Hebron University
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Isolation of *Beauveria bassiana* from Soil and Evaluation of its Entomopathogenic and Biocontrol Efficacy Against the Mediterranean Fruit Fly (*Ceratitis capitata*)

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Dedication

DEDICATED TO MY PARENTS

Acknowledgement

Thanks to Allah for granting me strength to live and work on this research

Deepest Thanks are extended to Prof. Dr. Radwan Barakat, without his great supervision and financial support, this work would have never saw light.

Thanks also to my colleagues and friends in the Faculty of Agriculture Salem Nassr, Saleh Seekh, Mohammad Adam, Omar Nasser and Mohammad Almasri, your support was so appreciated.

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List of Abbreviations

CFU	Colony forming unit
CM	Centimeter
CRD	Completely randomized design
DW	Distilled Water
EPPO	European and Mediterranean Plant Protection Organization
g	Gram
GPA	Glucose Peptone Agar
GPAD	Glucose Peptone Agar Dodine
L	Liter
LC_{50}	Lethal concentration required to kill 50% of insect pest population
LSD	Least significant difference
LT_{50}	Lethal time required to kill 50% of tested insect pest population.
mg	Milligram
PPM	Part per million
RH	Relative humidity
SDW	Sterile distilled water

Abstract

The biocontrol potential of the Entomopathogenic fungus *Beauveria bassiana* against the insect pest *Ceratitis capitata* was investigated. Fifty eight (58) *B. bassiana* isolates were recovered from 225 soil samples collected mostly from warm areas of the West Bank. The isolates were recovered by dilute plate technique on the modified semi selective medium Glucose Peptone Agar (GPA) amended with 90 mg /l Dodine. The optimum temperature for *B. bassiana* growth and reproduction was in the range of 25 – 30 ºC. *B. bassiana* isolates proved to be very virulent against *C. capitata* adult flies (up to 90% mortalities) but not very effective against *C. capitata* immature stages (less than 12% mortalities on larvae). Lethal concentration of *B. bassiana* conidia required to kill 50% of *C. capitata* population was in the range of 3.8 – 10.5 log conidia /ml after 5 days post inoculation. LT_{50}, however, ranged between 3.9 – 5.6 days at the conidial concentration of 10^8 conidia /ml. In the bioassay study, *B. bassiana* isolate Bv 32 was able to reduce *C. capitata* infestation to peach fruits by 73% compared to the control when 10^8 conidia /ml inoculum was used. The study revealed important potential of *B. bassiana* native isolates to control *C. capitata* adult flies when applied as conidial suspension. Further studies are needed in the field of formulation, shelf life, sustainability under field conditions, and modes of action of the fungus.

Chapter 1: General Introduction

1.1 *Ceratitis capitata*

1.1.1 Taxonomy of *Ceratitis capitata*

Over time, medfly has had several different synonyms (White and Elson-Harris, 1992). *Tephritis capitata* (Wiedemann, 1824), *Trypeta capitata* (Wiedemann, 1824), *Ceratitis hispanica* (De Brême, 1842) and *Pardalaspis asparagi* (Bezzi, 1924).

The taxonomic classification of *C. capitata* according to (Wiedemann, 1824):

Class: Insecta

Order: Diptera

Suborder: Brachycera

Infraorder: Muscomorpha (or Cyclorrhapha)

Family: Tephritidae

Subfamily: Dacinae

Tribe: Ceratitidini

Genus: *Ceratitis*

Subgenus: *Ceratitis*

Species: *capitata*

1.1.2 Description

The adult medfly is 4 to 5mm long (about two-thirds the size of a housefly). The general colour of the body is yellowish with a tinge of brown, especially the abdomen, legs, and some markings on the wings. The oval shaped abdomen is clothed on the upper surface with fine, scattered black bristles, and has two narrow, transverse, light coloured bands on the basal half. The female can be distinguished by its long ovipositor at the apex of the abdomen. The larval phase consists of three instars. The size of the larvae depend on their diet. The larvae are

typically elongate, cream coloured, cylindrical maggot-shaped. Their anterior end narrows and is somewhat recurved ventrally, with anterior mouth hooks, and a flattened caudal end. The length of the first larval stage is 1mm or less, and the body is mostly transparent; the second larval stage is partially transparent with the fruit in the gut visible; the fully grown third larval stage is 6 to 8mm long, with a body fully opaque white or the colour of ingested food. The pupae are cylindrical, approximately 3mm long, and dark reddish brown in colour (Mau and Kessing, 2005).

1.1.3 Habitat

Medfly has the ability to tolerate cooler climates better than most other species of fruit flies (Mau and Kessing, 2005). Thomas *et al.* (2001), stated that some adults may survive up to six months or more under favorable conditions of food (fruit, honeydew, or plant sap), water, and cool temperatures.

Broughton and De Lima (2002) explained that "In winter, the Medfly may become inactive in cold areas. Medfly can overwinter as adults, eggs and larvae (in fruit), or pupae in the ground. Adult Medflies are active in winter when temperatures exceed 12 ºC. " They also recommend that any control methods should begin in Spring, as the temperature increase allows overwintering flies to become active, to prevent population sizes from increasing rapidly to problematic levels.

1.1.4 The economic importance of *C. capitata*

C. capitata is an important pest worldwide and has spread to almost every continent recording the single most important pest species in the family Tephritidae. It is highly polyphagous and causes damage to a very wide range of unrelated fruit crops. In the Mediterranean region, it is particularly damaging citrus and peaches. It also transmits fruit-rotting

fungi (Cayol *et al.*, 1994). Both *C. capitata* and *C. rosa* are highly polyphagous, with *C. rosa* tending to displace C. *capitata* in some areas where both species occur (Hancock, 1989).

An economic feasibility study conducted in 1997 showed that in Israel, Jordan and Palestine, the total annual losses from Medfly amounted to nearly US $300 million, including loss of markets that discriminate against Medfly and pesticide residues (Enkerlin, 1997).

The infestation percentage of pear reached 90%, apple 52% and fig 35% in Jordan (Mustafa *et al.*, 1996). Hashem et al (1987) showed that the degree of Medfly infestation on different host fruits was extremely heavy ranging from 5.7% on Valencia orange to 74% on apricot in Egypt.

Cohen and Yuval (2000) pointed out that "*C. capitata* is polyphagous and, as such, uses the various hosts in its environment as stepping stones, moving from one to another as fruit mature throughout the season." This gives Medfly the ability to destroy an area's production of many fruits, meaning that the damage is not limited to just one fruit species, but also providing medflies with refuges from control efforts, serving as a source of reinfestation to surrounding private or commercial plots

1.1.5 Geographical distribution

The Mediterranean fruit fly was reported in the Middle East region, and was first detected in Palestine in the early 1900s (Freidberg, and Kugler, 1989). Worldwide, Mediterranean fruit flies were accidentally introduced into Hawaii from Australia around 1907. They are usually found at upper elevations, although breeding has occurred in lower elevations on Kauai (Vargas *et al.*, 1983). *C. capitata* originated in tropical Africa, from where it has spread to the Mediterranean area and to parts of Central and South America.

Throughout the EPPO region, Medflies were reported to be found in the southern parts, e.g. Albania, Algeria, Croatia (Kovacevic, 1965), Cyprus, Egypt, France (very limited distribution in the South only; Cayol and Causse, 1993), Greece (including Crete), Hungary (found but not established), Israel, Italy, Lebanon, Libya, Malta, Morocco, Portugal (including Azores and Madeira), Russia (southern, found but not established), Slovenia, Spain (including Balearic and Canary Islands), Switzerland (limited distribution), Syria, Tunisia, Turkey, Ukraine (outbreaks in the south; eradicated). Records in Northern or Central Europe (Austria, Belgium, Bulgaria, Czech Republic, Germany, Hungary, Luxemburg, Netherland, Sweden, UK) refer to interceptions or short-lived adventive populations only (Karpati 1983; Fischer-Colbrie and Busch-Petersen, 1989).

In Africa, Medflies were found in several countries (e.g. Algeria, Angola, Benin, Burkina Faso, Burundi, Botswana, Cameroon, Cape Verde Islands, Congo, Côte d'Ivoire, Egypt, Ethiopia, Gabon, Ghana, Guinea, Kenya, Liberia, Madagascar (*C. malgassa*), Malawi, Mali, Mauritius, Morocco, Mozambique, Niger, Nigeria, Réunion, Sao Tome and Principe, Senegal, Seychelles, Sierra Leone, South Africa, St. Helena, Sudan, Tanzania, Togo, Tunisia, Uganda, Zaire and Zimbabwe. Medflies were also reported in North and Central America, Caribbean, South America and Oceania. (Karpati, 1983), (Fig. 1).

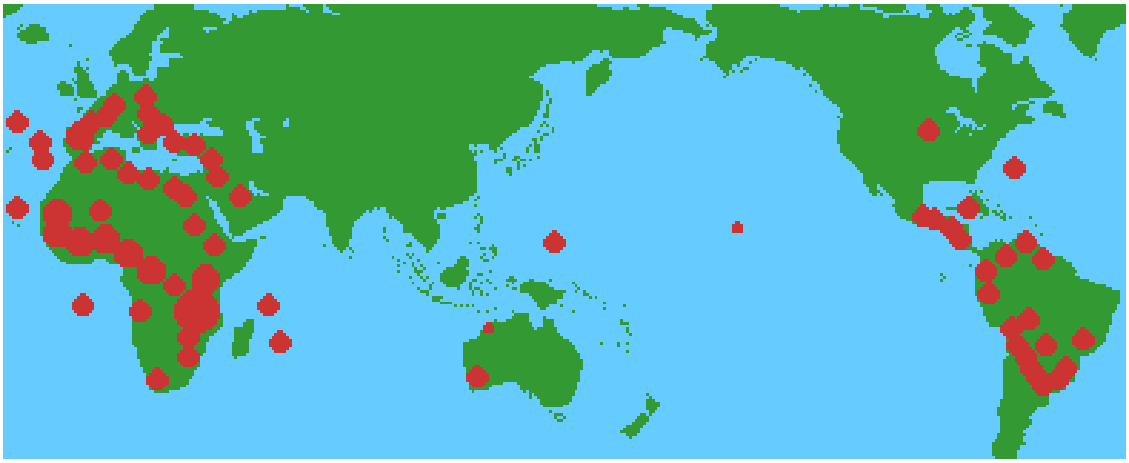


Figure 1. Medfly worldwide distribution

http://agspsrv34.agric.wa.gov.au

1.1.6 Life cycle of *Ceratitis capitata*

Eggs of *C. capitata* are laid below the skin of the host fruit. They hatch within 2-4 days (up to 16-18 days in cool weather) and the larvae feed for another 6-11 days (at 13-28°C). Pupariation occur in soil under the host plant and adults emerge after 6-11 days (24-26°C; longer in cool conditions) and adults live for up to 2 months (field-caged) (Christenson and Foote, 1960). *C. capitata* cannot survive under sub-zero winter temperatures.

1.1.7 Host range

C. capitata is a highly polyphagous species, having more than 300 host fruits (Liquido *et al.*, 1990). Among the most frequent host fruit species of the Medfly, the following stand out: from Rosaceae, plums (*Prunus domestica*), peaches (*Prunus persica*), apples (*Malus sylvestris*); from Rutaceae, oranges (*Citrus sinensis*), sweet lime (*Citrus aurantifolia*), grapefruit (*Citrus paradisi*); from Rubiaceae, coffee (*Coffea arabica*); from Anacardiaceae, mango (*Mangifera indica*); from Myrtaceae, feijoa (*Feijoa sellowiana*); from Lauraceae, avocado (*Persea americana*); and,

from Caricaceae, papaya (*Carica papaya*) (Krainacker *et al.*, 1987; Fimiani, 1989; Zucoloto, 1993; Carvalho and Aguiar, 1997; Papadopoulos *et al.*, 2002; Ovruski *et al.*, 2003; Medeiros, 2005).

1.2 The fungus *Baeuveria bassiana*

1.2.1 Taxonomy

Beauveria bassiana (Phylum Ascomycota: Order Hypocreales) was originally placed in the Family Clavicipitaceae (Arnold and Lutzoni, 2007; Sung *et al.*, 2007), but has recently been moved to the Family Cordycipitaceae (Ownley *et al.*, 2010). Considered an inducible mutualist (Carrol, 1988), *B. bassiana* is the anamorph stage of *Cordyceps bassiana* (Quesada-Moraga *et al.*, 2006; Sung *et al.*, 2007), an important traditional Chinese medicine, but the two forms are rarely seen together in nature.

1.2.2 History

Beauveria bassiana (Bals.) Vuill., the causative agent of white muscardine disease, was first described in 1835 by Bassi de Lodi who successfully demonstrated the fungus's pathogenic nature against silkworm, *Bombyx mori* (Tanada and Kaya, 1993). It was his discovery of *B. bassiana's* infectious nature that led to the development of the germ theory of disease (Kendrick, 2000). *B. bassiana* is categorized as a muscardine fungus, due to the characteristic mycotic stage exhibited on hosts after death when white, powdery-appearing hyphae cover the insect cadaver.

Beauveria species are found in the artificial class of Mitosporic fungi, class Deuteromycetes. Within the *Beauveria* genus, species are characterized by conidia born singly on a zig-zag or denticulate rachis (Tanada and Kaya, 1993; Humber, 1997).

Several species of *Beauveria* have been described, including *B. bassiana*, *B. tenella*, *B. brongniartii*, *B. amorpha*, and *B. velata* (Tanada and Kaya, 1993). Strains of the same species have been shown to exhibit variations in virulence and pathogenicity towards insect hosts (Todorova, 2000). *B. bassiana* can be distinguished from other *Beauveria* species by its globose conidia, which are usually larger than 3.5 mm in diameter (Humber, 1997). *B. bassiana* hyphae grows optimally at 23- 25 °C and conidia require relative humidity of 92% or higher to germinate.

1.2.3 Host range

The entomopathogenic fungus *B. bassiana* (Balsamo) is a natural enemy of a wide range of insects and arachnids and have a cosmopolitan distribution (Roberts and St. Leger, 2004; Rehner, 2005).

B. bassiana occurs worldwide (Steinhaus, 1949; Macleod, 1954), with a host range of more than 700 species (Li, 1987) including many insects of agricultural and medical importance (Hall and Papierok, 1982).

B. bassiana has been reported as a suppressive agent for several insect species worldwide, such as *Helicoverpa armigera* (Hübner), *Alphitobius diaperinus* (Panzer), *Plutella xylostella* (Linnaeus), *Laniifera cyclades*, *Prostephanus truncatus* (Horn), *Polyphagotarsonemus latus* (Banks) and *Bemisia tabaci* (Gennadius) (Dhuyo and Selman, 2007). *B. bassiana* has been extensively exploited under field conditions for the control of coffee berry borer, *Hypothenamus hampei* (Ferrari) (Coleoptera: Scolytidae) in many countries around the world (Neves and Hirose, 2005).

1.2.4 Ecology of *B. bassiana*

Although first recognized as an insect pathogen, *B. bassiana* can exist endophytically in many wild and cultivated plant species (Vega, 2008). *B. bassiana* colonization of several herbaceous and woody species may

provide a degree of bio-protection to the hosts (Go´mez-Vidal *et al.*, 2006; Posada and Vega, 2005; Quesada-Moraga *et al*., 2006; Ownley *et al.*, 2008; Ownley *et al.*, 2010).

More is known about the role of *B. bassiana* as an insect pathogen than as an endophyte in plants, but recent interest has begun to spur new research into the subject. The ecology of this organism is complex and not well understood. It is ubiquitous in soils and can exist in multiple phases infecting members of multiple kingdoms (Bing and Lewis, 1993).

1.2.5 Effect of physical factors on growth and development of *B. bassiana*

The effectiveness of *B. bassiana* is influenced by a number of abiotic factors, including ultraviolet light, temperature, rainfall and moisture. The propagules of *B. bassiana* are damaged by solar radiation, and they are especially susceptible to the ultraviolet B (UVB) light. Susceptibility differs between species and among strains (Fargues *et al.*, 1996). A degree of protection is offered when fungal propagules are not directly exposed to solar radiation, such as within plant canopy, although even in such protected circumstances, the propagules will ultimately be killed (Smits *et al.*, 1996).

While most entomopathogenic fungi can tolerate a wide range of temperatures, the temperature range required for infection, growth and sporulation is often considerably narrower. In field trials to determine such effective ranges, it is a challenge to measure the macro- and micro-temperatures created by features of the landscape, weather, or the cage itself; therefore, most experiments on the effects of temperature on fungal development are conducted in controlled environment chambers. Because of this, results obtained in a laboratory may not necessarily reflect the true activity of the fungus. Such controlled experiments may also fail to

address the effect of insect behavior itself on the effectiveness of the fungus. A critical factor in the success of an entomopathogenic fungus is the presence of moisture. Dry conditions may inhibit spore production or spore germination. Although in dry weather, microhabitats may exist that harbor pockets of moisture in which fungi can complete the life cycle (Goettel *et al.*, 2000).

Free water or high relative humidity (> 90%) has been considered for many years as the most serious constraint for germination and growth. By contrast, a number of studies indicated that infection occurs independently of relative humidity (Ferron, 1977; Marcandier and Khachatourians, 1987; Ramoska, 1984; Schaerffenberg, 1964). High relative humidity is only required for fungal sporulation, which is necessary for horizontal transmission from cadavers (Ferron, 1978; Schaerffenberg, 1964). Humidity also affects the survival of conidia (Lingg and Donaldson, 1981), with long survival at low relative humidity.

Temperature affects both germination and mycelial growth. For most strains, the optimal temperatures are thought to fall between 23°C and 25°C (Ferron, 1978; Hall and Papierok, 1982). Temperatures above or below the optimum range prolong the lag phase and decrease the germination rate (Hywel-Jones and Gillespie, 1990). In this case, infection may be inhibited and mycosis retarded (Carruthers *et al.*, 1985; Fargues, 1972; Walstad *et al.*, 1970).

Physiological condition, temperature, nutritional stress and age may predispose insects to infection (Donegan and Lightheart, 1989; Ferron, 1978; Hall and Papierok, 1982); young developmental stages are usually most susceptible (Feng *et al.*, 1985; Quintela *et al.*, 1990). Insects may escape infection during molts (Fargues, 1972; Vey and Fargues, 1977) or

because of some protective structures such as the chorion and elytra that prevent fungal penetration (Hunt *et al.*, 1984; Vey *et al.*, 1982).

1.2.6 Nutritional factors

B. bassiana grows and sporulates profusely on various natural substrates, including bran, whole grains, potatoes, hay and straw (Fogal *et al.*,1986). Synthetic media used to stimulate sporulation contain either inorganic or organic nitrogen (Barnes *et al.*, 1975; Kucera, 1971). Various methods of liquid culture (submerged fermentation), solid culture (surface fermentation) or a combination of both (two phase systems) have been used for mass production (Roberts and Humber, 1981; Samsinakova *et al.*, 1981).

B. bassiana produces blastospores and conidia in liquid and solid cultures, respectively. Both propagules are infectious and can be formulated and applied like chemical pesticides (Auld, 1992; Goettel, 1992). Production of fungal conidia worldwide is carried out using simple technologies that demand low inputs (Ferron, 1978; Hussey and Tinsley, 1981; Alves and Pereira, 1989; Antía *et al.*, 1992; Jones and Burges, 1997). Most of the production of *B. bassiana* conidia in Colombia for coffee berry borer biocontrol is done using simple sterilization technique based on cooked rice placed inside a bottles. The conidia are mainly used for field spray applications (Posada, 1993; Bustillo and Posada, 1996).

Another methodology for conidial production involves the use of fermenters and artificial media. The advantages of this technology are that spores are easily harvested and can be used to prepare formulations. Some private companies have been trying to develop *B. bassiana* formulations as wettable powders and dispersible granules (Morales and Knauf, 1994; Marín *et al.*, 2000).

There is a high potential for the use of Hyphomycetes such as *Metarhizium* or *Beauveria* for biological control because such fungi can be cost-effectively, mass-produced locally, and many strains are already commercially available. For example, *B. bassiana* has been mass produced on different solid substrates, including sugarcane wastes (Somasekhar *et al.*, 1998), silkworm pupal powder (Chavan *et al.*, 1998), agar medium (Sergio *et al.*, 2003) and steamed rice (Feng *et al.*, 1994).

1.2.7 Use of *B. bassiana* as a biocontrol agent for pest control

The safety of *B. bassiana* as a biocontrol organism was evaluated based on impacts to nontarget insects and mammals including humans, and no safety concerns were identified (Zimmerman, 2007). Increasing levels of interest and new research are leading to new possibilities in various agricultural systems (Ownley *et al.*, 2008).

Inoculation with *B. bassiana* offers a novel organic and environmentally friendly method of reducing pest pressure and increasing the levels of natural products in economically important crops. Corn leaves inoculated with *B. bassiana* had effective and sustained biocontrol against the European corn borer (Wagner and Lewis, 2000).

Control of leaf hoppers on rice and tea crops has also been successful (Hussey and Tinsley, 1981). A product known as "Bovercin" developed from *B.bassiana* has been successfully and extensively used as a biocontrol agent for Colorado potato beetle and coddling moth across thousands of hectares in Russia (Ferron, 1981). Innoculation with *B. bassiana* has also displayed effectiveness in controlling various soilborne and foliar pathogens in many plant species (Renwick *et al.*, 1991) including *Fusarium* spp. (Reisenzein and Tiefenbrunner, 1997), *Rhizoctonia* spp. (Lee *et al.*, 1999; Ownley *et al.*, 2008), and *Pythium* spp. (Vesely and Koubova, 1994). Intentional inoculation has been

achieved with various methods, but the highest infection rates were observed using seed coating of conidia prior to germination (Ownley *et al.*, 2008; Quesada-Moraga *et al.*, 2006). This is likely due to the easy infection of young tender seedling tissues that lack well developed cuticles and the fact that germinating seedlings may not have developed environmentally- or developmentally-induced resistance mechanisms. Since it is a soil inhabiting fungus, seedling infection by *B. bassiana* may occur in nature.

Companies used *B. bassiana* to produce different kinds of commercial formulation (**Table 1)** to control crop insects.

Table 1. Commercial formulation of *B. bassiana* used as biological insecticides

Product	Formulation	Producer	Target host	Crops
Mycotrol O ®	Emulsifiable Suspension (ES)	Laverlam Int. Corp., USA	Whiteflies Aphids Grasshoppers Termites Potato beetle Bean beetle Weevil Cereal leaf beetle European corn borer	indoor/outdoor nursery, greenhouse, landscape ornamentals, field crops and vegetables
Botanigard®	ES	Mycotech Corp.,USA		
	Wettable Powder (WP)			
Naturalis TNO ®		Troy Biosciences, USA		

1.2.8 Infection Cycle

The entomopathogenic nature of *B. bassiana* is quite extensive as it possesses one of the largest host lists of entomopathogenic, imperfect fungi (Tanada and Kaya, 1993). This wide host range has enabled *B. bassiana* to become one of the most widely used fungal biological control agent.

The infective unit of imperfect entomopathogenic fungi such as *B. bassiana* is the conidium (Gillespie, 1988). Infection usually occur through the integument, but infection has also been observed through the gut and the oral cavity as well (Broome *et al.*, 1976). (Fig. 2)

Conidia of entomopathogenic Deuteromycetes attach to an insect cuticle mainly through high hydrophobic interaction forces between the conidial rodlet layer and the insect cuticle (Boucias *et al.*, 1988). Binding of recognition proteins to substances on the insect cuticle and production of enzymes (e.g. esterase, lipase, N-acetylglucosaminidase) by the pregerminating conidia may also be factors in attachment of conidia to the insect cuticle (Boucias and Pendland, 1991).

Once attached for a sufficient period of time under suitable conditions , a germ tube grows along the cuticle. Germinating conidia do require a useable source of carbon as well as a nitrogen source for hyphal growth. The enzymes produced by the conidia may play a role in providing this nutrition (Boucias and Pendland,1991; Tanada and Kaya, 1993).

Chemical and physical stimuli may orient the growth of the germ tube towards an appropriate infection site, but a well developed system of this type is not likely to be found in *B. bassiana*, as it can infect a large range of hosts, and does not necessitate a very specific infection site (St. Leger, 1993). It has been shown, however, that conidia of *B. bassiana* adhering to heavily sclerotized regions, produce germ tube until an easily penetrated area is reached (Pekrul and Grula, 1979).

With *Metarhizium anisopliae*, another entomopathogenic deuteromycete, once a suitable penetration site is found, the germ tube differentiates into several specialized penetration structures, including appressoria, penetration pegs and penetrant hyphae.

The germ tube forms appresoria, which serve as an attachment site and may provide the "fulcrum" that is needed for the remainder of the penetration process (St. Leger, 1993).

Appresoria are also the source of cuticle degradation providing some nutrition for the fungal spores (St. Leger, 1993). Formation of appresoria by *B. bassiana* has not been observed. A combination of physical and chemical chitin degrading forces most likely allow for the penetration of the fungi into the insect hemocoel (Ferron, 1981).

The insect cuticle provides a formidable mechanical barrier to the invading fungal spore. It may also further prevent successful breach through the melanization of cuticle cells surrounding the penetrating hyphae or through the increased production of lysozyme and other antimicrobial proteins (Vilcinskas and Gotz, 1999). This penetration event can serve to increase the vulnerability of the insect to bacterial infections, as tissue and cuticle surfaces are breached (Vey and Fargues, 1977).

Once inside the insect body, the fungus produces hyphal bodies, which multiply by budding and circulate briefly in the hemocoel before invading muscle and fat tissues (Tanada and Kaya, 1993). Production of hyphal bodies has been shown to reduce the number of insect hemocytes, resulting in immunosuppression of the insect (Hung *at al.*, 1993; Pendland *et al.*, 1993). Insect hemocytes recognize an invading fungus as non-self propagules and attempt to phagocytose the invading blastospores. This method is usually not effective against highly virulent fungi such as *B. bassiana* (Vey and Gotz, 1986).

Encapsulation of the invading fungal body may be more successful. Encapsulation occurs when hemocytes attach to the blastospore surrounding it in layers that eventually melanize, preventing the spore from producing further infective hyphae (Vey and Gotz, 1986).

The fungus may outgrow this barrier and cause subsequent death, or the insect may successfully prevent the infection, and the capsule can remain in the hemocoel for an extended period of time.

Studies have shown that hyphal bodies circulating in insect hemolymph may shed parts of cell wall or lack sugar residues that are integral in the activation of insect immune system (Pendland *et al.*, 1993; Vilcinskas and Gotz, 1999).

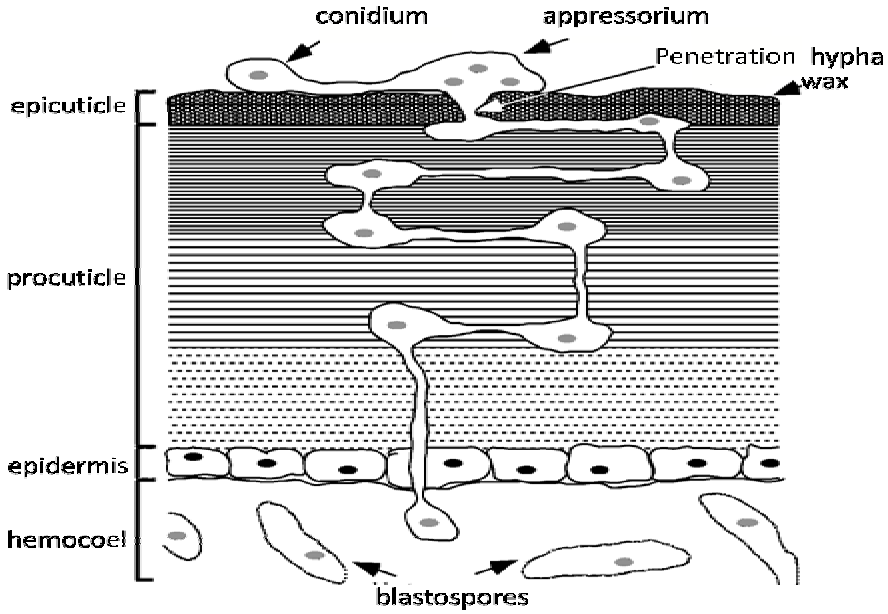


Figure 2. Structure of insect cuticle, and mode of penetration of entomopathogenic fungi (Adapted from Clarkson and Charnley (1996), St. Leger (1991)).

In later stages of mycosis, hyphal bodies have been found to possess a surface coat that mimics that of host cells, further evading detection by immune systems (Boucias *et al.*, 1995). There may also be immunosuppressive properties of *B. bassiana* spores that contribute to reducing the spread of hemocytes (Hung *et al.*, 1992).

B. bassiana also produces secondary metabolites which are suspected to play a role in their pathogenicity, although conclusive proof of their involvement has not been found. *B. bassiana* produces two families of secondary metabolites, beauverolides and cyclosporins (Vilinskas and

Gotz, 1999). High levels of beauvericin, a beauverolide could not be found in the hemolymph of *B. bassiana*-infected corn earworms before and leading up to the time of death.

Injections of beauvericin did not cause death. Charnplin and Grula, (1979) concluded that this toxin most likely does not play a role in the virulence of *B. bassiana*. It may, however, serve an anti-microbial function. Bassianolide, another beauverolide has been shown to be toxic when injected into lepidopteran larvae (Boucias and Pendland, 1988). Cyclosporins have also not been shown to have consistent insecticidal properties, but play a role in immune suppression in vertebrates (Vilinskas and Gotz, 1999).

Insect death from *B. bassiana* can result from hemocyte reduction, soluble nutrient reduction, susceptibility to toxins produced by the fungus, or from bacterial septicemia caused by penetration events. Once the host has expired, the fungus grows saprophytically. the mycelia eventually cover the insect body, and conidia are produced within a few days. (Boucias and Pendland, 1988).

Study objectives

1. **Development of selective media for isolation of *Baeuveria bassiana* from soil.**
2. **Study the distribution of the fungus *B. bassiana* in the West Bank soils.**
3. **Assess the virulence of *B. bassiana* isolates against *C. capitata*.**
4. **Study the factors that influence growth and development of *B. bassiana* and its application as a bioagent.**

Chapter 2: Materials and Methods

2.1 Development of modified growth media for the isolation of *B. bassiana* from soil

Glucose Peptone Agar (GPA) was used as growth media for the isolation of *B. bassiana* from soil after modification by adding different concentrations of the fungicide Dodine (inert). *B. bassiana* isolate used in this experiment was obtained from the "Plant Protection Research Center" fungal collection at Hebron University.

The GPA medium was composed of: 20 g glucose, 10 g peptone, 2 g yeast extract, 15 g agar, 0.3 g Chloramphenicol, all mixed into 990 ml deionized water (DW).

Different concentrations of Dodine (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 ppm) were added to the medium. *B. bassiana* conidia were harvested from 21 days old cultures. The number of conidia was determined by using a haemocytometer. The concentration of the conidial suspension was set to $4.5x10^7$ conidia/ml; 250 µl of the suspension was then added to 100 ml DW. The spore suspension was then added to 100 g autoclaved soil taken from a vegetable field. A total of 10 g of inoculated soil were added to 100 ml of DW. The mixture was then shaked for 24 hours on mechanical shaker. A volume of 200 µl were then seeded onto a GPAD plate and incubated at 25 ± 1ºC for 8 days under continuous light. The number of spores germinated was then determined as CFU/gm soil. The experiment was repeated twice and the design was completely randomized with three replicates per treatment.

2.2 Isolation of *B. bassiana* from the West Bank soils on Glucose Pepton Agar Dodine (GPAD) medium

Soil samples (300 g each) were collected from 225 different open fields usually grown with vegetables and field crops in the West Bank (Fig.4).

The Samples were collected from various governorates of the West Bank (54 Jenin, 23 Hebron, 120 Jericho, 16 Tulkarem and 12 Qalqelyia) (Table. 2). The samples were collected from soil at 5-15 cm depth, and kept in plastic bags immediately. A weight of 10 g of each collected soil sample was suspended in 90 ml DW (10^{-1} dilution) and shaked for 30 minutes at 200 rpm. A volume of 1 ml was then taken and suspended into 9 ml DW (10^{-2} dilution). Another dilution (10^{-3}) was also prepared in the same manner. A volume of 200 µl from each of the two dilutions (10^{-2} and 10^{-3}) were spread on GPAD medium with three replicates/each. Plates were then incubated at 25±1 ºC under continuous light and inspected daily for 6 days. *B. bassiana* colonies were identified by its growth habits and morphological characters (Huang and Fan, 2001). (Fig.3).

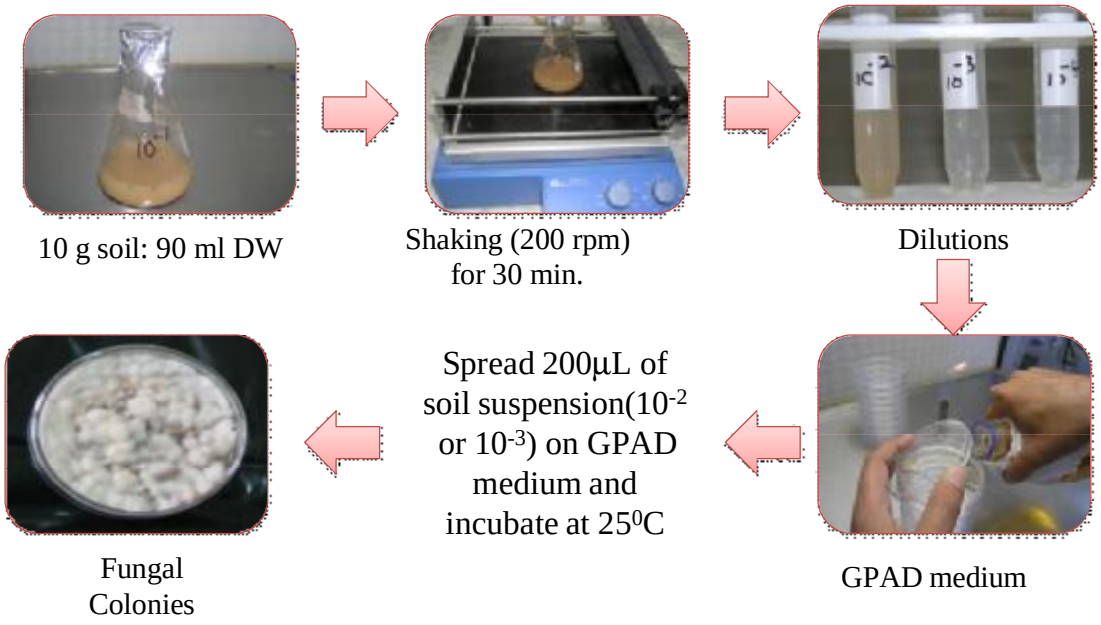


Figure 3. Dilution plate technique for the isolation of *B. bassiana* from soil using GPAD medium.

Table 2. Number and geographical origin of soil samples collected from various soils in the West Bank.

Soil samples	Site
Jenin (Total = 54)	
5	Kufrdan
5	Burqeen
13	Beer El Basha
1	Um Qaraqi
4	Ya'bad
2	Qabatia
15	Jalameh
4	Yamoun
5	Beit Qad
Tulkarem (Total = 16)	
2	Thennabeh
1	Joret Ghazleh
4	Anabtah
2	Dair Ghsoun
5	Attil
2	Khdouri
Qalqeliya (Total = 12)	
2	Azzoun
4	Ras Atteyah
5	Hablah
1	Fondoq
Jericho (Total = 120)	
2	Jericho Agricultural Station
2	Amman St.
2	Maghtas St.
1	Tourism Square
1	Awqaf plot
1	Ketf El wad
5	Seekh plot
1	Alquds St.
1	Marashaat plot
2	Khdawe
1	Akbat Church
3	Hisham Palace St.
2	Kharjah
1	Ein Sultan
11	Dyouk Tahta
6	Dyouk Fouka
8	Nwa'me Fouka
4	Nwa'me Tahta
7	Ein Baida

15	Bardala
4	Kardala
10	Marj Na'ja
5	Zbeidat
4	Marj Ghazal
13	Jeftlek
2	Fasayel
6	Auja
Hebron (Total = 23)	
2	Alarroub Agricultural Station
3	Alarroub University Station
16	Dura
2	Shyoukh

Figure 4. Origin of soil samples per governorate of the West Bank. (Source: GIS lab, Hebron University)

2.3 Effect of temperature on growth of selected *B. bassiana* isolates

Five native *B. bassiana* isolates (Bv.32, Bv.39, Bv.41, Bv.51 and Bv.52) described later as the most promising were used in these experiments. Growth rate, sporulation and spore germination of the five isolates were evaluated under several temperatures.

2.3.1 Growth rate

For measuring the mycelium growth rate, three plates of GPA medium were inoculated with single spore of each of the 5 isolates taken from 21-days old cultures. Plates were then incubated under different temperatures: 5, 10, 15, 20, 25, 30, and 35 ºC and continuous light condition. Measurements were taken as means of colony diameters after 4 and 7 days following inoculation. (Fig. 5)

The experiment was completely randomized with three replicates. The rate of increase in diameter was calculated by the formula:

Rate = D2 –D1 / T2 –T1

Where:

D2: the second reading of the growth diameter

D1: the first reading of the growth diameter

T2: the time of the second reading

T1: the time of the first reading.

2.3.2 Conidial germination

For measuring spores germination, conidia were harvested from 21 days old cultures of the five selected isolates (Bv.32, Bv.39, Bv.41, Bv.51 and Bv.52) with 3ml DW. Conidia concentrations were set to 700 spores/ ml. For each native isolate, aliquots of 500 µl of the above concentration were spread on three GPA medium plates and then incubated at (10, 15, 20, 25 and 30°C) and continuous light. The experiment was completely randomized with three replicates per each temperature. Number of colonies was determined 5 days after inoculation. (Fig 5.)

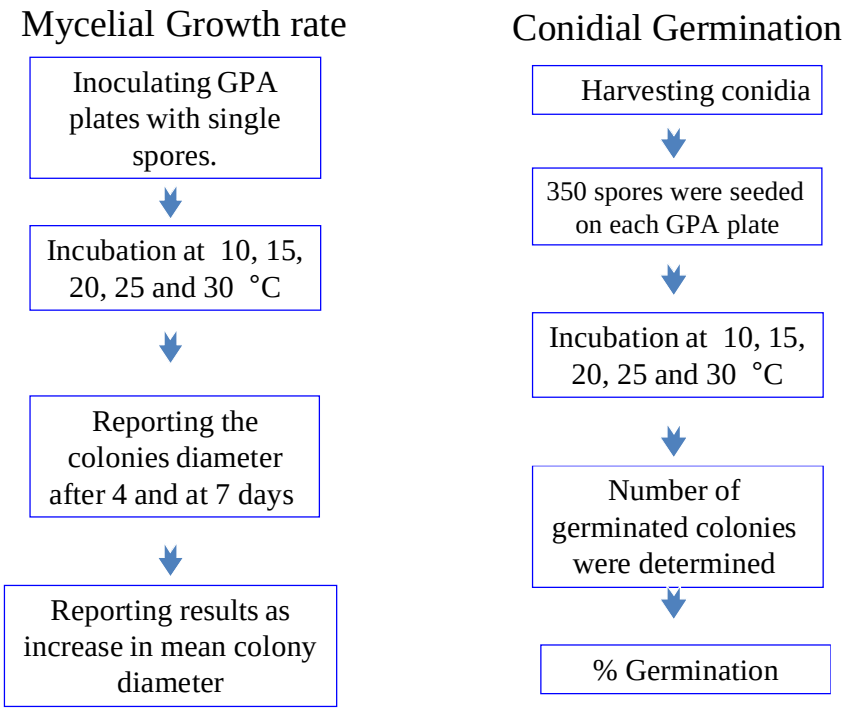


Figure 5. Evaluation of mycelial growth rate and conidial germination of *B. bassiana* isolates under different temperatures.

2.3.3 Sporulation of *B. bassiana*

For measuring spore's production, three 90 mm diameter plates of GPA medium were inoculated with mycelial discs (5mm) of each of the isolates (Bv.32, Bv.39, Bv.41, Bv.51 and Bv.52) taken from 21day old cultures.

Plates were then incubated under continuous light at various temperatures (10, 15, 20, 25 and 30 °C). After 21 days, mycelial disks (0.5cm diameter) were taken from each plate (replicate) and homogenized in 5ml of 95% ethyl alcohol. Haemocytometer was used to determine spores production per unit area of the plate. The experiment was completely randomized with three replicates per each treatment.

2.4 *Ceratitis capitata* rearing on artificial diet

Mediterranean fruit fly cultures were established from a pupae obtained from the lab of (Prof. S. Applebaum of the HUJ). Neonates were reared

later in lab on larval bran diet composed of (Bran 400g; Brewer's yeast 120 g; Sucrose 180 g; Nipagin (mold inhibitor) 6 g; HCl 24 ml(32%); and Water 760 ml) mixed together and kept in the refrigerator at 4 °C. Adults were reared in closed plastic boxes with circular openings at the front and back sides and covered with a mesh. Plastic dishes (15cm) were filled with 50 ml DW and placed under the openings in the bottom of the boxes. Adult females extend their ovipositors through the mesh openings and lay eggs. Eggs then fall into the water filled dishes. After one day eggs were collected using a 5ml pipette and filtered in cheese cloth. The cheese cloth was then placed in glass dishes (15cm, diameter) and covered with larval diet. Plates were then covered and incubated at 25 °C. Ten days later, the plates were taken and placed into larger plastic containers (40x30x25 cm) and covers were removed.

The plastic container contained fine vermiculite on which larvae could jump and pupate inside. A day later, vermiculite were filtered using a 2mm sieve, and pupae were transferred to plastic Petri dishes (9cm) and incubated at 25°C for 10 days.

Pupae plates were then transferred to the adult plastic containers. The containers contained moistened cotton amended with adult diet (1 part brewer's yeast and 4 parts sugar). Adult females started laying eggs after 4 days from emergence. Feeding was then continued and life cycles were repeated accordingly.

2.5 Bioassay (Infectivity Experiments)

2.5.1 Screening the Virulence of native *B. bassiana* isolates

Screening was done on 58 isolates of *B. bassiana*. The experiment was done 4 times using 14, 15, 15 and 14 isolates each time. In each experiment conidia of *B. bassiana* were harvested from 21 days old cultures as described earlier. A weight of 0.02g of dry conidia were then

placed in a test tube. Twenty adult fruit flies (5-10 days old) were placed inside each test tube and shaken gently for two minutes to inoculate the flies with conidia. Inoculated fruit flies were then transferred to the plastic cages.

The plastic cages were then supplied with a piece of moistened cotton which has a small amount of adult food, and incubated at 25 ±1 ºC.

Mediterranean fruit flies were supplied with water and food on a daily basis. Flies mortalities were recorded every day as well. The most virulent five isolates were then taken from each patch and further screened for virulence and other parameters. Each experiment was completely randomized with 3 replicates. (Fig. 6)

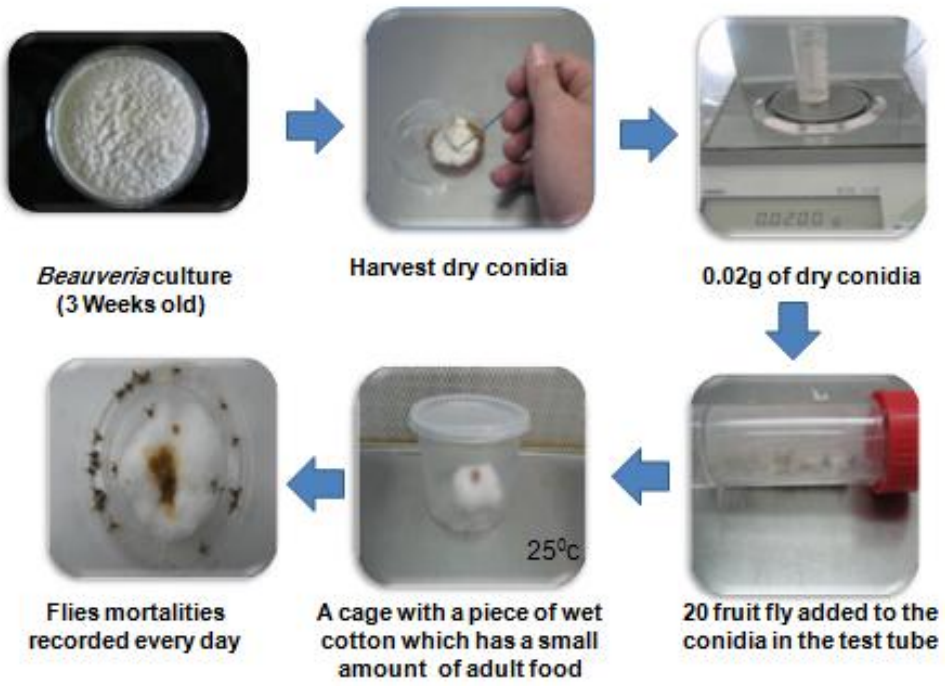


Figure 6. Procedure for screening virulence of native *B. bassiana* isolates against *C. capitata* adult flies.

2.5.2 Effect of *B. bassiana* on mortality of different *C. capitata* stages.

The objective of these experiments was to test the virulence of *B. bassiana* on morality of larval and pupal stages of *C. capitata* using two different inoculation procedures (feeding and contact):

2.5.2.1 Assessment of Larval mortality

In the first method (feeding), 20 larvae of *C. capitata* (3 days old) were obtained. *B. bassiana* conidia were harvested from a sporulating 21 days-old cultures with 3 ml DW. The concentration of conidia was set at $1x10^8$ conidia/ml. A volume of 19 ml of the conidial suspension were mixed with the larval diet composed of (10g bran, 3g Brewer's yeast, 4.5 g Sucrose and 0.6 ml HCl (32% normality). A total of 10 grams from this mixture were transferred to Petri plates (9cm diameter), and 20 larvae were added gently. The plates were then covered and incubated at 25 ±1ºC. Larval mortality was then assessed by counting the emerging living adults within 15 days.

In the second method (contact), 20 larvae from *C. capitata* (3 days old) were obtained and placed into a beaker (50 ml). *B. bassiana* conidia were harvested from a sporulating culture (21 days old) with 3 ml DW. The concentration of conidia was set at $1x10^8$ conidia/ml. A volume of 19 ml of the conidial suspension were poured into the larvae-containing -beaker and mixed gently for 30 seconds. The larvae were then removed gently from the beaker and transferred to a plate containing 10g larval diet. Plates were then covered and incubated at 25 ±1ºC. Larval mortality was then assessed by counting the emerging living adults within 15 days (Fig.7).

2.5.2.2 Assessment of Pupal mortality

In the first method (soil inoculation), 20 pupae of *C. capitata* (1 day old) were obtained. *B. bassiana* conidia were harvested from a sporulating culture (21 days old) with 3 ml DW. The concentration of conidia was set at $1x10^8$ conidia/ml. A volume of 10 ml of the conidial suspension were mixed with 50g autoclaved soil. A volume of 10 grams from this mixture were transferred to Petri plates (9cm diameter), and 20 pupae of *C. capitata* were added. The plates were then covered and incubated at 25 ±1ºC. Pupal mortality was then assessed by counting the emerging living adults within 9 days.

In the second method (contact), 20 pupae of *C. capitata* (1 day old) were obtained and placed into a beaker (50 ml). *B. bassiana* conidia were harvested from a sporulating culture (21 days old) with 3 ml DW. The concentration of conidia was set at $1x10^8$ conidia/ml. A volume of 20 ml of the conidial suspension were poured into the pupae-containing -beaker and mixed for 60 seconds. The pupae were then removed from the beaker and transferred to a plate containing 10g autoclaved soil. Plates were then covered and incubated at 25 ±1ºC. Pupal mortality was then assessed by counting the emerging living adults within 9 days (Fig.7).

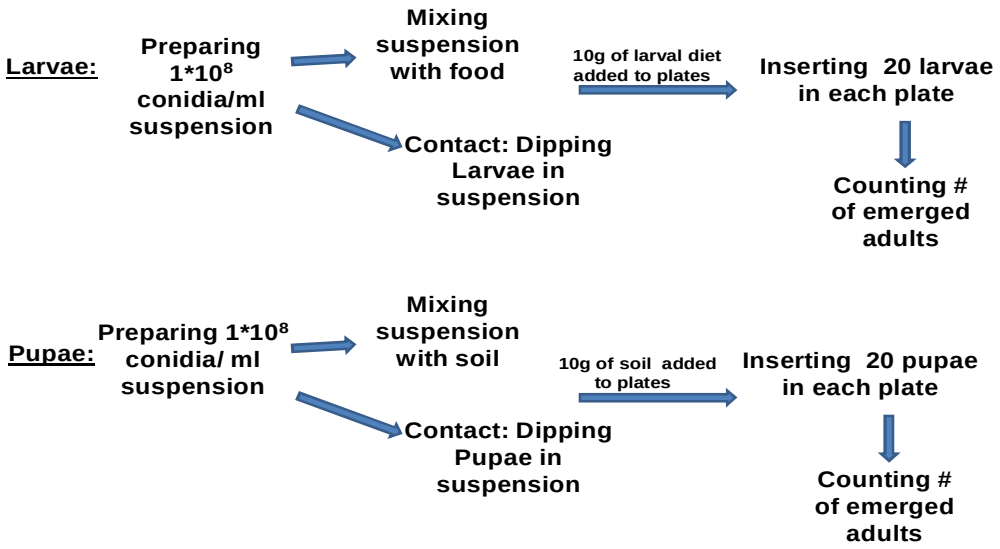


Figure 7. Procedure for evaluating the effect of *B. bassiana* on larval and pupal mortalities of *C. capitata* using different inoculation methods.

2.5.3 The effect of inoculum (conidia) concentration of *B. bassiana* on Mediterranean fruit fly mortality.

B. bassiana isolates (Bv32, Bv39, Bv41, Bv51 and Bv52) were used as the source of inoculum. *B. bassiana* conidia were harvested from 21 days old cultures. Five conidial concentrations (10^5, 10^6, 10^7, 10^8 conidia/ml) were used. For control treatment, SDW was used. A volume of 0.5 ml from each concentration was suspended into a test tube. A total of 20 adult fruit flies (5-10 days old) were inserted in the test tube and mixed gently together for twenty seconds. Flies were then transferred to plastic containers described earlier and incubated at 25 ±1ºC under continuous light (Fig.8). The number of dead Medflies was recorded daily. The experiment was completely randomized with three replicates in each treatment.

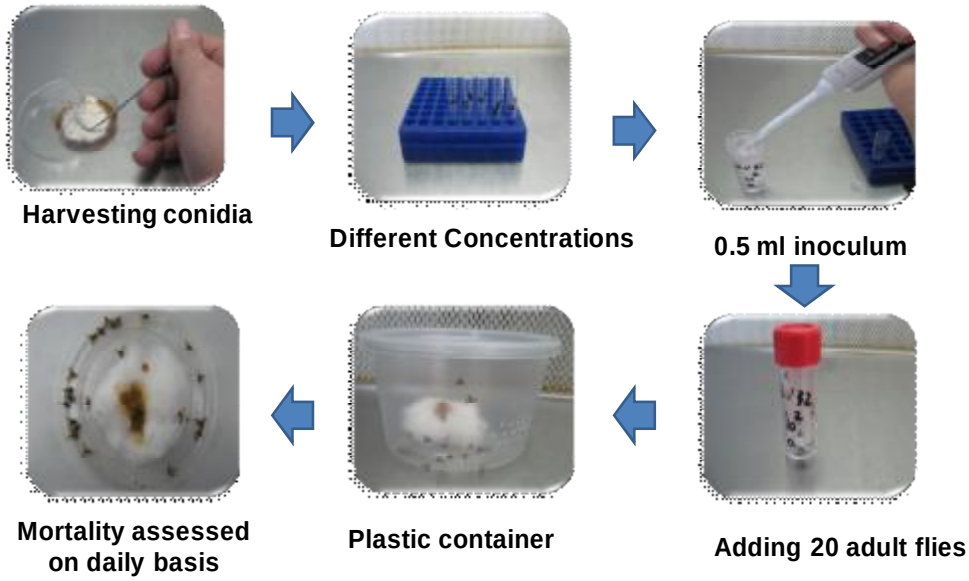


Figure 8. Protocol for testing the effect of *B. bassiana* conidial concentration on mortality of *C. capitata* in vitro.

2.5.4 The effect of temperature on virulence of *B. bassiana against C. capitata* adult flies.

B. bassiana isolates (Bv32, Bv39 and Bv51) were used as the source of inoculum. The conidia were harvested as described earlier from 21 days old cultures. The concentration of the conidia was set at $1x10^8$ conidia/ml. A volume of 0.5 ml of the conidial suspension was suspended into each test tube for each isolate. Twenty fruit flies (5-10 days old), were added to each test tube and mixed gently for twenty seconds and then transferred to plastic containers described earlier. Five plastic containers were incubated in five incubators set at different temperatures (10, 15, 20, 25 and 30°C). Number of dead Medflies was reported daily over a period of 8 days. The experimental design was completely randomized with three replicates (Fig. 9).

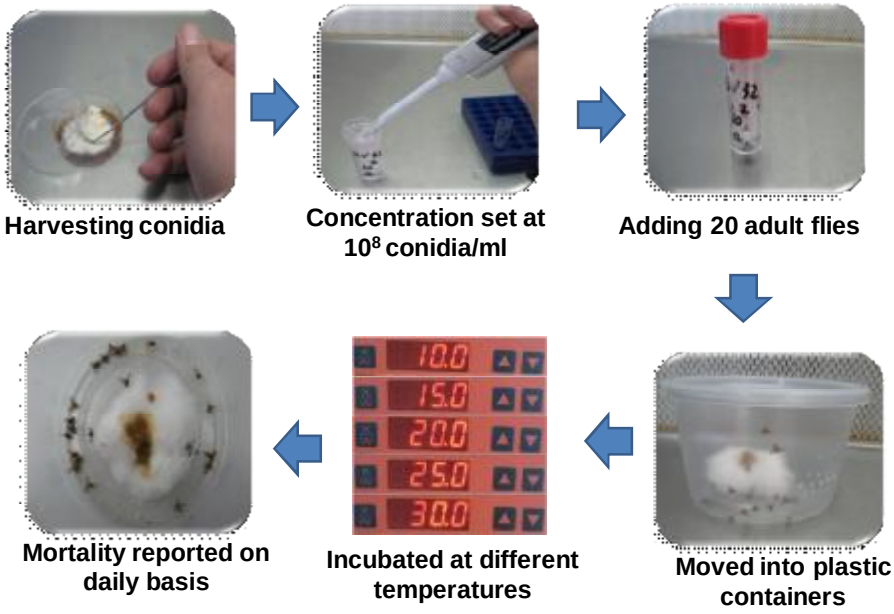


Figure 9. Protocol for testing the effect of temperature on virulence of *B. bassiana* against *C. capitata* in vitro.

2.5.5 Serial culturing of *B. bassiana* inoculum

This experiment was conducted to test the difference in virulence of two isolates (Bv32 and Bv39) following the serial culturing of the fungus. To have a basis for comparison, following the usual isolation and inoculation procedure, *B. bassiana* was first taken from dead Medfly. Conidial concentration was set to $1x10^8$ conidia/ml and used to inoculate 20 adult medflies (5-10 days old); inoculated flies were incubated in plastic cage at 25 ±1ºC. Number of dead Medfly was recorded on daily basis. In another related treatment, *B. bassiana* culture initially isolated from dead medfly was maintained under 25 ±1ºC and renewed again on GPA medium after 21 days. This re-culturing was repeated for 4 times over a period of 84 days. Conidia from the 4^{th} serial culture were harvested and concentration was set to $1x10^8$ conidia/ml . The inoculum was then used to inoculate another 20 adult medflies (5-10 days old). Inoculated flies were incubated in plastic cage at 25 ºC. Results (number of dead

Medflies) were reported on daily basis. The experiment was completely randomized with three replicates in each treatment.

2.5.6 *B. bassiana* method of application

Two methods of application (Feeding and contact) of five selected isolates of *B. bassiana* (Bv.32, Bv.39, Bv.41, Bv.51 and Bv.52) were evaluated in this experiment. The aim of this experiment was to determine the most effective method for using *B. bassiana* conidia to induce the highest mortalities of medfly.

In the first method (feeding), conidia of *B. bassiana* were harvested with 3 ml DW from 21 days old cultures. The concentration of conidia was set at $1x10^8$ conidia/ml. A volume of 0.5 ml of the suspension was mixed with 1g of adult flies' diet and placed on a piece of cotton inside the plastic container. A total of 20 adult flies (5-10 days old) were placed in the container. The container was then incubated at 25 ±1ºC under continuous light.

In the second method (contact), 0.5 ml of the inoculum $1x10^8$ conidia/ml was suspended in a test tube. and 20 adult flies of the same age (5-10 days old) were added to the test tube, mixed gently and placed in a plastic container containing adult flies' diet. All plastic containers were placed in the same incubator at the same temperature of 25 ±1ºC under continuous light. Mortality from both treatments (methods) was recorded on a daily basis. The experiment was completely randomized with three replicates in each treatment.

2.5.7 Bioassay on Peach fruits

The objective of this experiment was to investigate the ability of *B. bassiana* to control the medfly infestation on Peach fruits *in vitro*. Conidia of *B. bassiana* (Bv.32 and Bv.39) were harvested with 3 ml DW from 21 days old cultures. The concentration of conidia was set at $1x10^8$ conidia/ml. Three fully riped peach fruits (taken from an early fruiting cultivar) and obtained from the market were sprayed with the conidial suspension until saturation and left for one hour to dry. A control set of 3 fruits were sprayed with SDW.

The fruits were then placed in a plastic container ventilated from the upper side. The container included moistened cotton amended with med fly's adult diet. A total of 15 ovipositing medfly adult females and 6 adult males were inserted in the container. The container was then covered and incubated at 25 ±1ºC under normal light conditions. After 48 hours post insect introduction, medflies were removed from the cage. Seven days later, each peach fruit was opened and the number of medfly larval infestation was counted in each fruit and recorded. The experimental design was completely randomized with three replicates.

2.6 Statistical analysis

The data were stastically analysed using One-way repeated analysis of variance (ANOVA). Fishers LSD test ($P \leq 0.05$) was used for mean's separation. Linear regression was used to measure lethal concentrations (LC_{50}) and lethal time (LT_{50}) for *B. bassiana* isolates. (SigmaStat® 2.0 program, SPSS Inc. USA).

Chapter 3 :Results

3.1 Development of modified growth media for the isolation of *B. bassiana* from the soil

In this experiment, GPA medium was amended with several concentrations of Dodine for improving selectivity to the benefit of *B. bassiana* .

Numbers of *B. bassiana* colonies per plate increased with increasing Dodine concentration up to 50 ppm, after which it started to decline. The number of other fungal colonies kept at a steady paste at low Dodine concentrations (0 – 60 ppm) after which started to decline. The best Dodine concentration which provided selective advantage for *B. bassiana* over other fungi was 90 ppm (Fig. 10).

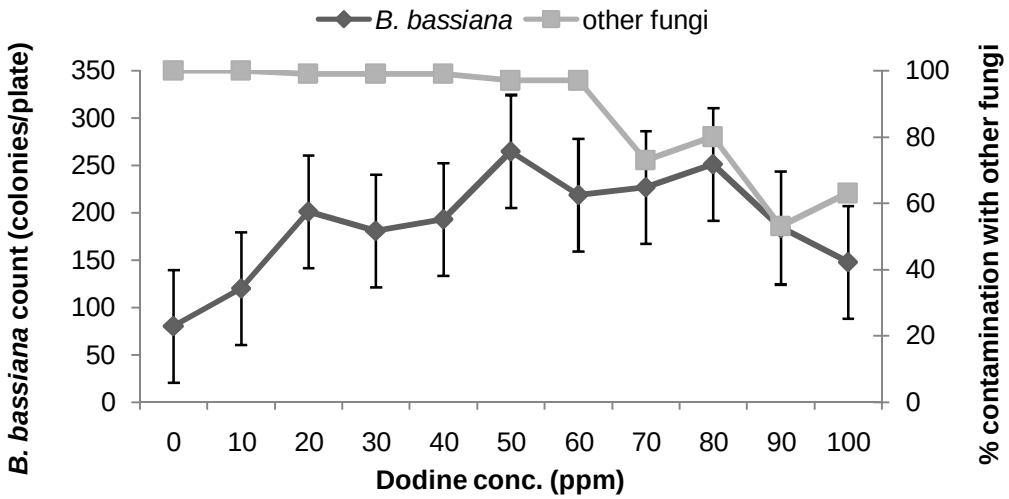


Figure 10. The effect of different Dodine concentrations on the selectivity of GPA medium for the isolation of *B. bassiana* from soil. (LSD = 59.5)

3.2 Isolation of *B. bassiana* from the soil of different agricultural sites in the West Bank

In this experiment, native isolates of *B. bassiana* were recovered from different sites of the West Bank. A total of 58 isolates of *B. bassiana* were recovered from a total of 225 soil samples collected earlier from various areas in the West Bank. The highest number of *Beauveria* isolates were collected from Jericho district (74.1 %) followed by Jenin district (13.8 %) and Hebron district (12 %), whereas, no *Beauveria* isolates were found in samples collected from the Tulkarem and the Qalqelya districts (Table 2, 3 and Fig. 4).

Table 3. Percentage of *B. bassiana* isolates recovered per each governorate of the West Bank

Governorate	% *B. bassiana* recovered from each governorate	% out of total soil samples
Hebron	12	10.2
Jericho	74.1	53.3
Tulkarem	-	7.1
Qalqelya	-	5.3
Jenin	13.8	24

Table 4. Origin of B. *bassiana* isolates recovered from agricultural soil of different sites of the West Bank

Site	Isolate	Site	Isolate
Bv1	Dura	Bv30	Kardala
Bv2	Dura	Bv31	Marj Na'ja
Bv3	Dura	Bv32	Marj Na'ja
Bv4	Dura	Bv33	Marj Na'ja
Bv5	Dura	Bv34	Marj Na'ja
Bv6	Burqeen	Bv35	Marj Na'ja
Bv7	Beer El Basha	Bv36	Marj Na'ja
Bv8	Beer El Basha	Bv37	Zbeidat
Bv9	Beer El Basha	Bv38	Zbeidat
Bv10	Jalameh	Bv39	Zbeidat
Bv11	Jalameh	Bv40	Marj Ghazal
Bv12	Yamoun	Bv41	Jeftlek
Bv13	Beit Qad	Bv42	Jeftlek
Bv14	Jericho station	Bv43	Jeftlek
Bv15	Seekh plot	Bv44	Jeftlek
Bv16	Marashaat plot	Bv45	Jeftlek
Bv17	Ein Sultan	Bv46	Jeftlek
Bv18	Dyouk Tahta	Bv47	Jeftlek
Bv19	Dyouk Fouka	Bv48	Jeftlek
Bv20	Dyouk Fouka	Bv49	Jeftlek
Bv21	Nwa'me Fouka	Bv50	Auja
Bv22	Nwa'me Tahta	Bv51	Auja
Bv23	Ein Baida	Bv52	Auja
Bv24	Ein Baida	Bv53	Auja
Bv25	Bardala	Bv54	Auja
Bv26	Bardala	Bv55	Auja
Bv27	Bardala	Bv56	Auja
Bv28	Bardala	Bv57	Shyoukh
Bv29	Bardala	Bv58	Shyoukh

Figure 11. Cultures (20 days old) for local isolates of *B. bassiana* (Bv32, Bv39, Bv41, Bv51, Bv52) recovered from different sites of the West Bank soils.

3.3 Effect of temperature on growth parameters of selected native *B. bassiana* isolates

3.3.1 Growth rate

This experiment was conducted to check the optimum temperature for mycelial growth of the five local isolates of *B. bassiana* (Bv32, Bv39, Bv41, Bv51, Bv52). All B. *bassiana* isolates were able to grow at the temperature range (10–30 °C). The optimum temperature for mycelial growth of all isolates was (25–30 °C). All isolates failed to grow 5 °C and 35 °C. However, there was no significant differences in general between the five isolates in this respect (Fig. 12 and 13).

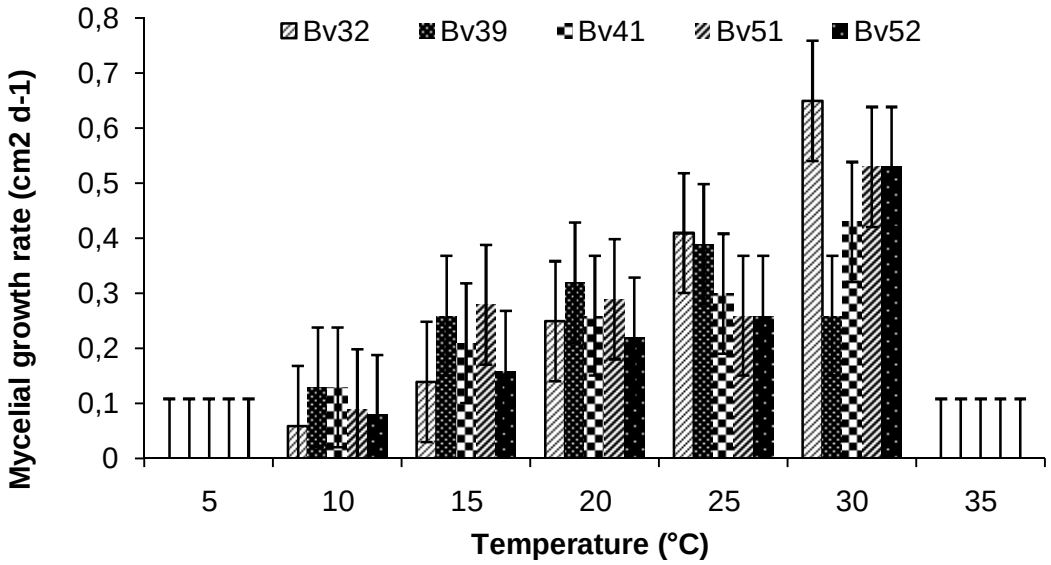


Figure 12. The effect of different incubation temperatures on mycelial growth rate of *B. bassiana* isolates. (LSD = 0.109)

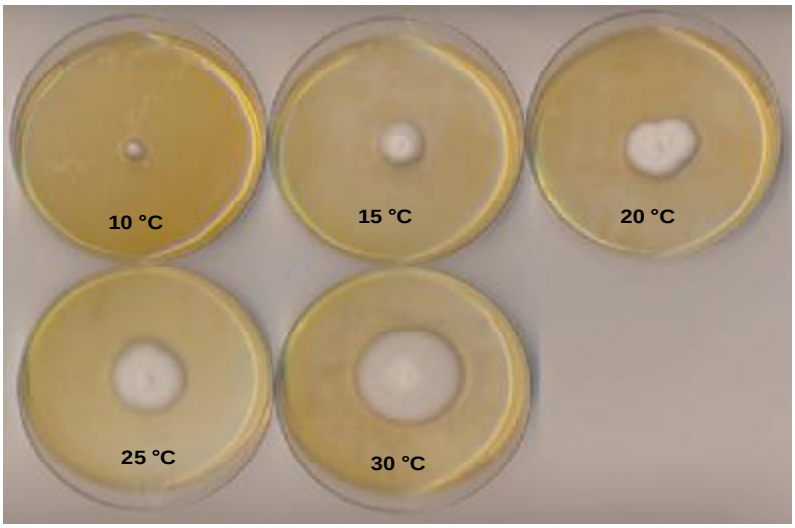


Figure 13. Effect of temperatures on mycelium growth rate of Bv32 growing on GPA after 9 days.

3.3.2 Conidial germination

This experiment was conducted to evaluate the optimum temperature required for conidial germination of *B. bassiana* isolates(Bv32, Bv39, Bv41, Bv51 and Bv52). Conidia of all isolates germinated well at temperatures ranging between 15-30 ºC but failed to germinate at temperatures below 15 and above 30 ºC. The optimum temperature for germination was in the range 15- 20 ºC without significant differences between isolates in general. (Fig. 14, 15)

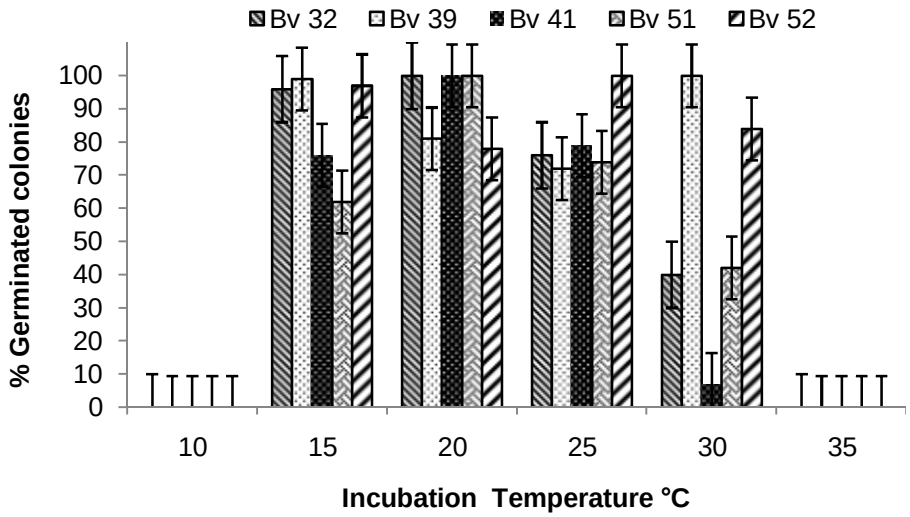


Figure 14. The effect of different incubation temperatures on conidial germination of *B. bassiana* 5 native isolates after 6 days of incubation under continuous light. (LSD = 9.457)

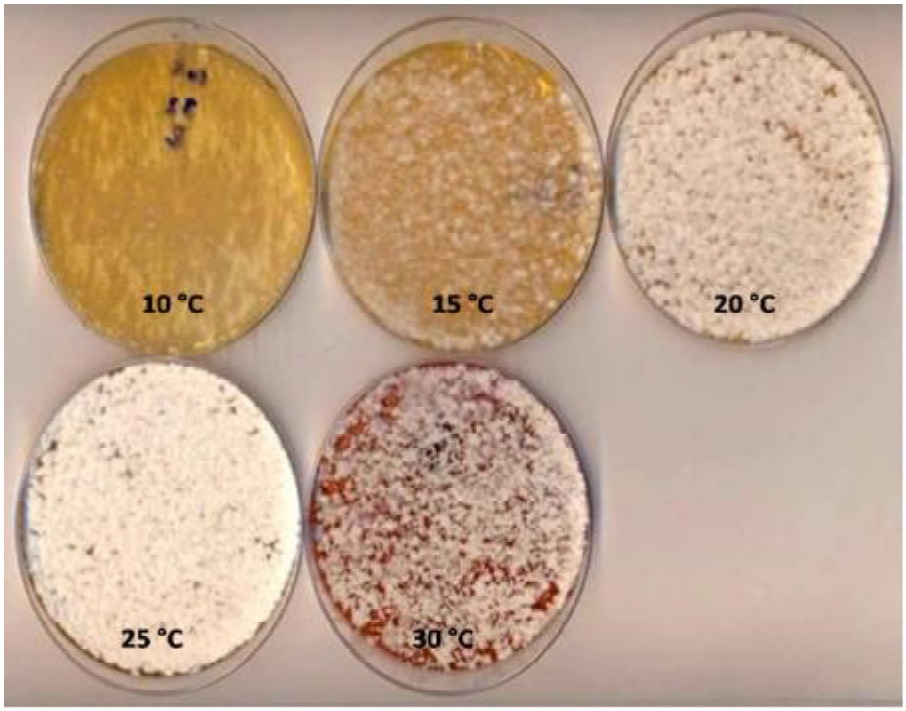


Figure 15. Effect of temperatures on conidial germination of Bv32 growing on GPA after 6 days.

3.3.3 Sporulation

This experiment was conducted to evaluate the effect of different temperature regimes on sporulation of *B. bassiana*. All isolates were able to produce conidia at the temperature range of 15-30 ºC. Number of conidia produced ranged from $19x10^7$ to $67x10^7$ conidia/cm^2 of mycelial colony under light. The favorable temperature which supported optimum conidiation was 25 ºC. Conidia production decreased at 30 ºC for all isolates and completely stopped at 35 ºC. Variation was obvious between isolates (Fig.16).

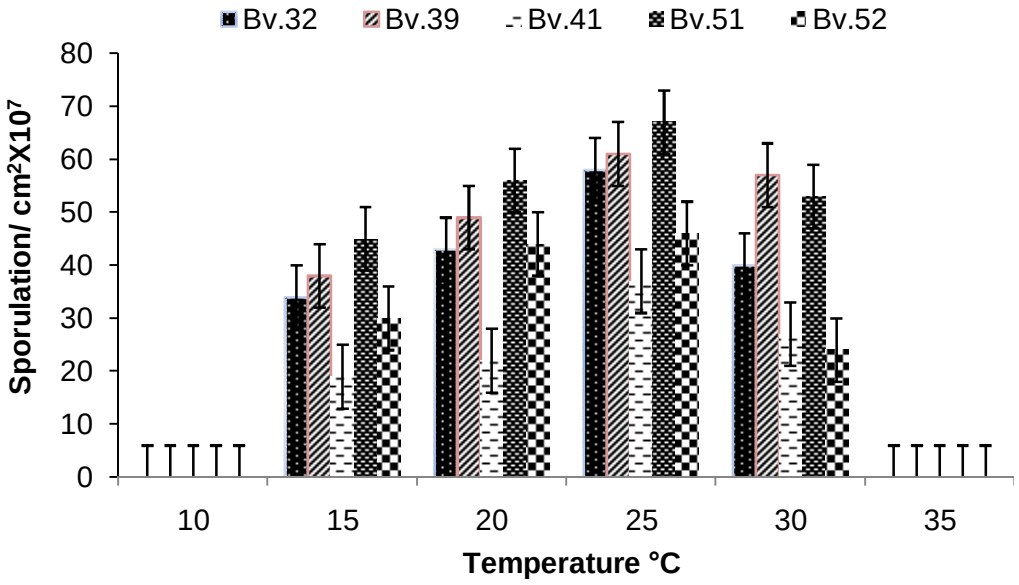


Figure 16. Effect of temperature on conidiation of 5 isolates of *B. bassiana* grown on GPA medium and incubated under light conditions after 21 days of inoculation.

3.4 Bioassay (Infectivity Experiments)

3.4.1 Screening the Virulence of native *Beauveria* isolates

The objective of this experiment was to assess the virulence of native *B. bassiana* isolates against *C. capitata* adult flies. The isolates were grouped into 4 categories (A, B, C and D). The most promising five isolates of *B. bassiana* from each group were identified. The most

virulent isolates from group A were (Bv.32, Bv.5, Bv.52, Bv.41 and Bv.9), group B (Bv.6, Bv.35, Bv.16, Bv.8 and Bv.48), group C (Bv.27, Bv.58, Bv.44, Bv.18 and Bv.37), and group D (Bv.39, Bv.23, Bv.17, Bv.51 and Bv.21) (Fig.17, 18, 19, 20 and 21).

Figure 17. *B. bassiana* Bv 32 infection symptoms on *C. capitata* adult flies.

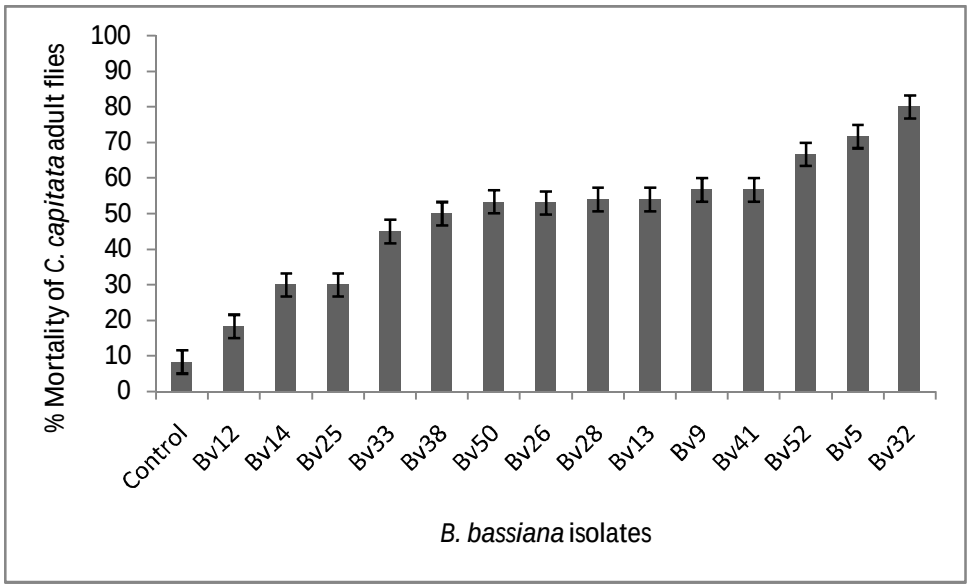
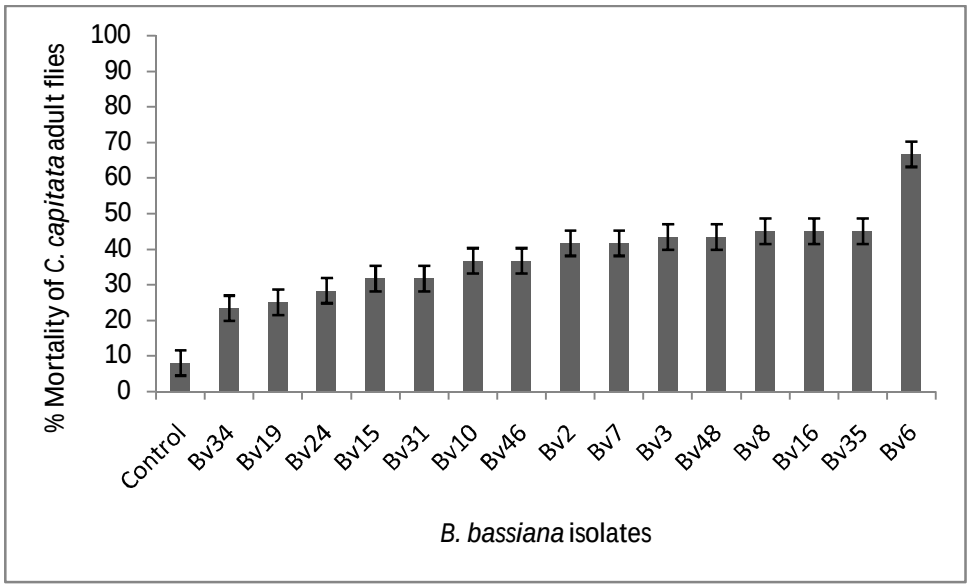


Figure 18. *C. capitata* adult mortalities caused by *B. bassiana* isolates after 3 days from inculcation: (batch 1), (LSD = 3.279).

A



B

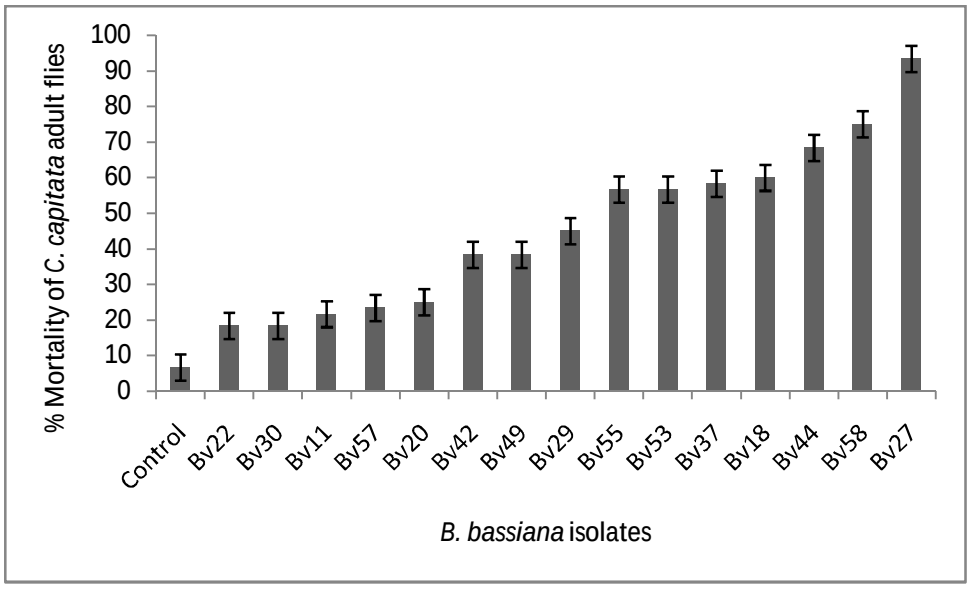


Figure 19. *C. capitata* adult mortalities caused by *B. bassiana* isolates after 3 days from inculcation: (A, batch 2, LSD = 3.58; B, batch 3, LSD = 3.68).

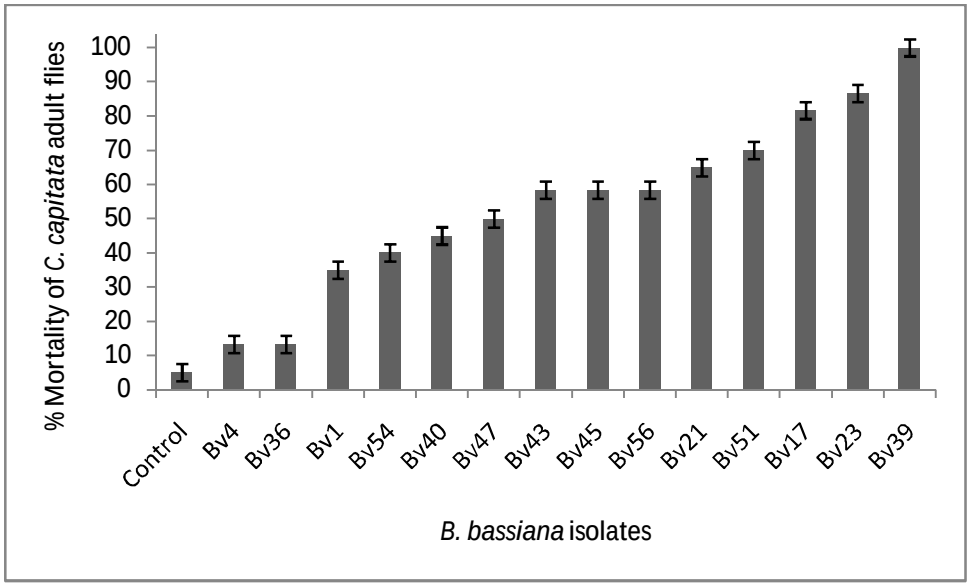


Figure 20. *C. capitata* adult mortalities caused by *B. bassiana* isolates after 3 days from inculcation: (batch 4), (LSD = 2.52).

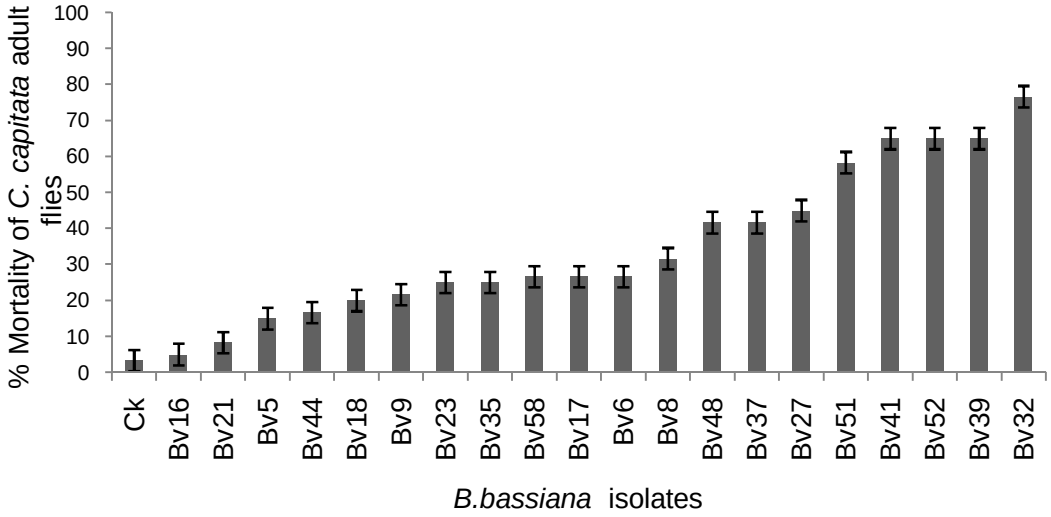


Figure 21. *C. capitata* adult mortalities caused by the most virulent *B. bassiana* isolates after 3 days from inoculation. (LSD = 2.97)

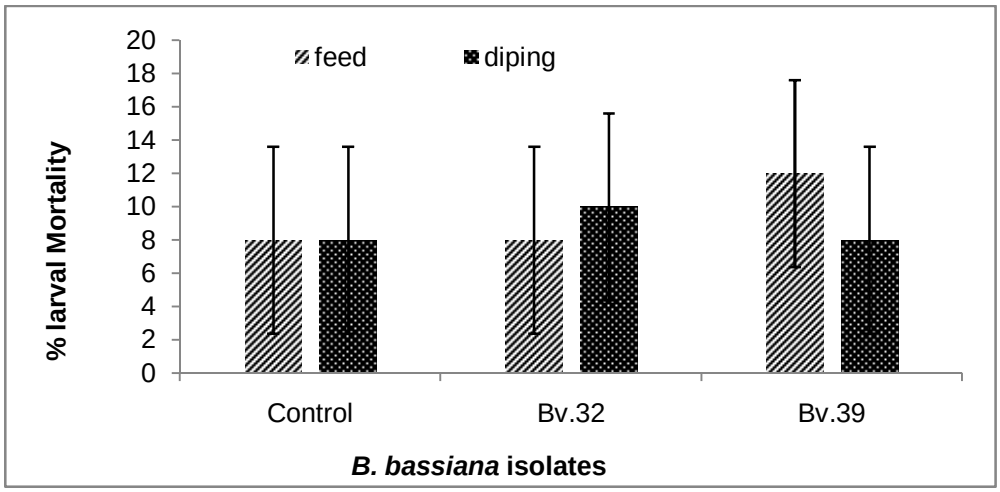
As shown above, results indicated significant differences in mortality percentages compared to the control treatment when more than 18 different *B. bassiana* isolates were used. Significant variation in mortalities was obvious among isolates suggesting a varying potential of local *B. bassiana* isolates to effectively control *C. capitata* population in vitro. The isolates Bv.32, Bv.39, Bv.52 Bv.41 and Bv.51 have induced

the highest mortalities (77, 65, 65, 65, and 58%, respectively) in *C. capitata* population. These isolates were therefore used for later experiments.

3.4.2 Effect of *B. bassiana* on larval and pupal mortalities of *C. capitata*

This experiment was done to investigate the efficiency of *B. bassiana* against the larval and pupal stages of the *C. capitata* using two different inoculation methods. *B. bassiana* was ineffective in inducing significant mortalities among the two immature stages. Larval mortalities have not exceeded 12% with no significant difference with the control treatment for both methods of *B. bassiana* application. *C. capitata* pupae, however, was totally unaffected by *B. bassiana* applications, with very low mortalities comparable to the control. (Fig. 22).

A)



B)

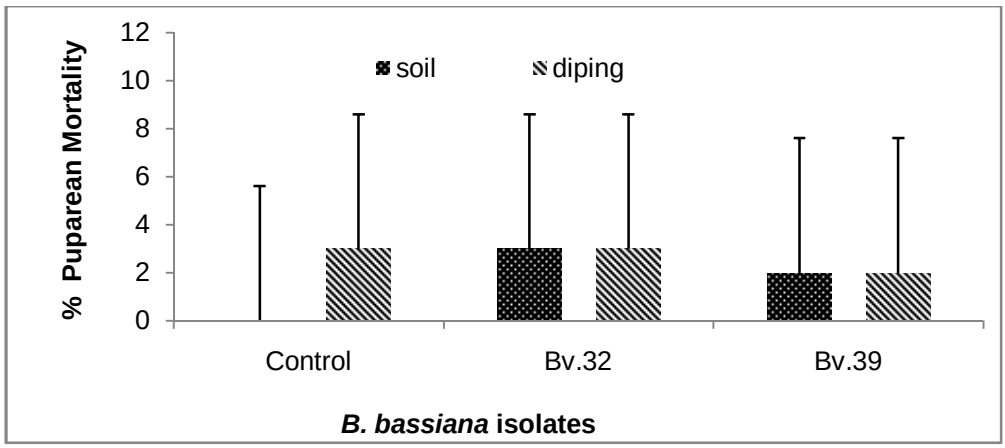


Figure 20. Virulence of *B. bassiana* (1×10^8 conidia/ml) on developmental stages of *C. capitata* (A): Larvae; (B): Pupae. (LSD = 5.617, 5.615, respectively)

3.4.3 The effect of *B. bassiana* conidial concentration on *C. capitata* mortality

The objective of this experiment was to assess the mortality of *C. capitata* adults induced by different conidial concentrations of *B. bassiana*. In general, morality rates increased with increasing concentrations of the *B. bassiana* conidial inoculum. At conidial concentration (10^5 conidia/ml), mortalities induced by isolates Bv.32, Bv.39, Bv.41, Bv.51, and Bv.52 were 78, 43, 30, 35, and 30%,

respectively. However, at the highest concentration tested (10^8), mortalities recorded for the same isolates were 100%, 100%, 68%, 67%, and 48%, respectively.(Fig. 21)

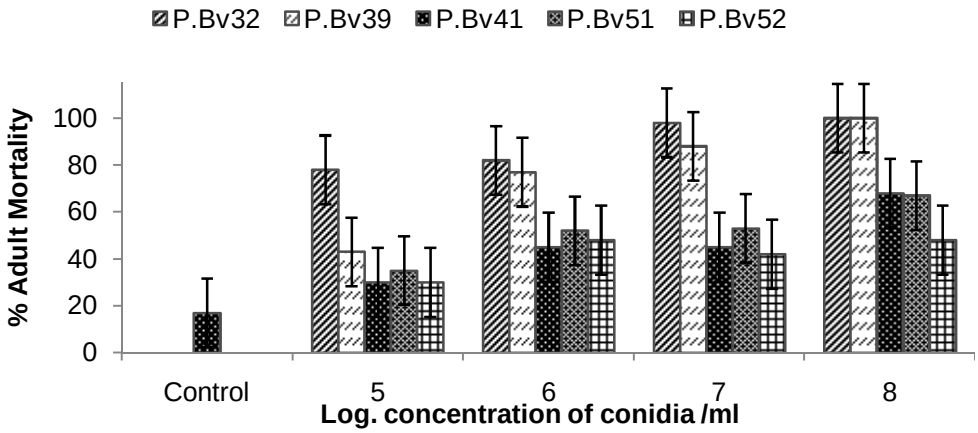


Figure 21. Adult mortality of *C. capitata* induced by *B. bassiana* isolates after 5 days from inoculation with different concentrations of conidia. (LSD = 14.643)

3.4.4 The effect of temperature on virulence of *B. bassiana*

The objective of this experiment was to evaluate the influence of temperature on virulence of *B. bassiana in vitro.* In general there were significant differences in *C. capitata* mortality rates at different temperatures.

The isolate Bv.32 has induced successful mortality rates (77-100%) under wide range of incubation temperatures (15-25 ºC). Other isolates, (e.g. Bv.39 and Bv.51) showed stronger infectivity at only high temperature (25 °C) . In general, slight significant variations were noticed between isolates under the high temperature regimes (Fig. 22).

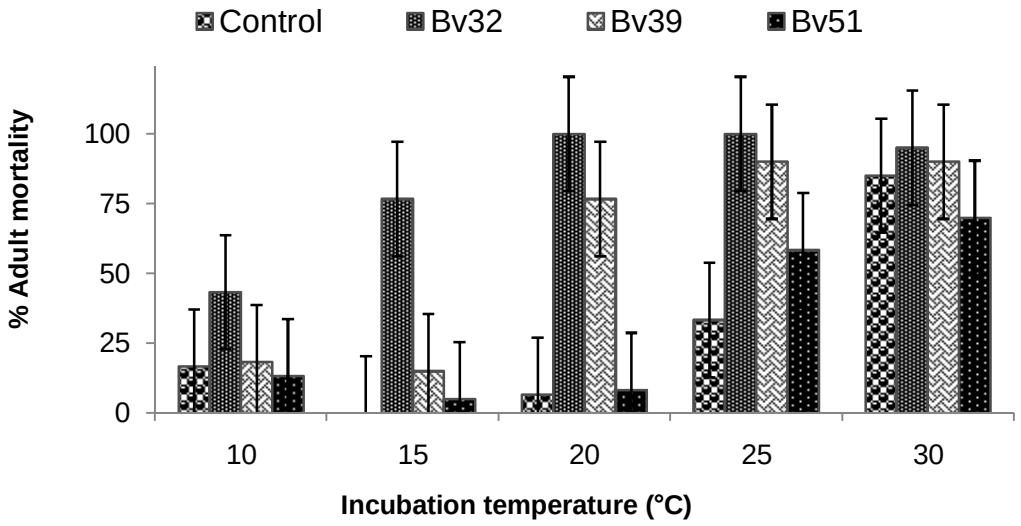


Figure 22. The effect of temperature on *C. capitata* mortality induced by *B. bassiana* after 4 days of incubation at various temperatures.

3.4.5 Lethal time and lethal concentration

This experiment was conducted to estimate the lethal time (LT_{50}) and the lethal concentration (LC_{50}) that is required to kill 50% of *C. capitata* adult population. The results showed that the time (LT_{50}) required ranged from 3.91 and 5.6 days depending on the isolate tested (Table 5). The lethal concentration (LC_{50}) required to kill 50% of tested *C. capitata* adult population ranged from 3.8 to 10.5 log conidia / ml. The most virulent isolate was Bv39 who was able to kill 50% of the tested *C. capitata* adult population only after 3.91 days and at the lowest inoculum concentration (4.3 log conidial / ml) (Table 5 and Table 6).

Table 5. Lethal Concentration required to kill 50% of *C. capitata* adult population.

Isolate	Regression equation	LC_{50} after the 5^{th} day post inoculation (log. conidia/ml)
Bv 32	y = 10.773x + 18.979	3.8
Bv 39	y = 10.387x + 10.99	4.3
Bv 41	y = 5.4897x + 12.454	7.9
Bv 51	y = 5.9072x + 14.082	7.3
Bv 52	y = 3.9691x + 16.361	10.5

Table 6. Lethal Time required to kill 50% of *C. capitata* adult population.

Isolate	Regression equation	LT_{50} at (10^8 conidia/ml)
Bv 32	y = 11.386x + 5.1389	3.94
Bv 39	y = 14.765x - 7.7315	3.91
Bv 41	y = 13.635x - 19.537	5.1
Bv 51	y = 12.685x - 19.769	5.5
Bv 52	y = 13.633x - 26.343	5.6

3.4.6 Serial culturing of *B. bassiana* inoculum

This experiment was conducted to test if the virulence of *B. bassiana* is influenced by long term culturing on artificial media, and to check if virulence of the fungus is enhanced by re-isolating the fungus from infected adult *C. capitata*. The results showed no influence of serial

culturing on virulence of *B. bassiana*. No significant differences were obvious in virulence of *B. bassiana* isolated directly from a dead fly and those exposed to serial in vitro culturing (Fig. 25).

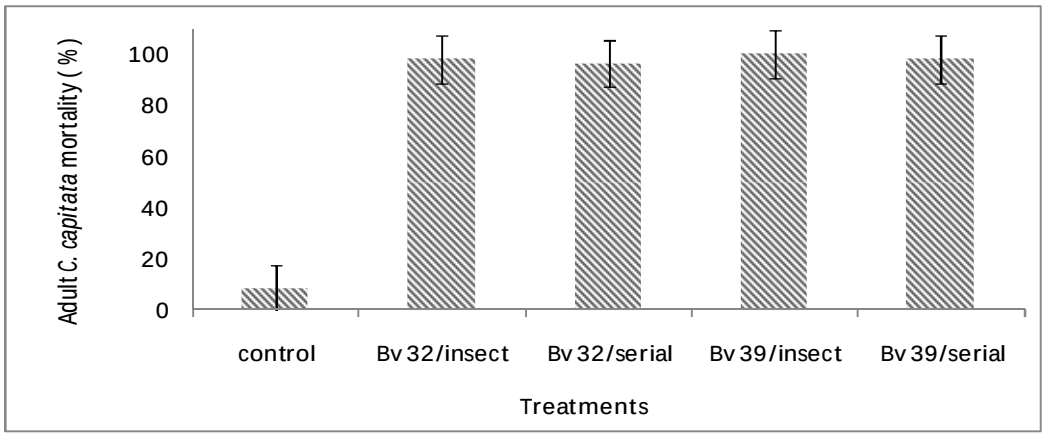


Figure 23. Mortality percentages induced by *B. bassiana* isolates (Bv.32 and Bv.39) subjected to serial culturing and those isolated directly from infected *C. capitata* after 6 days from inoculation. (LSD=9.271).

3.4.7 *B. bassiana* method of application

Two methods of *B. bassiana* applications were tested in this experiment against *C. capitata* adult flies. The results showed that the contact method of application recorded higher mortalities compared to the control and to the feeding method of applications.

In addition, *B. bassiana* (Bv.32 and Bv.39) recorded high mortalities (83% and 92%, respectively) after four days when the contact method of application was used compared to less than (20 %) mortalities for the other three isolates (Fig. 24).

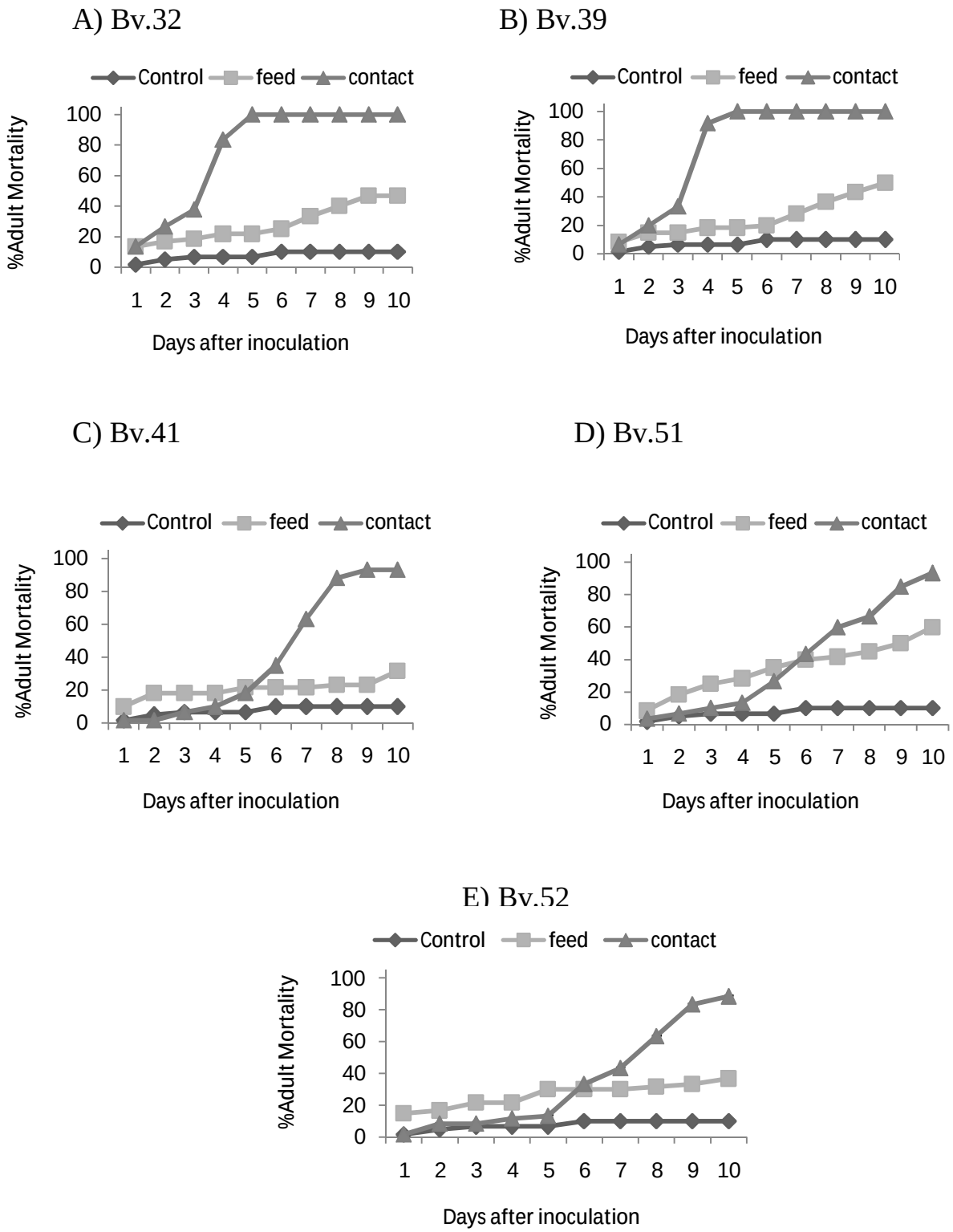


Figure 24. The effect of method of application (contact and feed) of *B. bassiana* (Bv32, Bv39, Bv41, Bv51 and Bv52) on *C. capitata* adult flies mortality. (LSD = 16.936)

3.4.8 Bioassay of peach fruits

This experiment was conducted to explore the biocontrol efficiency of *B. bassiana* on Peach fruits infested by *C. capitata* *in vitro* simulating similar field infestation conditions. *B. bassiana* isolates Bv32 and Bv 39 have reduced significantly the larval infestation compared to the control. Compared to the control treatment (an average of 33 larvae/fruit), only an average of 9 and 12 larvae/fruit were counted in Bv32 and Bv 39 treated fruits, respectively. No significant variation was noticed between both isolates used (Fig. 27).

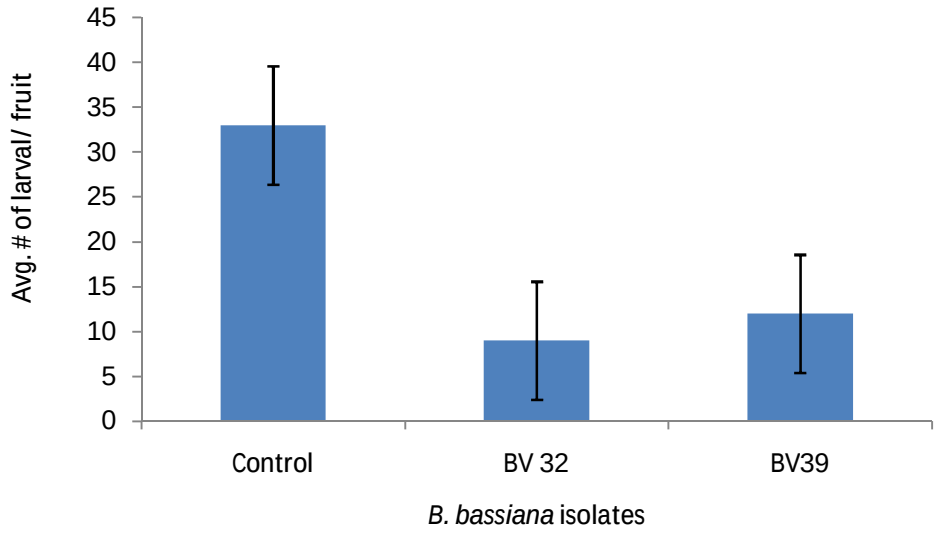


Figure 25. Number of *C. capitata* larval infestation of peach fruits following inoculation with two *B. bassiana* isolates (BV 32 & BV 39) at the concentration of 10^8 conidia/ml. (LSD = 6.586)

Chapter four: Discussion

The entomopathogenic fungus *B. bassiana* is the most common parasite of insects that has been isolated from the soil, litter, dead and moribund insects in nature (Thomas *et al.*, 1987).

In this study, *B. bassiana* was recovered from soil samples collected from Palestinian agricultural fields by using diluted plate technique on improved modified growth medium (GPAD) amended with 90 µg/ml Dodine. The fungicide at the concentration of 90 µg/ml suppressed the growth of other fungi whilst favoring the growth of *B. bassiana*. Several researchers have regularly used the fungicide Dodine at different concentrations in different media to isolate *B. bassiana* from soil and cadavers of insect (Beilhartz *et al.*, 1982; Chase *et al.*, 1986; Shimazu and Sato, 1996; and Tae-Young *et al.*, 2010). In some reports, dodine at the concentration 650 µg/ml incorporated in oatmeal agar medium (rolled oats 30 g, and agar 15 g in 1000 ml DW) had been successfully used to isolate *Beauveria* spp. from soil and cadavers of insect and suppressed the growth of non entomopathogenic fungi (Beilhartz *et al.*, 1982). However, Tae-Young *et al.*, (2010) reported that the optimal concentration of dodine added to sabouraud dextrose medium to isolate *B. bassiana* was 50 µg/ml. Dodine, therefore was fundamental for the isolation and purification of *B. bassiana* from soil, but the recommended concentration to be used might vary according to the sensitivity of the *B. bassiana* isolates and the type of nutrient media.

In this survey, a total of 58 native isolates of *B. bassiana* were recovered from various sites in the West Bank. Most of the isolates were recovered from warm weather areas (e.g. 90 % of the isolates were recovered from the Jordan valley and Jenin area). The monthly mean maximum air temperature in the Jordan Valley was 39.8 °C in Jericho (July, 2010) according to the Jericho Agriculture Station records. This actually

coincide with the in vitro temperature experiments which showed that the optimal temperature for mycelial growth (MGR) of the best *B. bassiana* isolates was 30 °C. It is worth mentioning here that the isolates Bv 32, Bv 39, Bv 41, Bv 52 were all recovered from the Jordan Valley area.

Furthermore, most of the isolates were recovered from the soil of vegetable-cultivated fields and soil is usually the conventional isolation site for several entomopathogenic fungi. Several species of *Beauveria* were reported to be found in both cultivated and natural habitats (Va¨nninen, 1996; Klingen *et al.*, 2002; Keller *et al.*, 2003; Meyling and Eilenberg, 2006). It was found that *B. bassiana* is affiliated with shaded and uncultivated habitats (e.g. forests) (Bidochka *et al.*, 1998). The communities of entomopathogenic fungi in the arable soil environments are different from communities of less disturbed habitats (Steenberg, 1995; Bidochka *et al.*, 1998; Meyling and Eilenberg, 2006) and less disturbance in the cropping system also affect the populations of the fungi.

Soil properties and environmental factors including soil texture, pH, soil moisture, UV solar radiation and temperature have been shown to affect the growth and survival of *B. bassiana* (Lingg and Donaldson, 1981; Groden and Lockwood, 1991; Fargues *et al.*, 1997; Inglis *et al.*, 2001; Meikle *et al.*, 2003). Indeed, temperature, moisture and UV-radiation seem to be most important for *B. bassiana* survival (Meikle *et al.*, 2003).

Concerning temperature preferences for *B. bassiana* growth, it was mentioned earlier that the optimum temperature for mycelial growth was 30 °C. However, the optimum temperature for conidial production and germination of *B. bassiana* best isolates were in the range of 15 – 25 °C, less than that required for mycelial growth. As for virulence, it depended largely on the isolate and the optimum was in the wider range of 15 – 30 °C. Similar results were found by Taylor and Francis, (2009) who

reported that the lowest radial growth occurred at 15ºC and 35ºC for all *B. bassiana* isolates except for the isolate Bb-01 which ceased to grow at 35ºC. The optimum temperature for sporulation and germination of the isolate Bb-01 was 20ºC . Furthermore, the optimum temperature reported for growth, germination, sporulation, and virulence of *B. bassiana* was in the range of 20 – 30 °C (Tanda and Kaya, 1993; Walstted *et al.*, 1970; Hajek *et al.*, 1990; Dimbi *et al.*, 2004). Parker *et al*, (2010) further showed that the highest production of *B. bassiana* conidia was obtained at 25 °C.

The results of this study showed that local *B. bassiana* isolates were virulent to cause mortalities on *C. capitata* adult flies in the range of 5 – 90% with wide variation between different isolates. Similar results were shown by Konstantopoulou and Mazomenos, (2005) who indicated that *B. bassiana*, induced 85.6% mortality in *C. capitata* population but was less effective against *Bactrocera oleae*. Similarly, Munoz, (2000) evaluated the pathogenic potential of 16 strains of *B. bassiana* against *C. capitata* adult flies and reported a mortality range of 20 - 98.7%. Quesada-Moraga *et al*., (2006) reported a mortality range of 30 - 100% when a conidial suspension was used at the concentration of 10^8 conidia/ml.

Virulence of native isolates of *B. bassiana* against immature stages of *C. capitata* were tested. Larval mortalities have not exceeded 12% with no significant differences from the control treatment. Pupal mortalities were very low as well and have not exceeded 3% compared to the control. These results are in agreement with those obtained by Aluja, (1993) and Hajek and St. leger (1994) who indicated that pupae of *C. capitata* is known to possess thick and completely sclerotinized cuticle, and a hard thick pupal capsule with a scaly surface. Hajek and St. leger (1994) considered these factors very important in explaining the null effect of *B.*

bassiana on insect pest mortality. It was further shown that mortality of pupae decreased with increasing pupal age, and the germ tube of *B. bassiana* was unable to penetrate the sclerotinized pupal integument (Ekesi *et al.*, 2002). Freidberg *et al.*, (1989) found that when larvae of *Rhagoletis cerasi* were sprayed with *B. bassiana* conidia at a concentration of 10^7 conidia/ml, the mortality % induced were only 13% and was attributed to the common molting cycles that hampers fungal penetration. Charnley *et al.*, (1991) reported also similar low medfly pupal and larval mortalities (maximum 11% and 8% respectively) when applying *B. bassiana* (isolate BCIP 1333) at a concentration of 10^8 conidia/ml.

Concerning lethal concentration of inoculum (conidia) effective against *C. capitata* adult flies, it was found that mortality was positively correlated with increasing concentration. Complete mortality (100%) was recorded after 5 days at a concentration of 10^7 conidia/ml.

This work revealed that lethal concentration is isolate dependent, and LC_{50} ranged between 3.8 to 10.5 log conidia /ml after 5 days post inoculation. Similarly and in the same direction, Martin *et al*, (1992) reported that when *B. bassiana* (isolate Bb – 1333) was used against *C. capitata* the LC_{50} was 6.3 log conidia / ml after five days.

Lethal time (LT_{50}) proved to be isolate dependent as well and ranged from 3.9 to 5.6 days at the concentration of 10^8 conidia / ml . In the same direction, Garcia *et al.*, (1984) evaluated the pathogenicity of *B. bassiana* against *C. capitata* under laboratory conditions, and reported an LT_{50} of 7.4 days.

Serial in vitro culturing of *B. bassiana* have not affected virulence of isolates . Similar results were obtained by (Hall, 1980; Iqtiat *et al.*, 2009; Morrow *et al.*,1989). The results of this study are in agreement with Marrow *et al.*, (1989) who concluded that the effect of repeated in vitro

sub culturing of entomopathogenic fungi could vary among species and pathotypes. However, attenuation in virulence of certain isolates of *B. bassiana*, *Metarhizium anisopliae* and *Verticillium lecanii* after serial culturing on artificial nutrient media was reported and documented, by Nagaich, (1973), who showed that serial in vitro culturing of *B. bassiana* have reduced virulence of these fungi on host insects.

The optimal method for *B. bassiana* application against adult flies of *C. capitata* was examined. Conidial suspension used gave high mortality rates with variation between isolates of *B. bassiana*. The variability in pathogenicity among strains may be related to the different attachment methods of conidia of each isolate onto the insect cuticle, mode of germination, as well as suppression of host immune system (Chandles *et al.*,1993). Extracellular enzymes produced by *B. bassiana* such as proteases and peroxidases may contribute positively to mortality rates (Bidochka and Khachatourians, 1990; Zayed and Zebitz, 1997).

At the bioassay level, *B. bassiana* isolates Bv32 and Bv 39 sprayed at the concentration of 10^8 conidia/ml on peach fruits reduced significantly numbers of larval infestation of fruits by 73% and 64% respectively, compared to the control . Similar results were reported on the effect of the fungus in bioassay; the effects seem to indicate not only a direct effect of the entomopathogenic fungus against *C. capitata* adults but also the indirect modes of action (Benuzzi and Santopolo, 2001). The entomopathogenic fungus might produce chemical compounds or fungal structures (e.g. hyphae) that reduce or inhibit medfly oviposition. Moreover, repellent volatile cues might be produced by *B. bassiana*, but the influence of these compounds on oviposition behavior of *C. capitata* is uncertain (Magnano *et al.*, 1989; Wright and Chandler, 1992). Moreover, the fungus produce metabolites repellent to insects (e.g.

peramine) (Rowan *et al.*, 1990; Daisy *et al.*, 2002). In the same direction, Feng *et al.*, (1994) reported that when apple fruits were sprayed with *B. bassiana* conidia at the concentrations of 10^0, 10^4, 10^5, 10^6 , 10^7 and 10^8 conidia/ml, the infestation rate were 100%, 73.6% , 73.2% , 51.7% , 40.5 % and 36.2%, respectively.

In conclusion, some *B. bassiana* native isolates proved to be quite virulent against adult flies of *C. capitata*. Further studies are necessary, however, in the areas of formulations, shelf life, sustainability in the field, host plants and modes of action.

5. Appendices

5.1 ANOVA table

Table 7. Summary of ANOVA table (SigmaStat® program)

Experiment	D.F treat	D.F residual	D.F total	SS Treat	SS Residual	SS Total	MS treat	MS residual	F
Effect of temp. on growth rate	34	70	104	3.2	0.3	3.5	0.1	0.005	
Spores germination	28	58	86	69239.1	1942	71181.1	2427.8	33.5	73.5
Screening the Virulence of native *Beauveria* isolates 56	63	128	191	12634	527.3	13161.3	200.5	4.1	48.7
Screening the Virulence of native *Beauveria* isolates20	63	128	191	12068.6	433.3	12501.9	191.6	3.4	56.6
The effect of inoculum (conidia) concentration of *B. bassiana* on Me fly mortality	20	42	62	36969	3316.7	40285.7	1848.5	79	23.4
The effect of temperature on virulence of *Beauveria bassiana*	19	40	59	53257.9	6133.3	59391.3	2803	153.3	18.3
Application method	63	128	191	51793.6	5350	57143.6	822.1	41.8	19.7
Effect of *B. bassiana* on larval mortalities of *C. capitata*	11	24	35	347.222	416.667	763.889	31.566	16.361	1.818
Effect of *B. bassiana* on pupal mortalities of *C. capitata*	5	12	17	27.778	133.333	161.111	5.556	11.111	0.5
Serial culturing of *B. bassiana* inoculum	29	60	89	152719.2	1933.333	154652.5	5266.2	32.4	163.4
Bioassay of Peach fruits	8	18	26	3556.519	265.333	3821.852	444.565	14.741	30.159

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Abstract in Arabic

تم دراسة اثر عزلات محلية من الفطر الممرض للحشرات *Beauveria bassiana* على ذبابة ثمار البحر الأبيض المتوسط *Ceratitis capitata*, حيث تم عزل 58 عزله من 225 عينة تربة جمعت معظمها من مناطق دافئة في الضفة الغربية. تم العزل بواسطة تقنية dilution plate technique تم تعديلها بإضافة المبيد الفطري Dodine بتركيز 90 جزء في المليون. كانت درجة الحرارة المثلى للنمو والتكاثر لفطر ال *B. bassiana* تتراوح من 25 إلى 30 درجه مئوية. واثبت الفطر *B. bassiana* انه قادر على إحداث موت في الحشرات البالغة من *C. capitata* (أكثر من 90% موت) ولكنها كانت غير فعالة على الأطوار غير البالغة للحشرة (اقل من 12% موت لليرقات). كما أظهرت النتائج أن التركيز اللازم لابواغ الفطر لقتل 50% من مجتمع الحشرة (LC_{50}) يتراوح من 10^4 إلى 10^{10} بعد 5 أيام من العدوى. والوقت اللازم لقتل 50% من مجتمع الحشرة (LT_{50}) يتراوح من 3.9 إلى 5.6 يوم عند استخدام تركيز الكونيديا 10^8 بوغ/ملم. وعند معاملة ثمار الخوخ بكونيديا الفطر بتركيز 10^8 بوغ/ملم انخفضت الإصابة بيرقات ذبابة ثمار البحر الأبيض المتوسط بنسبة 73% مقارنة مع تجربة الشاهد. كشفت الدراسة أن العزلات المحلية من ال *B. bassiana* قادرة على مكافحة ذبابة ثمار الفاكهة *C.capitata* عند استخدام ابواغها كمحلول للرش ولكن هذه الدراسات لا تزال بحاجة إلى دراسات حقلية ودراسات مفصلة تراعي تركيبة محلول الرش وقدرة الفطر على الصمود والتكاثر تحت الظروف الحقلية وآلية عمله ضد الحشرة المستهدفة.

جامعة الخليل
كلية الدراسات العليا والبحث العلمي
برنامج الوقاية النباتية

عزل الفطر الممرض للحشرات (بوفيريا باسيانا) من التربة وتقييم أثره على ذبابة ثمار البحر الأبيض المتوسط

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