BIOLOGICAL CONTROL OF THE COMMON HOUSEFLY (*MUSCA DOMESTICA L*) USING *BACILLUS THURINGIENSIS* (ISHIWATA) BERLINER VAR. *ISRAELENSIS* AND *BEAUVERIA BASSIANA* (BALS.) VUILLEMIN IN CAGED POULTRY FACILITIES

By

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ABSTRACT

The entomopathogenic fungus *Beauveria bassiana* and the bacterium *Bacillus thuringiensis* var. *israelensis (Bti)* have been widely studied for their role in biocontrol against many arthropods and extensively exploited for insect pest control. The purpose of this study was to evaluate the effect of four *B. bassiana* and two *Bti* formulations and their respective combinations, for the biological control of the common house fly, *Musca domestica* L., a major pest in poultry facilities.

In vitro screening was undertaken to select the best *B. bassiana* isolates from 34 *B. bassiana* isolates and two *Paecilomyces* isolates. All the isolates of *B. bassiana* were found to be effective against adult house flies, but were marginally effective in controlling fly larvae. The *Paecilomyces* isolates were non-pathogenic towards both adult house flies and larvae. The best four isolates R444, 7320, 7569 and 7771 caused >90% mortality within 2d and were subjected to dose-mortality bioassays. Microscopic studies using light and scanning electron microscopy indicated the different durations of the lifecycle of *B. bassiana* development on the house fly. High temperature was found to delay conidial germination. Spore germination and mycelial growth were also inhibited by high adjuvant concentrations.

Laboratory baseline bioassay data established, a dose-time response relationship using a water-dispersible granules (WDG) *Bti* formulation that demonstrated that the susceptibility of *M. domestica* larvae to a given concentration of *Bti* increased as the duration of exposure increased. In the laboratory studies, the LC_{50} and LC_{90} values of *Bti* for the larvae ranged between 65 - 77.4 and 185.1 - 225.9µg ml^{-1}, respectively. LT_{50} and LT_{90} values were 5.5 and 10.3d respectively. In the field, a concentration of 10g *Bti* kg^{-1} (bran formulation) of feed resulted in 90% reduction of larvae for 4wk post-treatment. A higher concentration (2g L^{-1}) of *Bti* in spray (WDG) applications was not significantly more effective than the lower concentration of 1g L^{-1}. Thus, adding *Bti* to chicken feed has potential for the management and control of house flies in caged-poultry facilities.

The impact of oral feed applications of a bran formulation of *Bti* and a commercial chemical larvicide, Larvadex®, were compared with respect to their efficacy on the control of house fly

larval populations in poultry manure. The sublethal effects were manifested in terms of decreasing emergence of adult house flies. Although Larvadex® reduced larval density and caused significant reductions in emergence of adult house flies, it generally exhibited weaker lethal effects than *Bti.* The reduction levels achieved as a result of feeding 250mg *Bti* kg^{-1} at 5wk were similar to those achieved as a result of feeding twice the amount of Larvadex® at 4wk to the layers.

From both an efficiency and economic perspective, comparisons to assess the impact of combining different concentrations of the two *Bti* formulations were carried out to evaluate their success in controlling house fly larvae and adults in poultry houses. The percentage mortality of larvae accomplished as a result of using a combination of 250mg kg^{-1} *Bti* in feed and 2g L^{-1} spray applications was equivalent to that obtained as a result of combining 500mg kg^{-1} *Bti* in feed and 1g L^{-1} spray application. The cost-benefit analysis (expressed in terms of mortality of larvae) indicated that the most effective combination for control of house fly larvae and fly emergence was the 500mg kg^{-1} in feed and 2g L^{-1} spray application combination that resulted in 67% larval mortality and 74% inhibition of adult house fly emergence. This study presents commercial users with possible combinations of applications of the two *Bti* formulations.

Comparisons of larval mortalities and house fly emergence resulting from the *Bti* - *B. bassiana* treatments with those from Larvadex® - *B. bassiana* treatments, showed better control levels compared to any of the individual agents alone. The *Bti* treatments were more effective at controlling larval populations and inhibiting the emergence of house flies than Larvadex®, even when Larvadex® was applied together with *B. bassiana*. The effects of the *Bti* - *B. bassiana* and the Larvadex® - *B. bassiana* interactions were additive. These trials suggest that the efficacy of *Bti* in the control of house fly larvae may be improved with frequent applications of *B. bassiana.*

PREFACE

DECLARATION

I .....**Lizzy A. Mwamburi.....** declare that:

(i) The research reported in this dissertation, except where otherwise indicated, is my original work.

(ii) This dissertation has not been submitted for any degree or examination at any other university.

(iii) This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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INTRODUCTION

The house fly, *Musca domestica* (L.), is the major pest in poultry facilities (Scott *et al.*, 2000). Concerns about animal health, public health and potential litigation all result from house fly activity. Furthermore, house flies have developed resistance to virtually every insecticide applied against them (Scott *et al.*, 2000), which has made efforts to control flies using biological control agents (BCAs) increasingly important.

Most arthropod pests of veterinary importance are largely controlled through spray and dip applications of chemical pesticides, but the development of resistance and the possibility of contaminating milk and meat are issues associated with most broad-spectrum pesticides. With increasing concerns over levels of insecticide residues in meat and other animal products, the agricultural industry is under increased market pressure to reduce its chemical use. There is a clear incentive for alternative biological control strategies to be developed.

The fungus *Beauveria bassiana*, the bacterium *Bacillus thurigiensis* var. *israelensis* (*Bti*) and the entomopathogenic nematodes (EPN), *Heterorhabditis* and *Steinernema spp,* have been used widely to control insects that affect crops or are vectors of human diseases. However there have been investigations into the effects of BCAs on insect pests of livestock and poultry.

Entomopathogenic microbes can serve as alternatives to broad-spectrum chemical insecticides. Numerous advantages can be found in the utilisation of entomopathogens, in addition to efficacy. Advantages include: safety for humans and other non-target organisms, reduction of pesticide residues in food, preservation of other natural enemies and increased biodiversity in managed ecosystems. However, many factors still limit the acceptance of entomopathogenic microbes by commercial users. In order to increase their utilisation, studies need to concentrate on: (a) pathogen virulence and speed of kill, (b) pathogen performance under challenging environmental conditions such as high temperatures, (c) ease of use and efficiency in existing production process, (d) improved formulations that enable ease of application, increased environmental persistence and longer shelf-life, (e) integration into managed ecosystems and interaction with the environment and other integrated pest management (IPM) components (Lacey *et al.*, 2001).

The aims of this study were to: -

a. Review the available literature on the use of entomopathogenic bacteria, fungi and nematodes for biological control of flies that affect chicken, dairy and pigs, with special reference to *Bti, B. bassiana*, and the EPNs *Heterorhabditis* and *Steinernema spp*.

b. Screen *Bti, B. bassiana*, and *Heterorhabditis sp.* for biological control against larvae and adult house flies.

c. Describe the external development of *B. bassiana* on the adult house fly using scanning electron and light microscopy.

d. Compare the larvicidal effects of a bran-formulated *Bti* and a commercial larvicide, Larvadex®, as feed additives for controlling house flies in caged chicken layers.

e. Compare the effectiveness of combined bran feed and water-dispersible granule (WDG) *Bti* formulations for control of house fly larvae and adults in poultry houses.

f. Combine the best *Bti* and *B. bassiana* treatments for the integrated biological control of house flies in poultry houses and compare their effects with those of *B. bassiana*-Larvadex® combinations.

h. Screen the effects of adjuvants and temperature on three selected *Beauveria* isolates (7320, 7569 and 7771).

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Chapter 1 LITERATURE REVIEW

1.1 Flies in agriculture

In livestock and poultry production systems, one important component is the management of arthropod pests. Insects, ticks, and mites attacking animals cause substantial direct economic losses. Many animal pathogens are vectored, or at least maintained, by arthropods, which necessitates suppression of these arthropod populations in order to reduce the incidence of livestock diseases and the corresponding economic losses. Furthermore, in many cases the arthropods affecting animal production also affect humans, either directly by annoyance and/or biting or by fostering the maintenance and spread of human diseases (Axtell, 1986). Public health economic losses cannot be accurately estimated but may be substantial. Concentrated animal production often increases the populations of arthropod pests affecting livestock and humans in an area. This is evident in the case of synanthropic filth flies (e.g., house flies, *Musca domestica* L.) (Axtell, 1986). This cosmopolitan fly is often the most abundant insect where livestock, poultry or companion animals are housed. Adults occur in almost all substrates surrounding the animals, including feed, faeces, vegetation, and the walls and ceilings of buildings. Adults also occur directly on animals where they feed on available blood, sweat, tears, and other body fluids. Throughout their ranges, these flies have adapted to a broad range of environmental conditions and cultural practices. Livestock and poultry production practices vary widely, and some practices enhance the development of large populations of house flies.

The nature of the arthropod pest problems is related to the three categories of livestock and poultry production systems, generally classified as: (1) pasture or range, (2) outdoor confined, and (3) indoor confined (Axtell 1986). In pasture systems, the discrete dung pats of beef and dairy cattle are the breeding habitats for horn flies (*Haematobia spp*.), face flies (*M. autumnalis*) and bush flies (*Musca sorbens* complex) (Axtell 1986). The flies lay their eggs on the very fresh dung and larval development is rapid, with pupation being in the drier fringes of the dung or adjacent soil. Destruction of the fly eggs and/or larvae by predation, parasitism, or alteration of the dung naturally tends to suppress the fly population (Axtell 1986).

In the outdoor confined production system, the high density of the cattle results in the dung being concentrated in areas of little or no vegetation. The manure accumulations along fences and manure-feed mixture around the feeders in the lots are conditions for the production of large numbers of house flies (*M. domestica*), biting stable flies (*Stomoxys spp*.) and assortment of other minor species of Muscidae and Calliphoridae (blow flies) (Axtell 1986).

Indoor confined systems are systems where problems of manure handling and disposal become intense. In these high-density confined systems, arthropod pest population can explode (Axtell, 1986). With the indoor confined systems for dairy, swine, and poultry, the fly problem intensifies, with the most abundant pest species being the house fly. Stable flies can also be major pests in situations where there is an accumulation of manure mixed with feed and/or bedding materials, such as in dairy calf pens. Therefore, efforts to control flies in this environment are increasingly important. The house fly, *M. domestica* L., and the stable fly, *Stomoxys calcitrans* (L.) readily exploit soiled bedding for oviposition and larval development (Schmidtmann, 1988). Additional frequently abundant manure-breeding flies in these systems are the false stable fly (*Muscina stabulans*), garbage flies (*Ophyra spp*.), little house flies (*Fannia spp*.), sphaerocerid dung flies (*Coproica, Leptocera),* and a variety of calliphorid blowflies (*Phaenicia, Calliphora, Phormia*). In highly automated systems with daily manure removal, levels of fly breeding are low. Manure from such systems may be flushed into a lagoon, which is commonly an ideal environment for the proliferation of Culex mosquitoes (Rutz and Axtell, 1978).

Among the confined animal production systems, poultry houses offer a highly managed system and often tremendous populations of muscoid filth flies build up, mostly *M. domestica* (Axtell, 1999), together with the lesser house fly, *F. canicularis* (L.) (Achiano and Giliomee, 2005). Farmers in poultry systems have to deal with the problem of manure handling, disposal and the concurrent problem of fly control (Wilhout *et al.*, 1991). These systems, especially caged layer houses, have been studied extensively (Geden, 1984; Rutz and Scoles, 1989; Axtell and Arends, 1990; Axtell, 1999; Scott *et al.*, 2000; Kaufman *et al.*, 2002).

To date, the primary BCAs used against muscoid flies in poultry houses have been the mites *Macrocheles muscaedomesticae, Fuscuropoda vegetans* and *Poecilochirus* sp., the beetle *Carcinops pumilio* (Geden 1984), and the hymenopterous parasites *Spalangia endues, S. cameroni, S. nigroaenea,* and *Muscidifurax raptor* (Axtell, 1986).

1.2 Economic impact of flies

Muscoid flies are universal pests of humans and livestock, on virtually every inhabited landmass on earth. Four species of muscoid flies, house fly, horn fly, face fly and stable fly are associated with livestock and poultry production, causing several billions of dollars per year in damage and control costs for livestock producers. Although they do not feed on blood, house flies are transmitters of pathogenic organisms of both humans and animals (Axtell and Arends, 1990; Kettle, 1995).

The common house fly, *M. domestica,* is actually a companion animal of livestock and humans and is not actually an ectoparasite, but it is far more important economically in many instances than any of the flies associated with livestock and poultry. The house fly becomes economically important to confined livestock by virtue of its biotic potential and synanthropic behaviour. These factors, coupled with the ability of house flies to exploit different developmental habitats on feedlots and dairies, have made this pest economically important (Barson *et al.,* 1994).

In cattle units, flies are attracted to cows' eyes, teats and open wounds. In addition to the diseases they transmit, extreme discomfort caused by constant fly attack can result in reduced weight gain and lower milk yield in cattle. Milk quality also suffers from the presence of excessive numbers of flies and production can be downgraded in the absence of adequate fly control (Lancaster and Meisch, 1986). Fly treatment has been shown to result in a significant increase in milk yield (Morgan and Bailie, 1980) and in meat production (Stork, 1979).

On poultry farms, heavy fly infestations can mean more time spent cleaning eggs to remove flyspecks, and possibly downgrading egg quality. Fly maggots have a negative impact on poultry manure quality due to liquefaction, which also fosters the release of ammonia, which is

responsible for increased chronic respiratory diseases (CRD), increased corrosion and increased ventilation costs. Fly treatment has been shown to result in a marked improvement in egg production (Abrams, 1976).

The major diseases transmitted by flies to humans and domestic animals are enteric diseases and eye infections. Flies also play an important role in the epidemiology of mastitis in cattle (Hillerton and Bramley, 1985). Various other infections and several parasitic diseases can also be carried and spread by flies. Flies are important vectors of several enteric infections affecting humans and domestic animals including *Salmonella*, *Shigella, Campylobacter, Escherischia coli, Klebsiella, Enterobacter, Chlamydia, Helicobacter, Klebsiella,* anthrax, parasitic worms, mastitis, pinkeye, amoebic dysentery, tuberculosis, cholera and Newcastle disease (Graczyk *et al*., 2001).

In cattle, the principal ophthalmic infection is infectious keratoconjunctivitis or pink eye (Greenberg, 1973; Stork, 1979). The causative agent here is *Moraxella bovis* and the major vector is the face fly, *Musca autumnalis* (Gerhardt *et al.*, 1982). Flies are implicated as vectors of various organisms causing both chronic and acute mastitis in cattle. Summer mastitis, due to *Corynebacterium pyogenes,* which is mainly spread by the head fly *Hydrotaea irritans* and the face fly *Musca autumnalis* (Hillerton and Bramley, 1985). Flies are also involved in the spreading and transmission of other forms of mastitis, due to other species of *Corynebacterium,* staphylococcal and possibly streptococcal infections (Braverman *et al.*, 1999; Yeruham *et al.*, 1999). Heifers from herds using fly control usually have a lower prevalence of mastitis than herds without fly control (Nickerson *et al.*, 1995).

Flies are frequent carriers of helminthes. Several nematodes, such as *Parafilaria bovicola, Thelazia spp*. and *Heterotylenchus automnalis,* are found in various fly species and especially in the face fly (Bech-Nielsen *et al.*, 1982; Geden and Stoffolano, 1982; O'Hara and Kennedy, 1989; Chirico, 1994). Thelaziosis, an eyeworm infection affecting cattle, sheep, horses and goats, is mainly transmitted by the face fly *Musca automnalis* (Greenberg, 1973; Stork, 1979; Lancaster and Meisch, 1986; O'Hara and Kennedy, 1991). Several fly species have also been found

carrying eggs of *Ascaris*, *Trichuris* and *Ancylostoma* and may therefore play a role in the transmission of helminthiasis (Dipeolu, 1982; Oyerinde, 1976; Umeche and Mandah, 1989).

The house fly is a vector of cestodosis in poultry (Abrams, 1976). In poultry, flies can transmit coccidiosis as well, although this is not the major way of spreading of the disease (Greenberg, 1973; Milushev, 1978).

Other human and animal diseases for which nuisance flies can also be considered as possible or potential vectors, although their role in the dissemination of these pathogens is usually not a major one include: *Helicobacter pylori* in humans (Grubel *et al.*, 1997), Aujesky's disease in pigs and sheep (Medveczky *et al.*, 1988), *Cryptosporidium parvum* in cattle (Grazyk *et al.*, 1999), foot-and-mouth disease in ungulates (Greenberg, 1973), mycobacterial infections in cattle and pigs (Fischer *et al.*, 2001), anthrax (Turell and Knudson, 1987), and bovine rhinotracheitis in cattle (Lancaster and Meisch, 1986). This list is far from being exhaustive. For several diseases, the role of flies as vectors is well established and well documented. For some others, flies may only play a minor role in transmission. However, given the huge size of fly populations, their role cannot be neglected from an epidemiological point of view.

In addition to their direct impact on livestock and poultry production, flies are frequently considered nuisances in urban and recreational environments. Because of the threat of disease and the nuisance impact of these flies, their control is mandated by law and overseen by local regulatory agencies responsible for assuring citizen health. Litigation due to house flies associated with livestock and poultry production has become a major issue in many parts of the country. Whenever fly populations reach plague proportions in locations close to human habitation, they represent a serious health and environmental hazard - not just to the farmer, but also to neighbours and local communities.

Fly problems have resulted in lawsuits in several instances and in severe legal limitations being placed upon agricultural producers. In a list of prioritized needs created in 2005 by the DAEA (KZN) and DoH (KZN) report of the intergovernmental fly task team, were

- changes to the way manure is managed;

- implementation of an effective and sustainable chemical fly control programme; and,
- implementation of a biological control programme.

Adequate fly control is therefore justified both from a public health and an economic point of view.

1.3 Potential for biological control of house flies

The development of pesticide resistance by the house fly (Scott *et al.*, 2000; Kaufman *et al.*, 2001b) has increased the willingness of producers to seek alternative methods for fly management. In light of existing problems with chemical control of house flies, research over the last two decades has been centred on the more highly destructive parasitoid and predatory species. For example, the encyrtid *Tachnaephagus zealandicus* Ashmead, five species of the pteromalid genus *Muscidifurax*, and *Spalangia sp*. were evaluated for their capabilities of attacking dipterous larvae and pupae in various breeding sources. They are believed to be capable of successful fly suppression, if the right species and strains are applied in the right locality (Legner and Brydon, 1966; Legner and Dietrick 1972; 1974; Morgan *et al.*, 1975, 1977; Olton and Legner, 1975; Pickens *et al.*, 1975; Morgan and Patterson, 1977; Rutz and Axtell, 1979; Gold and Dahlsten, 1981; Propp and Morgan, 1985; Axtell and Rutz, 1986; Legner, 1988b; Mandeville *et al.*, 1988; Pawson and Petersen, 1988).

Although partially successful, none of these strategies have become the leading method for fly control, and the wrong choice of a parasitoid strain may have detrimental results (Legner, 1988b). Instead, the focus is on integrated controls including other methods such as cultural practices, adult baiting, and aerosol treatments with short residual insecticides. However, it is generally agreed that existing predatory complexes exert great influences on fly densities (Legner *et al.*, 1975b; Geden, 1984; Geden *et al.*, 1987, 1988; Geden and Axtell, 1988), that many biological control agents of endophilous flies have not been thoroughly surveyed, and that their potential has not been thoroughly surveyed and adequately assessed (Mullens, 1986).

Bacillus thuringiensis serovar *israelensis* has been found to be toxic to the house fly (Indrasith *et al*., 1992; Hodgman *et al.,* 1993; Zhong *et al.,* 2000). Several other isolates of *B. thuringiensis* which are active against house fly larvae have also been found (Johnson *et al.*, 1998). Thuringiensin-containing preparations have been used to control larvae of *M. domestica* (Mullens and Rodriguez, 1988; Mullens *et al.*, 1988a). It was also been reported that by Carlberg *et al.* (1991) that nuisance flies in cattle sheds, slaughter houses and latrines could be successfully controlled by applying *Bt* var *thuringiensis* to larval breeding sites.

Few fungi have been evaluated for control of house flies. To date, few field studies have evaluated entomopathogenic fungi, other than *Entomophthora muscae* (Cohn) Fresenius, for control of the house fly (Geden *et al.*, 1993; Mullens *et al.*, 1987; Watson and Petersen, 1993). Under laboratory conditions *E. muscae* (Cohn) Fresenius readily infected house flies (Kramer and Steinkraus, 1981). Occassionally, *E. muscae* epizootics devastate fly populations (Mullens *et al.*, 1987; Watson and Petersen, 1993; Steinkraus *et al.*, 1993), and laboratory-infected house flies have been used to induce epizootics in the field (Kramer and Steinkraus, 1987; Geden *et al.*, 1993; Steinkraus *et al.*, 1993). However, the potential of *E. muscae* for house fly suppression is limited by short-lived conidia, intolerance to high temperatures, by the apparent requirement for large populations of flies to sustain epizootics (Geden *et al*., 1995) and by the fact that it is difficult to grow artificially. Few other studies are available documenting the use of *B. bassiana* in controlling the house fly. Steinkraus *et al.* (1990) first reported the natural occurrence of *B. bassiana* (Balsamo) Vuillemin on the house fly. The entomopathogenic fungus *B. bassiana* (Balsamo) Vuillemin has potential as a biological control component of an integrated fly management program. Irrespective of its extensive host range, few reports of fly-derived *B. bassiana* strains exist (Humber, 1992). Steinkraus *et al.* (1990) reported that *B. bassiana* infected 1% of house fly adults under natural conditions in central New York. Despite the low prevalence of disease, strains collected by Steinkraus were virulent in subsequent laboratory studies (Watson *et al.,* 1995). One strain (P89), when formulated in water and a surfactant, induced 99% mortality in house flies (dose $1x10^8$ conidia per cm^2) within 6d of exposure.

Paecilomyces lilacinus is a soil saprophytic hyphomycete with a broad-based distribution (Samson, 1974). The efficacy of the fungus *P. lilacinus* has been widely demonstrated against

root knot nematodes, cyst nematodes and other important plant and animal parasitic nematodes. This fungus has also been reported as a human pathogen (Takayasu *et al*., 1977). Until recently, there were no reports of the use of the nematophagous fungus *P. lilacinus* in the control of insect pests (Fielder and Sosnowska, 2007).

Nematodes of the genera *Heterorhabditis* Poinar and *Steinernema* Travassos, in conjuction with their symbiotic bacteria species are pathogenic on many insect pests. Several studies have addressed the efficacy of entomopathogenic nematodes against the house fly, *M. domestica* L., in the laboratory and in confined poultry environments (Renn *et al.* 1985; Geden *et al*. 1986; Belton *et al*. 1987; Mullens *et al*. 1987a, b; Renn and Wright, 2000).

1.4 *Bacillus thuringiensis*, *Beauveria bassiana* and entomopathogenic nematodes as BCAs of livestock pests.

1.4.1. Bacillus thuringiensis Berliner

The species *Bacillus thuringiensis* Berliner (*Bt*) is a Gram-positive, aerobic, endospore-forming bacterium. Although *Bt* can be isolated from many environmental sources and that it is typically referred to as a 'soil bacterium', it has several features indicating that its principal ecological niche is insects (Meadows, 1993). The species *Bt* was first isolated from diseased larvae of the silkworm, *Bombyx mori*, in Japan by Ishiwata in 1901. It was not officially described, however, until it was re-isolated by Berliner in 1915 from diseased larvae of the Mediterranean flour moth, *Anagasta kuehniella*, in Thurungia, Germany, hence the derivation of the name *thuringiensis*. He was able to show that the bacterium was toxic when the spores were fed to insects. This suggested that the bacterium could be used to control insects.

Though commonly referred to as *Bt*, the species *B. thuringiensis* as currently recognized as a complex of subspecies, all of which are recognized by the production a parasporal body during sporulation. The parasporal body is the principal characteristic used to differentiate this species from closely related species, such as *B. cereus* and other bacilli. The parasporal body contains one or more proteins, typically as crystalline inclusions, and most of these are highly toxic to one

or more species of insects. The inclusions are composed of crystal (*Cry*) proteins that are toxic to a wide variety of insect species. In the insecticidal isolates, the toxins are known as endotoxins and often occur in the parasporal body as protoxins that after ingestion dissolve and are converted to active toxins through by proteolytic enzymes in the host gut. Crystal proteins have been classified as CryI to CryVI (Table 1.1), depending on host specificity (Höfte and Whiteley, 1989; Tailor *et al.*, 1992). These are located on large conjugative plasmids (Carlton and Gonzalez 1985), hence providing a mechanism for the transfer of the genes between and within different subspecies (Cooper, 1994). It is common for individual *Bt* strains to synthesize more than one toxin, but each acts specifically on susceptible insects (Höfte and Whiteley, 1989; Dulmage *et al.,* 1990).

In addition to the crystal—associated toxic proteins linked to sporulation, some *Bt* isolates synthesise other unrelated proteins during vegetative growth. These proteins, termed vegetative insecticidal proteins (or Vips), have demonstrated insecticidal activity against a wide spectrum of lepidopteran insects (Estruch *et al.*, 1996; Schnepf *et al.*, 1998). Another secretory product, nonproteinaceous β-exotoxin, known to be particularly active against dipteran species (Pinnock, 1994), is not insect specific. The β-exotoxin is an adenine nucleotide analogue and because it interferes with protein synthesis, it is considered harmful to mammals and is therefore unlikely to be approved for registration (Glare and O'Callaghan, 2000). An interesting aspect of *Bt* general biology is that, unlike most other insect pathogens, it rarely causes natural epizootics in insect populations (Federici, 1999).

Table 1.1 Host specificy of Cry protoxins[1]

Homology group	Host specificity	Variations in host specificity
CryI	Lepidoptera	CryIA(b)[2] and CryIC[3] may confer toxicity against Diptera, CryIB[4] may confer toxicity against Coleoptera
CryII	Lepidoptera and Coleoptera	CryIIA – Lepidoptera and Diptera CryIIB and IIC – only Lepidoptera
CryIII	Coleoptera	
CryIV	Diptera	
CryV	Lepidoptera	
CryVI	Nematodes	

[1]Lereclus *et al.,* 1993; [2]Haider *et al.*, 1987; [3]Smith *et al.*, 1996; [4]Bradley *et al.*, 1995.

1.4.1.1 Mechanism of action of Bacillus thuringiensis

The bacterium *B. thuringiensis* produces a heterogenous range of insecticidal, nematocidal and acaricidal toxins, most notably the crystal (δ-endotoxin) proteins and thuringiensin (β-exotoxin) nucleotide. The *B. thuringiensis* toxins, other than the δ-endotoxins, are generally less host specific. The most notable of these toxins, thuringiensin, is highly toxic to dipteran insects and in particular, the house fly, *M. domestica* (Bond *et al.*, 1971). The host spectrum of these toxins varies considerably according to their different modes of action. The insect, usually the larval stage, must ingest the crystal protoxin for them to be effective (Schnepf *et al.,* 1998). The protoxins are converted to toxins that exert their effect on the host by causing lysis of midgut epithelial cells, which leads to gut paralysis, cessation of feeding and eventual death of the host. Upon ingestion, the crystals are solubilized by the alkaline pH of the midgut and the protein protoxins are processed by midgut proteases to release the active toxins (Lecadet and Dedonder, 1967). Binding of activated protein toxins to specific cell surface receptors on the midgut epithelia leads to formation of pores in the apical membranes, leading to an influx of ions and water, causing gut lysis and insect death (Gill *et al.,* 1992; Theunis *et al.,* 1998) within a day or two.

1.4.1.2 Bacillus thuringiensis as a BCA

Most studies have concentrated on the effect of *Bt* on insects that affect crops or are vectors of human diseases. Preparations containing *Bt* are widely used in the forestry and horticultural industries (Kellar and Langenfruch, 1993; Navon, 1993; Rajakulendran, 1993; Teakle, 1994). Pesticide formulations containing *Bt* subsp. *israelensis* are used for control of medically important dipteran pests of the suborder Nematocera (mosquitoes and blackflies) (Mulla, 1990; Becker and Margalit, 1993; Becker, 1997). However, few have investigated the toxic effects of *Bt* on insect pests of livestock (Gough *et al.*, 2005) (Table 1.3). Interest in this research area has been brought about by the high economic importance of these pests and by the concern over their increasing resistance to chemical insecticides. For many of these pests, *Bt* has shown great potential for development as a control agent (Pinnock, 1994), and research on the toxins and their mode of action against livestock pests is in progress in many countries. Strains with activity

against the sheep louse (Drummond and Pinnock, 1992; Pinnock, 1994), the sheep blowfly (Pinnock, 1994), and hornfly (Gingrich and Haufler, 1978; Temeyer, 1984, 1990, 1994) have also been reported. To date, no commercial preparation of *Bt* has been released for the control of insect pests of livestock (Gough *et al.*, 2005). One of the limiting factors has been the availability of specific strains with high toxicity to insect pests of sheep and cattle (Gough *et al.*, 2002; Brar et *al*., 2006).

Table 1.3 *Bacillus thuringiensis* in use as a microbial agent of pests of livestock and poultry

Strain of *Bacillus thuringiensis*	Target organism	Method of application	Comments	Reference(s)
Bt subsp. *kurstaki* strain WB3S16	*Bovicola ovis*, (sheep biting louse)	Feeding assays	Paralysis and death of *B. ovis* occurred between 8-12 h postfeeding.	Hill and Pinnock, 1998.
Bt strain 4412 , HD-1 and HD-2, *Bt* subsp. *tenebrionis*, *Bt* subsp. *israelensis*	*Lucilia cuprina* *Chrysomya albiceps* (tropical blowflies) *M. domestica*	Feeding assays	The addition of spores to the δ-endotoxin was, however, essential to inducing significant mortality in larvae of *Chrysomya albiceps* (Weid.) and enhanced mortality in the other species tested.	Johnson *et al.*, 1998
Bt strain YBT-226	*M. domestica*, *Chrysomela scripta* cottonwood leaf beetle), *Manduca sexta* (tobacco hornworm).	Feeding assays	Bioassays resulted in significant mortality at low to moderate concentrations to larvae of the house fly (*M. domestica*, Diptera), cottonwood leaf beetle (*Chrysomela scripta*, Coleoptera), and tobacco hornworm (*Manduca sexta*, Lepidoptera).	Zhong *et al.*, 2000
96 *Bt* strains – 19 of which with known activity against Diptera	*L. cuprina* (Weidemann) (sheep Blowfly); *Bovicola ovis* (Schrank) (sheep louse); *Haematobia irritans exigua* (de Meijere) (buffalo fly)	Feeding assays	Some *Bt* isolates were found to have toxic activity against larvae of the two fly species and moderate activity against the sheep louse.	Gough *et al.*, 2002
Several strains of *Bacillus thuringiensis*	*L. cuprina* Wiedemann.		*Bt* isolates producing Cry1Ba were toxic to *L. cuprina* larvae and provided protection against flystrike for up to 6 weeks.	Heath *et al.*, 2004
A series of 410 *Bt* isolates	*Haemonchus contortus*, *Trichostrongylus colubriformis* and *Ostertagia circumcincta* (Livestock parasitic nematodes)	Growth medium	Two strains inhibited larval development of *H. contortus, T. colubriformis and O. circumcincta*. Adult *H. contortus* and *O. circumcincta* showed complete cessation of movement within 2 and 4 days, respectively.	Kotze *et al.*, 2005
Several isolates of *Bt*	*L. cuprina* (Wieldmann)	Feeding assays	These isolates were highly toxic to feeding larvae in both in vitro bioassays and in vivo on sheep.	Gough *et al.*, 2005
Bt strain L366	*Haemonchus contortus*	*In vitro* larval development and migration assays	Significantly more toxicity in migration assays than development assays, some fully developed *Bt*-exposed larvae were less able to migrate than controls, and hence compromised in their ability to infect sheep.	O'Grady *et al.,* 2007

Table 1.4 *Beauveria bassiana* use as a microbial agent of pests of livestock and poultry

Strain of *B. bassiana*	Target organism	Method of Application	Comments	Reference(s)
B. bassiana (HF88)	*M. domestica* L.	*B. bassiana* conidia formulated as a dust or aqueous solution were applied to plywood surfaces, to which adult lies were exposed.	Starch dust formulations were more effective than aqueous suspensions. Adult flies were susceptible (94-100% mortality) when conidia applied to boards was 10^7 conidia /cm^2.	Geden *et al.*, 1995
B. bassiana (P89, L90)	*M. domestica* L. *Stomoxys calcitrans* L. (stable fly)	*B. bassiana* conidia formulated as a dust or aqueous suspension were applied to plywood surfaces, to which adult flies were exposed.	Adult house fly mortality was dose dependent, with ≥90% dying at $1x10^8$ conidia/cm^2 for Strains P80 and L90 formulated as dusts. A dose of $1x10^8$ conidia/cm^2 killed only 70 and 84% of adult stable flies. Aqueous formulations were less effective for controlling both species of adult flies. *B. bassiana* was most effective against the house fly larvae at $1x10^{10}$ conidia/cm^3, at which 56 and 48% died following treatment with Strains L90 and P89 respectively.	Watson *et al.*, 1995
B. bassiana (Balsamo) Vuillemin	*M. domestica* L.	Conidial spray formulations were applied to the inside of hutch walls.	The prevalence of *B. bassiana* in the adult fly population was significantly greater in hutches sprayed with conidia than in untreated control hutches. Maximum weekly recovery of *B. bassiana* was 43 and 47% of the collected fly populations at two treatment farms.	Watson *et al.*, 1996
B. bassiana (HF88, HF89, WV, NC)	*Alphitobius diaperinus* Panzer), (The lesser mealworm)	Forced-contact bioassays with either 1 ml of aqueous or 0.1 g of starch dust inoculum.	Starch dust formulations were more effective than aqueous suspensions. Young larvae were more susceptible than adult beetles.	Geden *et al.*, 1998
B. bassiana (90517)	*Ambylomma americanum* Linnaeus (the lone star tick), *A.. maculatum* Koch	Ticks were submerged for ~30 s in spore suspensions (ranging from 10^4 to 10^8 cells/ml)	Fungal suspensions of conidia harvested from potato dextrose plates containing 10^8 conidia /ml caused greater than 90% mortality in adult *A. maculatum* but less than 10% mortality in adult A. americanum over a 28 day time course.	Kirkland *et al.*, 2004
B. bassiana product, balEnce	*M. domestica* L.	Applications were made using backpack mistblowers calibrated to allow a coarse fog of 40 µm or larger.	Adult house fly populations were lower in *B. bassiana*-treated facilities during the spray and post-spray periods. The numbers of house fly larvae recovered in *B. bassiana*-treated facilities were less than one-half that of the pyrethrin-treated facilities.	Kaufman *et al.*, 2005
B. bassiana (IHEM 18747)	*Psoroptes ovis* Hering (parasitic mite)	Adult females immersed into increasing concentrations of conidia (10^4– 10^9 conidia ml^{-1}).	Egg laying was not reduced by the fungal infection but both the hatchability of the eggs and the life span of the emerging larvae were significantly reduced. One hundred percent of healthy mites exposed to infected cadavers or surfaces acquired the infection.	Lekimme *et al.*, 2006

1.4.2 Beauveria bassiana (Balsamo) Vuillemin

White muscardine, *B. bassiana* (Balsamo) Vuillemin (Hyphomycetes), is a ubiquitous fungus occurring naturally in many areas of the world (Roberts *et al.*, 1981; Feng *et al.,* 1994, Humber, 1996). Isolated from over 700 species of insects from nine orders, it most commonly infects Lepidopteran and Coleopteran hosts (Fargues and Remaudiere, 1977; Li, 1988). *Beauveria bassiana* was the first reported insect pathogen, originally isolated from the silkworm, *Bombyx mori* L. (Lepidoptera Bombycidae), by Agostino Bassi in 1834 (Feng *et al.,* 1994), and has subsequently become the most extensively studied and exploited entomopathogen (Glare and Milner, 1991; Hajek and St. Leger, 1994). *Beauveria bassiana* is now exploited in greenhouse and outdoor crops as a tool for the control of many agricultural pest arthropods including whiteflies, aphids, thrips, psyllids, weevils and mealybugs (Shah and Goettel, 1999).

1.4.2.1 Mechanism of action of Beauveria bassiana

Pathogenesis of insect hosts due to *B. bassiana* occurs mainly through infection via the integument, though it may also enter through the respiratory system (Feng *et al.*, 1994). Penetration through the host cuticle is the mode of entry for most entomopathogenic fungi (Charnley, 1989). Host infection by most entomogenous fungi including *B. bassiana,* consists of conidial attachment to the cuticle (Boucias and Pendland, 1991), followed by germination and infection of the target insects, the penetration of hyphae through the activity of cuticle lytic enzymes produced by the fungus, proliferation inside the hemocoel and host death due to toxaemia (Khachatourians, 1991). Under favourable conditions, the fungus emerges and produces aerial conidia over the host cuticle (Feng *et al.*, 1994; Gupta *et al.*, 1995) and sporulation takes place approximately 4-6d after initial infection (*B. bassiana*) (Pell *et al.*, 2001).

The virulence of *B. bassiana* is attributed to the production of toxins including beauvericin, bassianolide and oosporein (Gupta *et al.*, 1995). Some toxins are proteases of high molecular weights, and they either directly damage the principal functions of the hemolymph or cause damage indirectly by producing a toxic by-product in the insect (Kučera and Samšiňáková, 1968).

1.4.2.2 Beauveria bassiana as a BCA

Several pests (Table 1.4) of livestock and poultry that have been targeted with the use of *B. bassiana,* include the lesser mealworm, *Alphitobious diaperinus* (Panzer) and the hide beetle, *Dermestes maculates* DeGeer (Crawford *et al.* 1998; Geden *et al.* 1998; Geden and Steinkraus 2003), and the stable fly, *Stomyxys calcitrans* L. (Watson *et al.*, 1995). Recent studies by Watson *et al.* (1995; 1996) and Kaufman *et al.* (2005) demonstrated that *B. bassiana* has potential for house fly control, even though the practical use of *B. bassiana* isolates as biological control agents was dependent on dosage and formulation (Watson *et al.*, 1995).

1.4.3 Entomopathogenic nematodes

Entomopathogenic nematodes (EPN) are small (less than 1-3mm) parasitic roundworms that occur most commonly in nature as parasites of soil-inhabiting insects. They have relatively simple and typical life cycles that, aside from the egg include four larval stages and one adult stage. Although known as EPN, but also referred to as beneficial or insecticidal nematodes, these nematodes are species in the genera *Steinernema* (family: Steinernematidae) and *Heterorhabditis* (family: Heterorhabditidae) of the Phylum Nematoda.

These nematodes are unusual, however, in that they have established a mutualistic relationship with bacteria that they harbour within their alimentary tracts, and these bacteria kill the insects rather quickly after the nematode invades the insect body. The bacteria have evolved specific relationships with individual species of nematodes (Ciche *et al.*, 2006). For example, the bacterial species *Xenorhabdis nematophilis* is associated with steinernematid *Steinernema carpocapsae* whereas *Photorhabdis luminescens* is associated with *Heterorhabditis bacteriophora* (Kaya and Gaugler, 1993). Both bacterial genera belong to Enterobacteriaceae. The nematodes employ the bacteria to help overcome the humoral and cellular defenses of insect hosts, to protect the insect cadaver against saprophytic microorganisms, bacteriovorous nematodes and scavenging insects, and as a substrate for growth and reproduction (Ciche *et al.*, 2006). The bacteria utilize the nematode vector for delivery into the insect hemocoel and to persist outside the insect host (Ciche *et al.*, 2006). Moreover, nematodes devoid of symbiotic bacteria usually fail to cause insect death, or if death occurs, they fail to grow and reproduce (Poinar *et al.*, 1977; Han and Ehlers, 1998).

Bacterial symbionts multiply rapidly within the host and produce a variety of anti-microbial compounds to suppress the growth of contaminants or competing pathogens (McInerney *et al.*, 1991a; 1991b). In addition, these nematodes also produce an unusual quasi-resistant larval stage, called 'dauer larva' – the infective juvenile (IJ), the insect infective stage of these nematodes, which is actually the third-instar juvenile surrounded by the molted cuticle of the second stage. In the life cycle of these nematodes, the dauer larva seeks out and infects an insect. Infective juveniles, small size, lack of appendages, limited sensory modalities and capabilities, and the size/mobility discrepancy between parasite and host constrain the host-seeking ability of entomopathogenic nematodes (Lewis, 2006).

1.4.3.1 Mechanism of action of entomopathogenic nematodes

Depending on the host and the nematode species, different routes of penetration are taken. One route of entry is through the mouth opening or the anus. Using the anus as an entry site represents the main route for infection of house fly maggots and leafminers (Renn, 1998). In grubs and sawfly larvae invasion is more successful via the mouth than via the anus (Georgis and Hague, 1981; Cui *et al.*, 1993). Alternatively, entomopathogenic nematodes may enter the tracheal system via the spiracles. In sawfly larvae, the spiracles are the most important route of entry by *S. carpocapsae* (Georgis and Hague, 1981).

Another site of penetration by entomopathogenic nematodes is the integument or the intersegmental membranes of an insect. Penetration through the integument was shown to be the main route of entry for *S. feltiae* into leatherjackets (Peters and Ehlers, 1994). Another port of entry to adult arthropods is the gonad openings. This is the main entry port for nematodes into ticks (Samish and Glazer, 1992).

Once within the hemocoel, the IJ nematodes begin to feed on hemolymph; while doing so they defacate, releasing the symbiotic bacteria (Ciche and Ensign, 2003; Martens *et al.*, 2004). These quickly colonize the insect, killing it within 1 to 3 days. The nematodes feed on the bacteria and tissues of the dead larva, maturing and undergoing from two to three generations within the dead insect's body over a period of 1 to 2 weeks. The final generation results in the formation of thousands of dauer larvae that leave the cadaver in search of a new host (Ciche *et al.*, 2006).

When penetrating into the insect's hemocoel, the IJ encounters the non-self response by the immune system of the host. Nematodes can be trapped in cellular or noncellular capsules. Encapsulation of entomopathogenic nematodes has been reported in Orthoptera, Coleoptera, Diptera, and Lepidoptera (Dowds and Peters, 2002). Nematodes may resist encapsulation in insects by either avoidance of being recognized (evasion), by overwhelming the immune system by multiple infections and disrupting encapsulation (tolerance), or by actively suppressing the encapsulation response (suppression) (Dowds and Peters, 2002). Nematodes themselves may also release an immune-depressive factor (Götz *et al.*, 1981).

The nematode-bacterium complex kills insects so rapidly that the nematodes do not form an intimate host-parasite relationship. This rapid mortality permits the nematodes to exploit a range of hosts that spans nearly all-insect orders (Grewal and Georgis, 1999). However, behavioural barriers restrict nematode efficacy to a few selected hosts (Gaugler, 1988) in the field.

1.4.3.2 Entomopathogenic nematodes as BCA

EPN are found under diverse ecological conditions including cultivated fields, forests, grasslands, deserts, and ocean beaches (Hominick *et al.*, 1996). They can be excellent biological control agents for soil-dwelling stages of many insect pests and are fast acting, killing target insect pests in 24-48h (Kaya and Koppenhöfer, 1999). In comparison, many other biological control agents take days or weeks to kill the target insect pest. EPN are safe to most non-target organisms and the environment, are easy to apply, and are compatible with most agricultural chemicals (Kaya and Gaugler, 1993). They also have a broad host range, ability to search for pests, and a potential to reproduce after application (Kaya and Gaugler, 1993). Table 1.5 shows some examples of the use of EPN's use as microbial control agents of insects.

Table 1.5 Some examples of Steinernematid and Heterorhabditid EPNs in use as a microbial agent of pests of livestock and poultry

Strain	Target	Method of Application	Comments	Reference
S. felitae (=bibionis), *H. megidis*	*M. domestica* L.	Diet medium	*S. feltiae (=bibionis)* killed 94% of house fly eggs and 90% of first instar larvae and 100% by D6. By D2, in the same medium, 1 000 000 encapsulated *H. megidis* had killed 71.4% of eggs and 90% of first instar larvae. This increased significantly (P 0.01) by D6, to 99.2% and 100% respectively.	Renn, 1995
S. felitae, H. megidis	*M. domestica* L.	Baits sprays	Significantly fewer flies were counted in houses baited with either *S. feltiae* or *H. megdis*. Significantly fewer flies were counted after spraying with *S. feltiae*.	Renn, 1998
Heterorhabditis Poinar *Steinernema* Travassos	*M. domestica* L.	Filter paper assays	None of the 22 strains of *Heterorhabditis* infecting maggots caused significant levels of mortality in filter paper assay but produced significant fly mortality in the manure substrate. 10 strains of *Steinernema* infected maggots, of which 7 strains (4 *S. carpocapsae* (Weiser), 2 *S. feltiae* (Filipjev), and 1 *S. scapterisci* Nguyen & Smart) caused significant mortality.	Taylor *et al.*, 1998
13 *spp* of *Steinernema* and *Heterorhabditis*	*Ixodes scapularis* (black-legged tick)	Feeding assays	*S. riobravis* (355) and *H. megidis* (M145) killed ticks most rapidly, with mean day of death postinfection of 2.5 and 3.5 days, respectively	Hill, 1998
Heterorhabditis sp	*Boophilus annulatus* *Hyalomma excavatum* *Rhipicephalus bursa,* *R. sanguineus*	Feeding assays	Ticks seem to be less susceptible to nematodes when feeding on a host. Preimaginal tick stages were less susceptible to nematodes than adult ticks. The mortality rate of unfed females was highest, followed by unfed males, and engorged females.	Samish *et al.*, 2000
H. bacteriophora *S. intermedia*, NC513 strain of *S. glaserii, S. anomali, S. riobrave, Steinernema* sp. and 5 strains of *S. feltiae*	*Lucilia sericata* Meigen, 1826) (Sheep blowfly)		None of the examined EPN species or strains showed larvicidal efficacy at 37^{o}C. At lower temperatures (20^{o}C and 25^{o}C) only strains of *S. feltiae* were found to be active.	Tóth *et al.*, 2005

1.5 Formulations and application methods

The development of a suitable formulation is a critical component in helping a BCA to germinate and infect the host (Brar *et al*., 2006). Additionally, commercial biopesticides must be economic to produce, have high residual activity, be easy to handle, stable in storage, mix and apply and be consistently effective in controlling the target pest (reviewed by Brar *et al*., 2006).

B. thuringiensis based biopesticides are formulated as concentrated liquids, oil-based flowables, wettable powders, water dispersible granules, and dusts (Boyetchko *et al.*, 1999). The principal mode of action of *Bt* biopesticides is based on target insect ingestion of the δ-endotoxin protein, which causes feeding inhibition and eventual toxemia to the mig-gut of susceptible larva (Boyetchko, 1999). Only a few species of insects in the families Lepidoptera, Coleoptera and Diptera are susceptible to the *Bt* proteins. Thus, these biopesticides have a relatively narrow insecticidal spectrum (Boyetchko *et al*., 1999).

Encapsulations are recent advances in bioinsecticidal formulations and provide protection from extreme environmental conditions (UV radiation, rain, etc.) and enhanced residual stability due to slow release of formulations (sustained delivery) (Brar *et al.*, 2006). Microbial propagules are encapsulated in a coating (capsule) made of gelatin, starch, cellulose and other polymers and even microbial cells (Barnes and Cummings, 1987; 1987b; Barnes and Edwards, 1989; Bok *et al.*, 1993).

A recent advance in encapsulations is the production of hydrocapsules that are of a shellcore type (water based), consisting of a polymer membrane surrounding a liquid center. These shells are produced by using UV radiation initiated free-radical copolymerization of functionalized prepolymers (silicones, urethanes, epoxys, polyesters, etc.) and/or vinyl monomers such as acrylates for better dispersion and UV radiation protection (Lelchelt-Kunze *et al.*, 2000; Toreki *et al.*, 2004).

Wastewater/wastewater sludge (WW/WWS) has been successfully used as a raw material for *Bt* biopesticide production with lower process costs (Sachdeva *et al.*, 2000; Tirado-Montiel *et al.*, 2001; 2003; Lachhab *et al.*, 2001; Tyagi *et al.*, 2001; Vidyarthi *et al.*, 2000; 2001; 2002; Yezza *et al.*, 2004a, 2004b). The WW/WWS based *Bt* biopesticides encompass advantages and drawbacks

over commercial biopesticides. Two principal problems associated with the use of wastewater sludge are the presence of toxic heavy metals and human pathogens (Brar *et al.*, 2006).

Different formulations of *Bt* registered in Canada/ North America are listed in Table 1.6. This list is not exhaustive as there are numerous unregistered formulations *Bt* formulations worldwide, especially in developing countries.

The three developmental stages of *B. bassiana* are conidia, blastospores and mycelia (Feng *et al.*, 1994). All the three developmental stages are formulated with several objectives in mind. Ingredients selected should improve spray coverage, including microsite targeting, rainfastness; increase safety (e.g. reduce dust inhalation, eye irritation); improve and simplify handling; improve storage stability (especially at moderate to high temperatures); improve field stability (especially under ultraviolet radiation); and improve efficacy (especially reduce ambient temperature requirements) (Feng *et al.*, 1994; Wraight and Carruthers, 1999).

Fungal pathogens possess a purely contact mode of action. Infectious propagules must be inoculated onto the target pest or onto substrates in the habitat from which secondary inoculation can be effected via pest movement or feeding (Faria and Wraight, 2001). The fungi may be applied directly to the insect as wettable powders, emulsions or dusts, with conventional equipment used for the application of synthetic chemical insecticides, amended into baits or traps, or added to soil (Feng *et al.*, 1994).

Various formulations of *B. bassiana* are listed in Table 1.7. So far dry, formulations are the forms that have been used for *B. bassiana* conidia, although oil- and water-based formulations are being tried in field experimental trials (Feng *et al.*, 1994). Oil diluents have contributed to multiple formulation objectives, thus offering several advantages. Oils are highly compatible with lipophilic conidia, as well as with the insect cuticle (Wraight and Carruthers, 1999). This compatibility reduces and eliminates the need wetting, sticking, or spreading agents (Prior *et al.*, 1988). Oils are also more effective carriers for low-volume applications than water that rapidly evaporates when applied as small droplets (Wraight and Carruthers, 1999). Moreover, Jarosnki (1997) reported that use of oil formulants significantly enhanced the high temperature stability of *B. bassiana* conidia. Formulation in oil has also been reported to reduce irritation to eyes of test

animals (Goettel and Jarosinki, 1997) and improve handling safety (Wraight and Carruthers, 1999).

Nematode formulations are developed with two main objectives: a means of delivery of live nematodes to the consumer and a means of extended product storage. During the past decade, significant progress was been made in developing nematode formulations with improved shelf stability, scalability, and ease of use (Table 1.8). Most of these formulations were based on the one fundamental principle of conseving IJ nematodes; limited stored energy reserves by either restricting their movement or reducing their oxygen consumption by inducing a state of partial anhydrobiosis (Grewal and Georgis, 1999). The first such formulations used activated charcoal to restrict nematode movement (Yukawa and Pitt, 1985). Kaya and Nelsen (1985) were the first to report on the encapsulation of EPN with calcium alginate.

Nematodes have also been successfully formulated in gel-forming polyacrylamides (Georgis, 1990), flowable gels (Georgis and Manweiler, 1994), wheat flour and attapulgite clay chips (Bedding, 1988).

A significant advancement was made with the advent of a water-dispersible granule (WDG) in which IJs are encased in 10-20mm diameter granule consisting of various types of silica, clays, cellulose, lignin and starches (Silver *et al.*, 1995). The development of WDG formulations has offered several advantages over existing formulations. For instance, it extended the nematode storage stability to several months at room temperature; enhanced nematode tolerance to temperature extremes and improved the ease of applications of nematodes (Grewal and Georgis, 1999).

Table 1.6 *Bacillus thuringiensis* products (formulations) registered for commercial use in Canada/ North America

Industry/ Company	Product	Active ingredient(s)	Target pest)s)
Valent Biosciences Corporation	Dipel (Wettable Powder)		Spruce budworm; gypsy moth; spring and fall cankerworms and cabbagelooper
	Thuricide 48LV (Liquid suspension)	*B. thuringiensis* Berliner ssp *kurstaki*	Bagworm; elm spanworm; fall spanworm; gypsy moth; spring and fall cankerworm; spruce budworm; jack pine budworm
	Vectobac-200G Larvicide (Granules)		
	Teknar Granules Larvicide		Mosquitoes
	Vectobac 200g (Granules)	*B. thuringiensis ssp. israelensis*	
	Vectobac 600L (aqueous suspension)		Fungus gnats
	Teknar HP-D Larvicide (aqueous suspension)		Mosquitoes and Black flies
	Novodor Flowable Concentrate	*B. thuringiensis* ssp *tenebrionis*	
	Foray 48B		Spruce budworm (Eastern and Western); gypsy moth; jackpine budworm; eastern hemlock looper; whitemarked tussock moth and forest tent.
	Foray 48B low volume		
	Foray 76B (aqueous concentrate)		
	Foray 96B		
	Dipel 2X DF (Dry Flowable)(Wettable Granules)	*B. thuringiensis* Berliner ssp. *kurstaki*	Spruce budworm; gypsy moth; bagworm; spring and fall cankerworm; fall webworms; elm spanworm; tent caterpillar; cabbage looper; leafroller and diamondback moth
Certis USA LLC	Thuricide-HPC High Potency Aqueous Concentrate		Spruce budworm; gypsy moth; bagworm; spring and fall cankerworms and cabbage looper
AFA Environment Inc.	Aquabac II XT (Liquid suspension)	*B. thuringiensis ssp. israelensis* (*B. thuringiensis* serotype H-14)	
	Aquabac 200G (10/14) (Granules)		Mosquitoes
	Aquabac 200G (10/14) (5/8)		
AFA Environment Inc.	Aquabac XT	*B. thuringiensis ssp. israelensis*	Mosquitoes and black flies
Abbott Laboratories Ltd.	Dipel 176 (Emulsifiable suspension)		Forest tent caterpillars; gypsy moth; spruce buworms; hemlock looper
Woodstream Canada Corporation	Safer's BTK (Liquid concentrate)	*B. thuringiensis* Berliner ssp. *kurstaki*	Gypsy moth; tent caterpillar and cabbage looper
AEF Global Inc.	Bioprotec Aqueous Biological (aqueous suspension)		Gypsy moth; eastern spruce budworm; western spruce budworm; jack pine budworm; forest tent caterpillar; eastern hemlock looper; bagworm; elm spanworm; fall spanworm; spring and fall cankerworm; satin moth and white marked tussock moth
	Bioprotec CAF		
	Aqueous Bioprotec HP		
	Bioprotec ECO		

Source: Brar *et al.*, 2006

Table 1.7 Summary of the storage stabilities of *Beauveria bassiana* formulations.

Stage	Formulation	Shelf life (viability after time at ^{0}C)	Reference
Conidia	Dry attapulgite clay	78% after 12 mo at 26^{0}C	Ward and Roberts, 1981
Conidia	Dry attapulgite clay	No viability loss after 12 mo at 26^{0}C	Chen *et al.*, 1990
Conidia	Dry, unformulated	70% after 12mo at 25^{0}C	Jarosnki, 1997
Conidia	Paraffinic oil	93% after 9 mo at 25^{0}C	Jarosnki, 1997
Blastospores	Dry xanthan/carob gel	100% after 12mo at 28^{0}C	Jung and Mugnier, 1989
Mycelium	Dry alginate pellets	100% after 5 mo at 22^{0}C	Knudsen *et al.*, 1990

Source: Wraight and Carruthers, 1999)

Table 1.8 Some commercially available formulations containing *Steinernema* and *Heterorhabditis* nematodes with expected shelf life

		Shelf life (m)	
Formulation	**Nematode species**	**Room Temp**	**Refrigerated**
Alginate gels[a]	*S. carpocapsae*	3.0 - 4.0	6.0 - 9.0
	S. feltiae	0.5 - 1.0	4.0 - 5.0
Flowable gels[a]	*S. carpocapsae*	1.0 - 1.5	3.0 - 5.0
Attapulginite clay chips[b]	*H. bacteriophora*	1.5 - 2.0	0
	S. feltiae	1.0 - 1.5	4.0 - 6.0
	S. carpocapsae	1.0 - 1.5	3.0 - 4.0
Water-dispersible granules[a]	*S. carpocapae*	4.0 - 5.0	9.0 - 12.0
	S. feltiae	1.5 - 2.0	5.0 - 7.0
	S. riobravis	2.0 – 3.0	4.0 – 5.0

[a] P.S. Grewal and R. Georgis (unpublished data)
[b] Bedding 1984.
Source: Grewal and Georgis (1999)

1.6 Commercialization

Most *Bt*-based bio-insecticide products are produced using naturally occurring strains of *Bt*, and utilize only a small fraction of the known *Cry* proteins. In agriculture, *Bt* products have been used successfully in the vegetable, cotton, and especially fruit crop markets and almost exclusively for the control of foliar-feeding lepidopteran pests (Baum *et al.*, 1999). Table 1.9 provides a listing of some better-known *Bt*-based insecticide products.

Table 1.9 Registered *Bacillus thuringiensis* based bioinsecticide products for agricultural use.

Product	Company	Strain background	Target insect Order
Able	Thermo Triology	*kurstaki*	L
Agree	Thermo Triology	*aizawai*	L
Biobit	Abbott	HD1 *kurstaki*	L
Bactospeine	Abbott	*kurstaki*	L
Condor	Ecogen	*kurstaki*	L
Costar	Thermo Triology	*kurstaki*	L
CRYMAX	Ecogen	*kurstaki*	L
Cutlass	Ecogen	*kurstaki*	L
Design	Thermo Triology	*aizawai*	L
Dipel	Abbott	HD1 *kurstaki*	L
Foil	Ecogen	*kurstaki*	L/C
Foray	Abbott	HD1 *kurstaki*	L
Florbac	Abbott	*aizawai*	L
Futura	Abbott	*kurstaki*	L
Javelin	Thermo Triology	HD1 *kurstaki*	L
Lepinox	Ecogen	*kurstaki*	L
MATTCH	Mycogen	Pseudomonas	L
MTRAK	Mycogen	Pseudomonas	C
MVP	Mycogen	Pseudomonas	L
Novodor	Abbott	*tenebrionis*[a]	C
Raven	Ecogen	*kurstaki*	L/C
Steward	Thermo Triology	HD1 *kurstaki*	L
Thuricide	Thermo Triology	HD1 *kurstaki*	L
Trident	Thermo Triology	*tenebrionis*	C
Vault	Thermo Triology	HD1 *kurstaki*	L
Xentari	Abbott	*aizawai*	L

[a]*tenebrionis* = subspecies *morrisoni*
L, lepidopteran-toxic, C, coleopteran-toxic
Source : Baum *et al.*, 1999

Table 1.10.*Beauveria bassiana* in production and registered or submitted for registration in the United States

Product name	Company	Formulation	Active ingedient	Principal market
[b]Mycotrol	Mycotech	ES	Conidia	Field crops
		WP	Conidia	Field crops
		OF	Conidia	Rangeland
[b]BotaniGard	Mycotech	ES	Conidia	Greenhouse
		WP	Conidia	Greenhouse
[b]Naturalis	Troy BioSciences	ES	Conidia	Field and greenhouse
[b]Conidia	Hoechst Schering AgrEvo	WDG		Field crops
[b]Bea-Sin	Agrobiológicos del Noroeste			Field and Greenhouse
[c]Ago Biocontrol Beauveria	Ago Biocontrol			Field and Greenhouse
[c]Boveril PM	Itaforte Bioprodutos			Greenhouse

[a]Emulsifiable oil suspension (ES); wettable powder (WP); oil flowable (OF); water-dispersible granule (WDG); aqueous suspension (AS).
Source: [b]Wraight and Carruthers, 1999. [c]Faria and Wraight, 2001

Commercial preparations of fungi that can be applied using conventional spray equipment and baits have been successfully developed for the control of cockroaches, grasshoppers (for field crops) and whiteflies, aphids and mites (in greenhouses). However, formulations for fly control in livestock and poultry production are only available on a limited basis (Kaufman *et al.*, 2005).

Several *B. bassiana* strains have been studied as potential biocontrol agents of insects. However, the strains available target whiteflies, locusts and beetle (Faria and Wraight, 2001) and none of the *B. bassiana* products currently available worldwide (Table 1.10) target livestock pests.

Table 1.11 Some available commercial products containing *Steinernema* and *Heterorhabditis* nematodes

Product name	**Nematode species**	**Company**	**Formulation**
Mioplant	*S. carpocapsae*	Norvatis, Vienna, Austria	Alginate gel
Boden-Nutzlinge	*S. carpocapsae*	Rhone-Poulenc, Celaflor, Germany	Alginate gel
BioSafe	*S. carpocapsae*	SDS Biotech, Minato-Ku, Tokyo, Japan	Flowable gel
Exhibit	*S. feltiae*	Norvatis Basel, Switzerland	Flowable gel
Stealth	*S. feltiae*	Norvatis, Macclesfield, Chester, UK	Flowable gel
Nemasys-H	*H. megdis*	MicroBio, Cambridge, UK	Flowable gel
LarvaNem	*H. megdis*	Koppert B.V., Berkel en Rodenrigs, Netherlands	Clay
Nemasys	*S. feltiae*	MicroBio	Clay
Entonem	*S. feltiae*	Koppert B.V.	Clay
Proactant Ss	*S. scapterisci*	BioControl, Gainesville, FL	Clay
Biosafe	*S. carpocapsae*	ThermoTrilogy, Columbia, MD	Water-dispersible granules
Biosafe-N	*S. carpocapsae*	ThermoTrilogy	Water-dispersible granules
BioVector	*S. carpocapsae*	ThermoTrilogy	Water-dispersible granules
Vector TL	*S. carpocapsae*	Lesco, Lansing, MI	Water-dispersible granules
Helix	*S. carpocapsae*	Norvatis, Mississauga, Canada	Water-dispersible granules
X-GNAT	*S. feltiae*	E.C. Geiger, Harleysville, PA	Water-dispersible granules
Magnet	*S. feltiae*	Amycel-Spawn Mate, Watsonville, CA	Water-dispersible granules
BioVector	*S. riobravis*	Thermotrilogy	Water-dispersible granules
Vector MC	*S. riobravis*	Lesco	Water-dispersible granules

Source: Grewal and Georgis, 1999

Mass production of entomopathogenic nematodes of the genera Steinernema and Heterorhabditis has been greatly facilitated by the ease with which the symbiotic bacteria and nematodes can be grown on a variety of artificial media *in vitro* (Georgis, 2002). Table 1.11 provides a listing of examples of some better-known commercially available products containing *Steinernema* and *Heterorhabditis* nematodes.

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CHAPTER 2

LABORATORY SCREENING OF INSECTICIDAL ACTIVITIES OF *BEAUVERIA BASSIANA*, *PAECILOMYCES LILACINUS* AND *HETERORHABDITIS SPP.* ON THE HOUSE FLY (*MUSCA DOMESTICA* L.) LARVAE AND ADULTS

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Abstract

The house fly, *Musca domestica* L., is a major pest in poultry facilities. It has been implicated as a mechanical vector of a wide range of enteric pathogens among animals and humans. Among potential microbial biocontrol agents, entomopathogenic fungi, bacteria and nematodes are the most promising candidates. Thirty-four fungal isolates of *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycetes) and two isolates of *Paecilomyces lilacinus* and a nematode *Heterorhabditis spp*. were evaluated *in vitro* for their entomopathogenic activity against the larvae and adults of the common house fly, *M. domestica*. In the first laboratory experiment, different isolates of *B. bassiana* and *P. lilacinus* were tested by infecting larvae and adults with conidial suspensions of 10^8 ml^{-1} concentration. Flies and larvae were exposed to fungi for at least 6d in the dark at a temperature of 25±1^oC. The *B. bassiana* isolates were pathogenic to the adult flies, causing mortality levels of 30-100% within 6d. The *P. lilacinus* isolates were non-pathogenic towards adult flies. All the fungal isolates were marginally effective in controlling house fly larvae. All the *B. bassiana* isolates sporulated on flies and 30-100% of the flies ended up being covered with mycelium. The best four *B. bassiana* isolates, that caused mortality of 90% or more within 2d, were subjected to dose-response mortality bioassays. These showed a dose-related pathogenicity on adult flies. The lethal concentration of *B. bassiana* isolates that caused 50% mortality (LC_{50}) ranged between 10^3-10^5 conidia ml^{-1}. The lethal time to 50% mortality (LT_{50}) values ranged between 0.44-1.3d. In order to evaluate the potential of entomopathogenic nematodes, isolates of *Heterorhabditis spp* were screened on fly larvae using a filter paper bioassay and in fresh bovine manure substrate. The highest mortalities observed were 85.5% and 5.4% on filter paper and manure respectively. The present results suggest that the *B. bassiana* Isolates 7320, 7569 and 7771 have excellent potential for the biological control of adult *M. domestica*.

2.1 Introduction

The house fly, *Musca domestica* (L.), is the major insect pest in poultry facilities (Scott *et al.*, 2000) and intensive animal units (Barson *et al.*, 1994). Large populations of flies create problems for animal and public health. Furthermore, house flies have developed resistance to most insecticides used against them (Scott *et al.*, 2000). Hence, efforts to control flies using biological control agents (BCAs) are increasingly important.

Most research on fly pathogens has concentrated on the fungal pathogen *Entomophthora muscae* (Cohn) Fresenius (Mullens *et al*., 1987b; Geden *et al*., 1993; Steinkraus *et al*., 1993). However, the potential of *E. muscae* for fly suppression is limited by short-lived conidia, intolerance of high temperatures, and by the requirement for large populations of flies to sustain epizootics (Geden *et al*., 1995). The broad host range of *B. bassiana* predicates the potential of this pathogenic fungus as a biological control agent (Humber, 1992). Discoveries in recent years of natural infections of house flies (Steinkraus *et al*., 1990) with the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin suggest that this pathogen is compatible with environmental conditions in animal agriculture production systems (Geden *et al*., 1995) and that *Beauveria bassiana* has potential for fly control (Steinkraus *et al*., 1990; Geden and Rutz, 1992; Watson *et al*., 1995; 1996; Kaufman *et al*., 2005). The entomopathogenic fungus *B. bassiana* is an attractive candidate for biological control of the house fly because the fungus is easily and economically produced and the conidia have a long storage life, and can be formulated and applied in a variety of ways (Feng *et al*., 1994). Furthermore, unlike *E. muscae*, the entomopathogenic fungus *B. bassiana* can be cultured on artificial media (Bell, 1974).

Paecilomyces lilacinus is a soil saprophytic hyphomycete with a broad-based distribution (Samson, 1974). The efficacy of the fungus *P. lilacinus* has been widely demonstrated against root knot, cyst nematodes and other important groups of plant and animal parasitic nematodes. This fungus has also been reported as a human pathogen (Takayasu *et al*., 1977). Until recently, there were no reports of the use of the nematophagous fungus *P. lilacinus* in the control of insect pests (Fielder and Sosnowska, 2007).

Nematodes of the genera *Heterorhabditis* Poinar and *Steinernema* Travassos, in conjunction with their symbiotic bacteria, *Photorhabdus spp*. and *Xenorhabdus spp*., are pathogenic to many insect pests (Poinar, 1979) and may be effective biological control agents for soil-associated insects (Klein 1990). Several studies have addressed the efficacy of entomopathogenic nematodes against the housefly, *M. domestica* L., in the laboratory and in confined poultry environments (Renn *et al.* 1985; Geden *et al*. 1986; Belton *et al*. 1987; Mullens *et al*. 1987a, 1987b).

The objectives of this study were: (1) To evaluate the effectiveness of 34 isolates of *B. bassiana* and 2 strains of *P. lilacinus* against laboratory-reared larvae and adults of *M. domestica* in the laboratory; (2) To select highly virulent strains that could be used in the development of a mycoinsecticide against *M. domestica;* (3) To compare *B. bassiana* isolates for relative virulence against adult house flies; (4) To determine the susceptibility of house fly larvae to *Heterorhabditis*.

2.2 Materials and methods

2.2.1 Fungi

A sample of 34 isolates of *B. bassiana* and two strains of *P. lilacinus* from plants of diverse orders and from diverse geographical origins (Table 2.1) was bioassayed against larvae and adult house flies. The investigations were also performed with two commercial strains of *B. bassiana* (R444 and 1174) and cultures of *P. lilacinus*. Isolates of *B. bassiana* and *P. lilacinus* were provided by ARC Plant Pathology Research Institute PPRI)[1] Pretoria. Commercial strains of *B. bassiana* were from Plant Health Products (PHP)[2] (Pty) Ltd.

Beauveria bassiana cultures were grown on potato-dextrose agar. Sporulating cultures (3 – 4 wk-old) were harvested by brushing the dry conidia from surface of the agar plate into sterile vials. Conidia were counted with aid of a haemocytometer to calibrate a dose of 10^8conidia ml^{-1}. An aqueous formulation was prepared by a surfactant (0.1% dilution of Tween 80) and 1 $x10^8$ conidia ml^{-1}.

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[2] Plant Health Products (Pty) Ltd. P.O. Box 207, Nottingham Road, South Africa

Table 2.1 Origin of fungal strains of *Beauveria* sp. used to study their toxinogenic activity

Strain	**Host**
B. bassiana 7274	Garden plots
B. bassiana 7284	Orchard
B. bassiana 7288	Wheat
B. bassiana 7291	Rooibos
B. bassiana 7292	Apricot
B. bassiana 7293	Oranges
B. bassiana 7296	Oats
B. bassiana 7297	Vineyard
B. bassiana 7299	Maize
B. bassiana 7309	Fallow land
B. bassiana 7311	Tea
B. bassiana 7313	Peaches
B. bassiana 7317	Tea
B. bassiana 7320	Fallow land
B. bassiana 7569	Disturbed soil
B. bassiana 7573	Vineyards
B. bassiana 7581	Below waterfall
B. bassiana 7586	Soil under tree
B. bassiana 7587	Soil, cane field
B. bassiana 7600	Tea
B. bassiana 7618	Tea
B. bassiana 7647	Soil, treated field
B. bassiana 7762	Lawn
B. bassiana 7768	Oats
B. bassiana 7771	Disturbed soil
B. bassiana 7772	Sugarcane rows
B. bassiana 7775	Disturbed soil
B. bassiana 7781	Vineyards
B. bassiana 7791	Rooibos field
B. bassiana 7853	Canefields
B. bassiana 7864	Field
B. bassiana 7872	Banana
B. bassiana R444	-
B. bassiana 1174	-
P. lilacinus 7865	Field
P. lilacinus 7896	Banana

2.2.2 Nematodes

The entomopathogenic nematodes used were isolates of *Heterorhabditis spp*., supplied as infective juveniles (IJs) by PHP (Pty) Ltd.

2.2.3 Insect rearing

House flies were maintained at 28°C, 68% RH, with a 12:12 L:D photoperiod. Flies were given a maintenance diet of *ad lib* sugar, milk powder and water and were offered chicken liver as an oviposition medium on a twice weekly basis from the third week of postemergence. Larvae for replacement colonies were reared on a diet comprising 50g active dried yeast (DCL), 8g agar (Oxoid Bacteriological), 250ml distilled water, and 750ml UHT full cream milk, prepared by microwaving the milk to its boiling point (Johnson *et al*., 1998).

2.2.4 Laboratory evaluations of Beauveria bassiana against house fly larvae and adults

2.2.4.1 Bioassays

The first screening was conducted by spraying 30 cold-anesthetized house flies on Petri dishes. Treated flies were placed in cages provisioned with food and water, and held for observation for 7d. The cages with the treated insects were arranged in a controlled environmental chamber (25 ± 1°C, RH 80% and L:D 8:16h). The bioassays were set up in a completely randomized design with three replicates for each treatment. The controls were treated with an equal amount of water and 0.1% Tween 80. The dead flies were examined for red abdominal colouration, which is indicative of *B. bassiana* mycosis (Steinkraus *et al*., 1990). The cause of death was confirmed by placing all dead insects on moist filter papers to facilitate fungal sporulation. The number of insects that expressed mycoses was noted. The bioassay was repeated twice.

Batches of approximately 300 *M. domestica* eggs were placed on moist black paper within Petri dishes (90mm in diameter) and incubated at 25°C in darkness for 72h. Groups of approximately 150 neonate larvae were then transferred to 50g of a semisynthetic rearing diet within 250ml polyethylene pots with push fit lids containing a mesh insert for aeration. Larvae were reared at 20°C in darkness for 5d and then transferred for 2d to a preservative-free diet. Batches of 30 larvae were treated by immersion for 10s in 10ml of conidia suspension and then removed. Controls were sprayed with a dilution of 0.1% Tween in distilled water. Each batch of larvae was transferred to damp filter paper within a Petri dish on the laboratory bench for 1h and then

transferred to 15g of preservative-free diet in a 25ml polyethylene pot with a push fit lid (lid pierced with a dissecting needle for aeration).

The pots were placed within a polystyrene sandwich box (280 by 160 by 90mm), on damp paper towels to prevent the medium from drying out. Incubation was at 20^{o}C in darkness. Numbers of living and dead larvae (no movement when probed with a dissecting needle) were counted 5d post inoculations, and thereafter once every 24h for a total of 7d. Each batch of larvae was transferred to a pot of fresh food every 2-3d. Any dead larvae were removed daily and incubated on damp filter paper within Petri dishes (20±1^{o}C) for 7d and inspected for the presence of mycelium on the cadavers.

2.2.4.2 LC_{50} and LT_{50} assessment with selected strains

Four doses (10^5, 10^6, 10^7 and 10^8) of conidia from the four most promising fungal isolates were used in the bioassay. Conidia of sub-cultured slants were inoculated into 50ml of SMY liquid media in a 250ml-Erlenmeyer flask and incubated for 2-3d at 25^{o}C with rotary shaking of 200rpm. Ten milliliters of the culture were put into plates containing SMY solid medium and incubated for 10-15d at 25^{o}C. The conidia which developed in the flask were suspended in 15ml of a solution containing 0.2% Tween80 and 0.89% NaCl, and then the conidial suspension was filtered through a 2-layered-paper filter to remove mycelial fragments and aggregated conidia. The concentration of conidia was determined by using haemocytometer. Thirty flies were inoculated by spraying. The number of dead flies was checked every 24h. Each treatment was replicated three times. Lethal concentrations required to kill one-half of the adults was estimated using LC_{50} Probit analysis according to Finney (1971).

2.2.5 Laboratory evaluation of Heterorhabditis against house fly larvae

The virulence of *Heterorhabditis spp.* towards house fly larvae and adults was tested using a filter paper assay. Thirty third-instar larvae were placed in a 90mm Petri dish lined with a wet filter paper disk (70mm diameter) containing a nematode-water suspension (approximately 20, 40 and 100 nematodes per larva). The Petri dish was covered, sealed with parafilm, and incubated in

the dark at 25°C. Water was used as the control treatment. Larval mortality was observed after 24h. *Heterorhabditis spp.* was tested for pathogenicity towards fly larvae in fresh chicken manure. Manure samples (150g) collected from poultry houses at University Research Station at Ukulinga, were mixed and placed in plastic containers (100 x 120 x 12.5mm deep) with lids. Three replicates of 30 third-instars were inoculated with 600, 1200 and 3000 (approximately 20, 40 and 100 nematodes per larva) nematodes per container. Dead larvae were counted after every 24h up to 5d.

2.2.6 Statistical analysis

The cumulative insect mortality in each treatment was corrected for control mortality (Abbott, 1925). The number of insects with mycosis was estimated as percent proportion of dead insects. The mortality and mycosis values were arcsine-transformed to normalize the data (Gomez and Gomez, 1984) before analysis. The mean and standard error of all the replicates for mortality after 48h and mycosis were calculated and presented in tables as untransformed data. Mortality data are presented as percentage mortality, although actual mortality was used for statistical tests.

Logit transformation (Probit analysis GENSTAT) was used to estimate the lethal time to 50% mortality (LT_{50}) and the lethal concentration causing 50% mortality (LC_{50}) for the selected isolates. Percent mortality were arcsine-transformed and analyzed using a repeated measures model (GENSTAT) with isolate, dosage and time as main factors and isolate by dosage, isolate by day, dosage by day and isolate by dosage by time interactions. Least Significant Differences were used to compare the means of mortalities of adult flies caused by the four *B. bassiana* isolates.

For the nematode bioassays, mortalities of larvae grown on the filter paper and on manure were corrected for control mortality (Abbott, 1925). Mortalities of the larvae were arcsine transformed and analyzed using repeated measures ANOVA (GENSTAT) with number of nematodes per host and time as the main factors, and number of nematodes by time as the interaction. Probit analysis (GENSTAT) was used to estimate LC_{50} and LT_{50} for nematodes on filter paper and on manure.

2.3 Results

2.3.1 Screening bioassays against house fly adults and larvae

There were significant differences among fungal isolates in their virulence towards adult flies (F=544.61; P<0.001) and exposure time (F=2643.45; P<0.001). The interaction between fungal isolates and exposure time (F=32.27; P<0.001) was also significant. All 34 isolates of *Beauveria* were pathogenic to adult house flies and caused a mortality of 100% within 6d (Table 2.2), and 30-100% after 24h of exposure.

Significant differences were observed among fungal isolates in their virulence towards fly larvae (F=7.83; P<0.001) and exposure time (F=7.83; P<0.001). The interaction between fungal isolates and exposure time (F=3.43; P<0.001) was also significant. However, none of the fungal isolates were very effective in controlling house fly larvae (Table 2.3), and no fungus was observed growing on dead larvae. Percent mortality ranged between 0-36.7%. Isolate 7771 caused the highest larval mortality in 4d. None of the *P. lilacinus* isolates were pathogenic on adult flies or larvae.

Fungi were classified into four groups (Table 2.2) according to the number of days required to cause 90% or more fly mortality: Class I (2d or less), Class II (3-4 days), Class III (5-6d) and Class IV (>6 days). Fungi in Class I, which had four *B. bassiana* isolates, were selected as high pathogenicity isolates. Class IV contained the isolates non-pathogenic on the house fly. The two strains of *P. lilacinus* were placed in this class. Two isolates of *B. bassiana* caused mortality of 90% or more in 1d or less while two isolates caused similar mortality in 2d or less. Twenty-four isolates of *B. bassiana* caused 90% mortality in 3d and three isolates of *B. bassiana* killed 90% or more flies in 4d. Three isolates of *B. bassiana* killed 90% or more flies in 5 – 6d. High mortality rates were observed as a result of a formulated strain of *Beauveria* R444 and Isolates 7320, 7569 and 7771 (Table 2.3). No significance differences in infectivity were found among these three isolates of *Beauveria* (Table 2.3). These four isolates produced mortality rates that exceeded 90% within 2d or less and were submitted to dose response bioassays.

Table 2.2. Number of *Beauveria bassiana* isolates and *Paecilomyces lilacinus* classified by days required to kill more than 90% of house flies in bioassay.

Genus	**Total**	**Class I (2d or less)**	**Class II (3-4d)**	**Class III (5-6d)**	**Class IV (> 6d)**
B. bassiana	34	4	27	3	0
P. lilacinus	2	0	0	0	2

The entomopathogenic activity of *B. bassiana* was confirmed by the presence of fungal hyphae on the body of flies. Approximately 2d after death, saprophytic growth of mycelium was observed from intersegmental parts and subsequently, the fungus emerged from all parts of the integument (Fig. 2.1a). Adult flies treated with *B. bassiana* displayed a reddish coloration upon death, probably as a consequence of oosporein production (Fig. 2.1b).

Percent mycosis in the dead insects was significant (F=110.39; P<0.001) among the fungal isolates, with Isolate 7771 showing the highest percent mycosis (100%) (Table 3.2). Five *B. bassiana* isolates caused mycosis in 90% or more of the dead insects. Six isolates induced less than 50% mycosis in the dead insects. The two *P. lilacinus* isolates were found to be non-mycotic to adult flies.

Table 2.3 Percent mortality and mycosis values of larvae and adult *Musca domestica* 4d and 48h respectively, following exposure to 34 isolates of *Beauveria bassiana* and two strains of *Paecilomyces lilicanus* at a concentration of 10^8 conidia ml^{-1}.

Isolate	**Spray assay♠♦**	**Percent mortality of larvae after 4d ± se**	**Percent mortality of adults after 48h ± se**	**Percent mycosis of adult flies ± se**
B. bassiana 7274	3	10.00 ± 0.58 ^{bcde}	84.44 ± 0.33 ^{gh}	73.67 ± 1.86 ^{hij}
B. bassiana 7284	3	7.78 ± 0.33 ^{abc}	82.22 ± 0.58 ^{fg}	21 ± 0.58 ^{b}
B. bassiana 7288	3	8.89 ± 0.67 ^{abcd}	85.56 ± 0.67 ^{h}	72 ± 1.53 ^{hij}
B. bassiana 7291	3	7.78 ± 0.33 ^{abc}	85.56 ± 0.88 ^{hi}	97.67 ± 1.45 ^{m}
B. bassiana 7292	3	8.89 ± 0.67 ^{bcdef}	85.56 ± 0.33 ^{ef}	34.33 ± 2.33 ^{cd}
B. bassiana 7293	3	8.89 ± 0.33 ^{abcd}	68.89 ± 0.33 ^{b}	53 ± 1.15 ^{fg}
B. bassiana 7296	5	6.67± 0.58 ^{a}	70.00 ± 0.58 ^{b}	57.33 ± 1.45 ^{g}
B. bassiana 7297	3	8.89 ± 0.33 ^{abcd}	84.44 ± 2.19 ^{gh}	47.67 ± 1.45 ^{ef}
B. bassiana 7299	3	12.22 ± 0.33 ^{bcdefg}	74.44 ± 1.86 ^{c}	72 ± 1.53 ^{hij}
B. bassiana 7309	3	13.33 ± 0.58 ^{bcdefgh}	75.56 ± 0.88 ^{c}	69 ± 0.58 ^{hi}
B. bassiana 7311	3	11.11 ± 1.15 ^{bcde}	80 ± 0.58 ^{b}	29 ± 0.58 ^{bcd}
B. bassiana 7313	4	12.22 ± 0.88 ^{bcdefg}	76.67 ± 0.58 ^{cd}	67 ± 2.08 ^{h}
B. bassiana 7317	6	12.22 ± 1.20 ^{bcdefg}	22.22 ± 1.20 ^{b}	24.33 ± 2.85 ^{bc}
B. bassiana 7320	2	24.44 ± 2.03 ^{ij}	97.78 ± 0.33 ^{k}	84 ± 2.08 ^{l}
B. bassiana 7569	1	22.22 ± 1.45 ^{hij}	96.67 ± 0.58 ^{k}	81.33 ± 2.40 ^{kl}
B. bassiana 7573	3	15.56 ± 0.88 ^{bcdefgh}	84.44 ± 0.88 ^{gh}	51.33 ± 1.86 ^{fg}
B. bassiana 7581	3	23.33 ± 1.15 ^{ij}	76.67 ± 0.58 ^{cd}	99 ± 1.0 ^{mn}
B. bassiana 7586	4	18.89 ± 0.88 ^{efghi}	36.67 ± 0.58 ^{b}	25 ± 2.52 ^{bc}
B. bassiana 7587	3	20.00 ± 1.73 ^{efghi}	85.56 ± 1.20 ^{hi}	73.67 ± 0.88 ^{hij}
B. bassiana 7600	5	16.67 ± 1.53 ^{cdefghi}	57.78 ± 0.33 ^{b}	39 ± 1.53 ^{de}
B. bassiana 7618	4	17.78 ± 2.03 ^{defghi}	67.78 ± 0.33 ^{b}	52.67 ± 2.67 ^{fg}
B. bassiana 7647	3	8.89 ± 0.33 ^{abcd}	87.78 ± 0.33 ^{i}	98.33 ± 1.67 ^{mn}
B. bassiana 7762	3	10.00 ± 0.00 ^{bcde}	76.67 ± 0.58 ^{cd}	73.33 ± 1.20 ^{hij}
B. bassiana 7768	3	22.22 ± 1.86 ^{hij}	88.89 ± 0.33 ^{j}	77.67 ± 1.86 ^{jk}
B. bassiana 7771	1	36.67 ± 1.73 ^{k}	96.67 ± 0.58 ^{k}	100.00 ± 0 ^{n}
B. bassiana 7772	3	17.78 ± 2.85 ^{defghi}	78.89 ± 1.20 ^{de}	71.33 ± 1.86 ^{hij}
B. bassiana 7775	3	16.67 ± 2.00 ^{cdefghi}	87.78 ± 0.33 ^{ij}	81.33 ± 0.88 ^{kl}
B. bassiana 7781	3	16.67 ± 2.00 ^{cdefghi}	80 ± 0.58 ^{ef}	51.33 ± 1.33 ^{fg}
B. bassiana 7791	3	10.00 ± 0.00 ^{cde}	87.78 ± 0.67 ^{ij}	51.67 ± 0.88 ^{fg}
B. bassiana 7853	3	16.67 ± 2.00 ^{cdefghi}	76.67 ± 0.58 ^{cd}	76 ± 0.58 ^{ijk}
B. bassiana 7864	3	12.22 ± 0.67 ^{bcdefg}	85.56 ± 0.33 ^{k}	71.67 ± 2.85 ^{hij}
B. bassiana 7872	3	18.89 ± 0.88 ^{efghij}	84.4 ± 1.20 ^{gh}	45.67 ± 2.19 ^{ef}
B. bassiana 1174	3	21.11 ± 1.67 ^{ghij}	78.89± 0.33 ^{de}	75 ± 2.65 ^{ijk}
B. bassiana R444	2	28.89 ± 0.88 ^{jk}	95.56 ± 0.88 ^{k}	98.67 ± 1.33 ^{mn}
P. lilacinus 7865	>6	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}
P. lilacinus 7896	>6	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}
F-ratio		3.43	32.27	110.39
P value		<0.001	<0.001	<0.001
% CV		45.7	3.3	9.1
LSD		0.027	0.008	0.108

F and *P* values after arcsine transformation

Means followed by the same letter within the same column are not significantly different

♠Days required to kill more than 90% of the adult house flies

♦ Conidial suspension of $1x10^8$ conidia ml^{-1} was used to spray adult flies.

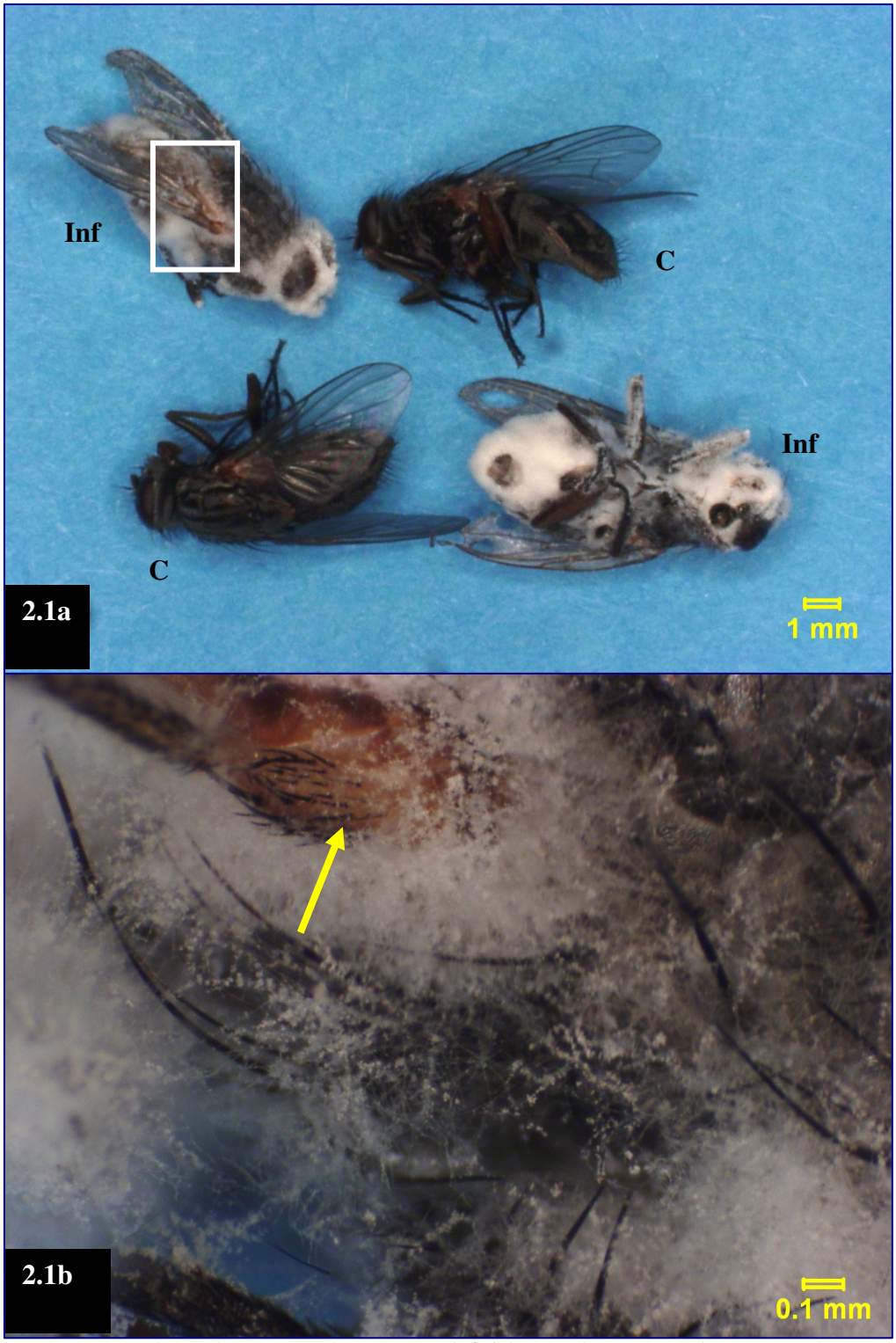


Fig 2.1a and 2.1b. Light microscope micrographs of; **3.1a)** mycelial growth of *Beauveria bassiana* (Isolate 7771) on infected adult house flies; (C = Control; Inf = Infected) **3.1b)** arrow showing detail of red abdominal colouration typical of *B. bassiana* infection.

2.3.2 LC_{50} assessment with selected strains of Beauveria bassiana

Three main effects caused significant differences in house fly mortality, namely isolates (F= 16.23; P<0.001), doses (F= 30.14; P<0.001) and time (F= 180.29; P<0.001) 4d post infection. The interaction effects between isolate and time (F= 3.27; P<0.004), dose and time (F= 6.41; P<0.001) and isolate and dose and time (F= 1.79; P<0.032) were also significant. However, no significant interaction effects were found between isolate and dose (F= 0.35; P< 0.951).

Adults of *M. domestica* were susceptible to all tested isolates of *B. bassiana*. This susceptibility was dose-dependent (Fig. 2.2, Table 2.4). The best of the four *B. bassiana* isolates killed 100% of target flies at a dose of 10^6 conidia ml^{-1}, by D4 after exposure.

Isolate 7569 killed over 80% of the adult flies by D2 after flies were exposed to a conidial concentration of 10^5 (Table 2.2). No significant differences were observed in fly mortality between Isolates 7771 and 7569 at all conidia concentrations (Table 2.4). The dose-mortality relationship curves of Isolates 7771 and 7569 were also similar (Fig. 2.2). However, significant differences were observed in fly mortality between Isolates 7771 and 7320 and between Isolates 7569 and 7320 at 10^5, 10^6 and 10^7 conidia ml^{-1}. One hundred percent mortalities of flies caused by Isolates 7771 and 7569 were attained at the dose of 10^5 conidia ml^{-1} (D4) (Fig. 2.2, Table 2.4). Of the remaining isolates, 100% mortality was attained at the dose of 10^6 conidia ml^{-1} on D4. There were no significant differences in fly mortality between the four best isolates of *B. bassiana* at 10^6, 10^7 and 10^8 conidia ml^{-1} on D4.

LC_{50} as determined by Probit analysis, were from 10^3-10^4 (Table 2.5). The LC_{50} value of Isolate *B. bassiana* 7771 was lower than those of the other isolates, followed by Isolates 7569, 7320 and finally R444 (Table 2.5). There were no significant differences in mortalities caused by Isolates 7771 and 7569. Thus, *B. bassiana* Isolates 7771 and 7569 had the highest pathogenicity among the isolates tested. The three most virulent isolates were Isolate 7771 (Log (LC_{50}=3.58) (95% CI = 2.02-4.31), slope = 0.817±0.11), Isolate 7569 (Log (LC_{50}=3.62) (95% CI = 2.25-4.27), slope= (0.94±0.14) and Isolate 7320 (Log (LC_{50}=4.31) (95% CI=3.15-4.90), slope= (0.82-0.08), which were selected for further study. In addition to being highly virulent, the three isolates all have

desirable colony growth characteristics. They produce fast-growing cultures with a compact, dense mycelial mat and produce high yields of conidia that are easily harvested from the surface of the culture.

The speed with which the insects were killed, as indicated by LT_{50}, increased with increasing dose up to a concentration of 10^8 (Fig 2.2). *Beauveria bassiana* Isolate 7569 achieved the shortest time to attain 50% mortality (LT_{50}=0.45d) (95% CI = 0.15-0.74). This was followed by Isolates 7771 (LT_{50}=0.64d (95% CI = 0.23-0.92), R444 (LT_{50}=1.29d (95% CI = 0.96-1.54) and 7320 (LT_{50}=1.30d) (95% CI = 1.06-1.49).

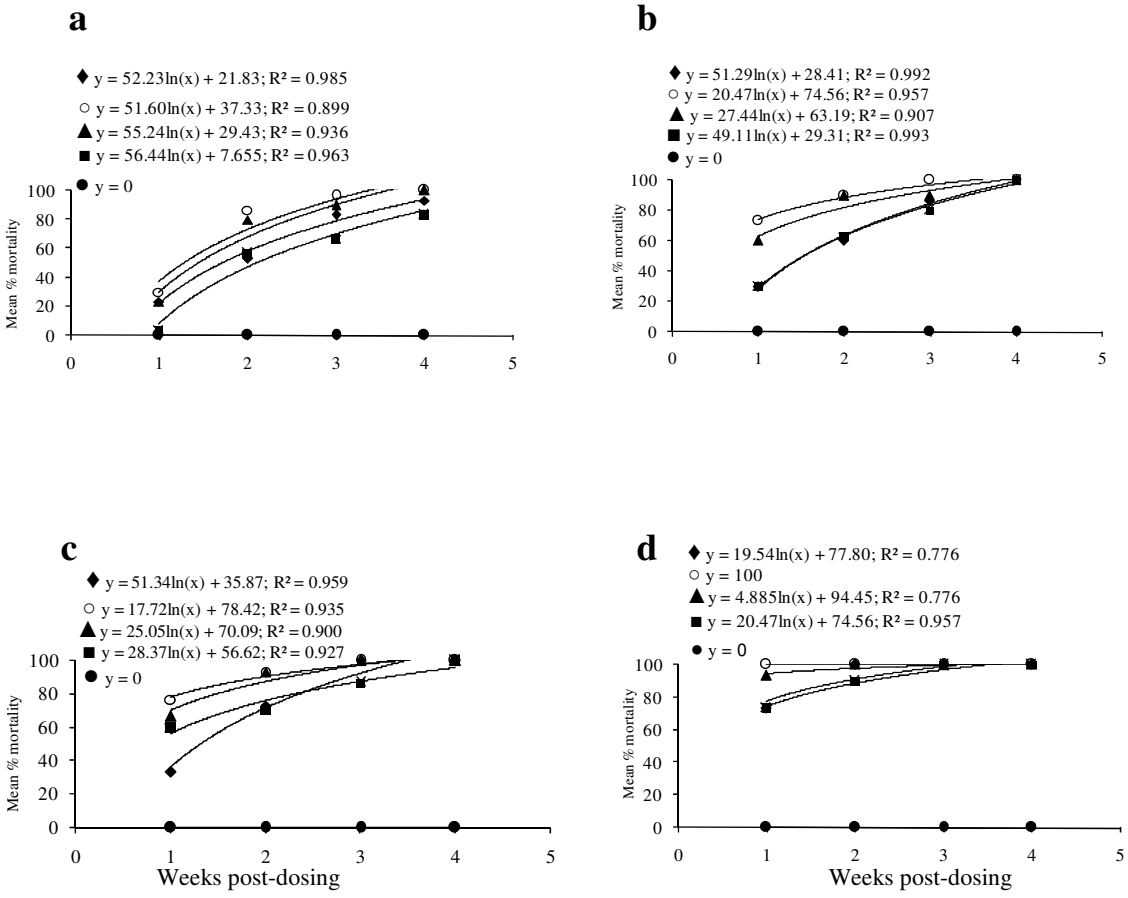


Fig. 2.2 Mean percent mortality of adult flies (*Musca domestica)* infected with (**a)** 10^5, (**b)** 10^6, (**c)** 10^7 and (**d)** 10^8 conidia ml^{-1} of four strains (♦ 7320, O 7569, ▲ 7771, ■ R444 and ● Control) of *Beauveria bassiana* for 4 days.

Table 2.4 Mean percent mortality of adult *Musca domestica* after infection with four isolates of *Beauveria bassiana*

Time (days)	*Isolate*	Mortality 4d after exposure to conidial concentration♠ (Log dose)			
		5	**6**	**7**	**8**
1	7320	23.33 ± 1.2 ^{ab}	30.0 ± 0 ^{abc}	33.33 ± 2.4 ^{abcde}	73.33 ± 0.67 ^{fghijk}
	7569	30.0 ± 1.73 ^{abcd}	73.3 ± 1.2 ^{fghijk}	76.67 ± 0.88 ^{fghijkl}	100.0 ± 0 ^{p}
	7771	23.33 ± 1.33 ^{ab}	60.0 ± 2.52 ^{efghi}	66.67 ± 0.33 ^{efghi}	93.33 ± 0.67 ^{nop}
	R444	3.33 ± 0.33^{a}	30.0 ± 1.15 ^{abcd}	60.0 ± 0 ^{cdefgh}	73.33 ± 0.33 ^{fghijk}
2	7320	53.33 ± 1.2 ^{bcdef}	60.0 ± 0.58 ^{cdefgh}	73.33 ± 1.76 ^{ghijklm}	100.0 ± 0 ^{p}
	7569	86.67± 0.33 ^{ijklmno}	90.0 ± 0.58 ^{klmnop}	93.33 ± 0.67 ^{nop}	100.0 ± 0 ^{p}
	7771	80.0 ± 1.0 ^{ijklmno}	90.0 ± 1.0 ^{mnop}	93.33 ± 0.33 ^{mnop}	100.0 ± 0 ^{p}
	R444	56.67 ± 0.33 ^{bcdefg}	63.33 ± 0.33 ^{defghi}	70.0 ± 0 ^{fghij}	90.0 ± 5.77 ^{klmnop}
3	7320	83.33 ± 0.67 ^{hijklmn}	86.67 ± 1.33 ^{lmnop}	100.0 ± 0 ^{p}	100.0 ± 0 ^{p}
	7569	96.67 ± 0.33 ^{p}	100.0 ± 0 ^{p}	100.0 ± 0 ^{p}	100.0 ± 0 ^{p}
	7771	90.0 ± 0.58 ^{klmnop}	90.0 ± 0.58 ^{klmnop}	100.0 ± 0 ^{p}	100.0 ± 0 ^{p}
	R444	66.67 ± 0.33 ^{efghi}	80.0 ± 0^{fghijklm}	86.67 ± 0.67 ^{jklmno}	100.0 ± 0 ^{p}
4	7320	93.36 ± 0.67 ^{nop}	100.0 ± 0 ^{p}	100.0 ± 0 ^{p}	100.0 ± 0 ^{p}
	7569	100.0± 0 ^{p}	100.0 ± 0 ^{p}	100.0 ± 0 ^{p}	100.0 ± 0 ^{p}
	7771	100.0 ± 0 ^{p}	100.0 ± 0 ^{p}	100.0 ±0 ^{p}	100.0 ± 0 ^{p}
	R444	83.33 ± 0.33 ^{hijklmn}	100.0 ± 0 ^{p}	100.0 ± 0 ^{p}	100.0 ± 0 ^{p}

♠Values are means of three replicates of assays each containing 30 adult flies ± se. Number of spores mixed with water and 0.02% Tween and 0.89%NaCl
Means followed by the same letter are not significantly different (P > 0.05) according to the LSD test (LSD = 0.38; CV% = 17.2).

Table 2.5 Lethal concentration (LC_{50}), lethal time (LT_{50}) and respective Confidential Intervals (95% C.I.) and slopes (± standard error) demonstrated as conidia ml^{-1} and days after infection with four strains of *Beauveria bassiana* towards on house fly (*Musca domestica)* for four days.

Isolate	**LC_{50} (95% CI) Log (conidia ml^{-1})**	**LT_{50} (95% CI) (Days)**	**Slope ± SE**
7320	4.31 (3.15 - 4.90)	1.30 (1.06 - 1.49)	0.82 ± 0.08
7569	3.62 (2.25 - 4.27)	0.45 (0.15 - 0.74)	0.94 ± 0.14
7771	3.58 (2.02 - 4.31)	0.65 (0.23 - 0.92)	0.82 ± 0.11
R444	4.89 (4.26 - 5.29)	1.29 (0.96 - 1.54)	0.62 ± 0.07

2.3.3 Laboratory evaluation of Heterorhabditis against house fly larvae

Overall, significant differences were observed in the number of *Heterorhabditis spp*. per larvae required to cause mortality of fly larvae in the filter paper assay (F= 16.81; P<0.001) and in days of exposure of larvae to nematodes (F= 137.49; P<0.001). The interaction between number of IJs per larvae x days of exposure of larvae to the nematodes was also significant (F= 2.96; P=0.041). Mortality generally increased with time of exposure and number of IJs per larvae (Table 2.6; Fig. 2.3a). After 2d of exposure, there were no significant differences observed in larval mortality, irrespective of number of the nematodes per larvae. The highest mortalities were 64, 70 and 85% for 20, 40 and 100 nematodes per larvae, respectively, after 4d of exposure (Table 2.6).

In the manure bioassays, significant differences were observed in the number of IJs infecting fly larvae (F= 240.71; P<0.001) and time of exposure of maggots to nematodes (F= 185.39; P<0.001). However, the interaction between number of IJ nematodes per larvae x time of exposure to nematodes was not significant (F= 2.68; P=0.068). *Heterorhabditis spp*. failed to infect maggots in the manure. Furthermore, the highest mortalities attained were 2.5, 3.7 and 5.4% for 20, 40 and 100 nematodes per larvae after 4d of exposure (Table 2.6, Fig. 2.3b).

Table 2.6. Mean percent mortality house fly (*Musca domestica*) larvae infected with *Heterorhabditis* infective juveniles on filter paper and in manure bioassays

Number of nematodes per larvae	Mean percent mortality ♠							
	Filter paper bioassay				Manure bioassay			
	1d	2d	3d	4d	1d	2d	3d	4d
20	24.75 ± 4.39 [a]	40 ± 2.55 [bc]	54.75 ± 2.69 [d]	64.75 ± 3.64 [ef]	0.13 ± 0.09 [a]	1.33 ± 0.09 [a]	1.8 ± 0.13 [a]	2.48 ± 0.19 [a]
40	26.5 ± 3.38 [a]	40.5 ± 2.06 [bc]	57.75 ± 4.89 [de]	70.5 ± 1.71 [fg]	0.9 ± 0.15 [a]	2.25 ± 0.06 [a]	2.58 ± 0.15 [a]	3.7 ± 0.25 [a]
100	34.75 ± 3.12 [a]	49.25 ± 5.86 [cd]	76.75 ± 2.56 [g]	85.5 ± 1.71 [h]	2.05 ± 0.16 [a]	3.55 ± 0.06 [a]	4.48 ± 0.09 [a]	5.4 ± 0.25 [a]

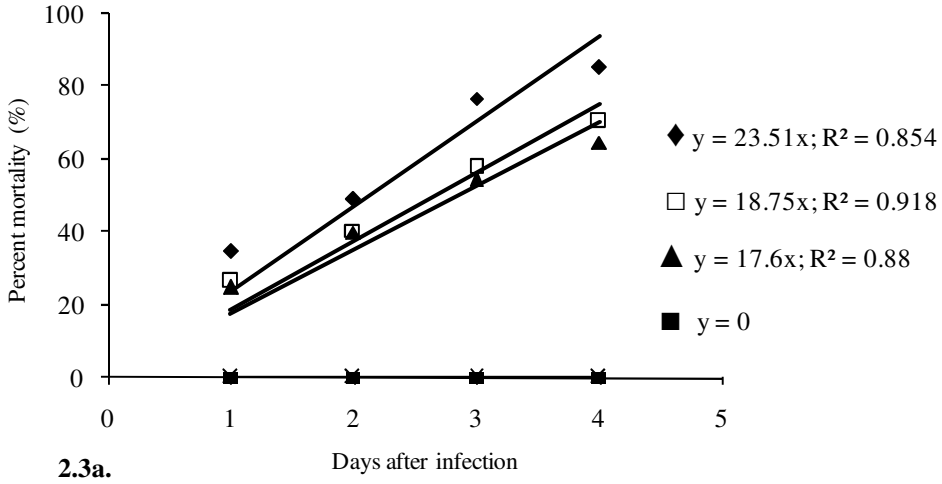
♠Values are means of three replicates of assays each containing 30 larvae ± se.

Statistical analysis of ANOVA at the 0.05 significance level of the of interaction effect between number of *Heterorhabditis* infective juveniles per larvae and time on mortality.

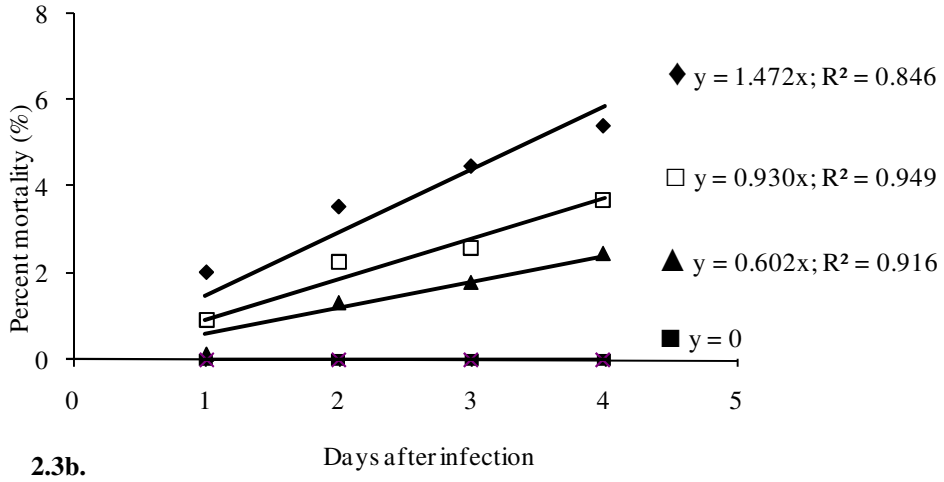
Means followed by the same letter within the same bioassay are not significantly different at $P < 0.05$

(Filter paper bioassay: P=0.041; LSD = 0.126; %CV = 12.7)

(Manure bioassay: P=0.068; LSD = 0.005; %CV = 11.8)



2.3a.



2.3b.

Fig.2.3a and 2.3b. Percent mortality of *Musca domestica* larvae after application of (■) 0, (▲) 20, (□) 40 and (♦) 100 *Heterorhabditis* infective juveniles per larvae on **(a**.) filter paper and **(b**.) in manure.

Table 2.7 Lethal concentration (LC_{50}), lethal time (LT_{50}) and respective Confidence Intervals (95% C.I.) and slopes (± standard error) demonstrated as conidia ml^{-1} and days, after infection of house fly larvae with *Heterorhabditis* infective juveniles for four days.

Assay	LC_{50} (95%CI) ♣	LT_{50} (95%CI) (days)	Slope±SE
Filter paper	42.3 (25.7 – 55)	2.35 (2.18 – 2.52)	0.67 ± 0.052
Manure	449.9 (239.1 – 1995)	12.33 (8.05 – 54.69)	0.38 ± 0.158

♣ Number of infective juveniles per larva

The LC_{50} values for Heterorhabditis *spp*. in the manure bioassay were higher than those on the filter paper. Moreover, the LC_{50} values Heterorhabditis *spp*. on the filter paper bioassay (42.3 nematodes per larva) (Table 2.7), was about one-tenth (449.9 nematodes per larvae) of the value observed in manure.

The time taken (LT_{50}) for *Heterorhabditis spp.* to kill 50% of the larvae in the manure (12.33d) was about six times the time taken to kill the same percentage of larvae on filter paper (2.35d).

2.4 Discussion

Entomopathogenic fungi such as *B. bassiana,* as well as entomopathogenic nematodes such as *Heterorhabditis spp.,* are two groups of naturally occurring biological control agents. However, few studies have been reported on the use of these organisms for biological control of house flies. The present study evaluated the pathogenicity of 36 fungal isolates and one nematode species against *M. domestica* larvae and adults. Despite the variability in virulence, the experiments clearly demonstrate that *Beauveria* can be an effective biocontrol agent against *M. domestica*.

In the bioassay to evaluate the pathogenicity of 36 isolates of entomopathogenic fungi, including *B. bassiana* and *Paecilomyces spp*, from different hosts and sources, the virulence of the fungi towards larvae and adult flies varied greatly. All *Beauveria* isolates tested were able to infect adult flies in the laboratory, but there were differences between the isolates. This agrees with previous studies that showed a similar pattern of differences in insect pathogenicity between

different fungal species and strains (Geden *et al*., 1995; Watson *et al*., 1995; Lecuona *et al*., 2005). Flies infected with *B. bassiana* died within 6d of exposure. Comparable studies by Geden *et al*., (1995) reported that adult flies infected by *B. bassiana* died within 5d of exposure, while Watson *et al*. (1995) reported fly mortality occurring within 7d.

None of the studied isolates of *P. lilacinus* were pathogenic to adult flies. These results suggest that *M. domestica* is not a susceptible host for *P. lilacinus*.

The entomopathogenic activity of *B. bassiana* was confirmed by the presence of fungal hyphae on the body of flies. Fungal growth was evident as early as 2d after exposure to 10^8 conidia ml^{-1}. The flies exhibited signs of infection such as post-emergence of the fungus followed by conidiation on the surface of the cadavers, if adequate humidity was present, making it possible to initiate epizootics.

Beauveria bassiana Isolates R444, 7320, 7569 and 7771 were identified as the most pathogenic isolates to adult flies, causing mortalities of 90% or more within 2d, at a conidial concentration of $10^8 ml^{-1}$. Three of these isolates (7320, 7569 and 7771) were isolated from the soil. In contrast, no pathogenic effects were noted on fly larvae. None of the fungal isolates were very effective in controlling house fly larvae, despite maximization of fungus-host contact by immersion of larvae in a conidial suspension. Steinkraus *et al*. (1990) reported 52-73% mortality in third instars infected by *B. bassiana*. Similarly, Watson *et al*. (1995) reported mortality rates between 48-56% in second instars with high doses (10^{10} ml^{-1}). In the present study, none of the fungal isolates was pathogenic towards fly larvae. Likewise, Geden *et al*. (1995), Watson *et al*. (1995) and Lecuona *et al.* (2005) reported that *B. bassiana* failed to infect larvae. These differences in outcomes may be the result of differences in strain virulence (Lecuona *et al*., 1996; Lecuona *et al*., 2005), in assay methods, conidial doses or culture methods (Lecuona *et al*., 2005). Furthermore, the insect cuticle acts as a barrier for fungal penetration and its thickness increases with every moulting so that differences in the susceptibility of larvae to entomopathogenic fungi can be explained by their cuticular properties (Boucias and Pendland, 1991).

Dose-mortality bioassays showed that mortality rates of *M. domestica* adults were a function of conidial concentration. Mortality responses observed were proportional to concentrations of conidial suspensions. Initially, a certain threshold of conidial concentration has to be exceeded to induce mortality (Butt and Goettel, 2000). In our experiments, low mortalities were observed at conidial concentrations below 10^5 conidia ml^{-1}. For Isolates 7569 and 7771 the theoretical maximum of 100% mortality was induced at the dose of 10^5 conidia ml^{-1} at 4d and therefore, no further increase was possible with time. With the isolate 7569, the maximum 100% mortality leveled off at 3d at a conidial concentration of 10^6 conidia ml^{-1}. Dose-dependent mortality was demonstrated previously by Watson *et al*. (1995) using two strains of *B. bassiana* (P80 and L90) formulated as a dust. This dose effect of *B. bassiana* conidia concentrations on infection levels and rates may have several reasons. First, it is possible that only certain conidia are able to infect the host (Butt and Goettel, 2000). Alternatively, it is possible that a certain minimal number of attached conidia are needed before the fly cuticle can be penetrated. Such positive correlations between the number of infective spores and mortality have been obtained when using *B. bassiana* against other arthropods (Kaaya and Munyinyi, 1995; Devi and Rao, 2006; Lekimme *et al*., 2006; Santoro *et al*., 2008).

Four of previously assayed strains were selected for further bioassays. These strains were Isolates 7320, 7569, 7771 and R444, which caused the greatest levels of mortality among adult flies. Subsequent bioassays indicated that Isolates 7771 and 7569 had the lowest LC_{50} values; Isolate 7569 had the lowest LT_{50} value and the greatest slope. These findings were consistent with earlier assays that indicated that Isolates 7569 and 7771 were the most virulent strains.

The use of *B. bassiana* as a BCA against house flies would be attractive for several reasons. First, it would provide an alternative to chemical management of the adult stage of the fly. Second, use of *B. bassiana* as a BCA on flies is attractive because the user can achieve 100% control. This is not the case when a traditional insecticides program is followed and producers are often faced with repeated application of insecticides to suppress house flies, but without achieving complete control. Furthermore, there is little chance of flies developing resistance to *B. bassiana.*

Evaluation of *Heterorhabditis* nematodes against fly larvae revealed that fly larvae were highly susceptible to nematodes when hosts were confined in Petri dishes containing nematode-treated filter paper. However, the maggots were not susceptible to *Heterorhabditis* in manure media. When the fly larvae were exposed to 100 nematode IJs per host on filter paper, *Heterorhabditis* caused far higher mortality (85%) than in the manure media (5.4%). Although moderate levels of mortality were observed on Petri dish filter paper assays with lower nematode numbers per host, virulence was greatly reduced when house fly larvae were exposed inside chicken manure substrates. Other studies have documented the pathogenicity of several strains of entomopathogenic nematodes toward house fly larvae on filter paper assays (Renn *et al*., 1985; Geden *et al*., 1986; Belton *et al*., 1987; Georgis *et al*., 1987; Mullens *et al*., 1987a) and likewise the inability of the nematodes to survive in chicken manure. Georgis *et al*. (1987) demonstrated that nematodes were severely hampered by poultry manure and exposure of IJs of *H. bacteriophora* to manure resulted in a high nematode mortality (70 – 100%) within 18h. Nematodes exposed to manure for more than 6h had drastically reduced efficacy against maggots of *M. domestica*. In contrast, Taylor *et al*. (1998) found that none of the 22 strains of *Heterorhabditis* nematodes screened caused significant mortalities of house fly maggots on filter paper, but they produced significant fly mortality in a manure substrate. Despite these differences, few species of nematodes have been screened against houseflies, and conflicting results have been obtained for some of the nematode species, possibly because of differences in strain pathogenicity (Gaugler, 1988). Furthermore, treatment of chicken manure with calcium alginate-encapsulated *H. megdis* nematodes resulted in 35-98% reduction in adult house fly emergence (Renn, 1995). These results indicate that use of advanced formulation that are able to protect the EPNs from toxins, such as ammonia, in chicken manure may enhance the efficiency of *Heterorhabditis spp*. against house fly larvae in poultry houses.

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CHAPTER 3

LABORATORY AND FIELD EVALUATION OF FORMULATED *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* AS A FEED ADDITIVE AND USING TOPICAL APPLICATIONS FOR THE CONTROL OF HOUSE FLY (*MUSCA DOMESTICA* L.) LARVAE IN CAGED-POULTRY MANURE

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Abstract

Infestations of house flies, *Musca domestica* L., are a continual problem around poultry establishments. The high levels of resistance developed by flies toward organochlorine and organophosphorous compounds have made the efforts to control flies using biological control agents (BCAs) increasingly important. Prior to conducting field evaluations, laboratory baseline bioassay data were established on a dose-time response relationship, closely simulating poultry house conditions in the laboratory. Acute toxicity of two *Bacillus thuringiensis* var. *israelensis* (*Bti)* formulations (water-dispersible granules (WDG) and bran formulation) was evaluated against larvae in the laboratory, and against natural populations of *M. domestica* larvae in the field, applied in oral feed to chickens and as topical applications in the poultry houses. Baseline bioassay data demonstrated that susceptibility of *M. domestica* larvae increased to a given concentration of *Bti* as the duration of exposure increased. In the laboratory studies, the LC_{50} and LC_{90} values of *Bti* for the larvae ranged between 65 - 77.4 and 185.1 - 225.9µg ml^{-1} respectively. LT_{50} and LT_{90} values were 5.5 and 10.3d respectively. In the field, a concentration of 10g *Bti* kg^{-1} of feed resulted in 90% reduction of larvae for 4wk post-treatment. Furthermore, as the exposure duration increased, the level of *Bti* required to cause a significant mortality decreased. A higher concentration (2g L^{-1}) of *Bti* in spray applications was not significantly more effective than the lower concentration of 1g L^{-1}. Adding *Bti* to chicken feed is potentially an efficient measure for the management and control of house flies in caged-poultry facilities.

3.1 Introduction

Infestations of house fly, *Musca domestica* L., are a continual problem around poultry establishments where caged laying hens are maintained. Accumulation of undisturbed droppings beneath the cages for lengthy periods of time provides an excellent breeding medium for house fly larvae (Thomas and Skoda, 1993). The house fly is ubiquitous and rapidly infests newly accumulating poultry manure, often reaching enormous numbers. The behaviour of this pest is typically synanthropic and, because of its high reproductive rate and ability to prosper in a wide range of environments, it possesses a high capacity for dissemination of metaxenic and other types of important diseases, therefore posing a potentially serious health problem.

Besides being costly, insecticidal control of the house fly has many serious drawbacks. If not used properly, insecticides can be toxic to animals and humans, contaminate feed and water, and destroy the biological control agents (BCAs) of flies. Furthermore, house fly populations have developed a high level of resistance toward organochlorine and organophosphorous compounds (Scott *et al*., 2000), prompting a need for more effective solutions. Larviciding and source reduction have a major advantage in that they control flies before they disperse and transmit disease.

Preparations of *Bacillus thuringiensis* (*Bt*) are widely considered as safe and effective pesticides in horticulture and forestry (Kellar and Langenfruch, 1993; Navon, 1993; Rajakulendran, 1993; Teakle, 1994) and to control mosquitoes and blackflies (Mulla, 1990; Becker and Margalit, 1993; Ritchie, 1993; Hershey *et al.,* 1995; Becker, 1997; Brown *et al*., 1998a, 1999, 2002). However, to date, no commercial preparation of *Bt* has been released for the control of insect pests of livestock (Gough *et al*., 2005; Brar *et al*., 2006).

Bacillus thuringiensis var. *israelensis* (*Bti*) is a Gram-positive, spore-forming bacterium with entomopathogenic properties. During sporulation it produces protein crystals (δ- endotoxins) with insecticidal activity (Höfte and Whiteley, 1989). Its mode of action involves ingestion and solubilization of crystals in the gut of the target insect, followed by the cleavage of protoxins, activation of toxins and interaction with the cells of the midgut epithelium of susceptible larvae

(Gill *et al*., 1992). These toxins act synergistically to produce full toxicity, thereby making it difficult for insect populations to develop resistance to this entomopathogen. These toxins are highly specific to their target insect, are safe to humans, vertebrates and plants, and are completely biodegradable. Therefore, *Bti* is a viable alternative for the control of insect pests in agriculture and important vectors of human disease (Bravo *et al*., 2005, 2007).

Safety studies have so far shown no harmful effects on bees, vertebrates including man, and most beneficial insects are unharmed even at enormous doses (de Barjac, 1978; WHO, 1979). In a number of studies, the acute toxicity and pathogenicity of commercial *Bt* formulations have been evaluated on young bobwhite quail (*Colinus virginianus*) (Beavers *et al*, 1989a; Lattin *et al*., 1990a, 1990b; Beavers, 1991b) and young mallards (*Anas platyrhynchus*) (Beavers *et al*., 1989b; Lattin *et al*., 1990c,d; Beavers, 1991a), when administered orally daily at high dosages. The *Bt*-treated birds showed no apparent toxic or pathogenic effects by the *Bt*. In those studies that also evaluated feed consumption and weight gain, the *Bt*-treated birds showed no effect when compared with the non-treated controls.

The present study reports on the evaluation of two formulations of *Bti* for *M. domestica* larvae control under laboratory and field conditions. In the field *Bti* was evaluated for control of house fly larvae in two ways: as a feed-through larvicide to broiler chickens and as a spray application over the manure in poultry houses.

3.2 Materials and methods

3.2.1 Bacterial strains

Two *Bti* formulations (water-dispersible-granules (WDG) and a bran formulation) were obtained from Plant Health Products (PHP)[1] (Pty) Ltd.

[1] Plant Health Products (Pty) Ltd. P.O. Box 207, Nottingham Road, South Africa

3.2.2 Insect rearing

Adult house flies were maintained at 28°C, 68% RH, with a 12:12 L: D photoperiod. Flies were given a maintenance diet of *ad lib* sugar, milk powder and water and were offered chicken liver as an oviposition medium on a twice weekly basis from the third week of postemergence. Larvae for replacement colonies were reared on a diet comprising 50g active dried yeast (DCL), 8g agar (Oxoid), 250ml distilled water, and 750ml UHT full cream milk, prepared by microwaving to boiling point (Johnson *et al*., 1998).

3.2.3 Laboratory bioassay for Bti toxicity against house fly (Musca domestica) larvae

3.2.3.1 Viable spores quantification and preparation of spore-crystal suspension

The concentrations of viable spores of WDG and bran formulations were estimated. A sample of 1g of the WDG *Bti* formulation was suspended in 9ml sterile water and shaken for 30min. The bacterial suspension was subjected to thermal shock (80°C for 12min) to kill vegetative cells. After being sequentially diluted, 100µl samples were plated on Petri dishes containing nutrient agar and incubated at 28°C for 24h. The colonies formed were counted and expressed as colony forming units (c.f.u.) ml^{-1}. For microscopic observation of morphological features of spores and parasporal bodies, 1ml samples of *Bti* obtained from 3-4d old cultures were incubated in a flask containing 50ml TSB medium at 30±1°C for 72h and then stained according to Ammons *et al.* (2002).

Approximately 1ml of the bacterial culture obtained from the plates was inoculated into 50ml nutrient broth (Biolab) supplemented at 1ml l^{-1} with mineral salts (Stewart *et al*., 1981) to aid sporulation in 250ml flasks. Liquid cultures were incubated in an orbital shaker at 30° ±1°C and 200rpm for 72h. The crystals were harvested by centrifugation at 4000rpm for 15min at 4°C (Beckam Coulter Avanti J-E, SW32 rotor). The pellet was washed twice with cold 0.1M NaCl and twice with cold water before being resuspending in deionized water to give final concentration of 1µg, 10µg, 50µg and 100µg ml^{-1}. These preparations were stored in small aliquots at –20°C for use in the toxicity assays.

3.2.3.2 Toxicity assays

Chicken manure was collected from poultry houses from the Ukulinga University Research Farm. Three replicates of 50g manure for each treatment were placed into plastic lunch boxes (120 x 100 x 100mm) with screened holes in the lids. Aliquots of 1ml of *Bti* stock solution were pipetted evenly over the surface of the manure. Homogenous groups of thirty 2nd instar larvae were exposed to four different concentrations of the product in triplicates. Three lunch boxes remained untreated for control purposes. The lunch boxes were incubated at 28^{o}C and 60%RH. Larval mortality was quantified by counting live larvae remaining every 24h. Larvae that failed to react to gentle prodding with a glass pipette were considered to be dead (Brown *et al*., 1998b). The larvae were allowed to feed on the compost mixture for 7d. Larvae in control boxes developed to the third instar and pupal stages.

Mortality of the larvae in the treated boxes was corrected against control mortality (Abbott, 1925). The corrected mortality was subjected to Logit Probit regression analysis and median lethal concentration (LC_{50}) and 90% lethal concentration (LC_{90}) and associated 95% confidence intervals were calculated using the GENSTAT program.

All percentage mortality data were square root-arcsine percent transformed. The data was analyzed using repeated measures ANOVA. The (LSD) method was used to separate and compare means within the treatments.

3.2.4 Field trials

3.2.4.1 Spray trials

The poultry houses at Ukulinga were used for spray application trials. A few days before beginning the spray trial, the manure beneath the poultry houses was completely removed. Selected areas were 7m x 1.25m (8.75m^2) and supported 40 broiler chickens housed in cages (300 x 400 x 500mm). Similar unsprayed sites were run as controls for comparison. WDG-formulated *Bti* was applied at 0.11 g m^{-2} and 0.23 g m^{-2}. To prepare the solution for spraying, 1g and 2g of

Bti powder was suspended in 1L of water to cover the 8.75 m^2 of floor surface (114 ml m^{-2}). The *Bti* formulation was applied using hydraulic hand sprayers.

Post-treatment populations of house fly larvae were monitored after every 7d. The *Bti* application were carried out every 7d. Data were analyzed to measure reduction in the fly larvae numbers in comparison with untreated controls. The trial was run for 6wk.

3.2.4.2 Feed trials

For field evaluation experiments, forty-eight x 72wk old commercial broilers (Hyaline Brown) were obtained from the University Research Farm, Ukulinga. Broilers were evaluated upon receipt for signs of disease or other complications that could affect the outcome of the study. Following examination, broilers were randomly allocated into 48 hanging pens (440 x 420 x 610mm) in parallel back-to-back rows of twelve pens per row. Pens were separated by wire-mesh on all sides. Each pen was equipped with a removable feed trough and a nipple drinker. Each pen had a removable tray for individual manure collection. Broilers were housed in an environmentally controlled shed fitted with fans and 16:8h (L: D) at the University Research Farm at Ukulinga.

3.2.4.3 Diet preparation for broilers and administration

Bacillus thuringiensis var. *israelensis*-formulated bran was mixed into the broiler mash in varying quantities (0.1g, 0.5g, 1.0g, 5.0g and 10g per kg of broiler mash). The control did not contain any *Bti*-formulated bran. The rations were stored in trash cans lined with plastic bags until fed to the chickens. There were eight broilers per treatment. Feed and drinking water were provided *ad libitum*. Broilers were fed their respective dietary treatments for 5wk.

3.2.5 Arthropod sampling

For the feed trials, excreta from each bird was collected every 7d. Sampling of arthropods was started a week after the administration of the different feeds in order to give the broilers time to adjust to the different treatments. House fly larvae were monitored using a gardeners bulb planter (400ml) to collect two manure cores from each tray/bird/diet. Larvae was extracted using Berlese

funnels and enumerated. For the spray trials, ten samples of 400ml were taken every 7d during the trial.

3.2.6 Statistical analysis

Percentage mortality of larvae in the treated trays was calculated and corrected to the reduction, if any, in controls using the formula of (Mulla *et al*., 1971) as given below:

$$\% \text{ Reduction} = \frac{[C1 - C2]}{C1} \times 100$$

where; $C1$, number of larvae in untreated manure;

$C2$, number of larvae in treated manure.

The percentage values were normalized by square root-arcsine transformation. An analysis of variance (repeated measures) was performed by taking the percent reduction (square root arcsine values) as the dependent variable and dose as the factor. The LSD method was used to find significant differences between means.

3.3 Results

3.3.1 Evaluation of toxic activity and concentration of viable spores

The presence of *Bti* spores and parasporal bodies was evident in stained specimens taken from the WDG and bran formulations (Fig. 3.1a and 3.1b).

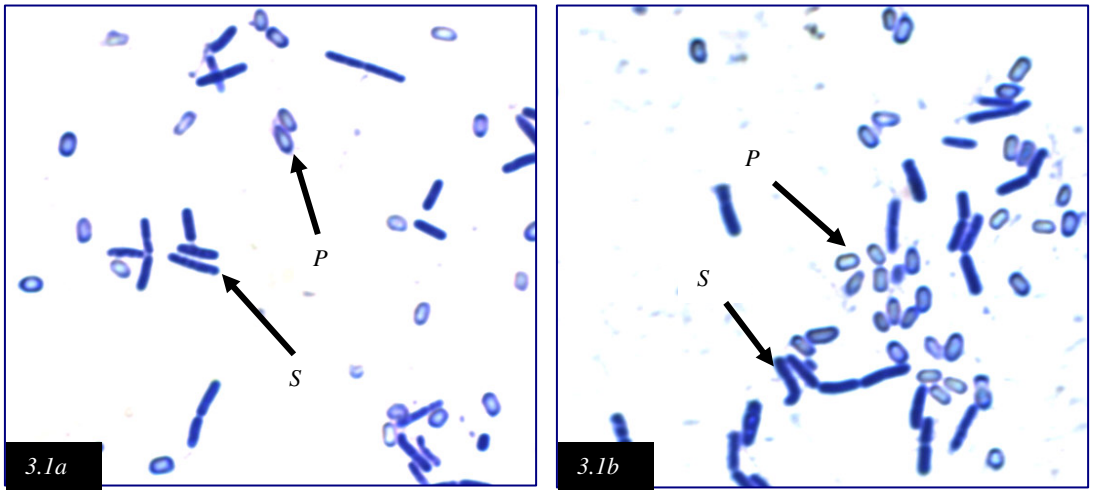

Fig. 3.1a and 3.1b. Light microscopy images of (a) WDG and (b) bran formulations of *Bacillus thuringiensis* var. *israelensis* stained with Coomassie Blue stain (0.133% in 50% acetic acid). (S = Spore; P = Parasporal body).

Table 3.1. Probit analysis of *Bacillus thuringiensis* var. *israelensis* evaluated in a bioassay against 2nd instar larvae of *Musca domestica* larvae and corresponding viable spore concentration

LC_{50}[a] (µg ml^{-1}) Average ± se[c] (95% CI)	LC_{90}[b] (µg ml^{-1}) Average ± se (95% CI)	Slope	Viable spores (c.f.u. ml^{-1})[d]
70.70 ± 3.17 (65 – 77.4)	203.1 ± 10.42 (185.1 – 225.9)	0.4640± 0.02	6.23 x 10^9

[a] LC_{50} = concentration lethal for 50% larvae
[b] LC_{90} = concentration lethal for 90% larvae
[c] Average of three bioassays ± standard error
[d] c.f.u. ml^{-1} = colony forming units per millilitre

The LC_{50} and LC_{90} values of *Bti* against *M. domestica* larvae are shown in Table 1. The analysis of the confidence intervals (95%) indicated LC_{50} and LC_{90} values were 65-77.4 and 185.1-225.9µg ml^{-1} respectively. The concentration of viable spores, as estimated by the number of colonies, was 6.23 x 10^9 spores g^{-1}.

Laboratory bioassays with the WDG formulation of *Bti* against second instar larvae of *M. domestica* showed that after 24h of exposure, poor control was recorded and no mortality was

observed as a result of any of the treatments (Table 3.2; Fig. 3.2). After 4d of treatment, there was a sharp decline in *M. domestica* larval densities in all the treatments. At D4 post-treatment, the mortality of *M. domestica* larvae was moderate in all five treatments (Fig. 3.2) and there was at least 50% mortality in three of the dosages (10, 50 and 100µg *Bti* ml^{-1}); the 1µg *Bti* ml^{-1} produced 36% mortality. While the minimum mortality observed was 42% (1µg *Bti* ml^{-1}) at D5 post-treatment, the rest of the dosages; 10, 50 and 100 µg ml^{-1} produced 58, 67 and 76% larval mortalities respectively. At D7 post-treatment, the highest larval mortality observed was 86%, while 10 and 50µg *Bti* ml^{-1} produced 66 and 74% mortality respectively and the mortality produced by 1µg *Bti* ml^{-1} was just below 50%. (Table 3.2; Fig. 3.2).

Table 3.2. Mortality (%) of house fly (*Musca domestica*) larvae in manure treated with different concentrations of *Bacillus thuringiensis* var. *israelensis* for 7d.

Dose µg ml^{-1}	Mean larval mortality (%) ♣						
	1d	2d	3d	4d	5d	6d	7d
1	0(0)	0(0)[a]	26.67(0.54)[a]	36.67(0.65)[a]	42.22(0.71)[a]	44.44(0.73)[a]	46.67(0.75)[a]
10	0(0)	10(0.32)[b]	43.33(0.72)[b]	50.00(0.79)[b]	58.89(0.87)[b]	61.11(0.90)[b]	66.67(0.96)[b]
50	0(0)	20(0.46)[c]	45.56(0.74)[b]	60.00(0.89)[c]	67.78(0.97)[c]	71.11(1.01)[c]	74.44(1.04)[b]
100	0(0)	30(0.58)[d]	56.67(0.85)[c]	66.67(0.96)[c]	76.67(1.07)[d]	82.22(1.14)[d]	86.67(1.20)[c]
F-Ratio		72.22	20.22	17.77	36.30	29.79	27.64
P-Level		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
LSD		0.096	0.093	0.103	0.0823	0.103	0.116
CV%		15.1	6.9	6.7	4.9	5.8	6.3
Effect		**	**	**	**	**	**

♣ Values are means of three replicates of assays each containing 30 larvae. Values in parenthesis are square root-arcsine transformed values of means.
Means followed by the same letter within the same day are not significantly different at $P < 0.05$
** Significant at $P \leq 0.001$

There were significant differences in mortality levels of *M. domestica* larvae between the *Bti* concentrations (F= 58.0; P <0.001), the time of exposure (F= 1072; P<0.001) and the interaction effects between dosages and days (F=12.05; P <0.001), indicating a change in the level of larval

reduction on post-treatment days with a change in the dosage. Significant differences between the treatments were apparent after D3 post-treatment (Table 3.2).

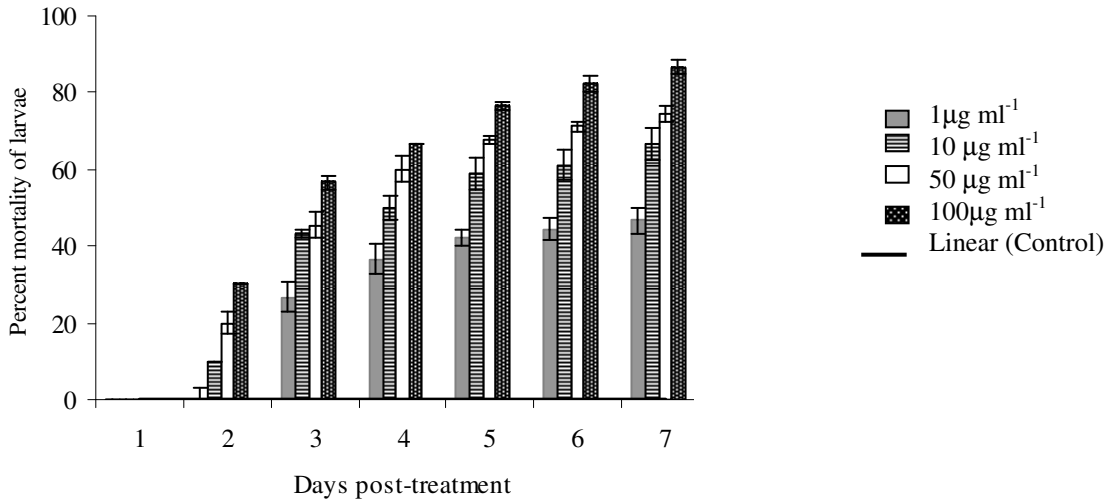

Fig. 3.2. Mean percent mortality of *Musca domestica* larvae treated with four (1, 10, 50 and 100µg ml^{-1}) concentrations of formulated *Bacillus thuringiensis* var. *israelensis* for 7d.

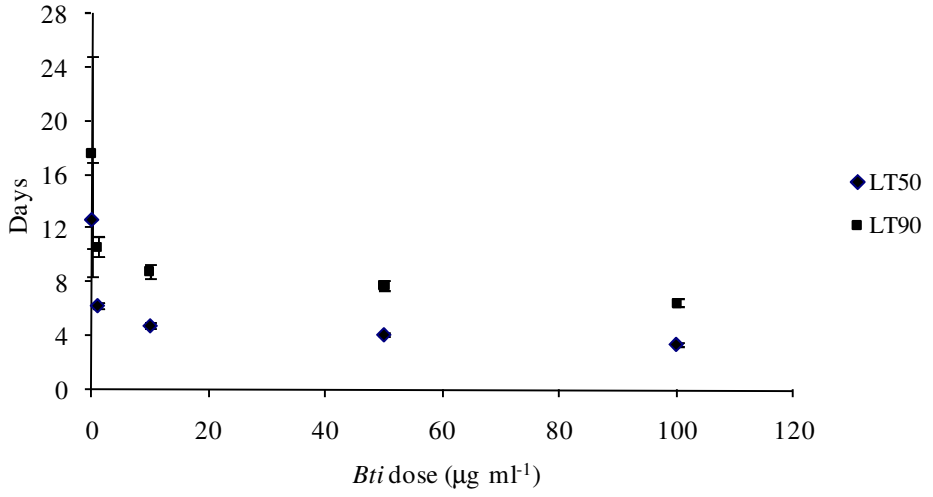

Fig. 3.3 LT_{50} and LT_{90} of *Musca domestica* larvae infected with different concentrations of *Bacillus thuringiensis* var. *israelensis.*

There was a dose-dependent relationship in the mortality of larvae (Table 3.2; Fig. 3.2). This was confirmed by Probit analysis. Comparisons between LT_{50} and LT_{90} values showed that the mortality of larvae exposed to 1-100µg *Bti* ml^{-1} differed significantly from the control ($P<0.001$). The lowest LT_{50} was obtained with the 100µg *Bti* ml^{-1} dose. Indeed, at the 100µg ml^{-1} dose, *Bti* killed 50% of the larvae in 3.42d and 90% of the larvae in 6.51d while 50% of the controls survived for 12.7d and 90% for 17.68d (Fig. 3.3).

3.3.2 Spray trials

The mean number of house fly larvae collected from the manure core samples in the poultry houses during the 6wk sampling period are presented in Fig. 4. After 2-4wk of spraying, the density of larvae in the manure was reduced by at least 20% in both the 1g L^{-1} and 2g L^{-1}dosages (Fig. 3.5). After 4wk, there was a sharp decline in larval densities at both dosages. Whereas there was a 50% reduction in larvae by 2g L^{-1} after 5wk, a larval reduction of just below 50% reduction was obtained with 1g L^{-1} only after 6wk. At 6wks post-treatment, 2g L^{-1} produced 52% mortality, while the reduction of larvae by the 1g L^{-1} treatment was 47% (Fig. 3.5; Table 3.3).

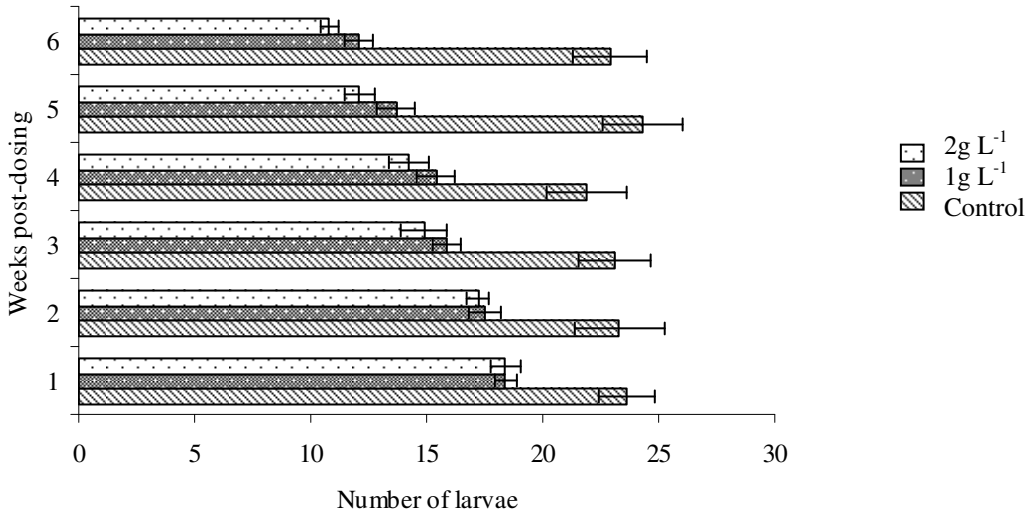

Fig. 3.4. Weekly mean number of house fly larvae in the manure of broilers sprayed for six weeks with two concentrations (1 and 2g L^{-1}) of a WDG formulation of *Bacillus thuringiensis* var. *israelensis.*

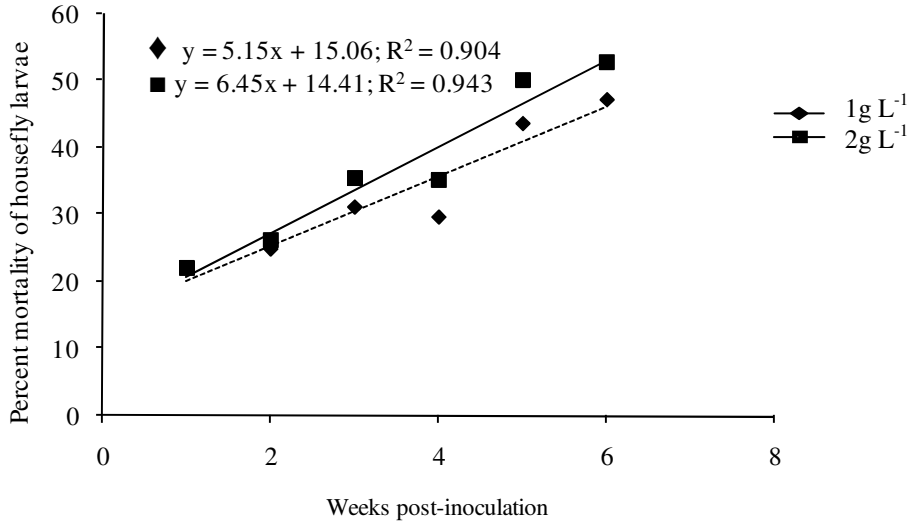

Fig. 3.5 Percent mortality of house fly (*Musca domestica*) larvae in the manure of broilers sprayed for six weeks with two concentrations (1 and 2g L^{-1}) of a WDG formulation of *Bacillus thuringiensis* var. *israelensis.*

Table 3.3. Percent mortality of house fly (*Musca. domestica*) larvae in manure of broilers sprayed for 6wk with two concentrations (1 and 2g L^{-1}) of a WDG formulation of *Bacillus thuringiensis* var. *israelensis.*

	Percent reduction in number of larvae					
	Weeks					
Bti g L^{-1}	1	2	3	4	5	6
1	22.03 (0.48)[a]	24.89 (0.52)[a]	31.17 (0.59)[a]	29.68 (0.57)[a]	43.62 (0.72)[a]	47.16 (0.76)[a]
2	22.03 (0.48)[a]	26.18 (0.53)[a]	35.50 (0.63)[a]	35.16 (0.63)[a]	50.21 (0.79)[a]	52.84 (0.81)[a]
F ratio	0.01	0.19	0.56	1.09	2.32	3.27
P Level	0.94	0.67	0.46	0.31	0.15	0.09
LSD	0.09	0.09	0.12	0.12	0.09	0.07
CV%	19.5	18.7	20.4	21.8	13.1	9.0
Effect	NS	NS	NS	NS	NS	NS

Values in parenthesis are square root-arcsine transformed values of means.
Means followed by the same letter within the same week are not significantly different at $P < 0.05$
F and *P* values after square root-arcsine transformation
NS = not significant

While the two dosages (1 and 2g L^{-1}) caused significant reductions in the number of larvae ($P<0.05$), they did not differ significantly in reducing the number of larvae ($F = 2.27$; $P = 0.149$).

Although there was a significant effect of weeks (F = 33.24; P <0.001), the interaction effect of dosages and week was not significant (F = 0.43; P = 0.752) indicating that a change in the level of larval reduction on post-treatment days did not change with a change in dosage. No significant differences were observed between the two dosages (1 and 2g L^{-1}) in their reduction of larvae during the study period (Table 3.3).

3.3.3 Feed trials

Following the continuous feeding of *Bti* in the broiler mash, the house fly larvae declined rapidly in the manure as a result of the five doses (Fig. 3.6). After 1wk of feeding chickens with *Bti* feed, the larval reduction, as a result of the 0.1, 0.5 and 1.0g *Bti* kg^{-1} feeds, was at least 20%, while the larval reduction as a result of the 5 and 10g *Bti* kg^{-1} feed was approximately 40% (Table 3.4). After 1wk of feeding broilers with bran-formulated *Bti*, larval populations were reduced by at least 25% as a result of all the dosages (Table 3.4). Percent reduction in larval numbers was almost doubled after 2wk of feeding with three dosages (0.1, 0.5 and 1.0g kg^{-1}). After 2wk of using *Bti* as a feed additive, the number of larvae was reduced by at least 50% as a result of four of the five dosages. By Wk3 of feeding, the reduction was at least 70% as a result of the 5 and 10g *Bti* kg^{-1} doses, whereas at 0.5 and 1.0g *Bti* kg^{-1}, the reduction was about 60%. After 4wk of treatment, the percent reduction of maggots was 91% at 10g *Bti* kg^{-1}. In the case of other dosages: 0.1, 0.5, 1.0 and 5g *Bti* kg^{-1}, the reductions were 62, 75, 78 and 88%, respectively (Table 3.4).

The two main effects, dose and time, were significant [(F=13.26; P<0.001), (F=73.05; P<0.001) respectively]. However, the interaction effect between them was not significant (F=0.38; P=0.959). The level of reduction of larvae as a result of dose did not differ significantly in the first week of feeding formulated *Bti* to chickens (Table 3.4), but significant differences were apparent between the dosages in subsequent weeks of feeding (Table 3.4).

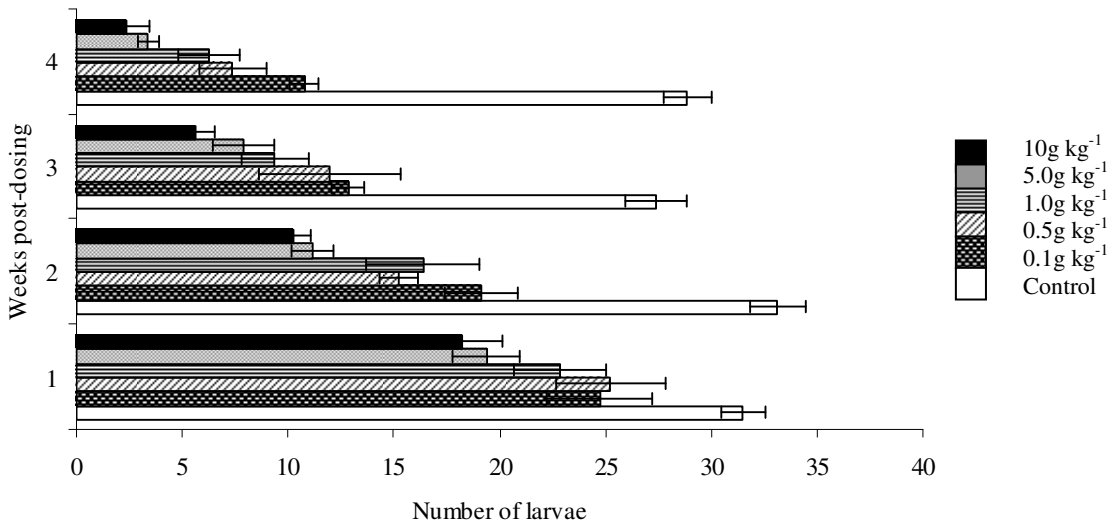

Fig. 3.6. Weekly mean number of house fly (*Musca domestica*) larvae in the manure of broilers fed for four weeks with five concentrations (g kg^{-1}) of a bran formulation of *Bacillus thuringiensis* var. *israelensis.*

Table 3.4. Percentage mortality in number of house fly (*Musca domestica*) larvae in the manure of broilers fed for 4wks with five concentrations (g kg^{-1}) of a bran formulation of *Bacillus thuringiensis* var. *israelensis.*

	Percent reduction of number of larvae			
	Weeks			
Bti (g kg^{-1})	1	2	3	4
0.1	21.95 (0.43)^{a}	41.62 (0.71)^{a}	52.17 (0.82)^{a}	62.42 (0.92)^{a}
0.5	20.04 (0.44)^{a}	53.15 (0.83)^{ab}	57.64 (0.92)^{ab}	74.81 (1.06)^{ab}
1	26.95 (0.52)^{ab}	50.29 (0.76)^{ab}	65.62 (0.96)^{abc}	78.09 (1.12)^{bc}
5	38.53 (0.66)^{ab}	65.78 (0.96)^{b}	70.94 (1.03)^{bc}	88.40 (1.25)^{cd}
10	42.15 (0.70)^{b}	68.68 (0.98)^{b}	78.38 (1.11)^{c}	91.56 (1.36)^{d}
F ratio	2.37	4.31	3.52	7.0
P level	0.07	0.01	0.02	<0.001
LSD	0.24	0.17	0.17	0.18
CV%	41.9	19.3	17.5	15.8
Effect	NS	*	*	**

Values in parenthesis are square root-arcsine transformed values of means.
Means followed by the same letter within the same week are not significantly different at $P < 0.05$
F and *P* values after square root-arcsine transformation
* Significant at $P< 0.05$
** Significant at $P\leq 0.001$
NS = not significant

3.4 Discussion

Baseline bioassay data demonstrated that *M. domestica* larvae exposed to different concentrations (1, 10, 50 and 100 µg ml^{-1}) of *Bti* were susceptible to the bacterial toxins. Indeed, 86% of fly larvae exposed to a concentration of 100µg of *Bti* formulation ml^{-1} (10^8 spores ml^{-1} of *Bti*), were dead within 7d. These results are comparable to those of Lonc *et al*. (1991). These authors reported a 70% mortality of house fly larvae as a result of a *Bti* spore concentrations of 2 x 10^9 ml^{-1}. However, since there is not always a correlation between spore concentration and the toxicity of a formulation (Skovmand *et al*., 2000; de Araújo *et al*., 2007), the toxicity of the *Bti* formulation in this study was not based on spore concentration. It is difficult to directly compare the results of this study with other publications on other *Bti* formulations, because application rates are expressed in many different ways (de Barjac, 1990) and the potencies of various test formulations are different.

In the laboratory, all the four test dosages produced significant effects towards controlling larval numbers. Mean LT_{50} and LT_{90} values for the WDG formulation showed increased susceptibility of *M. domestica* larvae to a given concentration of *Bti* as the duration of exposure increased. However, as the exposure duration increased, the amount of *Bti* required to cause significant mortality decreased, with the sharpest decline of larval numbers occurring after 4-5d exposure period. A lower mean LT_{50} value indicates that a particular dose is effective at shorter periods of time, which indicates that the larvae are more susceptible to the treatment. The lowest LT_{50} was obtained with the 100µg *Bti* ml^{-1} concentration (3.4d). Hence, the highest dosage, 100µg *Bti* ml^{-1} (with respect to active ingredient), could be used to calculate the dose of the choice for field application, since 90% control of larvae was achieved in 7d.

Spray treatment in the chicken houses of 1g L^{-1} once a week was suggested. Considering that LC_{50} values observed in the laboratory are often not enough to effect larvicidal activities in the field (de Araújo *et al*., 2007), a higher dose of 2g L^{-1} was the second choice for field application and treatment at this dosage once a week was necessary to cause >90% reduction of fly larvae. The time of exposure to treatment and the interaction effects of dosages by days after exposure were significant, indicating a change in the level of larval densities on post-treatment days with a

change in the dosage. The fact that time of exposure was significant suggests that repeated applications would be more effective in controlling the larval density. However, application of any larvicide twice a week in an operational programme may not be feasible nor cost-effective.

Results from the field spray trials with *Bti* WDG showed that a higher concentration of 2g L^{-1} applications was not significantly more effective than the lower concentration of 1g L^{-1} in causing larval mortalities. Indeed, significant differences between the two dosages were only observed in the first two weeks of application. Such low application dosages offer the possibility of keeping operational costs low. Moreover, both *Bti* concentrations were equally effective at lowering larval densities in the manure and a reduction of about 50% was achieved after 6wk post-spraying.

Results further showed that under laboratory conditions, *M. domestica* larvae were more susceptible to *Bti* WDG formulation than in the field. These results indicated that under static laboratory conditions, it is possible to overestimate the longevity of *Bti* spores in the fluctuating field environment (Yousten *et al*., 1992). The results also suggest that the potency of a product may not be an accurate indicator of its performance in the field, so far as the persistence of the larvicidal activity is concerned (Vilarinhos and Monnerat, 2004; de Araújo *et al*., 2007). This lack of persistence of *Bti* in the field has been observed in other arthropods (Karch *et al*., 1991; Gelernter and Schwab 1993; Kroeger *et al*., 1995; Fillinger *et al*., 2003; Russell *et al*., 2003).

Several environmental factors may influence *Bti* formulations effectiveness in the field (Ignoffo *et al*., 1981; Mulla *et al*., 1990; Becker *et al*., 1992), including solar radiation (Pusztai *et al*., 1991; Obeta, 1996; Nayar *et al*., 1999; Thiéry *et al*., 1999; Melo-Santos *et al*., 2001; Vilarinhos and Monnerat, 2004), high temperature (Ignoffo, 1992) and high larval densities outside of the bioassay rings (Yousten *et al*., 1992; Glare and O'Callaghan, 2000). Exposure of spores to prolonged periods of high temperatures has been reported to result in decreased activity of *Bt* products, especially in the tropics, where temperatures frequently exceed 30^{o}C (Morris, 1983). In addition to possible inactivation of the *Bt* toxins, high temperatures also reduce feeding by some insects (Han and Bauce, 2000).

Reduced persistence of *Bti* in the field may also be attributed to the existence of compounds in chicken litter that may inhibit the growth of some microorganisms (Himejima *et al*., 1992; Asukabe *et al*., 1994; Kim *et al*., 1995; Amaral *et al*., 1998; Adams *et al*., 2002). Moreover, the alkaline pH of chicken manure favours solubility of crystals, making protoxins more prone to degradation (de Araújo *et al*., 2007). The association of such factors would contribute to reducing the larvicidal activity of *Bti* products (de Araújo *et al*., 2007).

On the contrary, the prolonged larval control observed in the feeding trials with the bran formulation of *Bti* suggests that feeding *Bti* to the chickens might compensate for the partial loss of toxic crystals caused by UV and other factors. Furthermore, >90% reductions of *M. domestica* larvae were observed with a dose rate of 10g of bran formulation kg^{-1} of standard broiler mash. The bran formulation was approximately two and a half times as effective as the WDG formulation, achieving approximately 80% mortality at 4wk with 1g kg^{-1} and 30% mortality with 1g L^{-1}, respectively. Such variation in product efficacy has been reported previously (Brown *et al*., 2001; Fillinger *et al*., 2003; Russell *et al.,* 2003) and may be related to formulation characteristics.

These observations were similar to those observed by Labib and Rady (2001), who found that adding *Bti* to chicken food significantly reduced maggot numbers. In large-scale programmes, for any formulation to be considered for incorporation, it should have residual activity so that the application and thereby operational costs can be minimized. In the present study, the bran formulation was shown to be the most effective formulation for use in controlling *M. domestica* larvae in poultry houses. Specifically, it is easily applied, and lasted for long periods without a reduction in the levels of activity.

The results of our studies on the evaluation of *Bti* against *M. domestica* indicate that biological control of *M. domestica* larvae in poultry facilities may be possible. The highest mortality obtained with the bran formulation of *Bti* was 91% after 4wk of feeding 10g of the formulation per kg of basal diet to chickens. Successful results were also obtained with the WDG *Bti* formulation, causing *M. domestica* larvae mortality levels of 53% with 0.23g m^{-2} and 47% with

0.11g *Bti* formulation m^{-2}. Adding *Bti* to chicken feed is an effective measure for the management and control of house flies in caged-poultry facilities.

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CHAPTER 4

EXTERNAL DEVELOPMENT OF THE ENTOMOPATHOGENIC FUNGUS *BEAUVERIA BASSIANA* IN THE HOUSE FLY (*MUSCA DOMESTICA*)

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Abstract

Light and scanning electron microscopy (SEM) were used to view the external development, mode of penetration and to establish comparisons between three isolates (Isolates 7320, 7569 and 7771) of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) on the surface of the common house fly (*Musca domestica* L.). The host was sprayed with a conidial suspension of *B. bassiana* (10^8 conidia ml^{-1}). The specimens used in the SEM investigation were collected at particular periods after spraying and prepared for viewing using standard methods. *Beauveria bassiana* conidia attached to the host cuticle after 6h and germination tubes developed 12-48h after applying *B. bassiana* conidia to the host. Densities of *B. bassiana* conidia varied considerably on the body regions of the host, with the highest densities occurring on the intersegmental regions, the legs and the base of setae, with observable lower densities of conidia on body regions without setae. Penetration of the cuticle was effected after formation of appressori. Extensive growth of mycelium on the surface of the insect's integument followed colonization of the host. After 72-120h, post colonization, the first sporulation was observed on the cuticle. After 144h, post- spraying with *B. bassiana,* the cuticle of the host had completely degraded. Although all three *B. bassiana* isolates killed the flies within 24h, Isolate 7771 colonized the cadavers faster than Isolates 7320 and 7569.

4.1 Introduction

House flies are among the most important pests in poultry and other confined animal operations where they breed in the manure, animal feed, and other organic materials (Axtell, 1986). In addition to the direct damage these flies inflict upon poultry, their presence in confined poultry operations is generally a nuisance, especially when the flies enter the vicinity of human habitations and urban environments. House flies have been implicated as mechanical vectors of a wide range of pathogens of animals and humans (Greenberg, 1973; Graczyk *et al*., 2001). In poultry production, house flies transmit *Salmonella* among flocks; and spot eggs with 'fly specks', reducing the eggs' market value. The fact that house flies have developed resistance to most insecticides applied against them (Scott *et al.,* 2000), has made efforts to control flies using biological control agents (BCAs) increasingly important.

Entomopathogenic fungi are unique compared to other disease-causing microorganisms because they infect their hosts through the insect cuticle and do not need to be ingested, therefore showing great potential for controlling insect pests. The entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin is one of the most widely researched and promising species of pathogenic fungi used for insect control, and is pathogenic on more than 200 species of insects (Feng *et al*., 1994; Hajek and St.Leger, 1994). Furthermore, several studies (Steinkraus *et al.,* 1990; Geden and Rutz, 1992; Watson *et al.,* 1995; 1996; Kaufman *et al.,* 2005) have reported on the potential of *B. bassiana* for house fly control.

In order to develop entomopathogenic fungi as biocontrol agents, it is crucial to understand their mode of action on their insect pest target. Scanning electron microscopy has been widely used for this purpose (Lopez-Llorca *et al*., 1999, 2002). Asexually produced fungal spores or conidia of entomopathogenic Hyphomycetes are generally responsible for infection and are dispersed throughout the environment in which the insect hosts are present. Penetration through the host cuticle is the mode of entry for most entomopathogenic fungi (Charnley, 1989). However, prior to penetration, conidial attachment to the cuticle must take place (Boucias and Pendland, 1991), followed by germination and infection of the target insect.

The insect cuticle is the first barrier that the fungus must overcome. There are a number of discrete stages leading to breaching the cuticle by insect entomopathogens, including spore attachment, spore germination, spore differentiation and then penetration of the cuticle by enzymatic and mechanical means (St. Leger *et al*., 1991). Infection by entomopathogenic fungi such as *Beauveria spp*. results from direct penetration of the cuticle, using a combination of enzymatic and physical mechanisms, without any requirement for ingestion.

The invasion of a host is determined by a few aspects of insect host surface and fungal structure and behaviour (Charnley, 1989). For instance, surface structure and the chemical composition of the host cuticle are believed to affect the adhesion of fungal propagules to the cuticle. Chemical components of the cuticle can also affect conidial development after adhesion by either causing production of non-penetrant germ tubes or by inhibiting germination altogether (Boucias and Pendland, 1991). Conidial distribution on the host cuticle can be region specific (Sosa-Gomez *et al.,* 1997); surface topography has also been shown to influence growth of deuteromycetes after adhesion (Boucias and Pendland, 1991). Orientation of germ tubes and attachment to epicuticle may determine the relative virulence of entomopathogenic fungi (St. Leger, 1993; Clarkson and Charnley, 1996). Highly pathogenic strains of *B. bassiana* germinate very quickly and can orientate on the cuticle (Charnley and St. Leger, 1991).

Studies related to the duration of the different phases of fungal development on insects are relatively rare. These studies can be conducted using bioassays and observation of the different phases of fungal infection, through scanning electron microscopy (SEM) (Neves *et al.,* 1996).

The objective of this work was to describe the external development cycle of *B. bassiana* on *M. domestica* using SEM, and to determine the duration of the different phases of fungal infection.

4.2 Materials and methods

4.2.1 Fungi

Three isolates of *B. bassiana* (Isolates 7320, 7569 and 7771) were used in this study. Strains of *B. bassiana* were provided by Plant Pathology Research Institute[1] (PPRI). *Beauveria bassiana* cultures were grown on potato-dextrose agar. Sporulating cultures (3-4 wk-old) were harvested by brushing the dry conidia from surface of the agar plate into sterile vials. Conidia were counted with the aid of a haemocytometer to calibrate a dose of $1x10^8$conidia ml^{-1}. An aqueous spore suspension was prepared with a surfactant (0.1% dilution of Tween 80) and $1x10^8$ conidia ml^{-1}.

4.2.2 Insect rearing

Musca domestica were maintained at 28°C, 68% RH, with a 12:12 L: D photoperiod. Adult flies were given a maintenance diet of *ad lib* sugar, milk powder and water and were offered chicken liver as an oviposition medium on a twice weekly basis from the third week of postemergence. Larvae for replacement colonies were reared on a diet comprising 50g active dried yeast (DCL), 8g agar (Oxoid Bacteriological), 250ml distilled water, and 750ml UHT full cream milk, prepared by microwaving it to boiling point (Johnson *et al*., 1998).

4.2.3 Infection bioassay

Ten cold-anesthetized house flies were transferred to plastic petri plates (90mm), lined with filter paper. The flies were inoculated by spraying with a suspension (1×10^8 conidia ml^{-1}) of *B. bassiana* isolates. Ten plates with 10 insects/ plate were used for each one of the fungal isolates, maintained at 25 ± 1°C and 70±10% RH. Five insects were removed from the plates 0, 6, 12, 24, 48, 72, 96, 120 and 144 hours after inoculation. The insects were killed in petri dishes with ethyl ether and mounted on SEM stubs. Five insects were prepared for each sampling time and fixation in glutaraldehyde/OsO_4/Sodium cacodilate buffer.

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4.2.4 Scanning electron microscopy preparation

For fixation in glutaraldehyde/OsO_4/Sodium cacodilate buffer, the insects were fixed by immersion for 6 hours in 4% glutaraldehyde with a 0.2M pH 7.2 sodium cacodilate buffer. Specimens were then fixed in 1% OsO_4 in a 0.1M pH 7.2 sodium cacodilate buffer for 1 hour. Fixation and dehydration were performed in plastic Eppendorff tubes. After fixation, specimens were dehydrated in a 10, 20, 30, 50, 70, 90, and 100% acetone series. The insects were finally washed three times in a 100% acetone solution. They were then critical point dried using CO_2. The insects were then mounted on stubs and coated with gold-palladium. Five insects were prepared for each sampling time. The insects were observed with a Phillips, XL30 ESEM. Durations of the different phases of the infection process were estimated from SEM observations and compared for the three fungal isolates.

4.3 Results

4.3.1 Conidial adhesion and germination

The conidia of *B. bassiana* adhered to the insect integument within 0-6h (Fig. 4.1a, b) after application. Densities of conidia by body region varied considerably after inoculation. The highest conidial densities occurred in the intersegmental regions, the legs (Fig 4.1a), the eyes (Fig. 4.1c), and the base of the setae (Fig. 4.1d), with observably lower densities on the segmental areas without setae. Conidial germination took place after 12-48h (Fig. 4.1e). Little variation in the timing was observed for the conidial germination phase between *B. bassiana* Isolates 7569 and 7771. In the case of both Isolates 7569 and 7771, germination occurred 12-24h after inoculation. However, with Isolate 7320, germination of conidia was only observed after 12-48h. Insect death occurred within 24h of inoculation with all the isolates.

4.3.2 Penetration of the cuticle

Penetration occurred 36-72h post infection. The primary penetration sites for *B. bassiana* were at the base of the setae in different regions of the fly body, but mainly on the head, thorax,

abdomen, legs and wings (Fig. 4.1f). A thickening of the extremity of the germ tube was observed to precede penetration by *B. bassiana* (Fig. 4.1f).

4.3.3 Fungal emergence and conidiogenesis

Fungal emergence occurred between 72-96h after inoculation. During this period mycelium grew out through the base of setae (Fig. 4.2a, b) or from natural openings. Isolate 7771 colonized the dead insects faster than Isolates 7320 and 7569 (72-96h). Dead insects had a reddish colouration (Fig. 4.2c) that is characteristic of oosporein activity, which is typical of insects killed by *B. bassiana.*

Conidiogenesis was initiated between 72-96h (Isolate 7771) after inoculation (Fig. 4.2d) and intensified between 96-120h (Fig. 4.2e, f) and reached a peak 120-144h after inoculation (Fig. 4.3a, b). After 144h, the whole insect body was completely covered by *B. bassiana* conidia (Fig. 4.3c) and *B. bassiana* sporulated abundantly all over the dead insect's body (Fig. 4.3d). Cuticle degradation occurred subsequently along the whole body of the insect (Fig. 4.3e, f). Observation of the development of the three isolates of *B. bassiana* showed that Isolate 7771 colonized the fly cadavers faster than Isolates 7320 and 7569 (Fig. 4.4). Although conidial adhesion happened within 6h with all the three isolates, germination of conidia took place earlier in Isolates 7569 and 7771 (12-24h). There was little variation in timing of the process of penetration of the germtube into the insect cuticle.With all the three isolates, penetration occurred within 36-72h after application of *B. bassiana* (Fig.4.4).

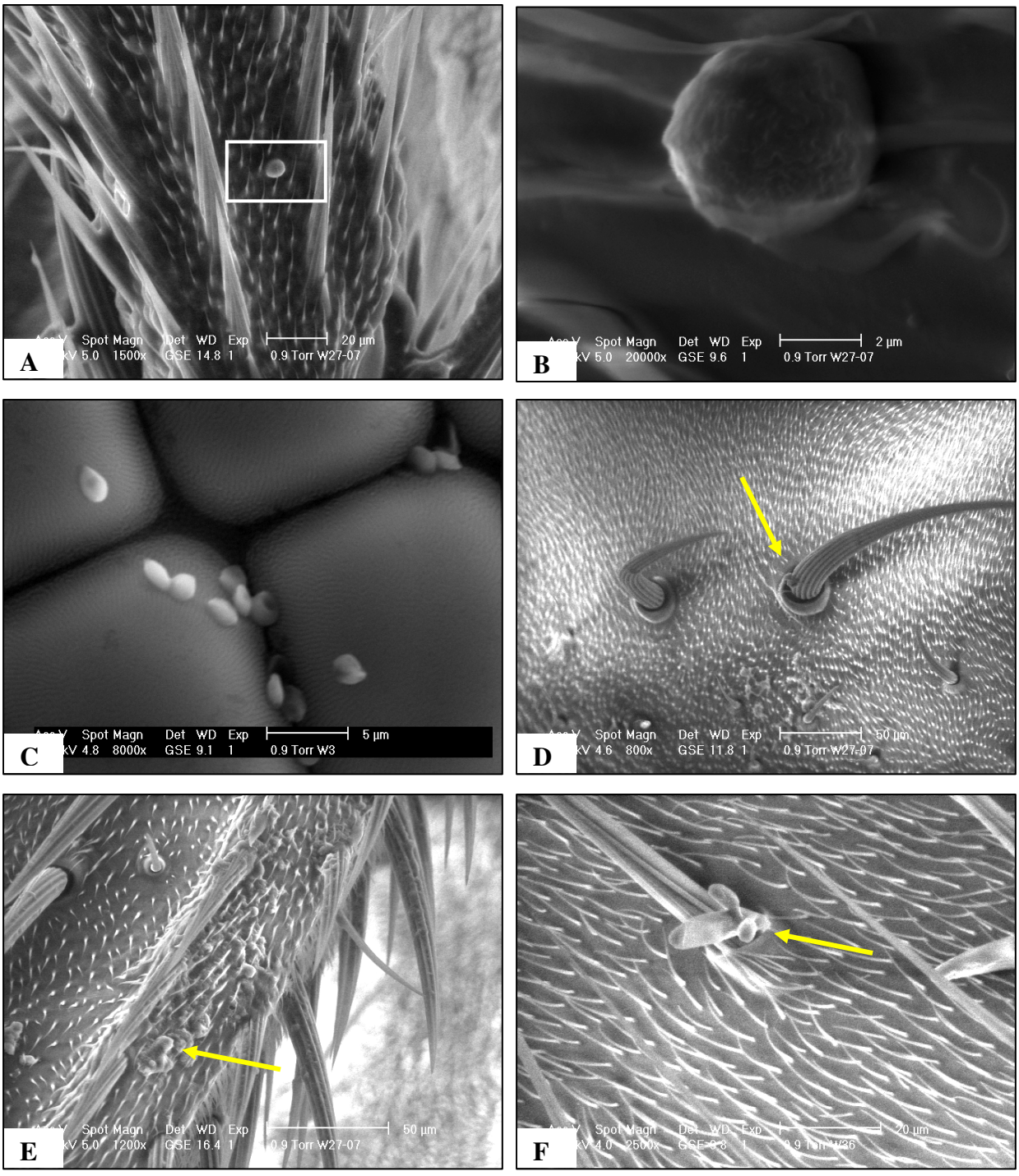

Fig. 4.1. Scanning electron microscope micrographs of the development of *Beauveria bassiana* on adult house flies (*Musca domestica*). A) A conidium adhering to an insect cuticle (1500X, 0h after inoculation; B) Close-up of a conidium (6500X); C) Conidia adhered to the eyes of a fly (8000X); D) Conidia adhering to the base of seta (800X, 24h); E) Germinating conidia and possible penetration at base of setae (1200X, 36h); and F) Penetration of a germ tube at the base of a setae (2500X, 48h).

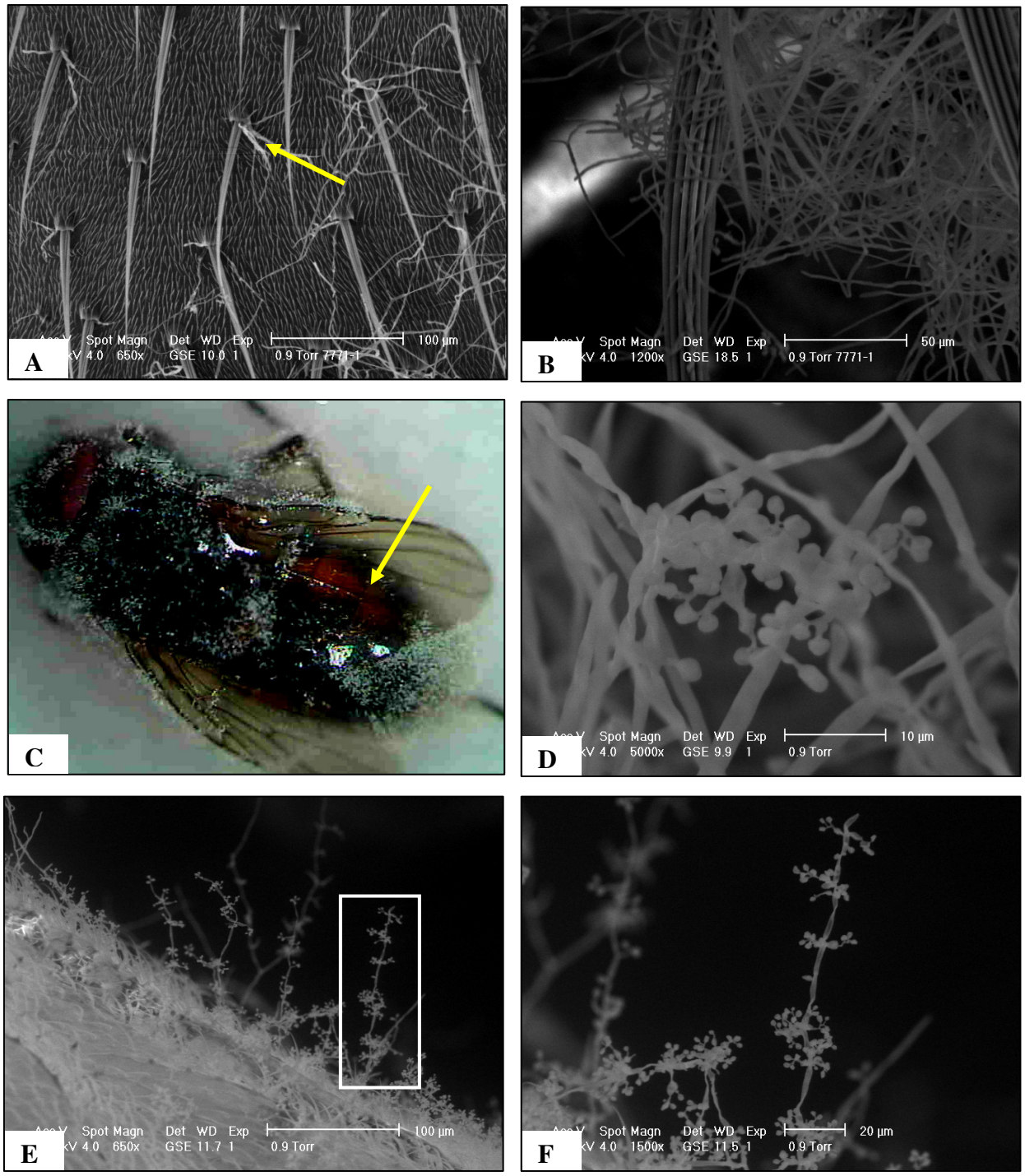

Fig. 4.2. Scanning electron micrographs of the development of *B. bassiana* on *M. domestica*. A and B) extrusion of mycelia from the base of setae and details of mycelial development (650X, 72h and 1200X, 96h, respectively); C) Reddish colouration typical of *B. bassiana* infection on the abdomen of dead insect; D and E) Conidiogenesis (5000X, 72h and 650X, 96h, respectively). F) Production of conidiophores and conidia (1500X, 96h).

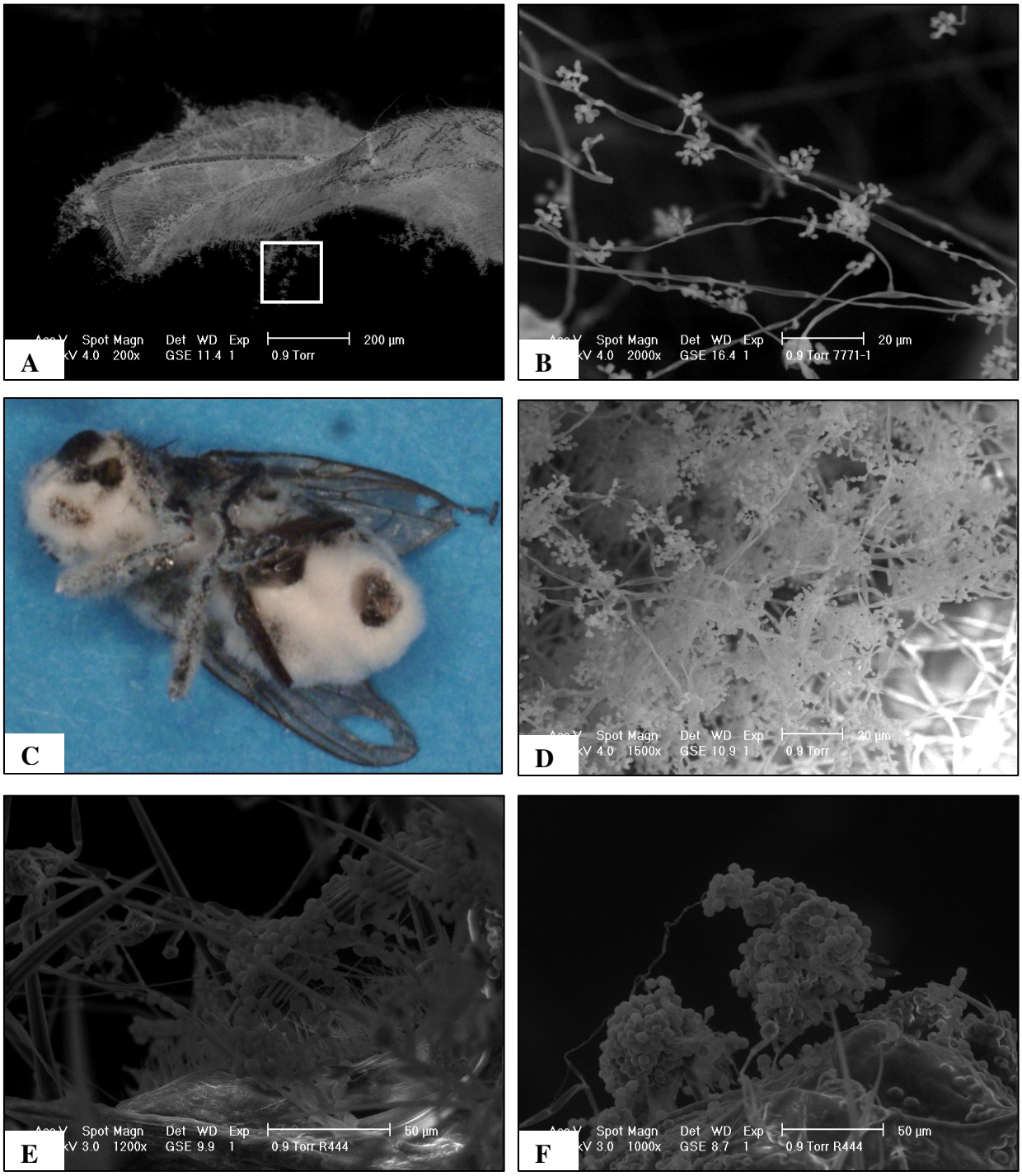

Fig. 4.3. Scanning electron micrographs of the development of *B. bassiana* on *M. domestica*. A) Conidiogenesis (200X, 120-144h). B) Detail of conidia chain (2000X, 120-144h); C) Mummified cadaver of *M. domestica* (144h); D) Massive sporulation of conidia (144h, 1500X). E and F) Cuticle degradation (1200X, 144h and 1000X, 144h respectively).

A. *Beauveria bassiana 7320*

	Time After Inoculation (h)									
Disease Phase	0	6	12	24	36	48	72	96	120	144
Conidial adhesion										
Germination										
Penetration										
Colonization										
Conidiogenesis										

B. *Beauveria bassiana 7569*

	Time After Inoculation (h)									
Disease Phase	0	6	12	24	36	48	72	96	120	144
Conidial adhesion										
Germination										
Penetration										
Colonization										
Conidiogenesis										

C. *Beauveria bassiana 7771*

	Time After Inoculation (h)									
Disease Phase	0	6	12	24	36	48	72	96	120	144
Conidial adhesion										
Germination										
Penetration										
Colonization										
Conidiogenesis										

Fig. 4.4 Duration of different developmental phases of three *Beauveria bassiana* isolates on adult house fly (*Musca domestica*).

4.4 Discussion

The present study describes the infection process of the common house fly *M. domestica* by three strains of *B. bassiana.* The following sequence of events in the infection of *M. domestica* adults by *B. bassiana* isolates:

- Attachment of *B. bassiana* conidia to the host,
- conidial germination and formation of germ tube,
- penetration and growth of the pathogen on the host,
- sporulation on the surface of the host's body.

These events are consistent with the commonly described sequence of events characterizing other entomopathogenic fungal infections (Charnley, 1989). They complement the detailed electron microscopical studies of other authors (Moino Jr. *et al*., 2002; Neves and Alves, 2004), who examined the ultrastructural aspects of the interaction between *B. bassiana* and insect hosts. The results of this study further demonstrate that insect death between germination and penetration of conidia.

During this study it was expected that conidia would be evenly deposited over the bodies of flies after application. On the contrary, individual conidia were found adhering particularly to the intersegmental regions, legs, eyes and base of setae, although equivalent numbers of conidia must have fallen over all cuticular zones. These results are consistent with results from previous studies with entomopathogenic hyphomycetes that also reported higher conidial densities in areas of the cuticle with spines (Boucias *et al*., 1988) or setae (Pekrul and Grula, 1979; Sosa-Gomez *et al*., 1997; Vestergaard *et al*., 1999; Hajek *et al.,* 2002; Hajek and Eastburn, 2003). It has been suggested that conidia stick to their hosts in these areas. It is easier to dislodge condia from areas without setae (Hajek and Eastburn, 2003).

It was anticipated that the abundant, long setae of *M. domestica* should have protected the flies to some extent from conidia landing on the cuticle, yet they seemed to increase the surface area for attachment of *B. bassiana* conidia. The effectiveness of setae in preventing infections has been questioned with respect to other species of Entomophthorales (Wraight *et al*., 1990; Hajek and Eastburn, 2003). On *M. domestica*, *B. bassiana* conidia were frequently found on the setae but this did not prevent conidia from germinating.

Germination of conidia occurred 12-24h after application. Rapid germination on hosts has also been documented for *B. bassiana* on termites (Boucias *et al.,* 1996; Moino Jr. *et al.,* 2002; Neves and Alves, 2004), *Entomophaga maimaiga* Humber on gypsy moth (Hajek *et al.,* 2002), *Erynia conica* (Nowakowski) Remaudière and Hennebert on black flies (Nadeau *et al*., 1996a), *Zoophthora* (= *Erynia*) *radicans* (Brefeld) Batko on leafhoppers (Wraight *et al*., 1990) and *Lecanicillium muscarium* (Petch) Zare and W. Gams on aphids (Askary and Yarmand, 2007). Rapid conidial germination and mycelial development of *B. bassiana* on *M. domestica* can be explained by the presence of stimulatory components in the chitin barrier of the hosts (Askary *et al.,* 1997, 1999).

Entomopathogenic fungi have been reported to invade the host cuticle shortly after germination or after limited hyphal growth (Wraight *et al*., 1990; St. Leger *et al*., 1991). This may take 24-48h under ideal conditions (Wraight *et al*., 1990). In this study penetration pegs were observed 36-72h after infection. Most often, pathogens produce penetration pegs from appresoria but occasionally hyphae may penetrate the cuticle directly (Schreiter *et al.,* 1994). There was no evidence of direct penetration of adult *M. domestica* by *B. bassiana.*

Not all areas of the insect cuticle are equally vulnerable to penetration by propagules of entomopathogenic fungi. The penetration pegs of *B. bassiana* were observed at the base of setae, which are covered by a thin cuticle and thus constitute zones of weakness (Davies, 1988). Penetration was accompanied with the formation of structures similar to appressoria. These structures occurred early in the developmental cycle of *B. bassiana* and may be the reason for the high virulence of the three *B. bassiana* isolates against the house fly. Moreover, most insects died within 24h of inoculation. Although a rapid germination rate has been recognized as an important determinant of virulence and pathology (Pekrul and Grula, 1979; Hassan *et al*., 1989; Yokohama *et al*., 1993), early penetration of the fungus into the host may also be associated with these traits (Neves and Alves, 2004). Furthermore, the production of penetration structures early in the developmental cycle of *B. bassiana* on the insect host may be an important trait for selection of isolates for use as a BCA.

Although all three *B. bassiana* isolates killed flies within 24h, Isolate 7771 colonized the cadavers faster than Isolates 7320 and 7569. The rates of conidial germination and penetration of the insect integument have been shown to be related to the virulence of the fungi (Pekrula and Grula, 1979; Fargues, 1984). Isolate 7771 may therefore have more potential as a BCA for the control of adult flies than Isolates 7320 and 7569.

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CHAPTER 5

COMPARISON OF LARVICIDAL EFFECTS OF BRAN-FORMULATED *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* AND LARVADEX® AS FEED ADDITIVES FOR CONTROLLING HOUSE FLIES (*MUSCA DOMESTICA*) LARVAE IN CHICKEN LAYERS

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Abstract

A field study was carried out over 6wk to compare the efficiency of two different larvicides for the control of house flies in poultry houses. The impact of oral feed applications of a bran formulation of *Bacillus thuringiensis* var. *israelensis* (*Bti)* and a commercial chemical larvicide, Larvadex®, were studied with respect to their efficacy on control of house fly (*Musca domestica*) larval populations in poultry manure. The sublethal effects of the larvicides were also assessed.

The 500mg kg^{-1} concentration had no advantage over the 250mg kg^{-1} oral feed application in causing mortality of house fly larvae. Although Larvadex® reduced larval density and caused significant reductions in emergence of adult house flies, it generally exhibited weaker lethal effects than *Bti.* The reduction levels achieved as a result of feeding 250mg *Bti* kg^{-1} at 5wk were similar to those achieved as a result of feeding twice the amount of Larvadex® at 4wk to the layers. Both *Bti* and Larvadex® had significant effects on the emergence of adult houseflies. The sublethal effects were manifested in terms of decreasing emergence of adult house flies. In the exposed larvae, the percentage of larvae that survived and succeeded to complete their life cycle decreased with the increase in the toxin concentration. By the end of the 6wk of study, the percent inhibitions of adult house flies caused by 250mg and 500mg kg^{-1} of *Bti* and Larvadex® were 56 and 66% for *Bti* and 57 and 67% for Larvadex® respectively.

5.1 Introduction

The accumulation of large quantities of organic wastes in poultry production systems provides an excellent breeding medium for house fly (*Musca* domestica) larvae and consequently adult fly production (Axtell 1986, 1999; Thomas and Skoda, 1993). House fly control is a major concern in poultry production (Axtell, 1986; Axtell and Arends, 1990) because these flies have an important role as vectors of many metaxenic pathogens. The house fly is typically synanthropic and, because of its high reproductive potential, feeding habits and ability to prosper in a wide range of environments, it breeds throughout the entire year.

Normally, chemical larvicides and adulticides are the primary means of nuisance fly control employed by poultry producers. Improper use of those products combined, with the house fly's short life cycle (Larsen and Thomsen, 1940) and high biotic potential produce conditions conducive to the development of resistance to insecticides. This has increased awareness of the need for effective of biological control agents (BCAs) to replace or complement existing house fly insecticides used by the poultry industry.

Bacillus thuringiensis is a Gram positive and soil inhabiting bacterium, which is environmentally safe and effective for the control of insects. The bacterium *B. thuringiensis* owes its insecticidal activity to the presence of parasporal crystalline proteinaceous δ-endotoxins. *Bacillus thuringiensis* has been found to be toxic to the house fly (Hodgman *et al.,* 1993). Several other isolates of *B. thuringiensis* have also been found which are active against larvae of the house fly (Johnson *et al.*, 1998) and thuringiensin-containing preparations have been used to control larvae of house flies (Mullens and Rodriguez, 1988; Mullens *et al.*, 1988a). Use of these biological control agents is safer than using chemical larvicides since they are species-specific and environmentally safe (WHO, 1999).

For *Bti* to be effective against flies, usually the larval stage must ingest the crystals, to be effective (Schnepf *et al.,* 1998). The crystals proteins exert their effect on the host by causing lysis of midgut epithelial cells, which leads to gut paralysis, cessation of feeding and eventual death of the host. Upon ingestion, the crystals are solubilized by the alkaline pH of the midgut and the protein pro-toxins are processed by midgut proteases to release the active toxins (Lecadet

and Dedonder, 1967). Binding by activated protein toxins to specific cell surface receptors on the midgut epithelia leads to formation of pores in the apical membranes, leading to an influx of ions and water, causing gut lysis and insect death (Gill *et al.,* 1992; Theunis *et al.,* 1998) within a day or two.

Insect growth regulators (IGRs) are a diverse group of insecticides, with a range of effects on insect specific phenomena, disrupting the growth and development of insects and other arthropods. They mainly affect the development of immature stages, and disrupt metamorphosis and reproduction (Graf, 1993; Retnakaran *et al*., 1985) and are becoming important in the management of insect pests (Grenier and Grenier, 1993). IGRs include various chemical categories including: juvenile hormones, chitin synthesis inhibitors, and triazine derivatives (Retnakaran *et. al*. 1985) with different modes of action.

Cyromazine (CGA 72662, N-cyclopropyl-1, 3, 5-triazine-2, 4, 6-triamine) represents a new class of IGRs derived from aziodotriazine herbicides (Shen and Plapp, 1990) and is commonly used to control immature house flies on poultry farms (Hogsette, 1979; Miller and Corley 1980; Miller *et al.,* 1981, Awad and Mulla, 1984). It was discovered by Ciba-Giegy Ltd. in the mid 1970s and originally developed under the trade name of 'Vetrazine', a blowfly control agent. In poultry farms it has been used for the control of manure-breeding flies, (especially house flies *M. domestica* L.) in two ways: as a feed-through larvicide or as a spray applied over the manure. For topical application Cyromazine is formulated as a water-soluble granule and a soluble powder (50%) ('Neporex'). A pre-mix (1%) Larvadex® is added to poultry food as a feed-through in poultry (Graf, 1993; Moreno-Mari *et al*., 1996).

Larvadex® does not kill fly maggots outright. It produces irreversible morphophysiological changes, which culminate in the death of the insects (Awad and Mulla, 1984). The effect varies according to the developmental stage of the insects. When housefly larvae are exposed to Larvadex®, deformations may be observed in the pupal stage, which result from interference with chitin digestion and synthesis. When applied at the prepupal stage, Larvadex® produces morphogenic aberrations in the adults, like absence of wings and underdevelopment of the genitalia in both males and females (Cerf and Georghiou, 1974).

This study was conducted to compare the effects of feeding chicken with bran-formulated *Bti* and Larvadex® on the populations of house fly larvae in poultry manure.

5.2 Materials and methods

5.2.1 Larvicides

The *Bti* formulation (a bran formulation) was obtained from Plant Health Products (PHP)[1] (Pty) Ltd. Larvadex® was obtained from the University Research Farm at Ukulinga.

5.2.2 Layers and housing

For field evaluation experiments, thirty-six x 26wk old commercial layers (Hyaline Brown) were obtained from the University Research Farm, Ukulinga. Layers were evaluated upon receipt for signs of disease or other complications that could affect the outcome of the study. Following examination, layers were randomly allocated into 36 hanging pens (44 x 42 x 61cm) in parallel back-to-back rows of 12 pens per row. Pens were separated by wire-mesh on all sides. Each pen was equipped with a removable feed trough and a nipple drinker. Each pen had a removable tray for individual manure collection. Layer chickens were housed in an environmentally controlled shed fitted with fans and 16:8h (L: D) at the University Research Farm at Ukulinga.

5.2.3 Diet preparation for layers and administration

The larvicides; Larvadex® and *Bacillus thuringiensis* var. *israelensis*-formulated bran were mixed into the layer mash in varying quantities (250 and 500mg kg^{-1} of layer mash). The control did not contain any Larvadex® or *Bti*-formulated bran. The rations were stored in trash cans lined with plastic bags until fed to the chickens. There were six birds per treatment. Feed and drinking water were provided *ad libitum*. Layers were fed their respective dietary treatments for 7wk, although house fly larvae sampling was started 2wk after treatment commenced to the layers time to adjust to their various feeds.

[1] Plant Health Products (Pty) Ltd. P.O. Box 207, Nottingham Road, South Africa

Post-treatment larval densities were monitored after every 7d. Application was carried out every 7d. Data were analyzed to measure reduction in the fly larvae in comparison with untreated controls.

5.2.4 Fly larvae sampling

For the feed trials, excreta from each bird were collected every seven days. Sampling of fly larvae began one week after the administering the different feed in order to give the layers time to adjust to the different treatments. Housefly larvae were monitored using a bulb planter (400ml) to collect two manure cores from each tray/bird/diet. Larvae was extracted in Berlese funnels and enumerated.

Larvae were maintained at 25^{o}C in the treated manure until adult house fly emergence and numbers were recorded. Adult house fly emergence rate was then calculated taking into account the number of exposed larvae.

5.2.5 Statistical analysis

Percentage mortality of larvae in the treated trays was calculated and corrected to the reduction, if any, in controls using the formula of Mulla *et al*. (1971) as given below:

$$\% \text{ Mortality} = \left[\frac{C1 - C2}{C1}\right] \times 100$$

where; $C1$, abundance of larvae in untreated manure;

$C2$, abundance of larvae in treated manure.

Adult house fly emergence inhibition was calculated using the following formula, on the basis of determining adult house fly emergence from the number of larvae that were obtained:

$$\% \text{ Mortality} = \left[\frac{C - T}{C}\right] \times 100$$

where; C = percentage of adult house flies emerging from the control manure and

T = percentage of adult house flies emerging treated manure.

The percentage values were normalized by square-root arcsine transformation. Analysis of variance test (repeated measures) was performed by taking the percent reduction (square root arcsine values) as the dependent variable and dose as the factor. The LSD method was used compare the effects of the treatments.

5.3 Results

Following continuous feeding of *Bti* and Larvadex® in the layer mash, the house fly larvae populations declined rapidly. The results of the study (Fig. 5.1) showed that when either *Bti* or Larvadex® was added to the poultry feed at 250 or 500mg kg^{-1}, few house fly maggots were present in the poultry manure at the end of the trial. After 6wk of using both feed additives, the number of house fly larvae was significantly reduced in the manure (Fig. 5.1).

Overall the two dosages of *Bti* (250 and 500mg kg^{-1}) caused significant mortality of larvae among treatments and controls ($P<0.05$). No significant differences were however evident between the two dosages of *Bti* on the reduction of fly larvae in the manure during the entire study period (Table 5.1) ($F=4.32$; $P=0.064$), except on one occasion at 2wk. While there was no significant interaction effect of dosages and time ($F=0.37$; $P=0.783$), the effect of time was significant ($F=169.12$; $P<0.001$).

The 500g *Bti* kg^{-1} feed additive had a marked effect on the larval stages of the house fly especially after the first 3wk of feeding. After 3wk of feeding chickens with *Bti* feed, the reduction in larval numbers as a result of the 250 and 500 mg *Bti* kg^{-1} feeds was at least 10% (Table 5.1), while the reduction in larval numbers as a result of 250 and 500mg kg^{-1} of Larvadex® in the feed was 6.5 and 11.2%, respectively (Table 5.3). Thereafter, the manure of the birds that were on 250 and 500 mg *Bti* kg^{-1} contained progressively fewer the house fly maggots. The number of larvae in the manure was reduced by at least 20% at 4wk. By Wk5 of feeding, the percent mortality of larvae was 29.9 and 33.8% as a result of the 250 and 500g *Bti* kg^{-1} doses

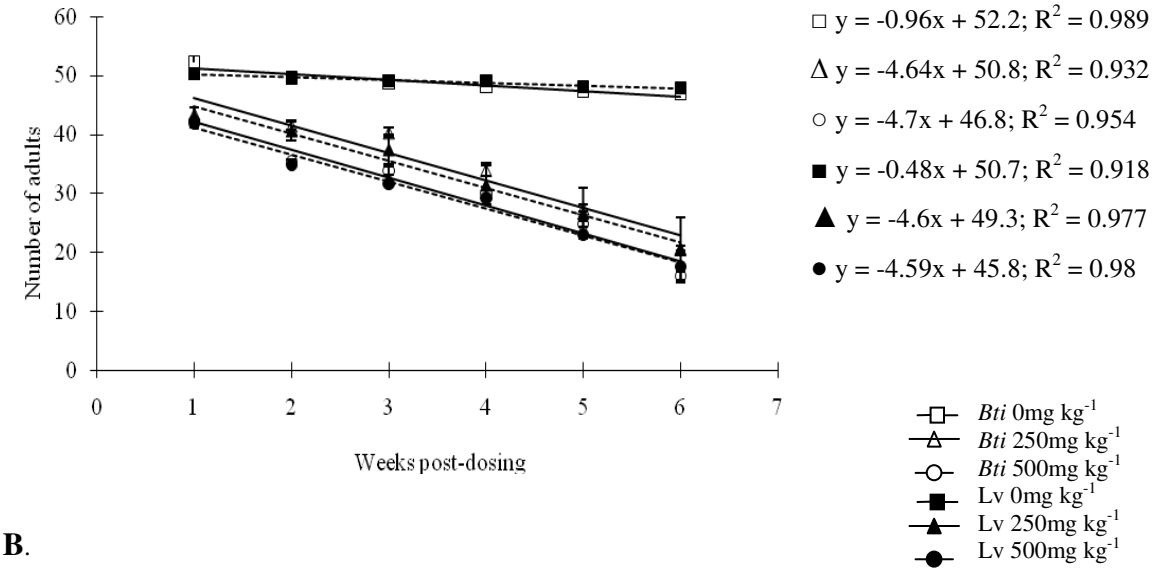
respectively, whereas the percent reduction of larvae from layers fed 250 and 500mg Larvadex® kg^{-1} was approximately 25.4 and 35.1% respectively. The reduction levels (29.9%) achieved as a result of feeding 250mg *Bti* kg^{-1} at 5wk were comparable to those achieved (30.1%) as a result of feeding layers to twice that amount of Larvadex® at 4wk (Table 5.1, 5.3).

Data on the emergence of adult house flies from larvae in manure obtained from layers fed with *Bti* are presented in Table 5.2. At both dosages, the total number of adult house flies was lower than for the control. The number of emerged house fly adults as a result of the two *Bti* dosages (250 and 500mg kg^{-1}) was significantly reduced ($P<0.05$) (59 and 66% respectively). The percentage emergence of adult houseflies as a result of the 250mg kg^{-1} and 500mg kg^{-1} *Bti* differed significantly throughout the study except Wk1 (Table 5.2).

The difference between the two dosages of Larvadex® was significant ($F = 29.64$; $P<0.001$). There were significant effects of time and interaction between treatment and time [($F = 309.78$; $P<0.001$), ($F = 5.8$; $P = 0.004$) respectively]. The main effects for Larvadex®, dose, time and the interaction between dosages and time, were significant [($F=47.71$; $P<0.001$), ($F=152.18$; $P<0.001$), ($F=2.25$; $P<0.001$) respectively]. The level of reduction of larvae as a result of the two dosages differed significantly in all except the last week of the study (Table 5.3). There was a dose-dependent relationship in the rate of mortality with Larvadex®. When lower levels of Larvadex® were added to the feed, control of the house fly larvae was reduced. However, percent mortality of larvae as a result of the 250 and 500mg kg^{-1} of Larvadex® were lower than those achieved by formulated *Bti*.

The dose of Larvadex® added to layer feed had a significant effect on the emergence of adult house flies ($P<0.05$) and there were significant differences between the two concentrations of Larvadex® ($F = 10.33$; $P = 0.009$). Similarly, time had a significant effect ($F = 134.44$; $P = 0.001$) on adult house fly emergence, while the interaction between treatment and time ($F = 2.49$; $P = 0.085$) was not significant (Table 5.4).

A.



B.

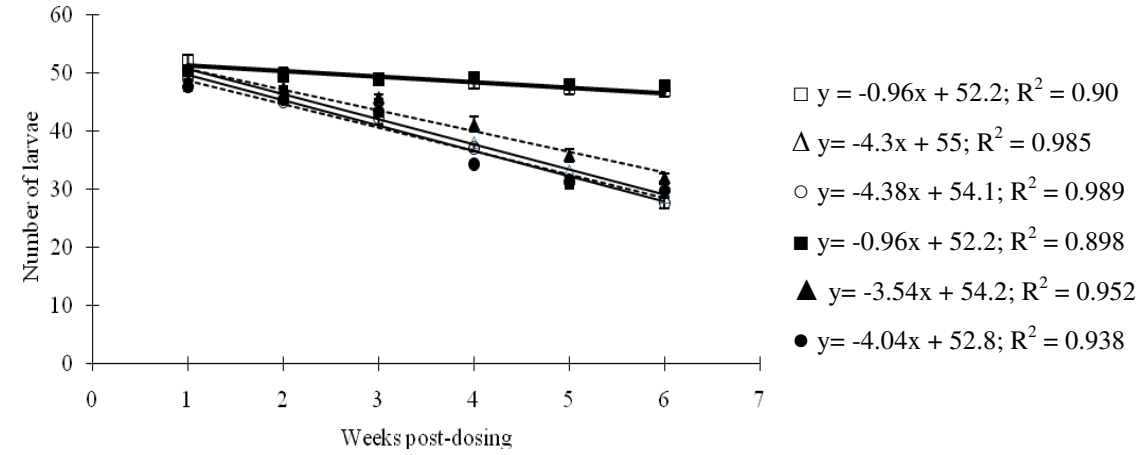


Fig. 5.1. Mean number of house fly (*Musca domestica*) **A**. adults and **B**. larvae in manure of layer chickens fed with two concentrations (250 and 500mg kg^{-1}) of a bran formulation of *Bacillus thuringiensis* var. *israelensis* (*Bti*) and Larvadex® (Lv) for 6wk.

Table 5.1. Percentage mortality in number of house fly (*Musca domestica*) larvae in manure of layer chickens fed for 6wk with two concentrations (250 and 500mg kg^{-1}) of a bran formulation of *Bacillus thuringiensis* var. *israelensis.*

	Percentage mortality of house fly larvae					
	Weeks					
Bti mg kg^{-1}	1	2	3	4	5	6
250	5.11(0.22) [a]	6.04(0.24) [a]	10.24(0.32) [a]	21.11(0.48) [a]	29.93(0.58) [a]	39.01(0.67) [a]
500	6.07(0.24) [a]	9.06(0.31) [b]	13.31(0.37) [a]	22.84(0.50) [a]	33.80(0.62) [a]	41.13(0.70) [a]
F-Ratio	0.47	6.46	1.07	0.73	1.97	0.85
P-level	0.51	0.03	0.33	0.41	0.19	0.38
LSD	0.07	0.05	0.10	0.06	0.07	0.05
CV%	21.8	15.1	23.4	9.2	8.7	5.9
Effect	NS	*	NS	NS	NS	NS

Values in parenthesis are square-root arcsine transformed
Means followed by the same letter in the same column are not significantly different at $P < 0.05$
NS – Not significant

Table 5.2. Emergence of adult house fly (*Musca domestica*) adults from larvae in manure of layer chickens fed for 6wk with two concentrations (250 and 500mg kg^{-1}) of a bran formulation of *Bacillus thuringiensis* var. *israelensis.*

	Percentage emergence of house fly adults					
	Weeks					
Bti mg kg^{-1}	1	2	3	4	5	6
250	16.61(0.42)[a]	16.44(0.42)[a]	17.41(0.48)[a]	29.41(0.57)[a]	41.90(0.70)[a]	56.03(0.85)[a]
500	19.81(0.46)[a]	28.86(0.56)[b]	30.72(0.59)[b]	37.72(0.66)[b]	47.54(0.76)[b]	65.96(0.95)[b]
F-Ratio	1.85	35.12	29.21	10.16	10.48	30.13
P-level	0.204	<0.001	<0.001	0.01	0.009	<0.001
LSD	0.072	0.057	0.06	0.063	0.039	0.042
CV%	12.7	9.0	10.1	7.9	4.2	3.6
Effect	NS	**	**	*	*	**

Values in parenthesis are square-root arcsine transformed
Means followed by the same letter in the same column are not significantly different at $P < 0.05$
* Significant at $P< 0.05$
** Significant at $P< 0.001$
NS – Not significant

Table 5.3. Percentage mortality in number of house fly (*Musca domestica*) larvae in manure of layer chickens fed for 6wk with two concentration (250 and 500mg kg^{-1}) of Larvadex® in standard chicken feed.

	Percent mortality of housse fly larvae					
Larvadex®	Weeks					
mg kg^{-1}	1	2	3	4	5	6
250	2.98(0.17)[a]	4.04(0.20)[a]	6.46(0.25)[a]	16.27(0.41)[a]	25.35(0.53)[a]	33.10(0.61)[a]
500	5.30(0.23)[b]	8.08(0.29)[b]	11.22(0.34)[b]	30.17(0.58)[b]	35.07(0.63)[b]	37.63(0.66)[a]
F-Ratio	9.96	18.52	5.13	18.93	9.28	3.03
P-level	0.01	0.002	0.047	0.001	0.012	0.112
LSD	0.04	0.04	0.08	0.089	0.079	0.061
CV%	15.6	14.1	21.1	13.9	10.6	7.4
Effect	*	*	*	**	*	NS

Values in parenthesis are square-root arcsine transformed values
Means followed by the same letter in the same column are not significantly different at $P < 0.05$
* Significant at $P< 0.05$
** Significant at $P< 0.001$
NS – Not significant

Table 5.4. Emergence of house fly (*Musca domestica*) adults from larvae in manure of layer chickens fed for 6wk with two concentrations (250 and 500mg kg^{-1}) of Larvadex® in standard chicken feed.

	Percentage emergence of house fly adults					
Larvadex®	Weeks					
mg kg^{-1}	1	2	3	4	5	6
250	12.58(0.35)[a]	15.82(0.39)[a]	24.49(0.51)[a]	35.59(0.64)[a]	43.75(0.72)[a]	57.14(0.86)[a]
500	16.23(0.41)[a]	29.63(0.58)[b]	35.37(0.64)[b]	40.68(0.69)[b]	52.08(0.81)[b]	67.07(0.92)[a]
F-Ratio	1.97	9.7	5.61	6.56	11.99	3.6
P-level	0.19	0.011	0.04	0.03	0.01	0.09
LSD	0.10	0.13	0.12	0.05	0.05	0.07
CV%	20	20.8	16.4	5.4	5.5	6.3
Effect	NS	*	*	*	*	NS

Values in parenthesis are square-root arcsine transformed.
Means followed by the same letter in the same column are not significantly different at $P< 0.05$
* Significant at $P< 0.05$
NS – Not significant

5.4 Discussion

The results of the present study reveal that both larvicides had significant effects on the mortality of house fly larvae and emergence of adult house flies. Although Larvadex® exhibited weaker lethal effects than *Bti* with respect to mortality of larvae and emergence of adult house flies, reasonable house fly control (>50%) was achieved after using both larvicides as feed additives.

The difference between the 250mg and 500mg kg^{-1} of the *Bti* formulation was statistically non-significant in inducing mortality of larvae. These observations confirm results from our earlier studies that the 500mg kg^{-1} concentration had no advantage over the 250mg kg^{-1} feed application in causing mortality of larvae. It is known that the potential of *Bti* might be limited due to its short persistence (Navon, 2000). Although *Bti* spores may not be involved in the toxicity (Skovmand *et al*., 2000), their continuous viability is an important factor. The persistence of *Bti* spores depends much on the characteristics of its own formulation as well as the environmental conditions of the breeding site (de Araújo *et al*., 2007). Several factors have been reported to influence the persistence of *Bti* including solar radiation and high temperature.

The reduction levels achieved as a result of feeding 250mg *Bti* kg^{-1} at 5wk were comparable to those achieved as a result of feeding twice the amount of Larvadex® to the layers after 4wk. For instance, a *Bti* formulation concentration of 250mg kg^{-1} was sufficient to achieve 29.9% mortality of house fly larvae at 5wks. This was equivalent to the 30.1% mortality obtained using 500mg kg^{-1} of Larvadex® at 4wks post-treatment. Similarly the 39% decrease in adult house fly emergence as a result of the 250mg kg^{-1} *Bti* was more than the 37.6% achieved using 500mg kg^{-1} Larvadex® at 6wk. The fact that a weekly application *Bti* yielded far better results than a double dose application of Larvadex® illustrates that there would be a need for a twice weekly application of Larvadex® in order to achieve similar mortality results as *Bti*.

Whereas the effects of sublethal amounts of *B. thuringiensis* on surviving individuals are not well understood (Kaelin *et al*., 1999), our results indicated that the larvae that survived exposure to *Bti* and completed their life cycle was significantly lower than in the controls. Furthermore, there were significant differences in mean number of emerged house fly adults as a result of the two *Bti*

dosages (250 and 500mg kg^{-1}). This suggests that some adverse biological effects were induced in house fly larvae by sublethal amounts of *Bti*. Limited data exist for comparing susceptibility of field populations of house fly larvae and adults to formulated *Bti* fed orally to chickens. However, authors (Labib and Rady, 2001) reported significant effects of concentration on reduction of maggot numbers and emergence inhibition of adult house flies when *Bti* was incorporated into chicken feed. Several other authors have also reported on the sublethal effects of *B. thuringiensis* on other arthropods (Salama *et al*., 1981; Keever *et al*., 1994; Staple *et al*., 1997; Adamczyk *et al*., 1998; Kaelin *et al*., 1999; Liu *et al*., 2001, 2005a, 2005b). Reduced feeding rates and partial starvation are consistent with *B. thuringiensis* toxicity (Gill *et al*., 1992) and may have contributed to these effects. By inducing many adverse biological effects in *M. domestica*, sublethal levels of *B. thuringiensis* can reduce house fly activity.

Larvadex® added to layer feed had significant effects on the emergence of adult house flies. Brake *et al*. (1991) observed mortality rates of 75.7% at 250ppm and 86.5% at 1000ppm. The authors observed that hens grown on 1000ppm produced manure that still caused more than 50% fly mortality 20wk after removal of feed additive. Several authors have also reported retention of larvicidal activity of manure from hens fed with Larvadex® after feeding the birds doses of 50 (Crespo *et al*., 2002) or 200 times (Brake *et al*., 1991) greater than the maximum recommended levels. These data demonstrated the dose-mortality response also exhibited in the present study and also by Tomberlin *et al*. (2002).

Although both Larvadex® and *Bti* caused significant inhibitions of adult house fly emergence, the effects of Larvadex® have been reported to result in deformations that are observable in the pupal stage, as a result from interference with chitin digestion and synthesis. When applied at the prepupal stage, cyromazine produces morphogenic aberrations in the adults, like absence of wings and underdevelopment of the genitalia in both males and females (Cerf and Georghiou, 1974). On the other hand, house fly larvae exposed to *Bti* are mostly affected in the larval stage as a result of the interaction of the bacterial toxin with the cells of the midgut (Gill *et al*., 1992). For these reasons, feeding the layer chickens with *Bti* reduced the development time of the house fly at the larval stage and, consequently, the adult house fly emergence rate as well. On the contrary, the observed reductions in adult house fly emergence rate in the manure of layers fed a

diet treated with Larvadex® may be as a result of a decrease in the reproductive potential of the house fly.

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CHAPTER 6

USE OF TWO FORMULATIONS AND TWO APPLICATION TECHNIQUES TO DELIVER *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* FOR THE CONTROL OF *MUSCA DOMESTICA* LARVAE AND ADULTS IN POULTRY HOUSES

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Abstract

A field study was carried out for 6wk to assess the efficiency of the combined effect of feeding and topical application strategies for larvae and adult house fly control in poultry houses using two formulations of *Bacillus thuringiensis* var. *israelensis* (*Bti*).

The objectives of this study were to compare, from both an efficiency and economic perspective, the impact of combining different concentrations of the two *Bti* formulations and to evaluate their individual and integrated success in controlling house fly (*Musca domestica* L) larvae and adults. There was no significant difference between the 1g and 2g L^{-1} spray applications of *Bti*. In the absence of spray applications, no significant differences in larval mortalities were observed between the 250mg and 500mg kg^{-1} feed applications. The percentage mortality of larvae accomplished as a result of using a combination of 250mg kg^{-1} *Bti* and 2g L^{-1} spray applications was equivalent to that obtained as a result of combining 500mg kg^{-1} *Bti* and 1g L^{-1} spray application. When only spray applications were used, house fly larval populations were reduced by 43%. When 250mg kg^{-1} feed application was used in conjunction with 2g L^{-1} spray application larval populations were reduced to 59%. A 500 mg kg^{-1} feed application used together with 1g L^{-1} spray application reduced larval populations by 57%. Treatment with *Bti* caused significant reductions in the emergence (up to 74%) of house fly adults compared to the control. The fact that the emergence of adult house flies was affected by *Bti* treatments implies that *Bti* has sublethal effects on house fly larvae. The cost-benefit analysis (expressed in terms of mortality of larvae breeding) indicated that the most effective combination for house fly larvae and adult house fly emergence control was the 500mg kg^{-1} of feed and 2g L^{-1} spray application combination that resulted in 67% larval mortality and 74% decrease in adult house fly emergence. This study presents commercial users with various alternatives for possible combinations of the two *Bti* formulations.

6.1 Introduction

The house fly, *Musca domestica* L., is a pest of medical and veterinary importance and a disease vector of significance for humans and animals (Moon, 2002). Organic wastes from intensive animal production provide excellent habitats for the growth and development of this insect (Thomas and Skoda, 1993). Currently, use of chemicals against larvae and adults is the major approach used to control or eliminate house flies employed by poultry producers. However, the widespread use of chemical insecticides reduces the impact of parasites and promotes the development of insecticide resistance in house flies (Scott *et al*., 2000). Therefore, it is desirable to look for strategies that are innovative, cost-effective and have a low environmental impact. For these reasons, alternative fly control methods, such as biological control, are of increasing importance. For instance, the use of biological control agents (BCAs) such as *Bacillus thuringiensis* could be employed in the management strategies for house fly control.

Bacillus thuringiensis Berliner is a spore forming bacterium, characterized by its ability to produce parasporal bodies (crystals), which contain specific insecticidal endotoxins. Among entomopathogenic bacteria, it is the most studied species. It is also the most widely used biopesticide in the world (Glare and O'Callaghan, 2000). The efficacy of some *B. thuringiensis* isolates against the house fly has been widely demonstrated (Indrasith *et al*., 1992; Hodgman *et al*., 1993; Johnson *et al*., 1998; Zhong *et al*., 2000).

Our prior research, however, identified a lack of persistence by a water-dispersal granule formulation (WDG) of *Bti* for the control house fly larvae in poultry houses. This observation has been reported by other authors working with other arthropods (Karch *et al*., 1991; Gelernter and Schwab 1993; Kroeger *et al*., 1995; Fillinger *et al*., 2003; Russell *et al*., 2003).

Moreover, results from our field spray trials with a WDG formulation of *Bti,* showed that applications of a higher concentration of 2g L^{-1} was not significantly more effective than the lower concentration of 1g L^{-1} in causing larval mortalities. Additionally, both *Bti* concentrations were equally effective at lowering larval densities in the manure and a reduction of about 50%

was achieved after 6wk of regular spraying. Such low application dosages have the advantage of keeping operational costs low.

Several environmental factors may influence *Bti* formulations effectiveness in the field (Ignoffo *et al*., 1981; Mulla *et al*., 1990; Becker *et al*., 1992). These include solar radiation (Pusztai *et al*., 1991; Obeta, 1996; Nayar *et al*., 1999; Thiéry *et al*., 1999; Melo-Santos *et al*., 2001; Vilarinhos and Monnerat, 2004), high temperature (Ignoffo, 1992) and high larval densities outside of the bioassay rings (Yousten *et al*., 1992; Glare and O'Callaghan, 2000). Exposure of spores to prolonged periods of high temperatures has also been reported to result in decreased activity of *Bt* products, especially in the tropics where temperatures frequently exceed 30^{o}C (Morris, 1983). In addition to possible inactivation of the toxin, high temperatures also reduce feeding by some insects (Han and Bauce, 2000). Reduced persistence of *Bti* in the field may also be attributed to the existence of compounds in chicken litter that may inhibit the growth of some microorganisms (Himejima *et al*., 1992; Kim *et al*., 1995; Amaral *et al*., 1998; Adams *et al*., 2002). Moreover, the pH of chicken manure favours solubility of *Bti* protein crystals, making protoxins more prone to degradation (de Araújo *et al*., 2007). The association of such factors would contribute to reducing the larvicidal activity of the product (de Araújo *et al*., 2007).

On the contrary, the prolonged larval control observed in the feeding trials with the bran formulation of *Bti* suggests that feeding *Bti* to the chickens might compensate for the partial loss of toxic crystals caused by UV and other factors. Furthermore, in our previous studies, >90% reductions of *M. domestica* larvae were observed with a dose rate of 10g of bran formulation kg^{-1} of standard broiler chicken mash. The bran formulation was approximately two and a half times as effective as the WDG formulation, achieving approximately 80% mortality at 4wk with 1g kg $^{-1}$ and 30% mortality with 1g L^{-1} respectively. Such variation in product efficacy has been reported previously (Brown *et al*., 2001; Fillinger *et al*., 2003; Russell *et al.,* 2003) and may be related to formulation characteristics.

Conceivably, combining a bran feed-through formulation of *Bti* with a spray application of the WDG formulation *Bti* would result in synergistic or additive interactions that would enhance the potential for biological control of house fly adults and larvae.

This field study was carried out over 6wk, to compare the effectiveness of combined bran feed and WDG spray formulations of *Bti* for the control of house fly larvae and adults in poultry houses.

6.2 Materials and methods

6.2.1 Bacterial formulations

Two *Bti* formulations (water-dispersible-granules (WDG) and a bran formulation) were obtained from Plant Health Products (PHP)[1] (Pty) Ltd.

6.2.2 Field trials

For field evaluation experiments, fifty-four x 26wk old commercial chicken layers were obtained from the University Research Farm, Ukulinga. Layers were evaluated upon receipt for signs of disease or other complications that could affect the outcome of the study. Following examination, layers were randomly allocated into 54 hanging pens (440 x 420 x 610mm) in parallel back-to-back rows of 12 pens per row. Pens were separated by wire-mesh on all sides. Each pen was equipped with a removable feed trough and a nipple drinker. Each pen had a removable tray for individual manure collection. Layers were housed in an environmentally controlled shed fitted with fans and 16:8h (L:D) at the Poultry section, University Research Farm at Ukulinga.

6.2.3 Treatments preparation and administration

Bacillus thuringiensis var. *israelensis*-formulated bran was mixed into the layer mash in varying quantities (250 and 500mg per kg of layer mash). The control did not contain any *Bti*-formulated bran. The rations were stored in trash cans lined with plastic bags until fed to the chickens. Feed and drinking water were provided *ad libitum*. Layers were fed their respective dietary treatments for 7wk.

[1] Plant Health Products (Pty) Ltd. P.O. Box 207, Nottingham Road, South Africa

WDG-formulated *Bti* was applied at the rate of 1g and 2g L^{-1}. To prepare the solution for spraying, 1g and 2g of *Bti* powder was suspended in 1L of water. The *Bti* formulation was applied using hydraulic hand sprayers.

The bran and WDG formulation application rates that were used in the field experiments are summarized in Table 6.1. In all the experiments, treatments consisted of either one concentration of bran formulation and /or WDG formulation. Treatments were arranged in a completely randomized design. The bran formulation was given as an oral feed while the WDG formulation was applied as a topical spray. Control layer hens were not offered any *Bti* in their feed and/ or their manure was not sprayed with *Bti*. There were six layers per treatment.

Post-treatment populations of house fly larvae were monitored after every 7d. Application was carried out every 7d. Data were analyzed to measure reductions in the fly larvae numbers in comparison with the untreated controls.

Table 6.1 Summary of combinations of treatments used field experiments testing efficacy of two (bran and a water-dispersible granule) formulations of *Bacillus thuringiensis* var *israelensis* (*Bti*)

Treatment	Bran Formulation mg kg^{-1}	WDG Formulation g L^{-1}
1	0	0
2		1
3		2
4	250	0
5		1
6		2
7	500	0
8		1
9		0

6.2.4 Fly larvae sampling

Excreta from each bird were collected every 7d. Sampling of house fly larvae began a week after the administration of the different feeds in order to give the layers time to adjust to the different treatments. House fly larvae were monitored using a gardener's bulb planter (400ml) to collect

two manure cores from each tray/bird/diet. Larvae were extracted using Berlese funnels and enumerated.

Larvae were maintained at 25^{o}C in the treated manure until adult house fly emergence. The numbers of emerged adults were recorded. Adult house fly emergence rate was then calculated taking into account the number of exposed larvae.

6.2.5 Statistical analysis

Percentage mortality of larvae in the treated trays was calculated and corrected to the reduction (Abbott, 1925), if any, in controls using the formula of Mulla *et al*. (1971) as given below:

$$\% \text{ Mortality} = \frac{[C1 - C2]}{C1} \times 100$$

where; $C1$, number of larvae in untreated manure;

$C2$, number of larvae in treated manure.

Percent reduction in adult house fly emergence was calculated using the following formula, on the basis of determining adult house fly emergence from the number of larvae that were obtained:

$$\% \text{ Reduction in adult house fly emergence} = \frac{[C - T]}{C} \times 100$$

where; C = percentage of house flies emerging from the control manure and

T = percentage of house flies emerging treated manure.

This experiment was a factorial design with three main factors (*Bti* feed treatment with three levels, *Bti* spray treatment with three levels and time) and six replicates. A three-way ANOVA on larval mortality and emergence of adult house flies was performed using GENSTAT statistical package after transforming the percent mortality by Arcsin $\sqrt{(\%100)}$ to normalize the data distribution and homogeneity of variances. The LSD method was used to find significant differences between means.

6.3 Results

Larval mortality was significantly affected by the *Bti* formulations (Feed (F=1298; P<0.001), Spray (*Bti* WDG) (F=1048; P<0.001) and interactions between formulations and Time (F=432, P<0.001) factors. There were also significant interactions between the Feed and Spray formulations (F=287; P<0.001), Feed and Week (F=3.68; P<0.001) and Feed and Spray and Time (F=22.37; P<0.001). However, no significant effects on the mortality of larvae were observed between Spray and Time interaction (F=1.15; P=0.327). These results are shown in Table 6.2.

The interaction between the Feed and Spray formulations consisted of additive effects in most instances. A larval population from the manure of birds treated with a combination of 250mg kg-1 and 1g L^{-1} was numerically lower than the population from birds fed with 250mg kg^{-1} alone, and was not significantly different from the population obtained as a result of using 1g L^{-1} spray treatment alone (Table 6.2).

Larval mortality within the same *Bti* feed formulation increased over time, irrespective of the concentration of the *Bti* in the feed (Fig. 6.1a, b, c). This effect was more pronounced after 2wk of feeding for all the concentrations of *Bti* feed. Larval mortality was higher as a result of 500mg kg^{-1} than with 250mg kg^{-1} for all the *Bti* spray concentrations (Fig. 6.1b, c).

There were no significant differences in larval mortalities between the 1g and 2g L^{-1} treatments when the layers were not given any *Bti* in their feed (0mg kg^{-1}) in the six weeks of study (Table 6.2). Likewise, no differences were observed between the larval population obtained as a result of the combined treatment of 250mg kg^{-1} with either 0g and 1g L^{-1} spray treatments. However, there were significant differences in larval mortalities when 250mg kg^{-1} was combined with either 1g or 2g L^{-1} manure treatments. These differences were evident throughout the study. By the end of 6wk, the percentage mortalities as a result of the 250g kg^{-1} feed using the 1 and 2g L^{-1} manure treatments were 46 and 59% respectively (Table 6.2).

Quite the reverse occurred when layers were fed with 500mg kg^{-1}. Significant differences with respect to larval mortalities were observed between of 0 and 1g L^{-1} spray treatments when combined with 500mg kg^{-1} oral feed, but no differences in larval mortalities were observed when the 500mg kg^{-1} was combined with 1g and 2g L^{-1} manure treatment, except at Wk6. At the end of 6wk of treatment, the larval mortalities as a result of combining 500mg kg^{-1} with the 0, 1 and 2g L^{-1} spray application were 50, 57 and 67% respectively (Table 6.2).

Likewise, no significant differences in larval mortalities were observed between the treatments 250 and 500mg kg^{-1} when the manure was either not sprayed (0mg L^{-1}) or treated with 2g L^{-1} *Bti* (Table 2). However, there were differences between the treatments (250 and 500mg kg^{-1}) in larval mortalities when combined with the 1g L^{-1} spray application. Furthermore, the percent mortality of larvae accomplished by using a combination of 500mg kg^{-1} *Bti* and 1g L^{-1} was as good as that achieved using a combination of 250mg kg^{-1} and 2g L^{-1} *Bti* topical spray. For instance, at Wk2, Wk3, Wk4, Wk5 and Wk6, the percentage larval mortalities achieved as a result of a combination of the 250g kg^{-1} *Bti* in the layer feed and 2g L^{-1} *Bti* topical treatment were 36.4, 43, 46.2, 52.3 and 59% respectively. For the same period of time, the percent larval mortalities attained as a result of a combination of the 500g kg^{-1} *Bti* feed and 1g L^{-1} *Bti* topical treatment were 35.5, 42.6, 46.2, 51.9 and 57% respectively (Table 6.2).

The main factors: *Bti* Feed formulation, *Bti* Spray formulation and Time, all significantly affected the percent emergence of adult house flies [(F=814.58; P<0.001), (F=686.11; P<0.001), (F=327.69, P<0.001)]. There were also significant interactions between the Feed and Spray formulations (F=204.74; P<0.001), Feed formulation and Time (F=6.39; P<0.001), Spray formulation and Time (F=1.95; P=0.039) and Feed and Spray formulations and Time (F=11.42; P<0.001).

Percentage reduction in the emergence of adult house flies increased over time, as a result of all the treatments (Fig. 6.1d, e, and f). Although there were instances where there were no significant differences between the 1g and 2g L^{-1} *Bti* topical applications, generally, the reduction of adult house fly emergence was higher in the manure treated with the 2g L^{-1} than for manure treated with the 1g L^{-1} spray application (Fig 6.1d, e, f).

Although both the 1g and 2g L^{-1} treatments significantly affected the emergence of adult house flies when the layers were not given any *Bti* (0mg kg^{-1}) in their feed, no significant differences were observed with respect to emergence of adult house flies between the two treatments (Table 6.3). At the end of 6wk, percent inhibition in emergence of adult house flies as a result of the 1g and 2g L^{-1} (0mg kg^{-1}) were 40.5 and 41.4%, respectively.

When layers were fed with 250mg kg^{-1} *Bti*, no significant differences were observed in adult house fly emergence as affected by manure sprays of the 0 and 1g L^{-1}; however, the 1g and 2g L^{-1} treatments resulted in significant differences. As such, the percent decline in adult house fly emergence achieved by the two spray treatments (1g and 2g L^{-1}) and a feed of 250mg kg^{-1} were 44.1 and 55.9%, respectively (Table 6.3).

Differences in emergence of adult house flies were evident between the layers fed with 500mg kg^{-1} *Bti* combined with either non-spray (0g L^{-1}) or sprayed with 1g L^{-1} *Bti*. These differences were observed throughout the study. This difference was not evident with manure of layers fed with 500mg kg^{-1} and treated with 1g L^{-1} and 2g L^{-1}. The percent reduction in adult of house flies as a result of the two combinations (250mg kg^{-1}- 1g L^{-1} and 500mg kg^{-1} - 2g L^{-1}) were 58.1% and 74.3% respectively (Table 6.3).

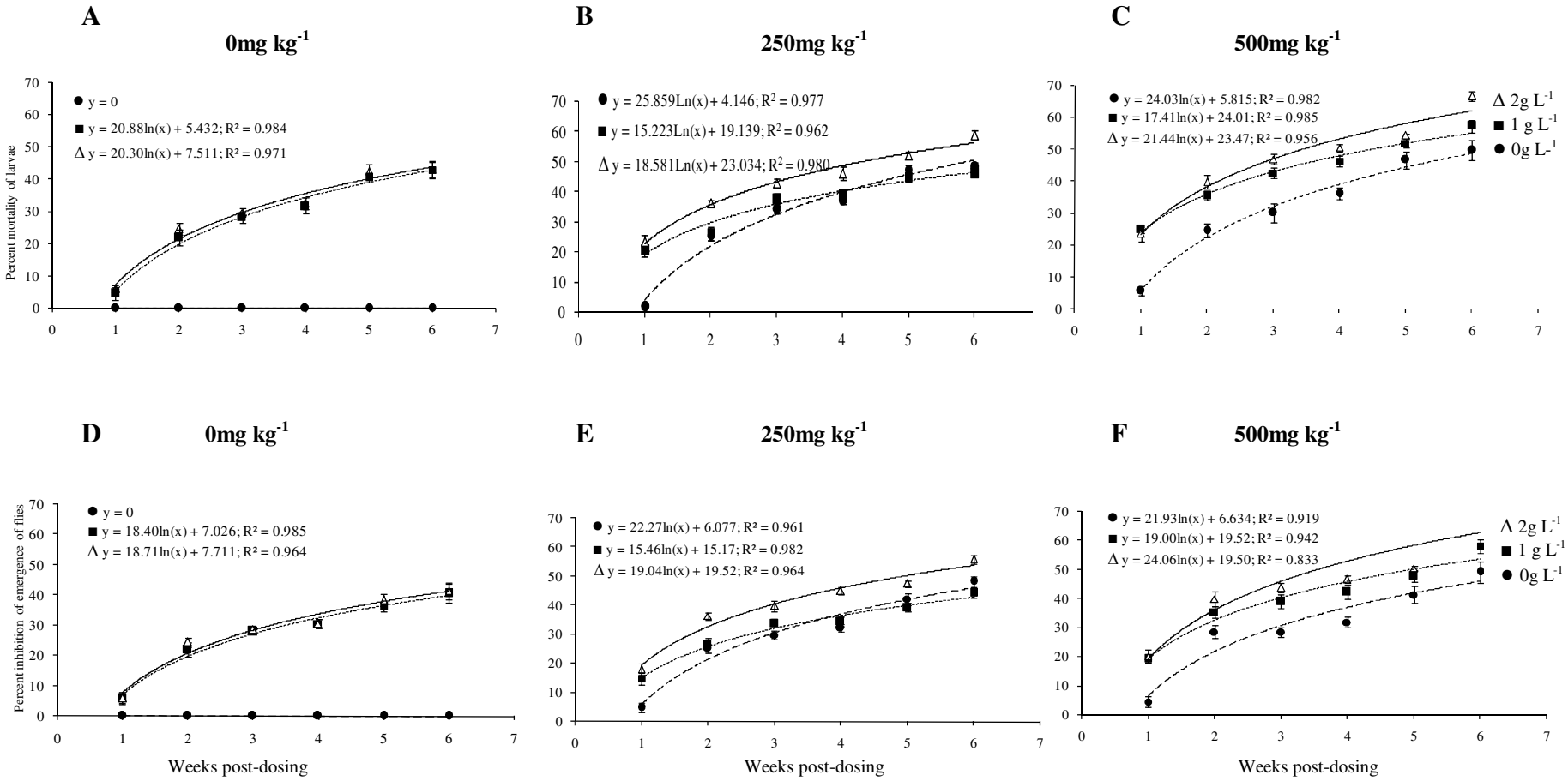

Fig. 6.1 Percent mortality of larvae (**A, B, C**) and percent emergence of adult house flies (**D, E, F**) in manure of layers sprayed with three concentrations (0 (●), 1(■) and 2(Δ) g L^{-1}) of a water-dispersal granules formulation of *Bacillus thuringiensis* var. *israelensis* (*Bti)* and fed with; **A**.and **D**. 0mg kg^{-1} **B**.and **E.** 250mg kg^{-1} **C**.and **F.** 500mg kg^{-1} bran formulation of *Bti* for 6wk.

Table 6.2 Percentage mortality of house fly (*Musca domestica*) larvae in manure of layers fed with three concentrations (0, 250 and 500mg kg^{-1}) of a bran formulation of *Bacillus thuringiensis* var. *israelensis (Bti)* and treated with three concentrations (0, 1 and 2g L^{-1}) of a *Bti* water dispersible granule (WDG) formulation in standard chicken feed for 6wk.

		Percent mortality of larvae					
		Weeks					
Bti(Feed) mg kg^{-1}	*Bti(Spray) g L^{-1}*	1	2	3	4	5	6
0	0	0 (0)^{a}	0 (0)^{a}	0 (0)^{a}	0 (0)^{a}	0 (0)^{a}	0(0)^{a}
	1	4.71 (0.19)^{bc}	21.93 (0.53)^{b}	28.32 (0.56)^{b}	31.54 (0.60)^{b}	40.49 (0.69)^{b}	43.0 (0.71)^{b}
	2	6.16 (0.25)^{c}	24.56 (0.52)^{b}	29.75 (0.58)^{bc}	32.62 (0.61)^{bc}	42.56 (0.71)^{bc}	43.0 (0.71)^{bc}
250	0	1.81 (0.11)^{b}	25.44 (0.57)^{bc}	34.41 (0.63)^{cd}	37.28 (0.66)^{d}	47.41 (0.76)^{de}	48.67 (0.77)^{de}
	1	20.65 (0.47)^{d}	26.32 (0.61)^{c}	37.63 (0.66)^{d}	39.07 (0.68)^{d}	44.99 (0.74)^{cd}	46.33 (0.75)^{bcd}
	2	23.55 (0.51)^{d}	36.40 (0.70)^{d}	43.01 (0.72)^{e}	46.24 (0.75)^{e}	52.25 (0.81)^{f}	59.0 (0.88)^{f}
500	0	5.80 (0.23)^{c}	24.56 (0.54)^{b}	30.11 (0.58)^{bc}	36.2 (0.65)^{cd}	46.72 (0.75)^{cd}	49.67 (0.78)^{de}
	1	25.0 (0.52)^{d}	35.53 (0.69)^{d}	42.65 (0.72)^{e}	46.24 (0.75)^{e}	51.91 (0.80)^{ef}	57.33 (0.86)^{f}
	2	23.51 (0.50)^{d}	39.91 (0.73)^{d}	46.95 (0.75)^{e}	50.54 (0.79)^{e}	54.33 (0.83)^{f}	66.67 (0.96)^{h}
F-Ratio		3.06	46.55	63.93	107.58	215.36	112.05
P value		0.03	<0.001	<0.001	<0.001	<0.001	<0.001
LSD		0.08	0.06	0.06	0.05	0.04	0.06
CV%		23.4	9	8.3	6.7	5.5	7.0
Effect		*	**	**	**	**	**

Values in parenthesis are square-root arcsine transformed
Means followed by the same letter within the same column are not significantly different at $P<0.05$
* Significant at $P<0.05$
** Significant at $P<0.001$

Table 6.3 Percentage reduction in emergence of adult house fly (*Musca domestica*) from manure of layers fed with three concentrations (0, 250 and 500mg kg^{-1}) of a bran formulation of *Bacillus thuringiensis* var. *israelensis (Bti)* and treated with three concentrations (0, 1 and 2g L^{-1}) of a *Bti* water dispersible granule (WDG) formulation in standard chicken feed for 6wk.

		Percent inhibition of emergence of adult house flies					
		Weeks					
Feed mg kg^{-1}	*Spray g L^{-1}*	1	2	3	4	5	6
0	0	0 (0)[a]	0 (0)[a]	0 (0)[a]	0 (0)[a]	0 (0)[a]	0 (0)[a]
	1	5.86 (0.22)[b]	21.93 (0.48)[b]	28.14 (0.56)[b]	30.57 (0.59)[b]	36.21 (0.65)[b]	40.54 (0.69)[b]
	2	5.86 (0.20)[b]	24.56 (0.52)[b]	28.57 (0.56)[b]	30.57 (0.59)[b]	38.37 (0.67)[bc]	41.44 (0.70)[bc]
250	0	4.95 (0.20)[b]	25.44 (0.53)[b]	29.87 (0.58)[bc]	32.32 (0.60)[b]	42.25 (0.71)[c]	48.20 (0.77)[cd]
	1	14.86 (0.39)[c]	26.32 (0.54)[b]	33.77 (0.62)[c]	34.5 (0.63)[b]	39.23 (0.68)[bc]	44.14 (0.73)[bcd]
	2	18.02 (0.44)[c]	36.40 (0.65)[c]	39.83 (0.68)[de]	44.98 (0.74)[c]	47.42 (0.76)[d]	55.85 (0.84)[de]
500	0	4.50 (0.17)[b]	28.67 (0.52)[b]	28.57 (0.56)[b]	31.88 (0.60)[b]	41.38 (0.70)[c]	49.10 (0.78)[d]
	1	19.37 (0.45)[c]	35.53 (0.64)[c]	38.96 (0.67)[d]	42.36 (0.71)[c]	47.85 (0.76)[d]	58.11 (0.87)[e]
	2	20.27 (0.46)[c]	39.91 (0.68)[c]	43.72 (0.72)[e]	46.73 (0.75)[c]	50.44 (0.79)[d]	74.32 (1.04)[f]
F-Ratio		0.55	40.59	86.75	102.03	141.23	70.5
P value		0.70	<0.001	<0.001	<0.001	<0.001	<0.001
LSD		0.12	0.06	0.05	0.05	0.05	0.07
CV%		35.4	10.3	7.4	6.9	6.7	8.5
Effect		NS	**	**	**	**	**

Values in parenthesis are square-root arcsine transformed
Means followed by the same letter within the same column are not significantly different at $P<0.05$
** Significant at $P<0.001$
NS – Not significant

The cost-benefit analyses of using a combination of three concentrations (0, 250 and 500mg kg^{-1}) of a bran formulation of *Bti* in chicken feed and three concentrations of a WDG formulation to control house fly larvae and adult house fly emergence for 6wk are given in Tables 6.4 and 6.5. *Bacillus thuringiensis* var. *israelensis* bran formulation retails at approximately ZAR200 kg^{-1}, while the WDG formulation retails at ZAR300 kg^{-1}. In our trial period (6wk), the cost was ZAR1.8 and ZAR3.6 for the 250mg kg^{-1} and 500 mg kg^{-1} doses respectively, while ZAR1.8 and ZAR3.6 were the costs for the 1g L^{-1} and 2g L^{-1} WDG applications (Table 6.4). Thus in the 6wks, ZAR0.04 and ZAR0.07 was spent to reduce larvae breeding by 1% using 250 and 500mg kg^{-1} alone respectively, compared to ZAR0.04 and ZAR0.08 for the WDG formulation (1g and 2g L^{-1}) to achieve the same unit of larvae breeding reduction. However, in terms of percent mortality of larvae, there was no significant difference between the 1g and 2g L^{-1}, even though it costs twice was much (ZAR0.008) to use the 2g L^{-1} to accomplish the same reduction as the 1g L^{-1} (Table 6.4).

Table 6.4 Comparison of cost-benefit analysis of larviciding on house fly larval mortality using a combination of three concentrations (0, 250 and 500mg kg^{-1}) of a bran formulation of *Bacillus thuringiensis* var. *israelensis (Bti)* and three concentrations (0, 1 and 2g L^{-1}) of a *Bti* water dispersible granule (WDG) formulation in layer feed for 6wk.

Feed concentration mg kg^{-1}	*Spray concentration g L^{-1}*	*Bti bran formulation consumed (6wk by 6 layers) (g)*	*Bti WDG formulation applied (6wk) (g)*	*Total cost (ZAR)*	*Percent mortality of larvae*	*Cost per dead larva(ZAR)*
0	0	0	0	0	0^{a}	0
	1	0	6	1.8	43.0^{b}	0.04
	2	0	12	3.6	43.0^{bc}	0.08
250	0	9	0	1.8	48.67^{de}	0.04
	1	9	6	3.6	46.33^{bcd}	0.08
	2	9	12	5.4	59.0^{f}	0.09
500	0	18	0	3.6	49.67^{de}	0.07
	1	18	6	5.4	57.33^{f}	0.09
	2	18	12	7.6	66.67^{h}	0.11

Percent mortality means followed by the same letter are not significantly different at $P<0.05$

Similarly, there was no significance difference in larval mortalities when either the 250mg kg^{-1} or the 500mg kg^{-1} *Bti* were used alone, although using 500mg kg^{-1} would cost twice (ZAR0.07) as much to accomplish what would achieved using a lower dose of 250mg kg^{-1} (ZAR0.04).

Likewise, the 500mg kg^{-1} bran formulation and 1g L^{-1} spray application combination costs the same (ZAR0.09) as the 250mg kg^{-1} feed treatment and 2g L^{-1} spray treatment combination (ZAR0.09), and there were significant differences between the two combinations with respect to larval mortality (Table 6.4).

A combination of 500mg kg^{-1} feed formulation and 2g L^{-1} spray treatment resulted in a 66.7% larval mortality at a cost of ZAR0.11. In the absence of a spray application, the 500mg kg^{-1} feed alone cost ZAR0.09 (49.7% control). With the 1g L^{-1} spray application, the cost was ZAR0.07 (57.3% control) (Table 6.4).

Table 6.5 Comparison of cost-benefit analysis of larviciding on reduction of adult house fly emergence using a combination of three concentrations (0, 250 and 500mg kg^{-1}) of a bran formulation of *Bacillus thuringiensis* var. *israelensis (Bti)* and three concentrations (0, 1 and 2g L^{-1}) of a *Bti* water dispersible granule (WDG) formulation in layer feed for 6wk.

Feed concentration mg kg^{-1}	*Spray concentration g L^{-1}*	*Bti bran formulation consumed (6wk by 6 layers) (g)*	*Bti WDG formulation applied (6wk) (g)*	*Total cost (ZAR)*	*Percent inhibition of adult flies*	*Cost per fly inhibited (ZAR)*
0	0	0	0	0	0^{a}	0
	1	0	6	1.8	40.54^{b}	0.04
	2	0	12	3.6	41.44^{bc}	0.09
250	0	9	0	1.8	48.20^{cd}	0.04
	1	9	6	3.6	44.14^{bcd}	0.08
	2	9	12	5.4	55.85^{de}	0.10
500	0	18	0	3.6	49.10^{d}	0.07
	1	18	6	5.4	58.11^{e}	0.09
	2	18	12	7.6	74.32^{f}	0.10

Trends similar to those observed with larval mortality were observed with the emergence of house fly adults. Moreover, there were no significant differences with respect to adult house fly emergence between the 1g L^{-1} and 2g L^{-1} spray applications in the absence of feeding *Bti* to the layers, although spraying 2g L^{-1} cost twice (ZAR0.09) as much as the 1g L^{-1} spray (ZAR0.04) application.(Table 6.5).

Likewise, the 500mg kg^{-1} feed treatment-1g L^{-1} spray application combination (ZAR0.09) and the 250mg kg^{-1}feed treatment-2g L^{-1} spray application combination (ZAR0.10) resulted in similar inhibition of adult fly emergence (Table 6.5).

The 500mg kg^{-1} feed treatment-2g L^{-1} spray application combination and the 250mg kg^{-1} feed treatment-2g L^{-1} spray application combination cost the same (ZAR0.10) in terms of inhibition of adult house fly emergence. However, the 500mg kg^{-1} feed treatment-2g L^{-1} resulted in a significantly higher inhibition of adult house fly emergence (74.32%) compared to the 250mg kg^{-1}feed treatment-2g L^{-1} spray application combination (55.85%) (Table 6.5)

6.4 Discussion

Our results show that compared to the effects caused by the formulations alone, combinations of feed and spray treatments did not always have an additive effect. Even so, elevated levels of control of house fly larvae and adults could be obtained by combining feed and spray treatments. For instance, no significant differences were observed in larval mortalities when layers were fed 250mg kg^{-1} feed formulation in the absence of *Bti* spray applications, and when the feed application was combined with a spray treatment of 1g L^{-1}. Similar observations were made with the emergence of adult house flies.

Although not statistically significantly different with respect to percentage reduction of adult flies, a feed concentration of 250mg kg^{-1} resulted in a 4% higher percentage inhibition of adult flies than when combined with a spray treatment of 1g L^{-1}. It can be inferred from the study results that using either the 250mg kg^{-1} feed application is sufficient to control house fly larval breeding and is in fact more effective than combining the feed treatment with 1g L^{-1} spray application.

Our results also suggested that when spraying *Bti* application alone, a *Bti* concentration of 1mg L^{-1} achieved 43% mortality for house fly larvae, which was similar to that achieved by 2g L^{-1}. Similar results were obtained during our earlier studies using broilers instead of layers, where the percent mortality of house flies caused by the 1g and 2g L^{-1} spray treatments were 47 and 52.8% respectively. In the present study, there was evidence that a concentration of 1g L^{-1} *Bti* spray treatment applied weekly resulted in an almost immediate drop in larval density, a trend similar to that shown by the spray of 2g L^{-1}, which was maintained throughout the study period.

There was little difference in the impact of the 1g L^{-1} or the 2g L^{-1} spray applications. A comparison of the cost-benefit analysis of using 2g L^{-1} spray application over 1g L^{-1} indicates

that although they achieve the same larval control, the 2g L^{-1} spray would cost twice as much (expressed in terms of each percentage reduction of larvae breeding) (ZAR0.08) as the lower dose (1g L^{-1}). Our findings confirm the observation that WDG *Bti* has a short persistence (Karch *et al*., 1991; Gelernter and Schwab, 1993; Kroeger *et al*., 1995; Fillinger *et al*., 2003; Russell *et al*., 2003) and we can therefore conclude that a dose of 2g L^{-1} as a topical spray application does not have any advantage over 1g L^{-1}. The bran formulation, on the other hand is expected to provide a greater residual larvicidal activity because of the longer persistence of the spores in the environment and their recycling potential in the gut of the larvae after dying (Becker *et al*., 1995).

Although not statistically significantly different, the addition of 250mg kg^{-1} to the 1g L^{-1} spray reduced larval breeding by approximately a further 3% (46.3%), but would cost approximately x2 more (ZAR0.08). However, using the 250mg kg^{-1} and 2g L^{-1} combination achieved higher mortalities (59%) at a cost ZAR0.09. A combination of 500mg kg^{-1} combined with a 1g L^{-1} spray treatment cost the same (ZAR0.09) but did not offer better control (57.3%).

The 500mg kg^{-1} feed and a 2g L^{-1} spray application provided the best control of larvae (66.7%). The cost–benefit analysis (expressed in terms cost per dead larva) showed that a combination of these two treatments cost ZAR0.11, which was the most expensive treatment, and was the least cost effective.

In the present study it had been hoped that simultaneous use of *Bti* formulations (bran and WDG formulations) could increase the efficiency of the bacterial pathogenic agents and, therefore allow a lower dose of their combination to be used for the control house fly larvae. Although the single use of either treatment was not always significantly different to the combined use of both *Bti* formulations, the highest combined applications of the two formulations resulted in higher levels of control of larval numbers and inhibition of adult house fly emergence. The combined applications of either 250mg kg^{-1} or 500mg kg^{-1} combined with a spray application of 2gL^{-1} were not always more cost-effective than the combinations of the two formulations applied at lower concentrations.

In terms of larval mortalities, the lowest level of control (43%) was as a result of using either the 1g L^{-1} (ZAR0.04) or the 2gL^{-1} spray application at twice the cost (ZAR0.08). Likewise,

there was no significant difference in terms of larval mortalities when using either the 250mg kg^{-1} feed or 500mg kg^{-1} feed applications. However, the higher dosage is almost twice as expensive (ZAR0.07) as the lower dosage (0.04). In these two cases, the user would opt for the same control at a lower cost. Similar trends were observed with adult house fly emergence. The highest control in terms of larval mortality (67%) was as a result of the 500mg kg^{-1} feed and $2gL^{-1}$ spray application. This was the most expensive treatment combination. However, in terms of fly inhibition, 500mg kg^{-1} feed and $2gL^{-1}$ spray application combination resulted in the highest control (74%), but at the same cost as the 250mg kg^{-1} feed and $2gL^{-1}$ spray application that resulted in a lower control (56%) of adult house fly emergence.

This study therefore presents commercial users with various possible combinations of application of the two *Bti* formulations. For instance, if there are a few flies about, no major disease outbreak and the chickens flock is stable, then the 250mg kg^{-1} feed treatment would be suitable and moderate control would be achieved at a low cost. In the mid summer, when flies are breeding fast and there are threats of chicken diseases, then the chicken farmer could go for a higher level of control provided by the 500mg kg^{-1} feed combined the $1gL^{-1}$ spray application or even the $2gL^{-1}$ spray application.

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CHAPTER 7

INTERACTION BETWEEN *BEAUVERIA BASSIANA* AND *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* FOR THE CONTROL OF HOUSE FLY LARVAE AND ADULTS IN POULTRY HOUSES

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Abstract

Field trials were carried out in the poultry houses to determine if a spray formulation of *Beauveria bassiana* (Balsamo) Vuillemin Isolate R444 would enhance the control potential of *Bacillus thuringiensis* var. *israelensis* (*Bti*) applied in chicken feed as a larvicide against house fly larvae and the emergence of adult house flies. The trials compared larval mortalities and adult house fly emergence that resulted from the *Bti* plus *B. bassiana* treatments with those resulting from a commercial larvicide, Larvadex® plus *B. bassiana* treatments. All treatments significantly reduced the house fly larval densities and adult house fly emergence compared to any of the agents acting alone. After 6wk of application, house fly larvae decreased by 11% as a result of *B. bassiana* treatment alone, 41% for 250mg kg^{-1} *Bti* alone and 42% for 500mg kg^{-1} *Bti* alone. Larval mortalities as a result of the combination treatments were 45% and 52% as a result of 250mg kg^{-1} *Bti* plus *B. bassiana* and 500mg kg^{-1} *Bti* plus *B. bassiana*, treatments respectively. House fly larval mortalities as a result of Larvadex® and *B. bassiana* were 30% for Larvadex® alone and 38% as a result of Larvadex® plus *B. bassiana*. The *Bti* treatments were more effective at inhibiting the emergence of adult house flies than Larvadex®, even when Larvadex® was applied together with *B. bassiana*. The interaction effects of *Bti* plus *B. bassiana* and Larvadex® plus *B. bassiana* were additive. These trials suggested that in the control of house fly larvae, the efficacy of *Bti*, applied as a larvicide may be improved with frequent spray applications of *B. bassiana* to the chicken manure.

7.1 Introduction

The house fly, *Musca domestica* L., is a key pest of poultry facilities and a vector of many metaxenic pathogens and can cause serious sanitary problems because of its high reproductive potential, feeding habits and ability to disperse. Organic wastes from intensive poultry farms provide excellent habitats for the growth and development of this insect (Thomas and Skoda 1993). Control recommendations for the house fly are currently limited to use of chemical insecticides to kill the house flies adults and larvae. Due to the problems associated with the development of pesticide resistance by the house fly (Scott *et al.*, 2000; Kaufman *et al.*, 2001b), as well as other environmental and regulatory concerns, research toward developing alternative control strategies is warranted. *Beauveria bassiana* (Balsamo) Vuillemin and *Bacillus thuringiensis* Berliner (*Bt*), which occur naturally as pathogens of *M. domestica*, are some of the potential alternatives.

Bacillus thuringiensis has been found to be toxic to the house fly (Hodgman *et al.,* 1993). Several natural isolates of *Bt* have also been found which are active against larvae of the house fly (Johnson *et al.*, 1998). Thuringiensin-containing preparations have been used to control larvae of *M. domestica* (Mullens and Rodriguez, 1988; Mullens *et al.*, 1988a). It has also been reported by Carlberg *et al.* (1991) that nuisance flies in cattle sheds, slaughter houses and latrines could be successfully controlled by applying *Bt* var *thuringiensis* to larval breeding sites.

The entomopathogenic fungus, *B. bassiana*, is a ubiquitous and important entomopathogen of several insect pests (Feng *et al*., 1994; Inglis *et al*., 2001; Lacey *et al*., 2001) and can be used effectively to suppress house fly populations. One approach to controlling house flies with *B. bassiana* would be to target the adult house flies as they fly around the poultry houses or rest on the walls. Steinkraus *et al.* (1990) reported that *B. bassiana* infected 1% of house fly adults under natural conditions in poultry houses. Despite the low prevalence of disease, strains collected by Steinkraus were virulent in subsequent laboratory studies (Watson *et al.,* 1995). One strain (P89), when formulated in water and a surfactant, induced 99% mortality in house flies (dose $1x10^8$ conidia/cm^2) within 6d of exposure.

Prior research, however, does not indicate great potential for *B. bassiana* to control the larval stage of house flies (Geden *et al*., 1995; Watson *et al*., 1995; Lecuona *et al*., 2005).

Furthermore, Geden *et al*. (1995), Watson *et al*. (1995), and Lecuona *et al.* (2005) found the virulence of *B. bassiana* to be relatively poor against *M. domestica.* Combining *B. bassiana* with other entomopathogens might result in synergistic interactions that would enhance the potential for biological control of *M. domestica* larvae.

Despite the importance of mixed infections there has been little investigation on interactions between diverse strains of *Bt* and entomopathogenic fungi (Navon, 2000; Wraight and Ramos, 2005). However, the use of mixtures of different control agents has a long history in insect control. Mixtures can provide improved pest control (Tompkins *et al*., 1986; Koppenhöfer and Kaya, 1997; Shapiro, 2000; Mendez *et al*., 2002; Wraight and Ramos, 2005; Oestergaard *et al*., 2006; Raymond *et al*., 2007) or may be used as a pesticide resistance management strategy (Tabashnik, 1989; Curtis *et al*., 1993; Roush, 1993; Corbel *et al*., 2002; Raymond *et al*., 2007b). Mixed infections lead to diverse interactions among natural enemies.

In studies targeting other insect pests, synergistic interactions have been observed from certain combinations of *B. bassiana* and *Bt* (Wraight and Ramos, 2005), independent (Lewis and Bing, 1991) or antagonistic (Ma *et al*., 2008).

Cyromazine (N-cyclopropyl-1, 3, 5-triazine-2, 4, 6- triamine) is an insect growth regulator commonly used to control immature house flies on poultry farms (Hogsette, 1979; Miller and Corley, 1980; Miller *et al*., 1981, Awad and Mulla 1984). Cyromazine is formulated as 1% pre-mix (Larvadex®), which is added to poultry food. Moderate to high levels resistance to cyromazine have been found to develop if the selection pressure is strong enough, as in the case of feed-through treatments (Keiding, 1999). Furthermore several authors (Bloomcamp *et al*., 1987; Sheppard *et al.,* 1992) reported resistance in flies from poultry farms where Larvadex® was used as a feed-through treatment. Combining Larvadex® with an entomopathogen such as *B. bassiana* may have the advantage of delaying or avoiding resistance, which extendthe life of this larvicide as a useful tool for house fly control.

A first objective of this study was to determine whether applications of *B. bassiana* combined with *Bti* enhanced the efficacy of *Bti* against *M. domestica*. A second objective was to evaluate the impact of *B. bassiana* combined with Larvadex®.

7.2 Materials and Methods

7.2.1 Biopesticides

The *Bti* bran and *B. bassiana* R444 formulations were obtained from Plant Health Products (PHP)[1] (Pty) Ltd. The Larvadex®- treated commercial feed was obtained from the University Research Farm at Ukulinga.

7.2.2 Layers and housing

For field evaluation experiments, sixty x 51wk old commercial-type layers (Hyaline Brown) were obtained from the University Research Farm, Ukulinga. Layers were evaluated upon receipt for signs of disease or other complications that could affect the outcome of the study. Following examination, the layers were randomly allocated into 80 hanging pens (440 x 420 x 610mm) in six chambers. Four chambers had 10 birds each while two chambers had 20 birds each. Pens were separated by wire-mesh on all sides. Each pen was equipped with a removable feed trough and a nipple drinker. Each pen had a removable tray for individual manure collection. Layers were housed in environmentally friendly chambers fitted with fans and 16:8h (light: dark) at the Poultry section, University Research Farm at Ukulinga.

7.2.3 Diet preparation for layers and administration

The *Bti*-formulated bran was mixed into the layer mash in varying quantities of 250 and 500mg kg^{-1} of layer mash. Larvadex® - treated feed contained 0.5% of Larvadex® kg^{-1} of layer mash. The control did not contain any Larvadex® or *Bti*-formulated bran. The rations were stored in trashcans lined with plastic bags until fed to the chickens. There were 10 birds per treatment. Feed and drinking water were provided *ad libitum*. Layers were fed their respective dietary treatments for 6wk.

Additionally, three chambers were treated with a wettable-powder formulation of *B. bassiana* R444 Isolate (1g powder (10^{10}) in 1L of water) at a rate of 250ml per chamber ($27m^3$) once a week. Applications were made using a Dyna-Fog® Cyclone ™ Ultra-Flex ULV sprayer. The treatments that were used in the field experiments are summarized in Table 7.1.

[1] Plant Health Products (Pty) Ltd, P.O. Box 207, Nottingham Road, South Africa

Post-treatment larval densities were monitored after every 7d. Data were analyzed to measure reduction in the house fly larvae in comparison with the untreated controls.

Table 7.1 Summary of combinations of *Bacillus thuringiensis* var. *israelensis*, *Beauveria bassiana* and Larvadex® for control house flies in poultry houses.

Chamber	*Components of treatment*
1	None
2	*B. bassiana*
3	Larvadex®
4	Larvadex® and *B. bassiana*
5	*Bti* (250 and 500mg kg^{-1})
6	*Bti* (250 and 500mg kg^{-1}) and *B. bassiana*

7.2.4 Fly larvae sampling

Excreta from each bird was collected every seven days. Sampling of house fly larvae began one week after the administering the different feed in order to give the layers time to adjust to the different treatments. House fly larvae were monitored using a bulb planter (400ml) to collect two manure cores from each tray/bird/diet. Larvae was extracted in Berlese funnels and enumerated.

Larvae were maintained at 25°C in the treated manure until adult house fly emergence and numbers were recorded. Adult house fly emergence rate was then calculated taking into account the number of exposed larvae.

7.2.5 Statistical analysis

Percentage mortality of larvae in the treated trays was calculated and corrected to the reduction, if any, in the control plots using the formula of (Mulla *et al*., 1971) as given below:

$$\% \text{ Mortality} = \frac{[C1 - C2]}{C1} \times 100$$

where; $C1$, abundance of larvae in untreated manure;

$C2$, abundance of larvae in treated manure.

Adult house fly emergence inhibition was calculated using the following formula, on the basis of determining adult house fly emergence from the number of larvae that were obtained:

$$\% \text{ Inhibition of adult house fly emergence} = \frac{[C - T]}{C} \times 100$$

where; C = percentage of adult house flies emerging from the control manure and

T = percentage of adult house flies emerging treated manure.

The percentage values were normalized by square-root arcsine transformation. Repeated measures ANOVAs were conducted using GENSTAT statistical package. Analyses included nominal variables representing application or no application of *Bti* and *B. bassiana* or Larvadex® and *B. bassiana* applications. The LSD method was used to find significant differences between the means of the treatments.

In order to determine the effects of *Bti* - *B. bassiana* and Larvadex®- *B. bassiana* treatments, the observed mortality rates were compared to the expected mortality rates under the assumptions of an independent effect. The expected mortality (BB_E) for the *Bti* -*B. bassiana* treatment was calculated as follows:

$$BB_E = B_T + B_B (1 - B_T)$$

Where B_T and B_B are the observed proportional mortalities caused by *Bti* and *B. bassiana* alone, respectively.

Similarly, the expected mortality BL_E for the Larvadex® plus *B. bassiana* combinations was calculated by:

$$BL_E = B_L + B_B (1 - B_L)$$

Where B_L and B_B are the observed proportional mortalities caused by Larvadex® and *B. bassiana* alone, respectively.

Results from a χ^2 test, were compared to the χ^2 table values ($df = 1$, $P \leq 0.05$).

$$\chi^2 = (O - E)^2 / E$$

where O is the observed mortality for either of the *Bti* doses plus *B. bassiana* or Larvadex® plus *B. bassiana* treatments and E is the expected mortality for either of the *Bti* doses plus *B. bassiana* or Larvadex® plus *Beauveria* treatments. Additivity would be indicated if $\chi^2 < 3.84$. If $\chi^2 > 3.84$, there would be reason to suspect that the interaction was non-additive, i.e. synergistic or antagonistic between the two agents (Finney, 1964). If $O < E$, the interaction would be considered antagonistic. Synergism would be indicated if $O > E$.

7.3 Results

Larval mortalities were significantly affected by the applications of *Bti* and *B. bassiana* Treatments (F= 48.99; P<0.001), Time (F= 45.37; P<0.001) and the Treatment x Time (F= 13.71; P<0.001). The three factors, Treatment (F = 84.88; P<0.001), Time (F = 278.38; P < 0.001) and Time x Treatment (F = 21.91; P <0.001), were also significant when Larvadex® plus *B. bassiana* were applied.

Table 7.2 Effects of combining two concentrations (250mg kg^{-1} and 500mg kg^{-1}) of *Bacillus thuringiensis* var. *israelensis* formulated feeds and spray applications of *Beauveria bassiana* on *Musca domestica* larval mortality

	Percent mortality of larvae					
	Weeks					
Treatment	1	2	3	4	5	6
Control +Bb	10.17 (0.31)^{a}	16.08 (0.40)^{a}	11.06 (0.32)^{a}	12.85 (0.35)^{a}	11.33 (0.33)^{a}	10.99 (0.32)^{a}
Bt1	10.48 (0.32)^{a}	12.99 (0.36)^{a}	15.25 (0.39)^{b}	25.16 (0.52)^{b}	33.78 (0.62)^{b}	41.21 (0.70)^{b}
Bt1 + Bb	12.74 (0.34)^{a}	21.87 (0.47)^{b}	24.17 (0.51)^{c}	33.40 (0.62)^{c}	41.22 (0.70)^{c}	44.64 (0.73)^{b}
Bt2	12.05 (0.35)^{a}	14.38 (0.38)^{a}	15.09 (0.40)^{b}	26.45 (0.54)^{b}	37.67 (0.66)^{bc}	42.09 (0.71)^{b}
Bt2 + Bb	11.84 (0.34)^{a}	22.47 (0.49)^{b}	26.96 (0.54)^{c}	37.47 (0.66)^{c}	46.56 (0.75)^{d}	51.54 (0.80)^{c}
F – ratio	0.83	7.67	17.34	46.20	115.2	162.53
P value	0.51	<0.001	<0.001	<0.001	<0.001	<0.001
% CV	25.5	22.5	23	14.2	11.3	10.2
LSD	0.05	0.06	0.06	0.05	0.04	0.04
Effect	NS	**	**	**	**	**

Values in parenthesis are square-root arcsine transformed
Means followed by the same letter within the same column are not significantly different at P<0.05
* Significant at P<0.05
** Significant at P<0.001
Bb = *B. bassiana*; Bt1 = 250mg kg^{-1} *Bti*; Bt2 = 500mg kg-1 *Bti*

The effects of applying *Bti* alone and when applied together with *B. bassiana* spray applications are shown in Table 7.2. Combinations of the two treatments resulted in higher larval control than either of the two treatments applied individually. However, there was no significant difference between the 250mg kg^{-1} and 500mg kg^{-1} feed when these were used alone. These resulted in 41% and 42% larval mortality, respectively. Similarly, no significant differences were observed between the 250mg kg^{-1} plus *B. bassiana* and 500mg kg^{-1} plus *B. bassiana* treatments, except in the last two weeks when the 500mg kg^{-1} plus *B. bassiana* treatment combination resulted in a significantly higher larval mortality than the 250mg kg^{-1} plus *B. bassiana* treatment (Table 7.2). Larval mortalities as a result of the combined applications of 250mg kg^{-1} plus *B. bassiana* and 500mg kg^{-1} plus *B. bassiana* treatments were 45% and 52%, respectively (Table 7.2). Use of *B. bassiana* alone resulted in low levels (11%) of larval control after 6wk.

Table 7.3 Effect of combining Larvadex® formulated feeds with spray applications of *Beauveria bassiana* on *Musca domestica* larval mortality

	Percent mortality of larvae					
	Weeks					
Treatment	1	2	3	4	5	6
Control +Bb	10.17 (0.31)[a]	16.08 (0.40)[ab]	11.06 (0.32)[a]	12.85 (0.35)[a]	11.33 (0.33)[a]	10.99 (0.32)[a]
Lv	9.96 (0.31)[a]	12.14 (0.34)[a]	13.67 (0.36)[a]	20.66 (0.47)[b]	24.11 (0.51)[b]	30.33 (0.58)[b]
Lv + Bb	10.48 (0.32)[a]	20.34 (0.46)[b]	18.69 (0.44)[b]	28.37 (0.56)[c]	31.44 (0.59)[c]	37.91 (0.66)[c]
F – ratio	0.11	6.43	4.92	26.59	68.32	103.56
P value	0.90	0.003	0.01	<0.001	<0.001	<0.001
% CV	31.2	26.8	33.2	19.4	15.5	15.0
LSD	0.06	0.07	0.08	0.06	0.05	0.05
Effect	NS	*	*	**	**	**

Values in parenthesis are square-root arcsine transformed
Means followed by the same letter within the same column are not significantly different at $P<0.05$
* Significant at $P<0.05$
** Significant at $P<0.001$
NS – Not significant
Bb = *B. bassiana;* Lv = Larvadex®

Application of Larvadex® together with *B. bassiana* spray applications resulted in significantly higher larval mortalities than either of the two treatments individually (Table 7.3). After 6wk of application, Larvadex® plus *Beauveria* treatments resulted in >15% more larval mortality than *Beauveria* alone and 7% more than Larvadex® alone (Table 7.3).

Regression lines of mortalities of larvae obtained as a result of the *Bti*, *B. bassiana* and Larvadex® treatments applied individually or in combination are presented in Fig 7.1a. Larval mortalities as a result of the *Bti* treatments plus *B. bassiana* achieved better control than mortalities obtained as a result of Larvadex® alone or when integrated with *B. bassiana* (Fig. 7.1). Larval mortalities obtained as a result of using *B. bassiana* alone were the lowest. The 250mg kg^{-1} and 500mg kg^{-1} *Bti* feed treatments were more effective (in terms of larval mortalities) than the Larvadex® treatment integrated with *B. bassiana* spray applications (Fig 7.1).

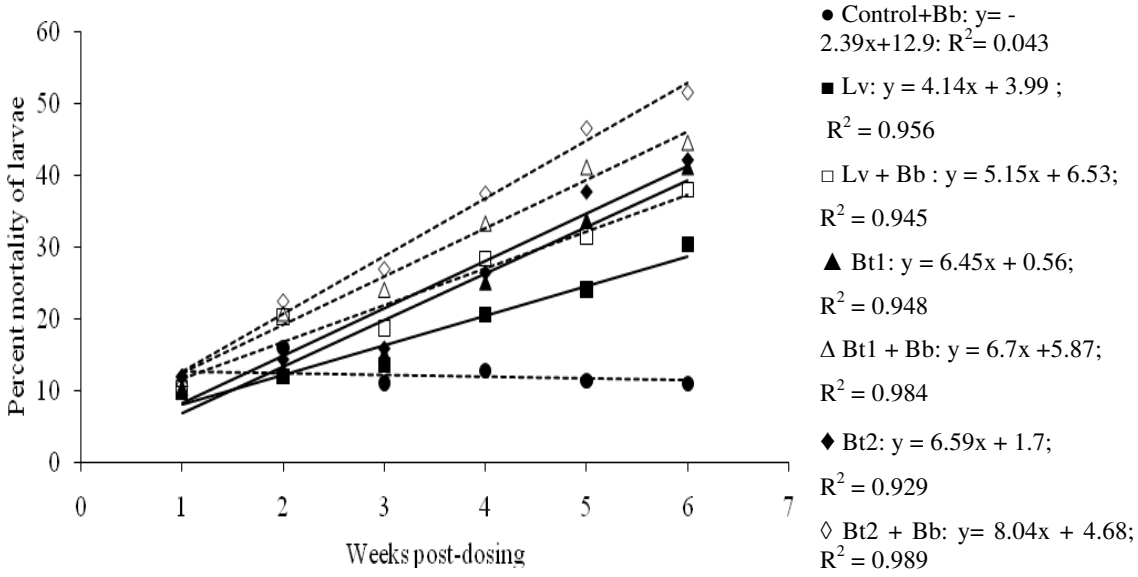

Fig. 7.1 Percent mortality of larvae in manure sprayed with or without *B. bassiana* (Bb) and layers fed with Larvadex (Lv), 250mg kg^{-1} *Bti* (Bt1) or 500mg kg^{-1} *Bti* (Bt2).

Table 7.4 Effect of combining *Bacillus thuringiensis* var. *israelensis* and Larvadex® formulated feeds integrated with spray applications of *Beauveria bassiana* on the mortality of *Musca domestica* larvae

Treatments	Week	Observed %	Expected%	χ^2	Interaction
Bt1 and B. bassiana	1	12.74	19.86	0.025	Additive
	2	21.87	26.98	0.009	Additive
	3	24.17	24.62	0.008	Additive
	4	33.40	34.78	0.001	Additive
	5	41.22	41.28	0.001	Additive
	6	44.64	47.67	0.193	Additive
Bt2 and B. bassiana	1	11.84	20.99	0.04	Additive
	2	22.47	28.15	0.01	Additive
	3	26.96	24.48	0.04	Additive
	4	37.47	35.90	0.02	Additive
	5	46.56	44.73	0.001	Additive
	6	51.54	48.45	0.197	Additive
Larvadex® and B. bassiana	1	10.48	19.11	0.04	Additive
	2	20.34	26.27	0.01	Additive
	3	18.69	23.22	0.007	Additive
	4	28.37	30.86	0.003	Additive
	5	31.44	32.71	0.003	Additive
	6	37.91	37.99	1.68	Additive

Although addition of *B. bassiana* to both *Bti* and Larvadex® resulted in significantly higher larval control than any of the agents acting alone, the interaction between *Bti* and *B. bassiana* and between Larvadex® and *B. bassiana* was additive and were based on the three agents acting independently for the entire 6wk trial (Table 7.4).

The emergence of house flies was significantly affected by *Bti* and *B. bassiana* Treatments ($F = 194.39$; $P < 0.001$), Time ($F = 193.87$, $P< 0.001$) and Treatment x Time ($F = 5.34$; $P< 0.001$). The three factors Treatment (F= 102.13; $P< 0.001$), Time (F= 64.93; $P< 0.001$) and Treatment x Time interaction (F= 4.40; $P< 0.001$) had significant effects on the emergence of adult house flies when Larvadex® and *B. bassiana* were applied together or singly.

The effects of applying *Bti* alone and when applied together with *B. bassiana* are shown in Table 7.5. The *Bti* treatments whether applied individually or together with *B. bassiana* significantly inhibited the emergence of adult house flies (Table 7.5). Application of *B. bassiana* alone inhibited the emergence of adult house flies by 24% after 6wk of application.

Table 7.5 Effect of combining two concentrations (250mg kg^{-1} and 500mg kg^{-1}) of *Bacillus thuringiensis* var. *israelensis* formulated feeds with applications of *Beauveria bassiana* on the emergence of house fly adults

	Percent inhibition of adult house fly emergence					
	Weeks					
Treatment	**1**	**2**	**3**	**4**	**5**	**6**
Control +Bb	11.22 (0.33) [a]	13.74 (0.39) [a]	18.37 (0.43) [a]	20.45 (0.46) [a]	18.22 (0.43) [a]	24.0 (0.51) [a]
Bt1	21.70 (0.48) [b]	27.37 (0.55) [b]	28.14 (0.56) [b]	28.48 (0.56) [b]	35.44 (0.64) [b]	46.33 (0.75) [b]
Bt1 + Bb	22.75 (0.50) [bc]	28.54 (0.56) [b]	32.55 (0.61) [bc]	37.04 (0.65) [c]	46.89 (0.75) [c]	52.11 (0.81) [c]
Bt2	26.52 (0.54) [c]	27.37 (0.55) [b]	29.11 (0.57) [b]	29.66 (0.58) [b]	39.22 (0.68) [b]	54.89 (0.84) [cd]
Bt2 + Bb	28.72 (0.56) [c]	31.10 (0.59) [b]	33.94 (0.62) [c]	39.40 (0.68) [c]	48.56 (0.77) [c]	59.33 (0.88) [d]
F - ratio	32.60	31.28	19.49	31.63	68.81	83.33
P - value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
% CV	14.9	13.9	14.1	11.5	11.3	9.5
LSD	0.05	0.05	0.05	0.04	0.05	0.05
Effect	**	**	**	**	**	**

Values in parenthesis are square-root arcsine transformed
Means followed by the same letter within the same column are not significantly different at $P<0.05$
** Significant at $P<0.001$
Bb = *B. bassiana*; Bt1 = 250mg kg^{-1} *Bti*; Bt2 = 500mg kg-1 *Bti*

There were no significant differences as a result of the 250mg kg^{-1} and 500mg kg^{-1} feed treatments on the emergence of adult house flies with or without *B. bassiana* spray application (Table 7.5).

Combining 250mg *Bti plus B. bassiana* inhibited the emergence of adult house flies by approximately 30% more than *B. bassiana* alone and 6% more than *Bti* alone. The 500mg kg *Bti* feed application reduced the emergence of adult house flies by 55%. This was 4% less than when the 500mg kg^{-1} feed treatment was combined with *B. bassiana* spray application (Table 7.5)

No significant differences were observed between the 250mg kg^{-1} *Bti* feed application and 250mg kg^{-1} plus *B. bassiana* treatment with respect to reducing adult house fly emergence for the first 3wk of application. Similar observations were made between the 500mg kg^{-1} *Bti* feed application and 500mg kg^{-1} *Bti* plus *B. bassiana* spray application in the first 2wk of application (Table 7.5). However, significant differences were observed thereafter between the treatments.

Use Larvadex® alone produced no significant reduction in adult house fly emergence during the first 3wk of the study. However, Larvadex® applied together with *B. bassiana* reduced adult house fly emergence by 48% after 6wk (Table 7.6), 10% more than Larvadex® alone.

Table 7.6 Effect of combining Larvadex® formulated feeds with applications of *Beauveria bassiana* on the emergence of adult house flies

	Percent inhibition of adult house fly emergence					
	Weeks					
Treatment	**1**	**2**	**3**	**4**	**5**	**6**
Control +Bb	11.22 (0.33)[a]	13.74 (0.39)[a]	18.37 (0.43)[a]	20.45 (0.46)[a]	18.22 (0.43)[a]	24.0 (0.51)[a]
Lv	17.3 (0.42)[b]	17.15 (0.41)[a]	22.45 (0.49)[a]	26.98 (0.54)[b]	31.44 (0.59)[b]	37.11 (0.65)[b]
Lv + Bb	15.83 (0.40)[ab]	26.94 (0.54)[b]	28.57 (0.56)[b]	35.12 (0.63)[c]	42.0 (0.70)[c]	47.89 (0.76)[c]
F – ratio	3.79	10.02	10.11	22.06	52.39	84.55
P value	0.029	<0.001	<0.001	<0.001	<0.001	<0.001
% CV	27.9	24.2	19.6	13.7	14.8	9.7
LSD	0.07	0.07	0.06	0.05	0.05	0.04
Effect	NS	**	**	**	**	**

Values in parenthesis are square-root arcsine transformed
Means followed by the same letter within the same column are not significantly different at $P<0.05$
** Significant at $P<0.001$
NS – Not significant
Bb = *B. bassiana;* Lv = Larvadex®

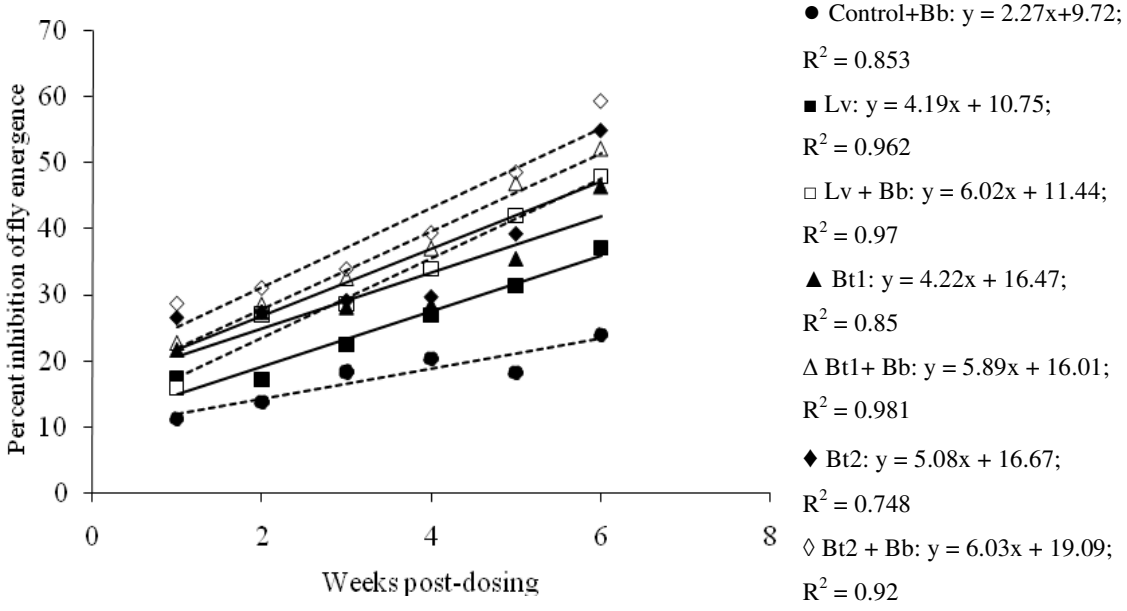

Fig. 7.1 Percent inhibition of adult house fly emergence in manure sprayed with or without *Beauveria* and layers fed with either Larvadex® (Lv), 250mg kg^{-1} *Bti* (Bt1) or 500mg kg^{-1} *Bti* (Bt2).

The *B. bassiana* formulation applied alone had the lowest impact on the emergence of adult house flies (Fig. 7.1). The *Bti* treatments were more effective than Larvadex® even when Larvadex® was applied together with spray applications of *B. bassiana*. The most effective combinations were *Bti* combined with *B. bassiana* spray applications (Fig. 7.1).

The interactions between *Bti* and *B. bassiana* and between Larvadex® and *B. bassiana* were additive effects during the entire study period (Table 7.7).

Table 7.7 Effect of combining *Bacillus thuringiensis* var. *israelensis* and Larvadex® formulated feeds with spray applications of *Beauveria bassiana* on the emergence of *Musca domestica* adult flies

Treatments	Week	Observed %	Expected%	χ^2	Interaction
Bt1 and B. bassiana	*1*	22.75	31.42	2.39	Additive
	2	28.54	36.87	1.88	Additive
	3	32.55	40.22	1.46	Additive
	4	37.04	49.92	3.32	Additive
	5	46.89	56.57	1.66	Additive
	6	52.11	63.60	2.08	Additive
Bt2 and B. bassiana	*1*	28.72	36.72	1.74	Additive
	2	31.10	38.94	1.58	Additive
	3	33.94	41.45	1.36	Additive
	4	39.40	51.79	2.96	Additive
	5	48.56	57.93	1.52	Additive
	6	59.33	69.09	1.38	Additive
Larvadex® and B. bassiana	*1*	15.83	25.27	3.53	Additive
	2	26.94	35.57	2.09	Additive
	3	28.57	36.69	1.80	Additive
	4	35.12	41.91	1.10	Additive
	5	42.0	52.57	2.13	Additive
	6	47.89	60.40	2.59	Additive

7.4 Discussion

Addition of *B. bassiana* spray applications to *Bti* or Larvadex® feed treatments offered some advantage to both agents in terms of larval mortality and inhibition of adult house fly emergence. Moreover, throughout the study, integrations of *B. bassiana* spray applications with either *Bti* or Larvadex® feed treatments resulted in better larval control than either *Bti* or Larvadex® acting individually. Furthermore, there was no evidence of antagonistic interaction between *B. bassiana* with *Bti* or Larvadex®. On the hand, *B. bassiana* on its own was not very effective in controlling larval populations or inhibiting adult house fly emergence. Our results confirm observations that weekly applications of *B. bassiana* are inadequate to achieve

effective insect larval control in the field (Galaini, 1984; Wraight and Ramos, 2002; 2005). Moreover, the fact that time of exposure was a significant factor suggests that repeated applications would be more effective in controlling larval densities.

A few studies (Johnson *et al*., 1992; Lobo-Lima *et al*., 1992; Johnson and Goettel, 1993; Inglis *et al*., 1996a) have also reported inconsistent results of *B. bassiana* with some insect hosts in the field, despite good control being obtained in the laboratory. In our earlier studies, *B. bassiana* Isolate R444 sprays resulted in >90% fly mortality within 2d in the laboratory.

The potential of *B. bassiana* in the field may be limited by high temperatures (Roberts and Campbell, 1977; Carruthers *et al*., 1985; Benz, 1987; Ferron *et al*., 1991; Fargues *et al*., 1992; Vestergaard *et al*., 1995; Ekesi *et al*., 1999). Also sunlight is known to rapidly inactivate *B. bassiana* conidia (Inglis *et al.,* 1997a), and house flies are known to thermoregulate, raising their body temperatures above ambient level (Watson *et al*., 1993), which may enable them to rid themselves of infection by basking in sunlight (Inglis *et al.,* 1997a).

The efficacy of *B. bassiana* could be enhanced by making applications in the evening (Delgado *et al*., 1999) because *B. bassiana* has been reported to achieve better control in cool temperatures (Inglis *et al.,* 1997b), owing to the prolonged life of exposed conidia, conceivably leading to germination and cuticle penetration by a greater number of conidia per insect and a higher effective inoculum (Delgado et al., 1999). Late evening applications of *B. bassiana* would allow more time for house flies to become exposed to fungal conidia through feeding on and/or contact with treated shed walls.

There have been numerous studies on the effect of combinations of microbial agents for insect control. Although the mechanism is not clearly understood, infections by more than one agent usually lead to an increase in mortality of the host population, especially when the two infections are spatially separated (Jacques and Morris, 1981). Our findings of an additive interaction between *B. bassiana* and *Bti* confirm the results of Sandner and Cichy (1967), Lewis and Bing (1991), Costa *et al*. (2001) and Ma *et al*. (2008). This interaction may be the result of the different routes of infection by the two pathogens (Ma *et al*., 2008). In our study, *B. bassiana* was sprayed in the chicken houses to reduce the number of breeding adult flies, while *Bti* was ingested by the larvae through the chicken manure.

A few authors have reported synergistic interactions between *Bt* and *B. bassiana.* For instance, Lewis *et al*. (1996) reported that application of *Bt* to maize enhanced the suppression of European corn borer by *B. bassiana* and Wright and Ramos (2005) reported synergistic interactions between *B. bassiana* strain GHA and *Bt tenebrionis* when applied in combination against larval populations of the Colorado potato beetle. The results of Lewis *et al*. (1996) and Wright and Ramos (2005) may have been as a result of *Bt* prolonging of the interval of time between molts, therefore providing *B. bassiana* more time to penetrate the cuticle before being shed off (Wright and Ramos, 2005). Also larvae feeding on *B. bassiana* may acquire infection via the gut (Ma *et al*., 2008). Miranpuri and Khachatourians (1991) found germinated *B. bassiana* conidia in the gut of larvae of the mosquito *Aedes aegyptii* 24-48h after exposure.

On the other hand, a study by Furlong and Groden, (2003) suggested that starvation might increase the susceptibility of larvae to *B. bassiana* infection, by prolonging the inter-molt period. This has been the suspected mechanism that would explain the increase in impact of *Bt* plus *B. bassiana* mixed treatments. However, it was also found that the insect growth regulator Larvadex® increased inter-molt period without significantly affecting susceptibility to the fungus (Furlong and Groden, 2001) and it was concluded that some unknown effect of starvation on host physiology was responsible for the change in susceptibility.

Despite not being able to understand the exact mechanism of *Bti* and *B. bassiana* interaction, this study has shown that enhancement of *Bti* efficacy by *B. bassiana* is possible and warrants further study in order to understand the effect of combining various dosage levels of each pathogen. Furthermore, microbial pesticides are costly and any strategies allowing for the reductions in the dosage levels make them attractive to large-scale users. *B. bassiana* is affected by environmental factors, and takes a long time to act. However, with frequent evening applications, *B. bassiana* might accumulate in the manure or even infect enough house flies to start an epizootic. This would result in long-term control, while the short residual *Bti* would provide rapid short-term control of larvae (Wraight and Ramos, 2005).

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CHAPTER 8

EFFECT OF ADJUVANTS AND TEMPERATURE ON GERMINATION AND VEGETATIVE GROWTH OF THREE *BEAUVERIA BASSIANA* ISOLATES

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Abstract

In previous studies, a number of *Beauveria bassiana* isolates that showed high levels of virulence to adult house flies were identified. These isolates were tested for their compatibility with three adjuvants: Tween20, Tween80 and Breakthru®. These adjuvants were screened for their effects on spore germination and mycelial growth rates and for their influence on spore germination at various temperatures. Higher spore concentrations resulted in greater germination. Although Tween20 and Tween80 were compatible with all the *B. bassiana* isolates in the germination studies, they inhibited germination at higher adjuvant concentrations, irrespective of the conidial concentrations. Breakthru® had an inhibitory effect on germination even at the lowest concentration of 0.1% on all the *B. bassiana* isolates. The effects of the adjuvants on spore germination did not fully correspond with their effects on colony growth. However, there were few exceptions in which the adjuvants caused non-significant increases in the mycelial growth rate. Conidial viability within the same formulation declined significantly with increases in temperature, irrespective of the adjuvant. Over 90% of conidia of all isolates germinated after 48h. The optimal temperature for conidial germination of *B. bassiana* isolates was approximately 25°C with an upper limit at 30°C. Isolate 7320 was identified as the least affected by the different adjuvants. This isolate was able to germinate rapidly in a broad temperature range of 25-30°C after 24h, this characteristic being an essential factor in controlling house fly populations in poultry houses.

8.1 Introduction

House flies (*Musca domestica* L.) are major pests of poultry production systems in tropical and subtropical countries. Up to now, control strategies have been dependent on the use of synthetic chemical insecticides. However, recognition of associated problems such as non-target effects, environmental pollution as well as the high economic costs involved, have prompted the development of alternative control strategies. *Beauveria bassiana* (Balsama) Vuillemin is one of the most ubiquitous and extensively studied entomopathogenic fungi (Feng *et al*., 1994; Hajek and St Leger, 1994). This entomopathogenic fungus has also been intensively studied with the aim of development of commercial mycopesticides for the management of insect pests (Burges, 1998; Butt *et al*., 2001).

During the course of our research, a number of *B. bassiana* isolates that showed high virulence to adult house flies were identified, but a range of factors need to be considered before selecting the isolates for further use. Prolonged conidial survival in the field would help to maximize mortality of target insects. However, prolonged exposure to high temperatures limits the survival of entomopathogenic fungi in the field (Roberts and Campbell, 1977; Carruthers *et al*., 1985; Benz, 1987; Ferron *et al*., 1991; Fargues *et al*., 1992; Vestergaard *et al*., 1995; Ekesi *et al*., 1999). The thermal constraints are not only as a result of ambient conditions, but also those achieved through host thermoregulation. For instance, some insects elevate their body temperature through basking in the sun (Chappell and Whitman, 1990) and such activity has been shown to reduce disease incidence of *Entomophthora muscae* (Cohn) Fres. in house flies (Watson *et al*., 1993), *Entomophaga grylli* (Fres.) Batko (Carruthers *et al*., 1992), *B. bassiana* (Bals.) Vuill. (Inglis *et al*., 1996a) and *Metarhizium flavoviride* Gams and Rozsypal (Fargues *et al*., 1997; Inglis *et al*., 1997a) in acridids.

The adjuvant used is also recognized as a critical component in assisting conidia of a pathogen to germinate and infect the target organism. The term adjuvant includes a wide range of compounds such as surfactants, stickers, inert carriers, antifreeze compounds, humectants, sunscreen agents, anti-evaporation agents and micronutrients (Prasad, 1993). Adjuvants can have a range of effects on fungal spore germination and mycelial growth. Therefore there is need for careful evaluation for compatibility of adjuvants with conidia prior to their use in formulations (Daoust *et al.,* 1983).

The objective of this study was to screen three *B. bassiana* isolates (7320, 7569 and 7771) formulated in different adjuvants, at three different temperatures.

8.2 Materials and methods

8.2.1 Fungal isolates

Three isolates of *B. bassiana* (Isolates 7320, 7569, 7771), selected in a previous study, were used in this study. These isolates were originally obtained from the PPRI[1], Pretoria.

8.2.2 Fungal cultures

The fungi were grown on Sabourand Dextrose Agar (SDA) in Petri dishes and incubated for 10d for fungal growth and conidial production. For viability tests, conidia were removed using a brush and then suspended in distilled water and different adjuvants, vortexed for 2min to produce a homogenous suspension. Conidia were mixed with adjuvants prior to addition of water to obtain homogenous suspensions. The stock formulation of each concentration was filtered using a sterile muslin cloth. All conidial formulations had the dose adjusted to 10^4, 10^5, 10^6, 10^7 and 10^8 conidia ml^{-1} using a Neubauer's chamber.

8.2.3 Adjuvants

Three adjuvants (Tween20, Tween80 and Breakthru®) were used to measure conidial germination and mycelial growth. For controls, water was used.

8.2.4 Effects of different adjuvants on B. bassiana conidial viability

A factorial design was set up consisting of three fungal isolates (Isolates 7320, 7569, and 7771), four conidial densities (10^5, 10^6, 10^7 and 10^8 conidia ml^{-1}), three adjuvants (Tween20, Tween80, Breakthru®) and water for a control, with five adjuvant concentrations (0, 0.1, 0.5, 1 and 5% v/v or w/v). The effects of adjuvants on conidial germination was evaluated by incorporating adjuvants directly into 1.5% water agar at 0, 0.1, 0.5, 1 and 5% (v/v or w/v).

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Agar discs (16mm diameter) were then cut with a cork borer and placed onto supporting slides. Drops (10µl) of each conidial suspension were placed on the discs and spread evenly on the surface. The glass slides supporting the agar discs were placed in Petri dishes and incubated at approximately 21^{o}C. After 24h of incubation, 12.5µl of lactophenol-cotton blue were placed on the agar discs to arrest germination of conidia. A conidium was considered to have germinated when the length of the germ-tube was greater than its width, or when a sessile appressorium was produced. Several randomly selected fields of view were examined using a compound microscope until a total of 300 conidia per replicate had been assessed.

8.2.5 Effects of different adjuvants on B. bassiana mycelial growth

To measure mycelial growth, Petri dishes containing 20ml PDA were amended with the same adjuvant concentrations. Each dish was inoculated with a 6mm diameter mycelial-agar plug obtained from the margin of a 7d-old culture. Dishes of each treatment were incubated for 10d at constant dark at 21±1^{o}C and were removed at 48h intervals for assessment of mycelial growth. Colony growth was recorded as mean perpendicular radius minus the diameter of the inoculum plug (6mm).

8.2.6 Effect of temperature on conidial germination

To investigate the effect of temperature on conidial viability, a conidial suspension (1ml) of each isolate (10^{8} conidia ml^{-1}) was mixed with the adjuvant and plated onto Petri dishes containing 20ml PDA that were amended with the same adjuvant. Plates were incubated in the dark at 25, 30 and 35 ± 1^{o}C. Conidial viability tests were carried out with readings after 24h, and 48h of incubation at 21^{o}C to allow time for conidia recovery from any adverse effects caused by temperature. Conidia were observed at 400x magnification and germination was recorded when the germ tube was visible. A minimum of 300 conidia per plate were evaluated.

8.2.7 Statistical analysis

The viability experiments had factorial designs with four factors. Analysis of variance (ANOVA) on conidial viability data was performed using GENSTAT, after transforming the

percent germination data to Arcsin √ (%/100) for normal distribution and homogeneity of variances. Means were compared using Least Significant Difference (LSD). The results are presented both as untransformed and transformed data.

The mycelial growth rate (K_r) was calculated in mm per 24h using simple linear regression and was used as the main parameter to evaluate the influence of temperature on fungal growth (Fargues *et al.,* 1992). ANOVA was performed on the growth rates and means were compared using LSD.

8.3 Results

8.3.1 Effects of different adjuvants on B. bassiana conidial viability

Conidial concentration, adjuvants, adjuvant concentration and the interaction of these factors affected germination of conidia (P<0.001) of isolates of *B. bassiana.* The analysis of variance summary is shown in Table 8.1.

Table 8.1. Analysis of variance summary for conidial germination by isolates of *Beauveria bassiana*

Source of variation	***F-ratio***	***P* - value**	**Effect**
Isolate (I)	6500.77	< .001	**
Conidial concentration (CC)	2718.13	< .001	**
Adjuvant (A)	2515.15	< .001	**
Adjuvant concentration (AC)	5045.72	< .001	**
I x CC	144.70	< .001	**
I x A	256.98	< .001	**
I x AC	274.23	< .001	**
CC x A	43.35	< .001	**
CC x AC	26.04	< .001	**
A x AC	182.30	< .001	**
I x CC x A	12.32	< .001	**
I x CC x AC	7.80	< .001	**
I x A x AC	182.41	< .001	**
CC x A x AC	14.22	< .001	**
I x CC x A x AC	8.57	< .001	**

** Significant at P≤ 0.001

The germination of conidia of three *B. bassiana* isolates in response to the three adjuvants (Tween20, Tween80 and Breakthru®), adjuvant concentration and conidial densities are shown in Fig 8.1. The adjuvants generally inhibited germination at higher adjuvant concentrations and at all conidial concentrations for all the three isolates of *Beauveria* (Fig. 8.1). Tween20 showed varied effects on the three *Beauveria* isolates. For Isolate 7320, conidial germination remained fairly constant initially, declining gently by at least 30% as the

concentration of Tween20 increased up to 1%, after which there was a more obvious decrease and germination dropped to 66, 56, 49 and 42% in 10^5, 10^6, 10^7 and 10^8 conidia ml^{-1} concentrations respectively.

For Isolate 7569, the addition of Tween20 did not stimulate conidial germination, even at low concentrations of 0.1%. Instead, the addition of Tween20 resulted in an immediate rapid decline in germination. For example, at low conidial concentrations of 10^5, 10^6 and 10^7conidia ml^{-1}, germination was reduced by 40 - 50%, while at a higher conidial concentration of 10^8, germination was reduced by approximately 20% with a concentration of 0.1% Tween20.. Increases of Tween20 concentrations beyond 0.1% resulted to a gradual decline in germination (Fig. 8.1). In the case of Isolate 7771, Tween20 stimulated conidial germination at 0.1% concentration, but inhibited conidial germination at higher concentrations (Fig. 8.1).

Tween80 inhibited germination of Isolate 7320 at 0.1% concentration, but stimulated germination at 0.5 and 1.0% concentrations. For example, at conidial concentrations of 10^6, 10^7 and 10^8, Tween80 had a stimulatory effect on germination, increasing by approximately 10% for 10^7 and 10^8 and 30% for 10^6 conidial concentrations. The same adjuvant concentrations had no effect on the 10^5 conidial concentrations. Conidial germination dropped sharply with a 1.0% Tween80 concentration, for all concentrations of conidia. Addition of 0.1% Tween80 had a slight stimulatory effect on germination of Isolate 7569 conidia, but concentrations between 0.1-0.5% had substantial inhibitory effects on germination. Tween80 concentrations higher than 0.5% had slight (5-12%) stimulatory effects on germination. For the Isolate 7771, 0.1% concentration of Tween80 stimulated germination; however, adjuvant concentrations higher than 0.1% had inhibitory effects on conidial germination (Fig. 8.1).

Breakthru® had an inhibitory effect on all the *B. bassiana* isolates at all concentrations. For Isolate 7320, although the 0.1% concentration caused a slight inhibition on germination, a significant decline of conidial germination was observed as Breakthru® concentration was increased, resulting in approximately 70% inhibition of germination with the 5% Breakthru® concentration. Although addition of Breakthru® inhibited conidial germination of Isolates 7569 and 7771, the trends differed from that observed with 7320. For both Isolates 7569 and 7771, addition of Breakthru® at a low concentration of 0.1% resulted in an immediate rapid drop of >40% of conidial germination (Fig. 8.1).

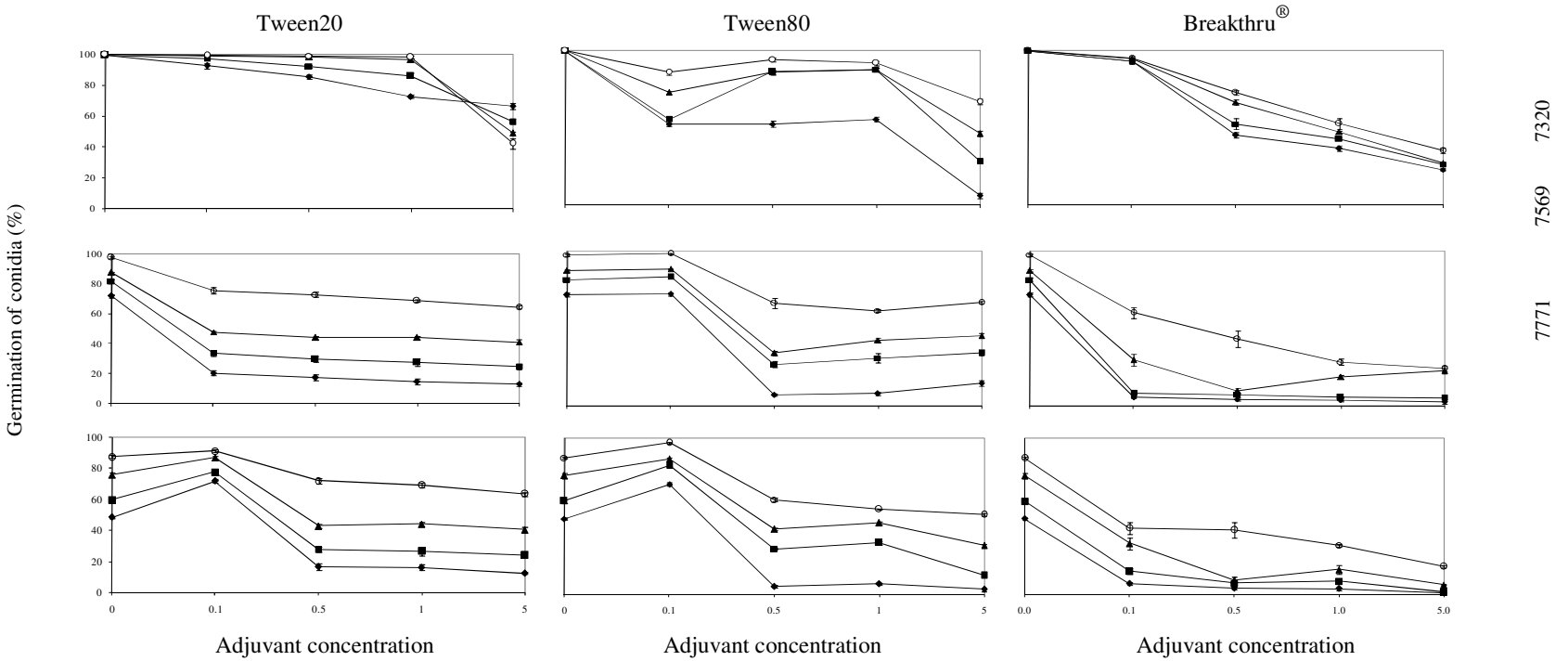

Fig 8.1. Effects of Tween20, Tween80 and Breakthru® at various concentrations (0, 0.1, 0.5, 1 and 5%) on the germination of conidia of three isolates of *Beauveria bassiana* at four conidial densities (10^5 (♦), 10^6 (■), 10^7 (▲) and 10^8 (○) conidia ml^{-1}).

Table 8.2 Effect of three adjuvants at various concentrations on the germination of conidia of Isolate 7320

Adjuvant	Adjuvant concentration	Mean germination of conidia (%) Log concentration of conidia 5	6	7	8
Tween20	0	99.4 (1.52)^{l}	99.0 (1.48)^{j}	99.6 (1.53)^{l}	99.8 (1.55)^{j}
	0.1	92.2 (1.29)^{j}	97.2 (1.43)^{j}	98.4 (1.46)^{jk}	99.0 (1.48)^{j}
	0.5	85.4 (1.18)^{i}	92.0 (1.29)^{h}	97.8 (1.42)^{j}	98.8 (1.47)^{i}
	1.0	72.4 (1.02)^{gh}	85.8 (1.19)^{f}	96.4 (1.38)^{i}	98.0 (1.45)^{i}
	5.0	66.2 (0.95)^{g}	56.4 (0.85)^{de}	48.8 (0.77)^{bc}	42.0 (0.70)^{ab}
Tween80	0	99.4 (1.52)^{l}	99.0 (1.48)^{j}	99.6 (1.53)^{l}	99.8 (1.55)^{l}
	0.1	51.8 (0.80)^{de}	55.0(0.84)^{d}	72.4 (1.02)^{e}	85.6 (1.18)^{f}
	0.5	52.2 (0.81)^{de}	86.0 (1.19)^{f}	85.6 (1.18)^{f}	93.8 (1.32)^{g}
	1.0	55.2 (0.84)^{ef}	87.4 (1.21)^{fg}	87.4 (1.21)^{fg}	91.6 (1.28)^{g}
	5.0	5.8 (0.22)^{a}	27.8 (0.56)^{ab}	46.0 (0.75)^{b}	66.6 (0.96)^{d}
Breakthru®	0	99.4 (1.52)^{l}	99.0 (1.48)^{j}	99.6 (1.53)^{l}	99.8 (1.55)^{j}
	0.1	92.6 (1.30)^{jk}	92.8 (1.30)^{hi}	94.0(1.33)^{h}	94.8 (1.35)^{gh}
	0.5	44.8 (0.73)^{d}	52.2 (0.81)^{d}	66.2 (0.95)^{d}	72.4 (1.02)^{de}
	1.0	36.4 (0.65)^{c}	42.4 (0.71)^{c}	47.0 (0.76)^{b}	52.4 (0.81)^{c}
	5.0	22.6 (0.49)^{b}	26.0 (0.54)^{a}	26.8 (0.54)^{a}	35.0 (0.63)^{a}
F-ratio		50.12	89.22	82.57	48.63
P- value		<0.001	<0.001	<0.001	<0.001
LSD		0.076	0.062	0.054	0.072
%CV		6	4.5	3.7	4.6
Effect		**	**	**	**

Values in parenthesis are square-root arcsine transformed
Means followed by the same letter within the same column (conidial concentration) are not significantly different at $P < 0.05$
** Significant at $P \leq 0.001$

There were significant differences among concentrations of the similar adjuvants within the same conidial concentration for Isolate 7320 (Fig. 8.2). Although germination in water was significantly higher than in the three adjuvants, in the higher conidial concentrations of 10^6 and 10^8, germination in 0.1% Tween20 were not significantly lower than germination in water.

There were no significant differences in germination using 0.5 and 1.0% concentrations of Tween80. In addition, these two Tween80 concentrations stimulated germination. Conidial germination using Breakthru® as an adjuvant presented significantly lower levels than with either Tween20 or Tween80 (Fig. 8.2).

Table 8.3 Effect of three adjuvants at various concentrations on the germination of conidia of Isolate 7569

Adjuvant	Adjuvant concentration	Mean germination of conidia (%) Log concentration of conidia 5	6	7	8
Tween20	0	71.6 (1.09)^{i}	81.4 (1.13)^{a}	87.2 (1.21)^{i}	97.4 (1.43)^{i}
	0.1	20.0 (0.46)^{gh}	33.4 (0.62)^{bc}	47.4 (0.76)^{gh}	75.4 (1.05)^{gh}
	0.5	16.8 (0.42)^{fg}	29.2 (0.57)^{de}	43.8 (0.72)^{fg}	72.6 (1.02)^{fg}
	1.0	14.4 (0.39)^{f}	27.0 (0.54)^{f}	43.8 (0.72)^{fg}	68.4 (0.97)^{efg}
	5.0	12.4 (0.36)^{f}	24.4 (0.52)^{h}	40.6 (0.69)^{f}	64.0 (0.93)^{de}
Tween80	0	71.6 (1.01)^{i}	81.4 (1.13)^{ab}	87.2 (1.21)^{i}	97.4 (1.43)^{i}
	0.1	72.0 (1.01)^{i}	83.2 (1.15)^{d}	88.2 (1.22)^{i}	98.0 (1.45)^{i}
	0.5	6.8 (0.26)^{cd}	26.6 (0.54)^{def}	33.8 (0.62)^{de}	66.0 (0.95)^{def}
	1.0	7.6 (0.28)^{de}	30.6 (0.58)^{fg}	42.2 (0.71)^{f}	61.2 (0.90)^{de}
	5.0	14.2 (0.38)^{f}	33.8 (0.62)^{h}	45.0 (0.74)^{fg}	66.6 (0.95)^{def}
Breakthru®	0	71.6 (1.01)^{i}	81.4 (1.13)^{ab}	87.2 (1.21)^{i}	97.4 (1.43)^{i}
	0.1	5.2 (0.23)^{bcd}	7.6 (0.28)^{de}	29.4 (0.57)^{d}	60.0 (0.89)^{d}
	0.5	4.0 (0.19)^{bc}	7.0 (0.27)^{ef}	9.4 (0.31)^{a}	43.0 (0.71)^{c}
	1.0	3.4 (0.16)^{ab}	5.2 (0.23)^{h}	18.4 (0.44)^{b}	28.0 (0.56)^{ab}
	5.0	2.2 (0.11)^{a}	4.8 (0.22)^{h}	22.4 (0.49)^{bc}	23.8 (0.51)^{a}
F-Ratio		52.22	72.45	66.53	22.22
P value		<0.001	<0.001	<0.001	<0.001
LSD		0.07	0.056	0.05	0.083
%CV		11.4	7	5.1	6.5
Effect		**	**	**	**

Values in parenthesis are square-root arcsine transformed
Means followed by the same letter within the same column (conidial concentration) are not significantly different at $P < 0.05$
F and *P* values after square root-arcsine transformation
** Significant at $P \leq 0.001$

Increases in adjuvant concentration generally lowered the conidial germination of the Isolate 7569, except in the case of Tween80. Among the three adjuvants tested, use of Tween80 resulted in the highest germination rates. Although germination was stimulated by low concentrations (0.1%) of Tween80, this germination was not significantly higher than germination in water (Fig. 8.3).

Tween20 caused more significant differences in conidial germination than the other two adjuvants while Breakthru® caused significantly lower levels of germination compared to germination in to water for Isolate 7569 (Fig. 8.3).

Table 8.4 Effect of three adjuvants at various concentrations on the germination of conidia of Isolate 7771

Adjuvant	Adjuvant concentration	Mean germination of conidia (%)			
		Log concentration of conidia			
		5	6	7	8
Tween20	0	48.2 (0.77)^{h}	59.4 (0.88)^{j}	75.6 (1.06)^{h}	87.2 (1.21)^{k}
	0.1	71.4 (1.01)^{j}	77.2 (1.07)^{l}	86.8 (1.20)^{j}	91.0 (1.26)^{m}
	0.5	16.4 (0.41)^{fg}	27.4 (0.55)^{fg}	42.6 (0.71)^{f}	71.6 (1.01)^{ij}
	1.0	15.8 (0.41)^{f}	26.4 (0.54)^{fg}	43.8 (0.72)^{f}	68.8 (0.98)^{hi}
	5.0	12.2 (0.36)^{f}	24.0 (0.51)^{f}	40.2 (0.69)^{f}	63.2 (0.92)^{gh}
Tween80	0	48.2 (0.77)^{h}	59.4 (0.88)^{j}	75.6 (1.06)^{h}	87.2 (1.21)^{k}
	0.1	70.2 (0.99)^{j}	82.0 (1.13)^{m}	86.4 (1.19)^{j}	96.8 (1.39)^{m}
	0.5	5.0 (0.22)^{cd}	29.0 (0.57)^{gh}	41.4 (0.70)^{f}	60.4 (0.89)^{g}
	1.0	6.6 (0.26)^{de}	32.6 (0.61)^{hi}	45.4 (0.74)^{fg}	54.4 (0.83)^{ef}
	5.0	3.0 (0.15)^{b}	11.8 (0.35)^{d}	31.2 (0.59)^{d}	50.8 (0.79)^{e}
Breakthru®	0	48.2 (0.77)^{hi}	59.4 (0.88)^{jk}	75.6 (1.06)^{hi}	87.2 (1.21)^{kl}
	0.1	6.6 (0.26)^{d}	14.6 (0.39)^{de}	32.4 (0.60)^{de}	42.2 (0.71)^{cd}
	0.5	3.6 (0.18)^{bc}	6.8 (0.26)^{b}	9.0 (0.29)^{ab}	41.0 (0.69)^{c}
	1.0	3.4 (0.17)^{bc}	8.2 (0.29)^{bc}	15.6 (0.40)^{c}	31.0 (0.59)^{b}
	5.0	0.8 (0.07)^{a}	1.2 (0.08)^{a}	6.0 (0.25)^{a}	17.8 (0.44)^{a}
F-ratio		55.28	67.15	34.23	46.86
P value		<0.001	<0.001	<0.001	<0.001
LSD		0.07	0.053	0.06	0.06
%CV		11.5	7	6.5	5.0
Effect		**	**	**	**

Values in parenthesis are square-root arcsine transformed
Means followed by the same letter within the same day are not significantly different ($P > 0.05$)
F and *P* values after square root-arcsine transformation
** Significant at $P \leq 0.001$

Adjuvant concentration of 0.1% of both Tween20 and Tween80 stimulated germination. Furthermore, no significant differences were observed between germination caused by the two adjuvants at this concentration. While higher concentrations of both adjuvants reduced germination significantly of the Isolate 7771, no differences in germination were observed between the higher concentrations (0.5, 1.0 and 5.0%) of Tween20 (Fig. 8.4).

Like Isolates 7320 and 7569, percentage germination as a result of using Breakthru® for Isolate 7771 were of significantly lower levels than the control (Fig. 8.4).

8.3.2 Effects of different adjuvants on B. bassiana mycelial growth rate

The analysis of variance summary on the effect of adjuvants and adjuvant concentration and their interaction on radial mycelial growth (K_r) of isolates of *B. bassiana* are shown in Table 8.5.

Table 8.5. Analysis of variance summary for mycelial growth by isolates of *Beauveria bassiana*

Source of variation	F - ratio	P- value	Effect
Isolate (I)	20.07	< .001	**
Adjuvant (A)	4.82	0.01	*
Adjuvant concentration (AC)	20.40	< .001	**
I x A	4.74	0.002	**
I x AC	2.60	0.013	*
A x AC	2.06	0.048	*
I x A x AC	1.20	0.281	NS

* Significant at $P< 0.05$
** Significant at $P\leq 0.001$
NS – Not significant

Tween20 caused a non-significant mycelial growth reduction at 0.1% concentration in 7320 and a significant inhibition after 0.1% concentration that remained fairly constant at higher concentrations (Fig. 8.2, Table 8.6). While an increase in concentration of Tween20 presented significant reductions in Isolate 7569, fewer differences were observed in Isolate 7771 (Fig. 8.2, Table 8.6).

The effect of Tween80 on the K_r of the *B. bassiana* isolates was similar to that of conidial germination. The growth of Isolate 7320 was inhibited by 0.1% concentration of Tween80, stimulated at 0.5 and 1.0% concentrations and inhibited at concentrations higher than 1.0%. Tween80 inhibited growth of Isolate 7569 at 0.1% concentration and non-significant reductions at higher concentrations (Fig. 8.2, Table 8.6). Growth of Isolate 7771 was not affected by Tween80 concentrations of up to 1% (Fig. 8.2).

For all the *B. bassiana* isolates, moderate reductions in mycelial growths were observed as a result of increasing concentrations of Breakthru®. However, fewer differences were observed for Isolate 7771 compared to Isolates 7320 and 7569 (Fig. 8.2, Table 8.6).

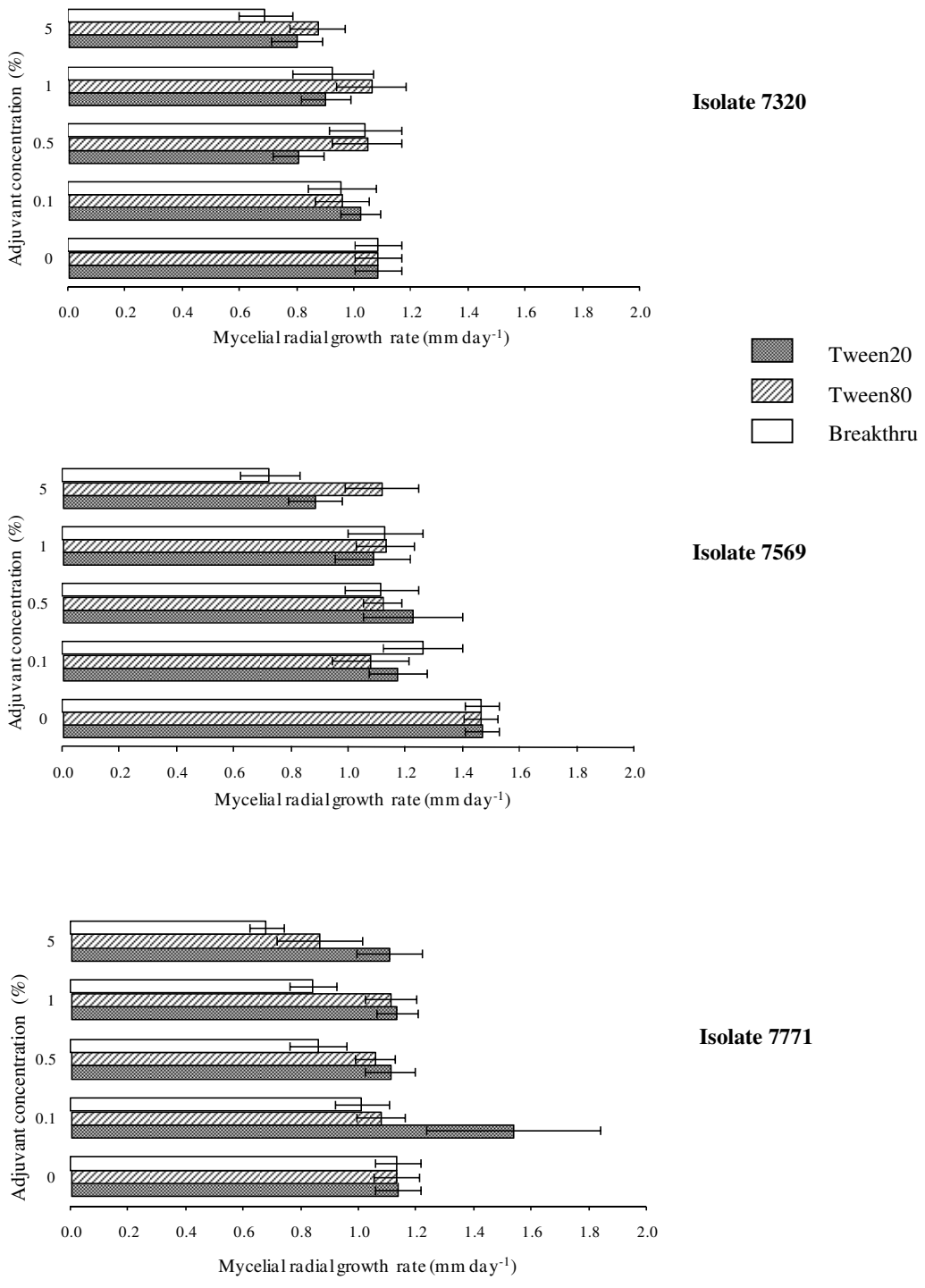

Fig. 8.2 Effects of Tween20, Tween80 and Breakthru® at various concentrations on the radial growth of mycelium of three isolates (7320, 7569 and 7771) of *Beauveria bassiana.*

Table 8.6. Effects of five concentrations of three adjuvants (Tween20, Tween80 and Breakthru®) on the radial growth rate (K_r) of three isolates (7320, 7569 and 7771) of *Beauveria bassiana*.

Adjuvant	Adjuvant concentration (%)	Radial mycelial growth rate (K_r in mm day $^{-1}$) ± se		
		Isolates		
		7320	7569	7771
Tween20	0	1.08 ± 0.08 ^{f}	1.46 ± 0.06 ^{f}	1.13 ± 0.08 ^{b}
	0.1	1.02 ±0.07 ^{def}	1.17 ± 0.1 ^{de}	1.54 ± 0.3 ^{c}
	0.5	0.8 ± 0.09 ^{ab}	1.22 ± 0.17 ^{e}	1.11 ± 0.09 ^{b}
	1	0.9 ± 0.09 ^{bcd}	1.08 ± 0.13 ^{cd}	1.13 ± 0.09 ^{b}
	5	0.8 ± 0.09 ^{ab}	0.88 ± 0.09 ^{b}	1.10 ± 0.11 ^{b}
Tween80	0	1.08 ± 0.08^{f}	1.46 ± 0.06 ^{f}	1.13 ± 0.08 ^{b}
	0.1	0.96 ± 0.1^{cdef}	1.07 ± 0.13 ^{c}	1.07 ± 0.09 ^{ab}
	0.5	1.04 ± 0.12^{def}	1.12 ± 0.07^{cd}	1.05 ± 0.07 ^{ab}
	1	1.06 ± 0.12^{ef}	1.13 ± 0.1 ^{cd}	1.11 ± 0.09 ^{a}
	5	0.87± 0.1^{bc}	1.12 ± 0.13 ^{cd}	0.86 ± 0.15 ^{ab}
Breakthru®	0	1.08 ± 0.08^{f}	1.46 ± 0.06 ^{f}	1.13 ± 0.08 ^{b}
	0.1	0.96 ± 0.12^{cdef}	1.26 ± 0.14 ^{e}	1.01 ± 0.09 ^{ab}
	0.5	1.04 ± 0.13^{def}	1.11 ± 0.13 ^{cd}	0.86 ± 0.1^{ab}
	1	0.92 ± 0.14^{bcde}	1.13 ± 0.13 ^{cd}	0.84 ± 0.08 ^{ab}
	5	0.69 ± 0.09^{a}	0.72 ± 0.1 ^{a}	0.68 ± 0.06 ^{a}
F-ratio		55.28	67.15	34.23
P value		<0.001	<0.001	<0.001
LSD		0.07	0.053	0.06
%CV		11.5	7	6.5
Effect		**	**	**

Means followed by the same letter within the same day are not significantly different ($P > 0.05$)
F and *P* values after square root-arcsine transformation
** Significant at $P \leq 0.001$

8.3.3 Effect of temperature of germination

The effects of adjuvant, temperature, incubation time and the interaction of these factors on the conidial germination of the various isolates of *B. bassiana* were examined, and the results are shown in Table 8.7.

Comparisons of the mean conidial germination levels between at each temperature within each adjuvant and between the isolates within each exposure time, 24 and 48h after incubation are shown in Table 8.8. Factorial analysis of variance revealed more significant differences among adjuvants at 24h than 48h. Conidial viability within the same formulation declined significantly with increases in temperature, irrespective of formulation. This effect was more pronounced for Isolate 7771 and less prominent for Isolate 7320 (Table 8.8).

Table 8.7. Analysis of variance summary for the effect of temperature conidial germination by isolates of *B. bassiana*

Source of variation	*F- ratio*	*P - value*	Effect
Isolate (I)	2853.79	< .001	**
Adjuvant (A)	33.63	< .001	**
Temperature (T)	2514.48	< .001	**
Incubation Time (IT)	19813.20	< .001	**
I x A	17.33	< .001	**
I x T	148.93	< .001	**
I x IT	521.81	< .001	**
A x T	15.30	< .001	**
A x IT	19.01	< .001	**
T x IT	2128.80	< .001	**
I x A x T	13.85	< .001	**
I x A x IT	10.18	< .001	**
I x T x IT	175.15	< .001	**
A x T x IT	14.33	< .001	**
I x Ax T x IT	14.48	<.001	**

** Significant at $P \leq 0.001$

All the isolates showed more than 90% germination after 48h at all temperatures. At a temperature of 30±1°C, <40% conidial germination was observed in all adjuvants for the tested isolates, except Isolate 7320. With this isolate >75% germination was observed at 30°C with all the adjuvants (Table 8.8). No delay in germination was observed at this temperature compared to germination at 25°C. At a temperature of 35 °C, all the isolates showed a significant delay or decrease in relative percentage germination after 24h but reached >90% after 48h. At a temperature of 35°C, all *B. bassiana* isolates failed to germinate within 24h. Only Isolate 7320 germinated, but the levels of germination were 0.18, 0.04 and 0.04% in water, Tween20 and Tween80, respectively.

The effect of water on germination can be separated into different responses with increasing temperature. While high germination levels were obtained with Isolate 7320 at 25°C, Isolates 7569 and 7771 exhibited moderate germination levels. Temperature of 30°C reduced conidial germination of Isolates 7320 and 7771 by 20%, and even less for Isolate 7569.

Tween20 and Tween80 stimulated germination of Isolate 7320 at 25°C, but inhibited germination of Isolates 7569 and 7771 at the same temperature. Breakthru® showed a similar trend to Tween20 and Tween80 on Isolate 7320. However, Breakthru® caused severe inhibition (<30% germination) of Isolates 7569 and 7771, even at a temperature of 25°C.

Table 8.8. Conidial viability (% ±se) of three isolates of *Beauveria bassiana* in different adjuvants, 24h and 48h after incubation at three temperatures (25, 30 and 35°C)

		Mean germination of conidia (%)					
		Isolates					
		7320		**7569**		**7771**	
Adjuvant	**Temperature**	***24h***	***48h***	***24h***	***48h***	***24h***	***48h***
Water	25	98.4 ±0.51 (1.46)^{e}	99.2±0.37 (1.50)^{a}	61.0 ±3.32 (0.90)^{e}	98.6±0.51 (1.47)de	63.8±3.46 (0.93)^{g}	98.6±0.51 (1.47)^{bc}
	30	77.2 ±0.86 (1.07)^{d}	98.4 ±0.51 (1.46)^{a}	0.80 ±0.12 (0.09)^{d}	98±0.32 (1.43)^{bcde}	48.4±1.08 (0.77)^{d}	98.4±0.51 (1.46)^{ab}
	35	0.18 ±0.09 (0.04)^{a}	98.4 ±0.40 (1.46)^{a}	0 ± 0 (0)^{a}	95.4±1.44 (1.36)^{abc}	0 ± 0 (0)^{a}	98.4±0.51 (1.46)^{a}
Tween20	25	98.4 ±0.51 (1.46)^{e}	98.6 ±0.40 (1.47)^{a}	34.6 ±1.86 (0.63)^{d}	98±0.32 (1.43)^{cde}	57.2±3.65 (0.86)^{i}	96.8±1.77 (1.42)^{bc}
	30	86.8 ± 1.07 (1.20)^{cd}	98.2 ±0.37 (1.44)^{a}	27.8 ±1.39 (0.55)^{de}	97.4±0.68 (1.41)^{abc}	38.6±2.73 (0.67)^{de}	95.6±1.5 (1.37)^{c}
	35	0.04 ± 0.02 (0.01)^{a}	98.6 ±0.40 (1.47)^{a}	0 ± 0 (0)^{a}	95.2±1.39 (1.36)^{ab}	0 ± 0 (0)^{a}	92.4±2.18 (1.30)^{c}
Tween80	25	98.0 ± 0.95 (1.46)^{e}	98.8 ±0.37 (1.47)^{a}	29.4 ±3.14 (0.57)^{f}	97.8±0.37 (1.42)^{e}	75.2±1.59 (1.05)^{h}	98±0.55 (1.44)^{c}
	30	83.0 ± 3.30 (1.15)^{bc}	98.2 ±0.20 (1.44)^{a}	31.2 ±1.85 (0.59)^{b}	95.2±1.39 (1.36)^{de}	40±3.54 (0.68)^{f}	98.8±0.37 (1.47)^{bc}
	35	0.04 ± 0.02 (0.01)^{ab}	98.2 ±0.37 (1.44)^{a}	0 ± 0 (0)^{a}	95±1.3 (1.35)^{abcd}	0 ± 0 (0)^{a}	98.8±0.37 (1.47)^{bc}
Breakthru®	25	97.2 ± 0.86 (1.42)^{e}	98.6 ±0.60 (1.48)^{a}	29.8 ±1.77 (0.58)^{de}	97.0±0.32 (1.4)^{bcd}	21.6±2.09 (0.48)^{c}	95.8±1.59 (1.38)^{ab}
	30	87.4 ± 2.50 (1.22)^{bc}	98.4 ±0.51 (1.46)^{a}	7.0 ±0.95 (0.27)^{c}	95.0 ±1.38 (1.35)^{ab}	15±1.92 (0.39)^{b}	98.2±0.37 (1.44)^{bc}
	35	0 ± 0 (0)^{ab}	98.0 ±0.32 (1.43)^{a}	0 ± 0 (0)^{a}	93.8 ±1.02 (1.32)^{a}	0 ± 0 (0)^{a}	97.2±0.73 (1.41)^{bc}
F-ratio		2.52	0.29	104.68	0.41	30.15	1.35
P- value		0.034	0.941	<0.001	0.868	<0.001	0.255
LSD		0.081	0.072	0.053	0.068	0.067	0.093
%CV		7.2	3.9	12	3.8	10	5.1
Effect		*	NS	**	**	**	NS

Values in parenthesis are square-root arcsine transformed
Means followed by the same small letter within the same column are not significantly different at $P<0.05$.
* Significant at $P< 0.05$
** Significant at $P\leq 0.001$
NS - Not significant

8.4 Discussion

While it is well documented that conidia of entomopathogenic fungi can germinate in adjuvants, the viability of conidia may also be influenced by adjuvant type (Boucias and Pendland, 1991; Milner *et al*., 1991; Prasad, 1994; Boyette *et al*., 1996). This study has shown that not only was conidial germination affected by the adjuvant but also by the adjuvant concentration and conidial concentration. The three adjuvants tested had different effects on the viability of conidia and mycelial growth. However, in both studies, there were enough exceptions to conclude that the concentration should be checked for individual adjuvants and individual isolates.

Our study generally showed that it is better to use low or moderate concentrations of adjuvants than high concentrations in order to avoid reduced conidial germination. In no case did the use of the three selected adjuvants result in improved germination in comparison to applying *B. bassiana* in water. The greatest germination occurred at the lowest adjuvant concentrations. Similar observations were noted by Zhang *et al*. (2003). For example, Tween20, which is commonly used for initial screening different fungi (Boyette *et al*., 1996), promoted germination at low adjuvant concentration of <1% in Isolate 7320, inhibited germination of 7569 at all concentrations tested and lowered germination at concentrations higher than 0.1% for Isolate 7771. Furthermore, it did not stimulate mycelial growth of any of the *B. bassiana* isolates except for Isolate 7771 at 0.1% concentration. Germination of Isolate 7320 was unaffected by Tween80 up to a concentration of 1% and then decreased significantly in higher concentrations. Isolates 7569 and 7771 were more sensitive to Tween80. Not all of the adjuvants were compatible with *B. bassiana* isolates *in vitro*. The Breakthru® series were inhibitory to all the isolates at all concentrations, confirming results of Milner *et al*. (1991), who described toxic effects for various wetting agents.

Tween20 increased mycelial growth of Isolate 7771 at 0.1%, while it decreased mycelial growth of all the other isolates. Tween80 and Breakthru® failed to stimulate mycelial growth of any of the *B. bassiana* isolates.

Greater germination was achieved with higher conidial concentrations. This relationship was checked because with similar fungi such as *Colletotrichum spp*., spore germination can be inhibited by high spore densities (Zhang *et al*. 2003). The observations of higher germination

rates with higher conidial densities in this study were consistent with our earlier observations and with other reports related to dose-mortality related studies of *B. bassiana* (Kaaya and Munyinyi, 1995; Watson *et al*., 1995; Lekimme *et al*., 2006; Devi and Rao, 2006; Santoro *et al*., 2008). In the field and poultry houses, inoculum at high densities are sprayed to target house flies and high germination rates of the inoculum may increase the overall effectiveness of the biocontrol agent.

Although this study only examined the effect of single adjuvants on germination and growth, there could be value in determining whether some of these adjuvants have a synergistic effect when combined into simple, invert or water emulsions (Auld *et al*., 2003; Hynes and Boyetchko 2006). Moreover, *B. bassiana* proved to be an effective biocontrol agent of house flies in our previous studies. By adding adjuvants at low concentrations or combining the adjuvants, the efficacy of *B. bassiana* could be increased, resulting in improved reliability.

The optimal temperature for conidial germination of *B. bassiana* isolates was approximately 25°C, with an upper limit at 30°C. A temperature of 25°C was reported to be optimal for *B. bassiana* by Fargues *et al*. (1992). In our study, all isolates showed >90% conidial germination after 24h of incubation at 21°C. Previous studies have shown that *Beauveria bassiana* is mesophilic, capable of growth at a wide temperature range (8-35^{o}C) with a maximum thermal threshold for growth at 37^{o}C (Fargues *et al*., 1997). High temperatures retarded the conidial germination process in *B. bassiana*. Similar delays were found in the same *B. bassiana* isolates (Luz and Fargues, 1997; Devi *et al*., 2005). This delay is possibly associated with the need to repair damages before germination occurs, as was previously demonstrated in *Bacillus* spores (reviewed by Nicholson *et al*., 2000).

Isolate 7320 was identified as the fungal strain that would be most suitable to formulate as a commercial product. Apart from being least affected by the different adjuvants, this isolate was able to germinate rapidly in a broad temperature range of 25–30°C after 24h, and this characteristic would be a crucial factor in suppressing house flies in poultry houses, where temperatures fluctuate markedly during the day and night. Also house flies multiply rapidly during the hot summer season; hence, higher germination and growth rates of *B. bassiana* at higher temperatures would be beneficial for house fly control. In addition, studies have also shown that entomopathogenic fungi may experience elevated temperatures through host thermoregulation (Olesen, 1985; Watson *et al.,* 1993; Kalsbeek *et al*., 2001). The authors

reported that infected house flies were capable of elevating their body temperatures through habitat selection or basking in the sun within the first few days of infection and, if the temperatures were high for a sufficiently long period, infected flies would be able to cure themselves from disease (Olesen, 1985; Watson *et al.,* 1993; Kalsbeek *et al*., 2001).

Our investigation was a laboratory study determining the influences of adjuvants upon the first two stages, germination, and mycelial growth, which had some limitations. For example, conidial behaviour was only studied on agar plates, whereas ultimately infection occurs on an insect cuticle where texture, exudates, and microflora have a role in the pre-infection stages. Also, some adjuvants may stimulate host defense responses and thereby reduce disease development in the host (Colson-Hanks and Deverall, 2000). Therefore, studies are recommended to evaluate the effect of these adjuvants on pre-infection stages and post-infection disease development on the host house flies.

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CHAPTER 9 GENERAL OVERVIEW

The entomopathogenic fungus *Beauveria bassiana* and the bacterium *Bacillus thuringiensis* var. *israelensis (Bti)* have been widely studied for their role in biocontrol against many arthropods and extensively exploited for insect control (Lacey *et al*., 2001). The studies of these two entomopathogens range from control of insects that affect crops to those that are vectors of human diseases. Additionally formulated products of these two species have been commercialized. However, although *B. thuringiensis (Bt)* was developed for commercial pest control and registered in 1961 (Glare and O'Callaghan, 2000), to date no commercial preparation of *Bt* has been released for the control of insect pests of livestock (Gough *et al.*, 2005). The effectiveness of *B. bassiana* against arthropods was described over 100 years ago, but to date commercial preparations of this fungus have focused only on insect pests of greenhouse and field crops (Shah and Goettel, 1999).

House fly populations have developed resistance to most synthetic organic insecticides (Scott *et al*., 2000; Kaufman *et al*., 2001b). Consequently the need of biological alternatives has increased in the last years.

Entomopathogens can serve as alternatives to broad-spectrum chemical insecticides. Efficacy and cost are usually the primary perspectives when comparing microbial control agents with conventional chemical pesticides. Numerous advantages can be found in the utilization of entomopathogens in addition to efficacy. Advantages include safety for humans and other non-target organisms, reduction of pesticide residues in food and conservation of other natural enemies (Lacey *et al*., 2001). However, many factors still limit the acceptance of entomopathogens by growers and general public, including pathogen virulence and speed of kill, pathogen performance under extreme environmental conditions, ease of application and lack of persistence in the environment and the integration into managed ecosystems and interaction with the environment and other integrated pest management (IPM) components (Lacey *et al.*, 2001). The aim of this thesis was to select pathogens that optimize the control of house fly larvae and adults and which can be applied with ease and integrated into the poultry environment.

The findings of this thesis resulted from the evaluation of 34 isolates of *B. bassiana*, two isolates of *Paecilomyces lilacinus*, two formulations of *Bti* and one isolate of *Heterorhabditis sp.* and the combinations of the most virulent entomopathogens for the biological control of house fly larvae and adults. Screening of the entomopathogens was carried out in the laboratory and experimental trials were conducted under controlled conditions in poultry houses at the University Research Farm at Ukulinga.

It was found that: -

- In the laboratory, *B. bassiana* isolates provided high levels of control of house flies but failed to control the larvae. Germination of *B. bassiana* conidia was delayed at high temperatures.
- *Bti* was effective in controlling house fly larvae. Addition of a *Bti* formulated bran formulation to chicken layer feed was more effective than spraying a water dispersible granular (WDG) formulation to manure for the control of house fly larvae in chicken manure.
- Dual applications of the two formulations of *Bti* resulted in higher levels of larval control than either of the two formulations alone.
- The larval mortality levels achieved as a result of feeding 250mg *Bti* kg^{-1} at 5wk were similar to those achieved as a result of feeding twice the amount of Larvadex® at 4wk to the layers. The sublethal effects of *Bti* and Larvadex® were manifested in terms of a decreasing emergence of adult house flies.
- Application *B. bassiana* as spray applications enhanced the efficacy of *Bti* feed treatments in controlling house fly larvae in manure.
- The interaction effect between applications of *Bti* as a feed treatment and *B. bassiana* as a spray application was additive.

Since *Bti* targets the larval stages of the house fly before they disperse, emphasis was given to the use of *Bti* formulations for house fly larval control. Formulations are stable, easy to handle and also to apply. Our initial screening results in the laboratory showed that the potency of *Bti* was not an accurate indicator of its performance in the field, so far as the persistence of the larvicidal activity is concerned (Vilarinhos and Monnerat, 2004; de Araújo *et al*., 2007) and therefore there is a high possibility of overestimating the longevity of *Bti* spores in the field environment (Yousten *et al*., 1992). A problem that needs to be addressed is the short

persistence observed in *Bti* formulations in the field. Reduced persistence of *Bti* formulations in the field has been attributed to several environmental factors (Ignoffo *et al*., 1981; Mulla *et al*., 1990; Becker *et al*., 1992), including high temperature (Ignoffo, 1992), solar radiation (Pusztai *et al*., 1991; Obeta, 1996; Nayar *et al*., 1999; Thiéry *et al*., 1999; Melo-Santos *et al*., 2001; Vilarinhos and Monnerat, 2004) and the presence of inhibitory substances in chicken litter (Himejima *et al*., 1992; Asukabe *et al*., 1994; Kim *et al*., 1995; Amaral *et al*., 1998; Adams *et al*., 2002). This lack of persistence of *Bti* in the field has been observed with other arthropods (Karch *et al*., 1991; Gelernter and Schwab, 1993; Kroeger *et al*., 1995; Fillinger *et al*., 2003; Russell *et al*., 2003).

Several ways to enhance the control of the *Bti* formulations towards house fly populations were pursued. Hence, the ability of dual applications of the two *Bti* formulations was to improve inoculum targeting, as well as to enhance their persistence in the chicken manure was evaluated. Our results showed that improved control of house fly larval populations resulted from the use of the two formulations of *Bti*. Several alternatives, with varying costs, were presented for large scale users that ranged from moderate to high levels of control of larvae and emergence of adult house flies. The most effective control was the use 500mg *Bti* kg^{-1} and 2g L^{-1} in spray applications.

The potential of *B. bassiana* to improve the efficacy of *Bti* in the control of house fly in poultry houses was investigated. Although the mechanism has not been plainly understood (Furlong and Groden, 2001) but positive interactions between entomopathogens has been used to improve pest control (Tompkins *et al*., 1986; Koppenhöfer and Kaya, 1997; Shapiro, 2000; Mendez *et al*., 2002; Wraight and Ramos, 2005; Oestergaard *et al*., 2006; Raymond *et al*., 2007). However, in as much as this mechanism is important, there has been little investigation on interactions between diverse strains of *Bt* and entomopathogenic fungi (Navon, 2000; Wraight and Ramos, 2005). In this study, the interaction between *Bti* and *B. bassiana* was additive, with both agents acting independently. However, some studies have reported synergistic (Wraight and Ramos, 2005), antagonistic (Ma *et al*., 2008) or even independent (Lewis and Bing, 1991) interactions between *B. bassiana* and *B. thuringiensis* in mixed reactions involving the two pathogens.

The observation that *B. bassiana* improved the efficacy of *Bti* against house fly larvae in poultry houses indicates that these two organisms have the potential to be used together for

control of house fly larvae in poultry houses. One problem of entomopathogenic fungi is a decreasing efficacy when exposed to high temperatures, low humidity and UV-light (Ignoffo, 1992). Spores lose their viability and therefore the ability to infect insect larvae. Hence, the effect of different adjuvants on the germination and vegetative growth of *B. bassiana* spores was evaluated. The performance of differently formulated conidia under high temperatures was also investigated. Results indicated that spore germination was inhibited by high adjuvant concentrations and high temperatures.

Furthermore, the compatibility of *B. bassiana* with the insect growth regulator Larvadex® and the potential of a combined treatment were investigated in order to examine the possibility of integration microbial control agents with chemical control strategies. The larval control levels obtained with this combination were significantly higher than using either agent individually but were less effective than those obtained with the *Bti* plus *B. bassiana* treatments.

Quo Vadis (The way forward…)

The success of biological control with *Bti* partially depends on the persistence of *Bti* toxins in the environment. Interaction studies enhanced the control levels of *Bti* against house fly populations in poultry environment. What remains to be resolved is a way to enhance the residual ability of the *Bti* toxic proteins. Brar *et al.* (2006) suggested improvement of *Bti* using encapsulation of the *Bti* spores or toxins. This mechanism has the advantage in that it would provide protection from extreme environmental conditions consequently enhancing the residual stability due to sustained releases of the bacterium (Brar *et al.,* 2006). When combined with frequent applications of *B. bassiana* to target adult breeding flies, this would provide long term control of house flies in poultry houses.

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