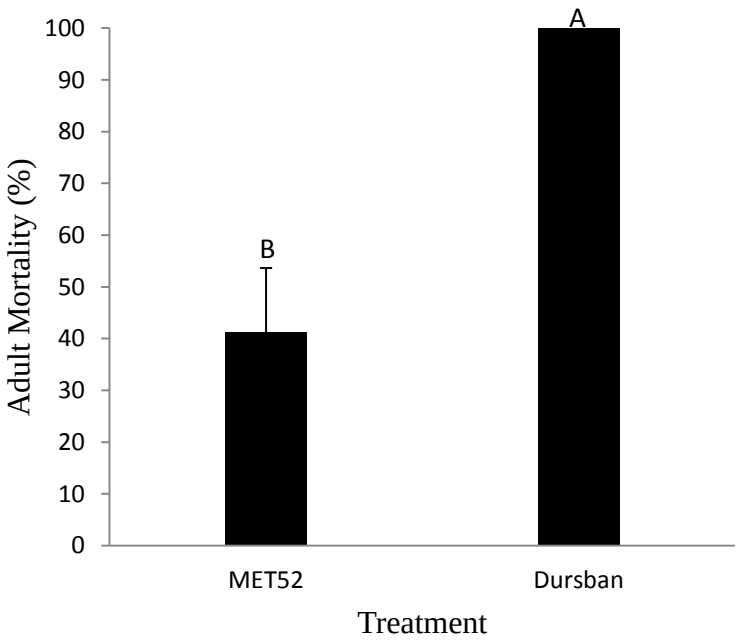


Figure 7.7 Mean (% ±SE) percentage adult mortality, n = 7 of *Dacus frontalis* treated with MET52 and Dursban at recommended doses (corrected data are shown). Bars with different letters have significantly different means based on Tukey's HSD test ($P < 0.05$) after Anova.

Figure 7. 8 Ants were observed on the surface of soil into fungi field cages one day after application.

7.5 Discussion

To my knowledge, there is no recent published research evaluating bait traps to attract *D. frontalis* adults. Brief information was mentioned in some taxonomic and ecological studies which indicated that males of *D. frontalis* are attracted to Cue-lure traps (Hancock, 1985; White, 2006). In the present trap and bait experiments, three different traps were evaluated for monitoring and capturing *D. frontalis* adults. The results showed only yellow sticky traps captured flies. A study conducted on another species of *Dacus*, the olive fly *Dacus oleae*, by Jones *et al.* (1983), found that yellow traps caught greater number than Delta traps. Only *D. frontalis* females were captured in the traps, suggesting that only females visit cucurbit crop for oviposition (Steffens, 1982). The attractiveness of YHE as food bait to *D. frontalis* adults was assessed using McPhail and Delta traps. In previous studies, other baits were evaluated for attracting species of fruit flies. Methyl eugenol and Cue-lure were successfully used for detecting males of *B. dorsalis* and *B. cucurbitae* (Vargas *et al.*, 2010a)**.** Roomi *et al.* (1993), reported that different extracts of *Ocimum sanctum* L. can attract flies of the genus *Dacus*, including *Dacus zonatus, D. cucurbitae, D. dorsalis* and *D. ciliatus* under field condition. Also, White (2006), indicated that males of many *Dacus* spp, have response to Cue-lure.

In the present study, baited McPhail traps performed much better than baited Delta traps. This means that the kind of trap plays a key role in efficiency of the bait. However, the baited Delta traps could be more practical procedure in monitoring the fly as they caught smaller number of flies which might be easy for farmers to detect and assess. Both males and females were captured with more females than males. Shafiq Ansari *et al.* (2012), reported that males of many fruit flies are attracted to protein baits. The present results suggest the potential use of yellow sticky traps and protein baited McPhail traps in monitoring and control strategies through mass trapping for *D. frontalis* management. Trapping strategies using different baits including protein have been successfully used in reducing fruit fly populations including some *Dacus* species (Shafiq Ansari *et al.*, 2012; Shelly *et al.*, 2014; Ishii *et al.*, 2015; Singh *et al.*, 2015).

The results of the semi field trial indicated that soil application with MET52 did not significantly reduce adult emergence of *D. frontalis*, but producing greater mortality than the untreated control in the emerging adult experiment. This is the first field evaluation of entomopathogenic fungi against *D. frontalis* under semi field condition.

In the pre-emergence treatment, percentage emergence of *D. frontalis* adult was not affected in MET52 treatment compared to insecticide treatment. This is probably because the biopesticide was not applied early enough before adult emergence. These results are consistent with the results of the laboratory experiments (Chapter 2) which indicated that MET52 granules did not cause a significant increase in mortality of *D. frontalis* pupae when they were applied two and eight days before adult emergence (section 2.4.1). Few studies on field application of entomopathogenic fungi against tephritid species have been conducted. In a field trial conducted by Lezama-Gutiérrez *et al.* (2000), a reduction in emergence rate of Mexican fruit fly, *Anastrepha ludens* was affected by the fungi used. The author found that percentage emergence of the adult flies in loam soil treated with a suspension of *M. anisopliae* Ma2 was significantly lower than in sandy loam soil treated and controls. Other authors were unable to control soil stages of *Delia* (Diptera: Anthomyiidae) by drench treatment of *M. anisopliae* in a field experiment (Chandler and Davidson, 2005). The fungus strain used in the present field trials (*M. anisopliae var anisopliae* strain F52) has been previously evaluated under field condition against other insect pests (Moorhouse *et al.*, 1993; Bruck and Donahue, 2007; Ansari and Butt, 2013), (Mauchline *et al.*, 2013) (Williams *et al.*, 2013)

In post-emergence treatment, soil application of MET52 was effective against *D. frontalis* adults inducing aproximetaly14% less adult mortality than the insecticide treatment and 26% more than in the control cages. The result support those of the earlier laboratory experiments (Chapter 2) which indicated that the adults were more suceptible to the fungus infection than the soil stages of *D. frontalis.* However, the mortality of inoculated adults was lower under field conditions, probably because of a low temperature average (21°C) recorded in November 2013 during the field trial. Few researchers have investigated the efficacy of insect fungal pathogens against fruit flies under natural condition. Dimbi *et al.* (2003), used a different inoculation method with *M. anisopliae* ICIPE 20 to show the possibility of control of adult of other fruit flies, *Ceratitis capitata* and *C. rosa* in cages under mango trees, causing 70-90% adult mortality of both species.

In the experimental cages not all of the flowers set and produced fruit, therefore fruit infestation and fly oviposition rates were not compared. The lack of set may be because the cages prevented access by pollinators.

Placing MET52 granules in the soil (for the pre-emergence treatment) and artificial diet on the top of all experimental cages (for the post-emergence treatment) attracted ants into the

experimental cages. This probably helped reduce the adult emergence rate in the fungal treatments and also increased the mortality post-emergence of *D. frontalis* adults in all the experimental cages. No attempt was made to identify the ants. The role of ants in control of several insect pests including fruit flies has been reported in several studies (Wong *et al.*, 1984; Way and Khoo, 1992; Van Mele *et al.*, 2007; Van Mele *et al.*, 2009). Various ant genera have been reported to be responsible for the disappearance of pupae of *Anastrepha* spp located at different depths in soil (Hodgson *et al.*, 1998). In a field and laboratory study, ants were the most common predators preying on pupae of *C. capitata* (Urbaneja *et al.*, 2006).

Insecticides have been used for controlling soil stages and adults of fruit flies species as previously reported (Vargas *et al.*, 2009; Vayssieres *et al.*, 2009; Liu *et al.*, 2015). In the present study, although Delfos and Dursban gave good control of *D. frontalis*, such chemical pesticides have a negative impact on non-target species and the environment (Croft, 1990) and application increases the costs.

In conclusion, the results suggested that a trapping strategy could be used in integrated management for *D. frontalis*. Using this control will decrease the fly population and consequently might increase the effect of entomopathogenic fungi on the population. Although MET52 showed poorer control of *D. frontalis* under semi field condition than under controlled condition, the results suggest that applying the fungus as granules to soil could be a valuable biological control, reducing overwintering stages and adult populations which could serve as future infection sources against new fly offspring in soil. Therefore, using MET52 in combination with other control agents such as sublethal doses of chemical pesticides or baited traps should be investigated to enhance fly control. Furthermore, early field application and persistence of MET52 should be investigated in reducing emergence rate and increasing adult mortality.

Chapter 8. General Discussion

Dacus frontalis is one of the most economically important insect pests of Cucurbitaceae in Libya causing economic losses over the last decade. Recently, new regions in North Africa and the Middle East have been reported as infested areas by the fly and this may raise concern about the continued spread of the fly to other areas in the world. Extensive use of pesticides has not successfully reduced the fly damage in Libya. Also, farmers' poor knowledge in identifying the fly, dealing correctly with the damaged cucurbit fruits and failing to use other control methods, such as field sanitation, trap crops (corn and sunflowers), bait traps and biological control, on their farms might be reasons for increasing losses of cucurbit fruits in the country. The damaged fruits are usually of low quality and unmarketable; therefore they are thrown to the sides of cucurbit fields or used as a food for farm animals. These losses reduce the total fruit production causing negative impacts on the farmer`s economy. Therefore, some farmers may be willing to pay for other control agents which could protect their crops. However, such methods are not currently available or known to them. There is therefore a need to give attention to farmers' education. Improved management might be achieved by enhancing the capacities of the growers with regard to the fly biology explaining the benefit of using more sophisticated control within integrated fly management programme. This could encourage the farmers to use different forms of pest control, thereby minimising the fly damage and increasing cucurbit production. Some studies have reviewed the importance of a farmer`s knowledge of planning a good procedure for fruit fly management. For example, a survey was conducted by Benjamin *et al*., (2012) to determine the level of the Ghanaian farmer`s knowledge with regard to fruit fly pests, who found that the majority of farmers showed poor knowledge in identifying economically important fruit fly species and 75% of the farmers did not take care regarding the disposal of infested fruits. The results of the survey also indicated that about 40% of the farmers had not taken any action against fruit flies although they were aware that these species can cause economically damaging losses to their crops. Some studies and reviews have demonstrated that farmer`s knowledge of using several effective management strategies against fruit flies played a fundamental role in limiting fruit fly damage. For example, Ekesi *et al.*, (2010) has reported that field sanitation can reduce fruit infestation by some African tephritid flies including *D. frontalis*. Also, spraying the edges of the cucurbit crop with insecticides, planting trap plants such as sunflowers at the edges of the crop, using bait traps, and uprooting wild host are effective procedures to control *D. frontalis* (Steffens, 1982). In a recent field assessment Hasyim and de Kogel (2013), found that populations of *Bactrocera tau* and the level of damaged fruits

were reduced when sanitation procedures were followed in passion fruit orchards. Thus, safer, effective approaches for *D. frontalis* management need to be developed. Entomopathogenic fungi are one of the alternative control agents for many insect pests; however, these pathogens have not yet been established in agricultural systems in Libya. The initial objective of the current project was to investigate the potential use of entomopathogenic fungi against the fly and to develop better strategies for using this approach in integrated fly management.

Insect pests have different susceptibilities to different strains of entomopathogenic fungi (Butt *et al.*, 1995). Therefore, investigation of pathogenicity is an essential step to select appropriate fungal strains. The pathogenicity of five commercial biopesticides, based on several strains of entomopathogenic fungi, *Metarhizium anisopliae, Beauveria bassiana* and *Paecilomyces fumosoroseus* were screened against different life stages of *D. frontalis* to identify the most promising entomopathogen candidate. This study confirmed for the first time the susceptibility of *D. frontalis* to entomopathogenic fungi. Based on the laboratory results presented in this thesis, *M. anisopliae var anisoplia*e strain F52 (MET52) was the most pathogenic against the fly. Soil application of MET52 granules was the best strategy to control the fly. The fly adult was more sensitive to the fungal infection than the soil stages. This could be because of the different characteristics of morphology, biology and behavior between the adults and the other stages.

The overall effectiveness of entomopathogenic fungi can be affected by application method. In the present study, MET52 was more effective when applied as a granule in soil against emerging adults than as a suspension and direct spray against adults. Also, prior application of the fungus in a granule form caused considerable mortality to larval-pupal stages compared with a drench application, indicating that prophylactic application may be an effective strategy to obtain considerable reduction in the fly emergence and should be taken into account during fly control programs. This might be because of the increased conidia density in the treated soil, but also the green conidia may be attractive to larvae as a food.

Persistence is an important factor which contributes to the successful use of fungal pathogens against insect pests which spend all or some of their life stages in soil. In the present study, MET52 persisted in soil over the period of 70 days and was able to reduce the emergence rate and cause post-emergence mortality of *D. frontalis* adults during the experimental period. The ability of the fungus to grow and recycle over this period tested (Figure 8.1.A) led to increasing the conidia density consequently inducing more mortality in the fly. This means

that applying one single application to soil in a season could be an effective approach for fly control. This is a good trait as it may reduce the cost of the application.

Soil application also gave more chances for MET52 to infect the fly at an early age. Consequently, once the adults emerged, they come into contact with MET52 conidia inducing sublethal effects including the adult`s mating behavior, oviposition time and reproduction. This will increase the total control efficacy and may reduce the fly progeny. In some cases, infected adults started to die two days after emergence. This could be because the fungus affected the adult in an early stage shortly before or after emergence from the pupae (Figure 8.1.B, C, D and E). A large amount of mycelium was seen growing on the infected flies and treated soil (Figure 8.1. F, G and H) and this could be an advantage to the fungus by increasing conidia density in the soil, therefore increasing the likelihood of causing future infections in a following season.

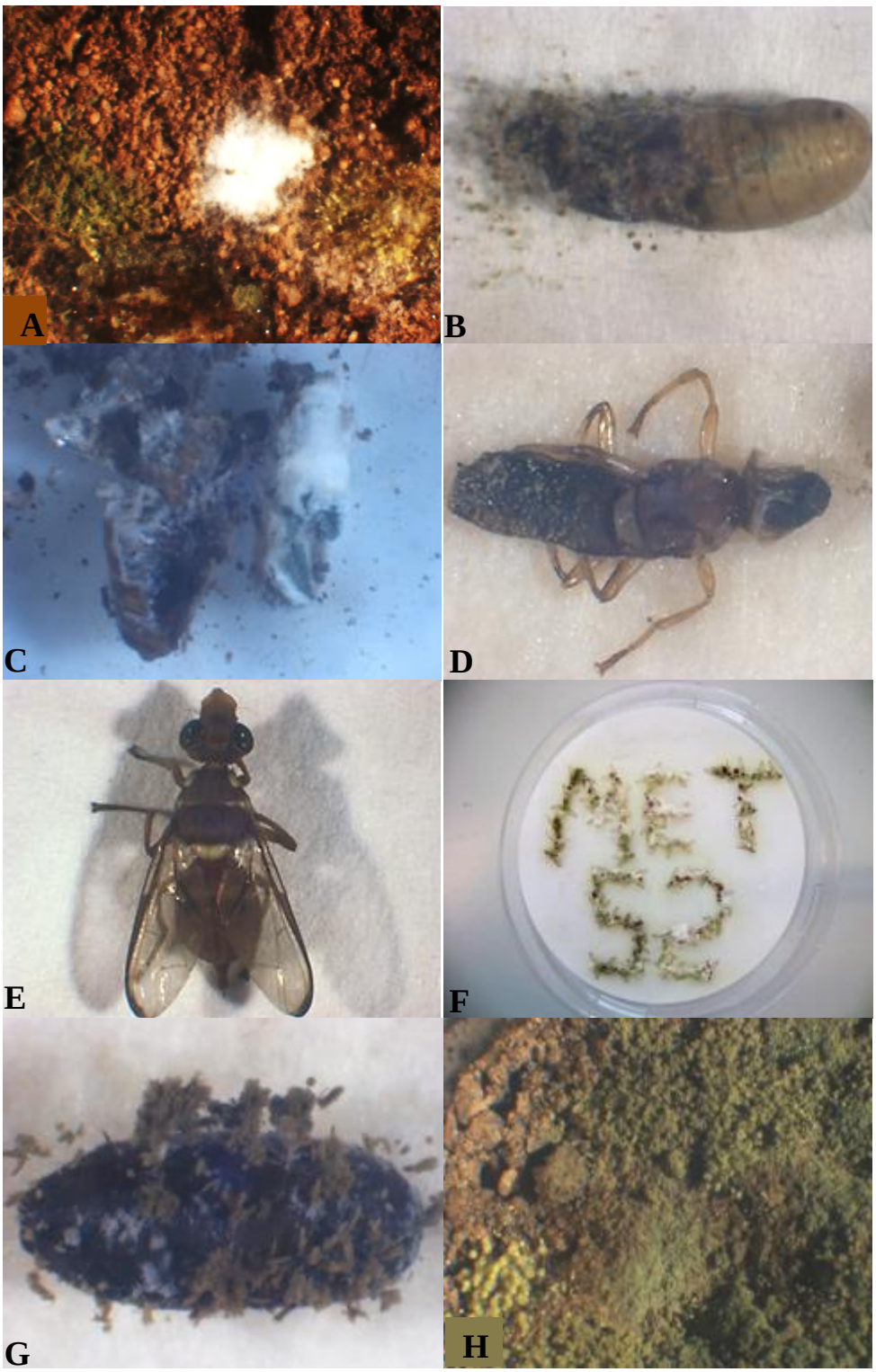


Figure 8.1 New mycelium and spores on of MET52 growing 70 days after the first treatment (A) Incomplete adult emergence infected with MET52 (B) Dead pupae of *Dacus frontalis* after being dissected showing growths of white mycelium of MET52 (C) Dead immature adult infected with MET52 before getting to a mature stage (D) An emerged adult covered with greenish spores of MET52 (E) A large number of *D. frontalis* adults infected with MET52 (F) A pupa infected with a mycelium of MET52 (G) Increasing growing of vegetative conidia of MET52 in soil (H). (Photographs © were taken by the author).

Although many studies have investigated conidia transmission of some entomopathogenic fungi, there is no research that has assessed potential fungal transmission through soil. This study demonstrated for the first time that *D. frontalis* adults that emerged from MET52 contaminated soil were able to transmit the fungal conidia to untreated individuals of the opposite sex. Transmission of conidia based on infecting adults emerging from soil could offer further benefits. For example, the newly emerging flies will be contaminated with conidia at an early stage, which means they have more chance to mate with other flies and cause early mortality before oviposition. Also, MET52 infection showed an effect on *D. frontalis* reproduction by reducing the number of pupae recovered from inoculated flies, resulting in a high reduction in progeny pupating. However, comparative investigations of the efficiency of other strategies (fungus contamination devices and baited stations) regarding the dissemination of MET52 to fly populations and the potential effect of the fungus infection on fertility and fecundity of fly are needed. Hence, inducing new infections among the fly population and reducing the adult's reproduction could increase the efficiency of MET52 leading to reduction of subsequent population size.

Undoubtedly, chemical pesticide application can still efficiently suppress many insect pest species. Recently, conventional insecticides have been successfully evaluated in combination with entomopathogenic fungi against several insect pests through integrated pest management programmes. Combined use of some of these pesticides with entomopathogenic fungi can produce synergic effect on insect mortality and reduce the amount of the chemical, minimising environmental contamination. However, such combinations could have deleterious effects on the occurrence and pathogenicity of the fungi. There is an increasing use of botanical insecticides in integrated pest management strategies as they are often safer than synthetic pesticides to the environment and and can be combined with other control agents providing a possible effective strategy for integrated fly management. This is the first report confirming that neem oil is toxic to *D. frontalis* larvae suggesting the product is a promising control agent against the fly. The potential advantages of combining entomopathogenic fungi with botanical insecticides have been evaluated against some insect pests (Santi *et al.*, 2011; Radha *et al.*, 2014). In order to improve the fungus efficacy against *D. frontalis* particularly larval and pupal stages, potential interaction effects between the fungus and some botanical extracts were examined. The current study found that neem oil and garlic extract were compatible with *M. anisopliae* inducing better control of the fly. However, different concentrations of the botanicals should be evaluated to avoid occurrence of

antagonistic effects. This is a desirable trait for a candidate control agent which could be involved within integrated fly management.

The laboratory results showed that MET52 was effective at a wide range of temperatures, humidities and soil moisture contents. Conditions of 25 ºC, with 75%-85% relative humidity and 70% soil moisture were optimal for MET52 to induce high adult mortality. Christias *et al.* (2001), indicated that insect pathogenic fungi should have a range of temperature and humidity when they can be used for insect control as different species of entomopathogenic fungi have different response to environmental conditions. In semi field cages experiments, MET52 showed lower pathogenicity against *D. frontalis*. The results were likely to be affected by some factors. For example, soil texture characteristics of the field; sandy soil with a high percolation might have a negative impact on the density of the fungus conidia consequently reducing the chance for the soil stages and newly emerged flies to come into contact with conidia. Another possible explanation for this is that the biopesticide was not applied early enough before adult emergence. Also, variable conditions of temperature and humidity in the field might be another reason for the lower mortality.

The above findings support potential entomopathogenic fungi as a valuable biological control candidate for the fly. MET52 has been used commercially in controlling black vine weevil larvae (*Otiorhynchus sulcatus*) and some other soil pests (http://www.fargro.co.uk/). However, the current study demonstrated for the first time the product is an effective biological agent against *D. frontalis*. Unfortunately, the current work did not include Libyan isolates of entomopathogenic fungi. To my knowledge, there is no work that has investigated Libyan isolates of entomopathogenic fungi. Therefore, indigenous strains should be isolated and evaluated against the fly in future research.

Conclusion

The purpose of the current study was to determine the potential use of entomopathogenic fungi against *D. frontalis*. This study has demonstrated susceptibility of the fly to these pathogens for the first time. MET52 was an effective biological control agent against the fly. The research has also shown that the fungus can induce new infections. The investigation of abiotic factors has shown that conditions of 25 ºC, with 75%-85% relative humidity and 70% soil moisture were optimal for MET52 to induce high adult mortality. This is the first study reporting an advantage of soil application in inducing new infections among the fly population reducing the adult's reproduction. MET52 fungus was able to persist in soil, reducing the adult emergence and subsequent fly population for more than two months after a single application. The present study confirms previous findings and contributes additional evidence that suggests entomopathogenic fungi are viable biological control agent against fruit flies and can be combined with other control agents providing a possible effective strategy for the integrated fly management. MET52 is a promising biological agent against the fly and better effective control could be achieved when the fungus is combined with neem oil within integrated fly management.

Future work

The results of the current work will open doors for further studies on entomopathogenic fungi in general and on the biological control against *D. frontalis* particularly in Libya through the National Integrated Fruit Flies Management Project.

The findings of the current thesis recommended further investigations in the following areas:

1- Ecology and biology studies on *D. frontalis* are very important for future fly management. For example, investigation of movement of the fly between sites would be valuable. So far, no research has been done to study *D. frontalis* generation times under typical conditions in Libya.

2- Survey of the occurrence of entomopathogenic fungi in different habitats or natural infections in Libya should be conducted to identify local isolates.

3- Pathogenicity of MET52 should be investigated on a complex of cucurbit pests (fruit flies, white fly, thrips and aphides).

4- It would be interesting to assess the possibility of compatible use of MET52 with other control agents such as chemical pesticides, nematodes and predators. This may enhance the fungus

efficiency when used at low concentrations and provide better control within integrated fly management.

5- A further trials to assess persistence of MET52 for the long-term to determine the ability of the fungus to induce new infection for the following generation under natural conditions.

6- Effect of MET52 on non-target species especially in soil.

7- More assessments of applying MET52 at an early time against larvae will be necessary in different regions in Libya which have different soil characteristics and conditions.

8- Economic feasibility of using MET52 compared to chemical pesticides.

Appendix I

Sequences of *Aspergillus ochraceus* strain CD1128

AAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGAGTGAG
GGTCCCTCGGGGCCCAACCTCCCACCCGTGTATACCGTACCTTGTTGCTTCGGCGA
GCCCGCCCCCTTTTTCTTTTAGGGGGCACAGCGCTCGCCGGAGACACCAACGTGA
ACACTGTCTGAAGTTTTGTCGTCTGAGTCGATTGTATCGCAATCAGTTAAAACTTT
CAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATA
ATTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCA
CCCCCTGGTATTCCGGGGGGTATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCA
CGGCTTGTGTGTTGGGTCGTCGTCCCCCCCCAGGGGGACGGGCCCGAAAGGCAGC
GGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTTGTAG
GCCCGGCCGGCTGCTGGCCGACGCTGAAAAGCAACCAACTATTTTTCCAGGTTGA
CCTCGGATCAGGTAGGGATACCCGCTGAACTTAA

Appendix II

Figure 1 Mean daily temperature and relative humidity readings during emergence rate assessment conducted in a field located in Almrazeek during experimental periods of September and October 2013. The climatic conditions were monitored by EL-USB-2 RH/TEMP DATA LOGGER.

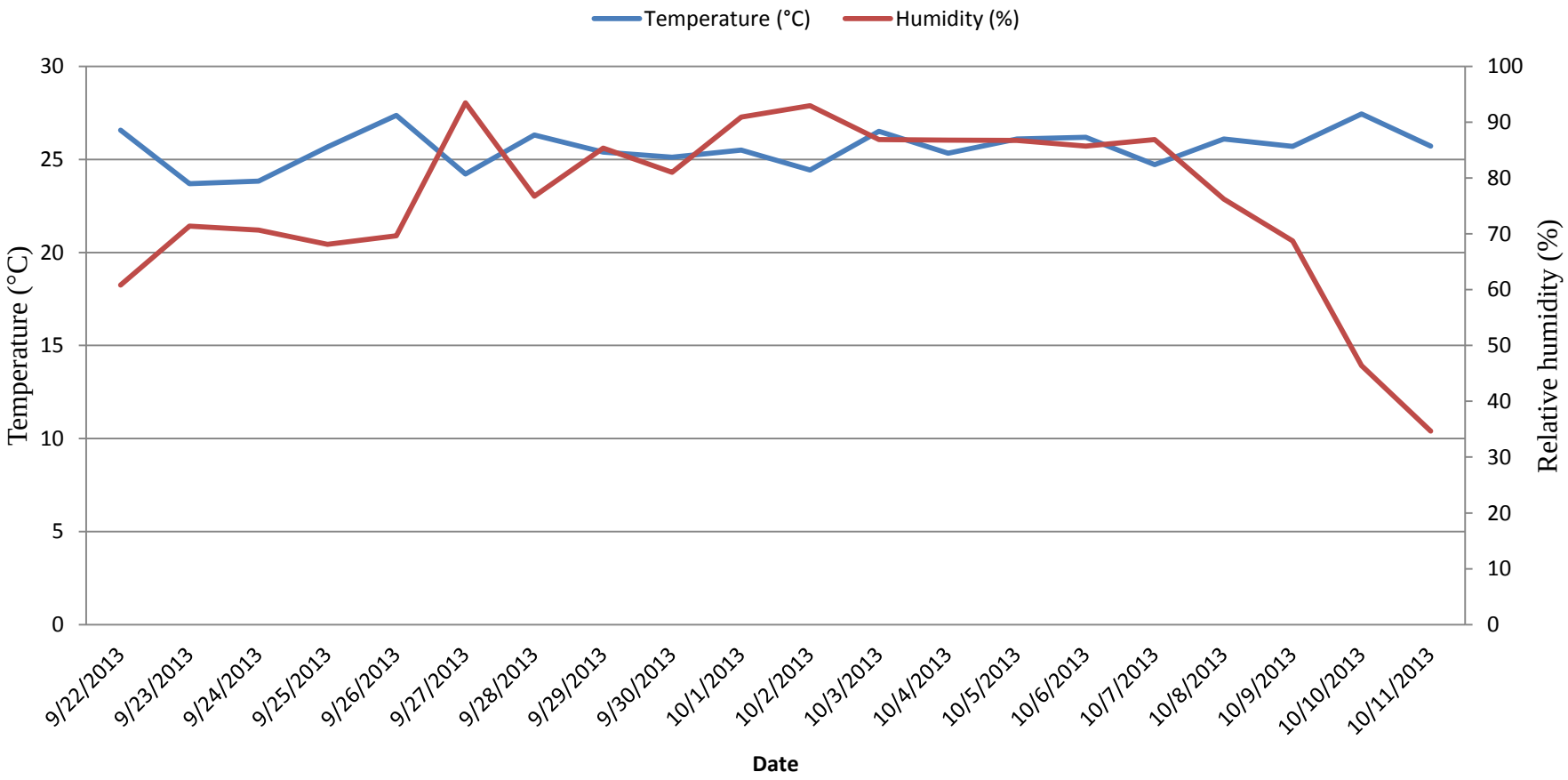
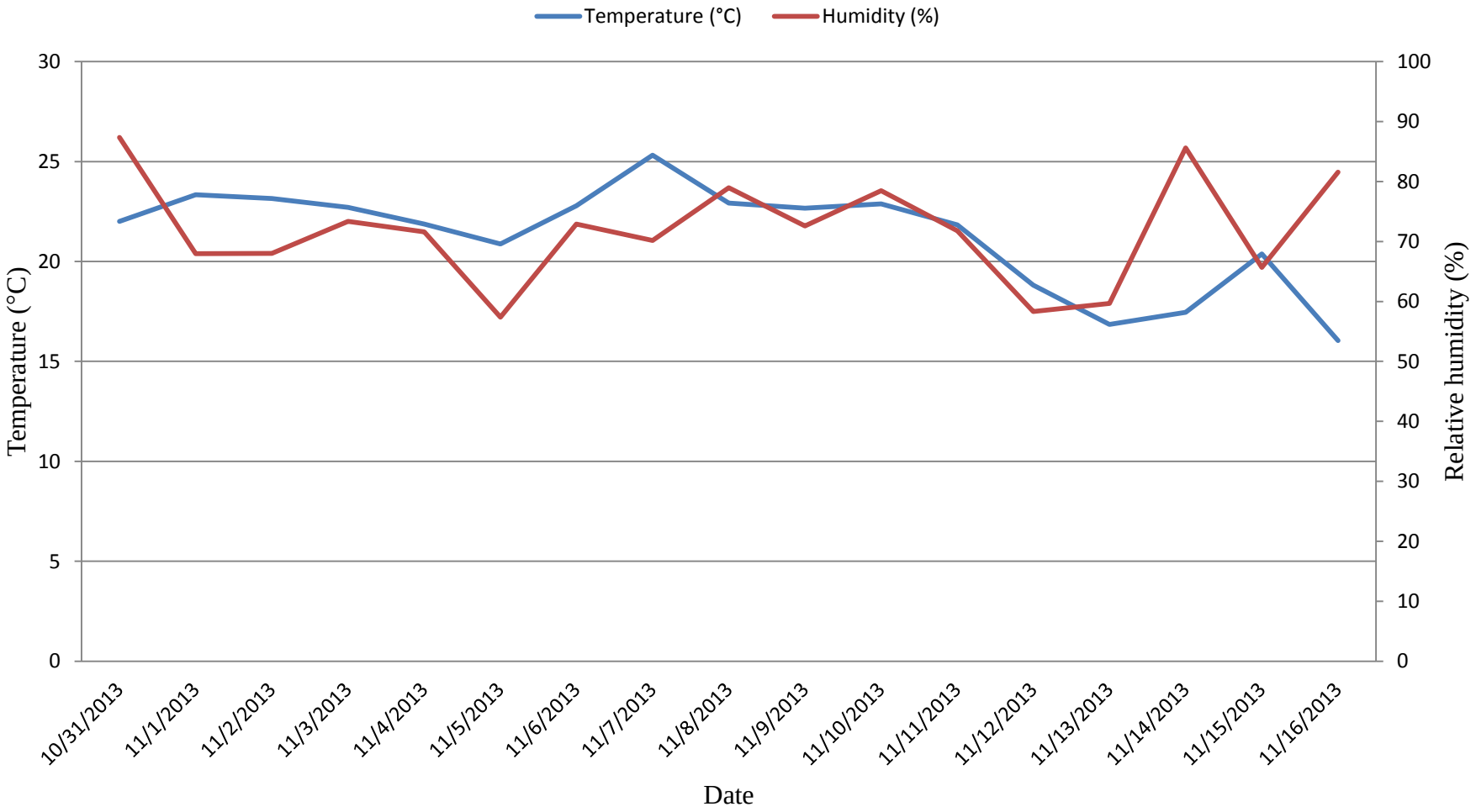


Figure 2 Mean daily temperature and relative humidity readings during adult assessment conducted in a field located in Ben Ashour area during experimental periods of November 2013. The climatic conditions were monitored by EL-USB-2 RH/TEMP DATA LOGGER.



Appendix III Preparation of Water Holding Capacity

A cylinder had a filter paper placed over its perforated base before filling with 100 g soil. The cylinder was weighed before and after filling with soil. The cylinder was immersed in a water bath so that the water level was lower than the top of the cylinder for two hours at room temperature. Then, the cylinder was submerged below the water level for an hour. After that, the cylinder was removed from the bath and placed on a tray to drain overnight. Next day, the cylinder containing the soil was weighed. Then, the soil was removed and dried in oven at 105 °C before being weighed again. Water Holding Capacity (WHC) was calculated by the following formula: $WHC = (ws – wt – wd/ wd) \times 100$.

ws = the weight of water-saturated soil + cylinder + filter paper

wt = the weight of the tare (cylinder + filter paper)

wd = the weight of dried soil

Appendix IV Abbreviations

Percent	%
Grams	g
Hours	h
Liter	L
Minute	min
Millilitre	ml
Light to Dark	L: D
Micro liter	µl
Degrees Celsius	ºC
Relative humidity	RH
Thermus aquaticus	Taq
Deoxyribonucleic acid	DNA
Colony Forming Units	CFU
Potatoes dextrose agar	PDA
Sterile insect technique	SIT
Water holding capacity	WHC
Internal transcribed spacer	ITS
Polymerase Chain Reaction	PCR
Integrated pest management	IPM
Yeast hydrolysate enzymatic	YHE
Met52 Granular bioinsecticide	MET52
Deoxynucleoside triphosphates	DNTP
Hexadecyltrimethylammonium bromide	CTAB
Lethal time to kill half of the population	LT50
Lethal time to kill 90% of the population	LT90
National Centre for Biotechnology Information	NCBI

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