Effects of a dual application of entomopathogenic nematodes and entomopathogenic fungi against a forest pest, *Hylobius abietis* L. (Coleoptera: Curculionidae) and a horticultural pest, *Otiorhynchus sulcatus* F. (Coleoptera: Curculionidae)

Roseanne Hennessy

M. Sc. **2012**

Master of Science by Research

Biology Department NUI Maynooth

Supervisor: Dr. Christine Griffin, NUI Maynooth.

Mr. Michael Gaffney, Teagasc.

Head of Department: Prof. Paul Moynagh

Declaration

This thesis has not been submitted in whole or in part to this or any other university for any other degree and is, except where otherwise stated, the original work of the author.

Signed _____

Roseanne Hennessy

Contents

Acknowledgements

Firstly I would like to thank my supervisors Dr Christine Griffin and Mr Michael Gaffney, who provided advice and guidance throughout my time at NUI Maynooth and were understanding and supportive supervisors.

Thanks to Teagasc Walsh Fellowship for funding the project.

Thanks to all in the Behavioural Ecology and Bio-control lab, NUIM for all the help and advice throughout the project: Dr. Chris Williams, Dr. Chris Harvey, Gail Maher, Ciaran Pollard, Adam Guy, Johan Val Vlaenderen and Louise McNamara.

Thanks to Dr. Aoife Dillion from Coillte for helping with field work sites.

Thanks to all the field workers: Laoise Kelly, Karen Morris.

Thanks to all in the Biology department and especially those who participated in numerous cups of tea.

Thanks to Prof. Martin Downes for all the advice throughout the project.

Thanks to my boyfriend, family and friends for their constant support.

Abstract

Entomopathogenic nematodes (EPN) and entomopathogenic fungi (EPF) are pathogens which require an insect host to fulfil their life cycle and are commonly used as biocontrol agents. Studies have shown that a combination of EPN and EPF can result in greater insect mortality when compared to the agents applied alone. This interaction could possibly reduce the cost and quantities of single dose applications, inferring a synergistic effect. However, combinations of these pathogens may also result in additive or antagonistic interactions which would be of no benefit to growers.

Hylobius abietis (large pine weevil) and *Otiorhynchus sulcatus* (black vine weevil) cause economic damage to forestry and horticultural crops, respectively. Large pine weevil larvae develop in the tree stumps, emerge as adults and feed on the bark of newly planted saplings, causing extensive mortality. Black vine weevil larvae kill plants by feeding on the roots and adults cause cosmetic damage.

In this investigation, laboratory and field trials were conducted against large pine weevil larvae to determine whether interactions occur if EPN and EPF were applied simultaneously. EPN and EPF have already been shown to interact synergistically against the vine weevil in laboratory conditions. These experiments were repeated to investigate if synergistic interactions could be reproduced.

Synergistic interactions were found with EPN and EPF against the large pine weevil in some laboratory experiments. However, antagonism and additivity were also recorded and synergism could not be predictably achieved. The field trial against the large pine weevil resulted in additivity. This indicates that the combined application of EPN and EPF would not be a better option than single applications.

Initial interaction experiments against the black vine weevil did not show similar results to previous publications. However, larvae used in previous publications were younger than in this investigation which may indicate that interactions are dependent on larval age.

List of Abbreviations

%	percentage
<	less than
=	equals
>	greater than
°C	degrees Celsius
ANOVA	Analysis of variance
Bb	*Beauveria bassiana*
Bc	*Beauveria caledonica*
bp	base pairs
cm	centimeter
ddH_2O	double distilled water
DF	degrees of freedom
DNA	deoxyribonucleic acid
e.g.	for example
EP	expected mortality
EPF	entomopathogenic fungi
EPN	entomopathogenic nematodes
et al.	and others
etc.	etcetera
Exp.	experiment
Fig.	figure
g	grams
Hb	*Heterorhabditis bacteriophora*
hr	hours
i.e.	that is
IJ	Infective juveniles
ITS	internal transcribed spacer
L	litre
LC_{50}	leathal concentraion at 50 %
Ma	*Metarhizium anisopliae*
Met52	*Metarhizium anisopliae* F52
$MgCl_2$	Magnesium chloride
min	minutes
ml	millilitre
mm	millimeter
N	number
OM	observed mortality
PCR	polymerase chain reaction
PDA	Potatoe Dextrose agar
rDNA	ribosomal deoxyribonucleic acid
s	seconds
Sc	Steinernema carpocapsae
SDA	Sabouraud Dextrose agar
Sf	*Steinernema feltiae*
sp(p).	species
TAE	Tris-Acetate-EDTA
Tween	Tween 80
v/v	volume/volume
w/v	weight/volume
µl	microliter
$\chi 2$	Chi-squared

Chapter 1 – Introduction

1.1 Biological Pest Control

Biological control (biocontrol) involves introducing living organisms (or a product from living organisms) into an area where a pest is present and reducing it to a tolerable level (DeBach & Rosen 1991). Currently synthetic pesticides (manufactured chemical pesticides) are used in vastly greater quantities than biocontrol agents. In 2000 only 0.2% of the global market consisted of biopesticides, however by 2009 this figure had dramatically increased to 3.8 % indicating that biopesticides are becoming more competitive in the market (Thakore 2006). Some synthetic pesticides have been shown to be very toxic and can pose a major threat to human health and the environment (Fantke *et al.* 2012; Lacey *et al.* 2001; Sifakis *et al.* 2011; World Health 2006). In 2009 the European Union brought in a new framework directive requiring that all member states should achieve a level of sustainable use of pesticides by December 2012 (European Directive 2009/128/EC). This new legislation will enable biocontrol agents to become more competitive in the market place and hence increase the demand for them. Biocontrol agents in many cases are an attractive alternative to synthetic pesticides as they can be more selective in targeting the pest, cause limited pollution to the environment, and pest resistance to most biocontrol agents is unlikely (Van Emden & Service 2004). However, there are reasons for biocontrol agents being underused in the global market: they can limit the effectiveness of other pesticides in later use, they usually take longer to act against the pest, they rarely exterminate the pest, they can be species specific (meaning that several may be needed for a complex of pests), they can have unpredictable results and mass rearing and transportation can be expensive (Van Emden & Service 2004). In order to solve some of these obstacles more research needs to be conducted into better understanding the interactions biocontrol agents have with the environments they are introduced into and investigating if they can be implemented into integrated pest management (IPM) systems (Ansari *et al.* 2008; Lacey *et al.* 2001; Shapiro-Ilan *et al.* 2004). Biological control agents include the biopesticides entomopathogenic nematodes (EPN) and entomopathogenic fungi (EPF). EPN and EPF have been shown to be effective agents against a wide range of insect pests including species in the order Coleoptera (Ansari *et al.* 2006; Dillon *et al.* 2006; Ennis *et al.* 2010; Ormond *et al.* 2010; Reay *et al.* 2008; Shah *et al.* 2008; Shapiro-Ilan *et al.* 2004; Wang *et al.* 1995).

1.2 Entomopathogenic Nematodes (EPN)

Entomopathogenic nematodes typically dwell in soil and are found thoughout the world. The two families which are studied for biocontrol are Steinernematidae and Heterorhabditidae (Grewal *et al.* 2006). They have a symbiotic association with a bacterium which they carry in their gut; this bacterium enables them to target a wide range of insect hosts. Steinernematidae carry the bacterium *Xenorhabdus* spp. and Heterorhabditidae carry the bacterium *Photorhabdus* spp. (Gaugler 2002; Grewal *et al.* 2006). The benefits of using nematodes include: the ability to produce them in large numbers, lower impact on non-target species, a wide range of species makes it easy to select for specific environments, application is safe and they can be implemented in an IPM system (Gaugler 2002; Georgis *et al.* 2006; Shapiro-Ilan *et al.* 2006).

1.2.1 Life cycle

The life cycle for both families is similar (Fig 1.1), the only difference is that heterorhabditids are hermaphrodites in the first generation (Dix *et al.* 1992) and steinernematids are mainly amphimictic, only *Steinernema hermaphroditum* from Indonesia has been found to be self fertilising (Griffin *et al.* 2001). Infective juveniles (IJ) are specialised third stage juveniles which seek out and infect an insect host (entering though mouth, anus, spiracles and cuticle). Once inside the insect haemolymph the IJ releases its symbiotic bacteria, which then break down the insect's defence system and begin to multiply, killing the insect usually within 48 hr. The IJ then feed on the bacteria and grow into adults. The adults reproduce and lay eggs in the host medium. This cycle is repeated until food source is fully consumed (several generations occur in a large host). The IJ then emerge from the host cadaver and seek a new host to repeat the life cycle. The whole cycle takes ca. 8-10 days for Steinernematids and 14-15 days for Heterorhabditids (Grewal *et al.* 2006; Kaya & Stock 1997; Lewis *et al.* 2006a).

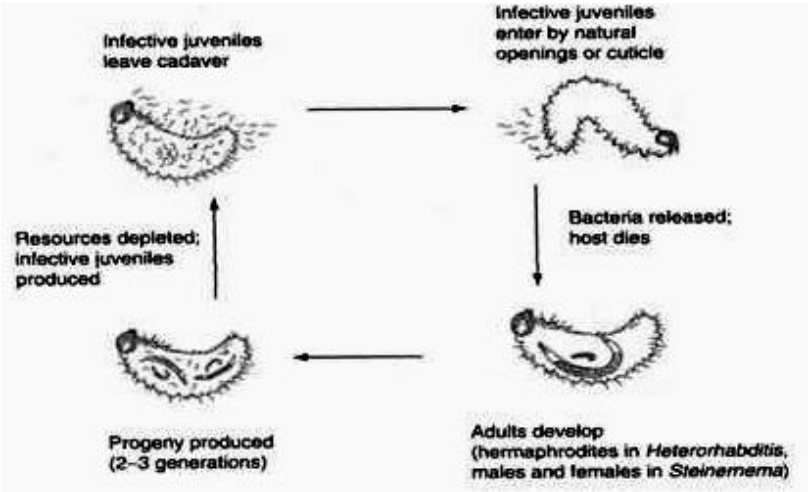

Fig 1.1 Generalised life cycle of EPN (Lewis *et al.* 2006b)

1.2.2 Symbiotic bacteria

Photorhabdus and *Xenorhabdus* are usually only associated with nematodes or infected insects but they can be grown on artificial media under laboratory conditions (Forst & Dowds 1997). However, when they were introduced to sterile soil a reduction in their survival was seen after seven days, indicating that the bacteria require nematodes in order to survive in soil environments (Morgan *et al.* 1997). Both *Photorhabdus* spp. and *Xenorhabdus* spp. are motile, Gram-negative and belong to the family Enterobacteriaceae (Burnell & Stock 2000). They multiply in the haemolymph where their enzymes break down and suppress the insects' immune system (Forst & Dowds 1997). Both bacteria can produce antibiotics that suppress competition from other organisms, reducing competition and enabling the nematodes to feed without contamination (Akhurst *et al.* 1993). The insect dies due to septicaemia or toxaemia from the bacteria (Forst & Dowds 1997). The main difference between the two bacteria is that some species of *Photorhabdus* emit luminescence from the cadaver of the insect (the reason for this is still unknown). In *Steinernema* spp. *Xenorhabdus* bacteria are kept in a special vesicle of the nematode's intestine while in *Heterorhabditis* spp. *Photorhabdus* is found in the anterior part of the intestine (Boemare *et al.* 1996). Both the nematode and bacteria benefit from the symbiotic association; the nematode enables the bacteria to survive outside the insect and find new hosts, while the bacteria suppress the immune system of the insect, eliminate competition from other organisms and degrade the insect enabling the nematodes to feed on the bacteria and the degraded insect (Grewal *et al.* 2006).

1.2.3 Steinernematidae

Steinernematidae consists of two genera, *Steinernema* and *Neosteinernema* (Kaya & Stock 1997; Lacey 1997). *Neosteinernema* contains only one species *N. longicurvicauda,* which was isolated in 1992 from a termite in Florida, U.S.A. (Burnell & Stock 2000; Nguyen & Smart Jr 1994). It differs from the *Steinernema* spp. in both biology and morphology (Nguyen & Smart Jr 1994), but it is currently not used as a biological control agent.

By the year 2002 the *Steinernema* genus contained 25 validated species and has global distribution (Gaugler 2002). To date only two of these species have been isolated in Ireland *Steinernema affine* and *Steinernema feltiae* (Blackshaw 1988; Griffin *et al.* 1991). Some species which are currently available commercially include *S. carpocapsae, S. feltiae*, *S. glaseri, S. kraussei* and *S. riobrave* (Grewal *et al.* 2006; Haukeland & Lola-Luz 2010; Weeden 2003). *S. carpocapsae* and *S. feltiae* have shown huge potential as biopesticides and they also infect a wide range of insect pests including the large pine weevil, black vine weevil, corn root worms, sweet potato weevil, codling moth etc. (Ansari *et al.* 2008; Dillon *et al.* 2006; Mannion & Jansson 1992; Unruh & Lacey 2001; Wright *et al.* 1993).

1.2.4 Heterorhabditidae

Heterorhabditidae consists of just one genus *Heterorhabditis* with nine validated species in 2002 (Gaugler 2002). They have a global distribution but *Heterorhabditis downesi* is the only species currently found in Ireland (Stock *et al.* 2002). Species of *Heterorhabditis* which are available commercially include *H. bacteriophora* and *H. megidis* (Long *et al.* 2000; Shapiro-Ilan *et al.* 2002; Sulistyanto & Ehlers 1996). *H. bacteriophora* is the most commonly studied *Heterorhabditis* spp. and has been shown to be an effective control agent against the vine weevil, white grubs, long-horned beetle and red palm weevil (Ansari *et al.* 2008; Atakan *et al.* 2009; Koppenhofer & Fuzy 2008; Susurluk *et al.* 2011). H*eterorhabditis downesi* is not commercially produced but it reduced the large pine weevil (*Hylobius abietis*) to reasonable levels in conifer plantations in Ireland (Dillon *et al.* 2006).

1.3 Entomopathogenic fungi

Entomopathogenic fungi (EPF) are usually found in the upper layer of the soil (Meyling & Eilenberg 2007). There are at least 700 known species of EPF, many with a global distribution (Lacey *et al.* 2001; Wraight *et al.* 2007). For the purposes of this study the focus was on the species *Metarhizium anisopliae* and *Beauveria bassiana* which belong to the artificial family Moniliaceae (Barnett & Hunter 1972). Both species can utilize a wide range of insects as hosts and cause death via toxicosis (Samson *et al.* 1988). *Metarhizium anisopliae* produces the toxins destruxins (>27 types), swainsinone and cytochalasin C (Kershaw *et al.* 1999; Vey *et al.* 2001) and *B. bassiana* produce bassianin, beauvericin, bassianolide, beauverolides and tenellin (Gillespie & Claydon 1989; Vey *et al.* 2001). These EPF are more appealing to use than synthetic pesticides as they are safer for sprayers to use, pest resistance to EPF is unlikely and they can be self-propagating (Van Emden & Service 2004). Both species have proven to be successful biological control agents and are commercially produced (Feng *et al.* 1994; Lacey *et al.* 2001; Shah & Pell 2003; Zimmermann 1993).

1.3.1 Life Cycle

The life cycle begins with conidial spores (asexual spores) landing on the surface of the insect (Fig 1.2). The spore germinates and penetrates through the cuticle of the insect via hyphal growth. Once inside the body the fungus produces toxins that suppress the insect's immune system and hyphae infiltrate the haemocoel. The insect then dies from toxicosis or obstruction of organs and the hyphae spread throughout the host consuming the nutrients. Once all nutrients have been used the hyphae penetrate out through the insect's cuticle, sporulate and produce conidia which are dispersed though various means (Inglis *et al.* 2001; Kaya & Stock 1997; Samson *et al.* 1988).

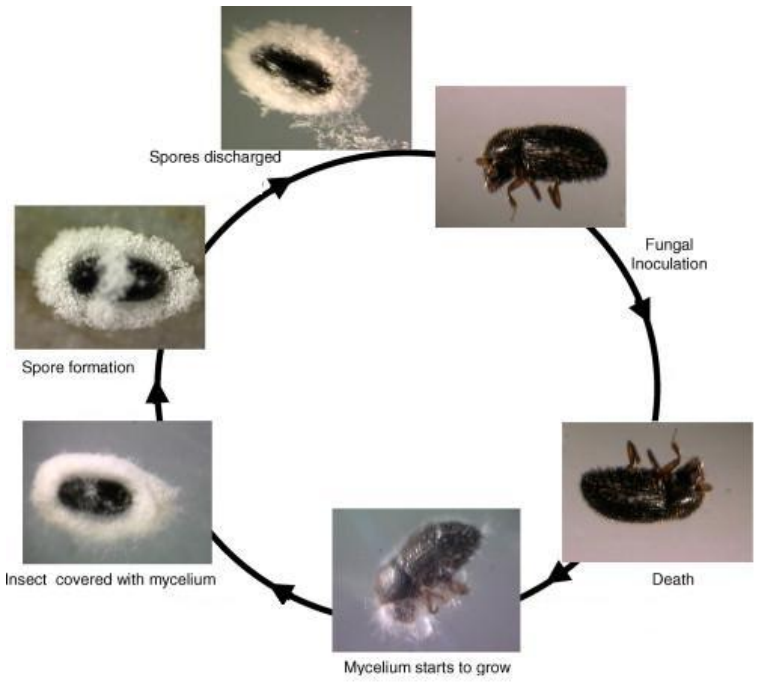

Fig 1.2 Generalised life cycle of EPF (Vega *et al.* 2008).

1.3.2 Ecology

Metarhizium spp. and *Beauveria* spp. have a global distribution and both have been recorded in Irish soil (Glare *et al.* 2008; Chandler *et al.* 1997). Their optimum growth temperature is between 20˚C and 25˚C and they can grow in temperatures as low as 10˚C (Fargues *et al.* 1997; Hallsworth & Magan 1999; Ouedraogo *et al.* 1997). Both EPF operate best in moderate – high humidity levels and low exposure to sunlight (ultraviolet light can kill spores) (Daoust & Roberts 1983; Doberski 1981; James *et al.* 1998; Morley-Davies *et al.* 1996; Wraight *et al.* 2000). In order for these EPFs to become established in an environment they rely on a healthy arthropod population to be present so they can infect a new host (Butt *et al.* 2001; Shah & Pell 2003). However, if conditions are unsuitable for regeneration they can form overwintering structures which can consist of compressed hyphae (sclerotia) or thick-walled resting spores (chlamydospores) which can remain dormant in the soil (Shah & Pell 2003). When conidial spores are produced they spread passively via wind, rain and host movement (Hajek 1997; Inglis *et al.* 2001; Shah & Pell 2003). *Metarhizium anisopliae* is more commonly found in arable soils while *B. bassiana* grows better in damp forest ecosystems, but both can be found in cultivated and uncultivated soils (Meyling & Eilenberg 2007; Ormond *et al.* 2010; Samson *et al.* 1988).

1.3.3 *Metarhizium anisopliae* and *Beauveria bassiana* as biopesticides

A lot of research has gone into using *M. anisopliae* and *B. bassiana* as biopesicide agents because of their ability to infect a wide range of hosts. *Metarhizium anisopliae* has effectively controlled insects such as the black vine weevil, the deer tick, locusts, mosquitoes and many more (Kaaya & Hassan 2000; Lezama-Gutierrez *et al.* 2000; Milner *et al.* 1998; Peng *et al.* 2008; Scholte *et al.* 2003; Shah *et al.* 2008; Wright *et al.* 2005; Zimmermann 1993). In 2011 a commercial strain of *M. anisopliae* F52 (sold commercially as Met52 and produced by Novozyme) became available for use in the Irish market and is mainly used for the control of the black vine weevil, *Otiorhynchus sulcatus*. *Beauveria bassiana* has been used as a biopesicide against insects such as the banana weevil, silverleaf whitefly, false-eye leafhoppers and the European cherry fruit fly, to name a few {Wraight, 2000; Feng, 1994 ; Godonou, 2000; Feng, 2004; Daniel, 2010}. Many products of *B. bassiana* are currently available on the market and they are produced in Europe and the U.S.A. The main pests these are used to control are the Colorado beetle, whiteflies and thrips (Whipps *et al.* 2001).

1.4 Integrating EPNs and EPFs

Biopesticides share a minimal amount of the global pesticide market. This is mainly due to their inconsistent results, lack of efficiency and the costs need to develop the products (Glare *et al* 2012.). More research is required in order to make it economical for growers to use them and one method that has been researched is to use a combination of EPN and EPF (Lacey *et al.* 2001). If a combination of EPN and EPF resulted in a synergistic interaction then the potential cost of using these biopesticides may be reduced. However, the combination of two or more agents can also give additive or antagonistic results (Berenbaum 1978) which would be of no benefit to growers.

1.4.1 Previous studies

A number of previous laboratory and field studies have used combinations of EPN and EPF against insect pests, but it is not always clear how the combination compares to the individual use of each agent. *Steinernema carpocapsae* was combined with *B. brongniartii* against *Exomala orientalis* (the oriental beetle) on golf courses in Korea. This combination of these two agents gave increased mortality when compared to the fungus alone, however the interactions could not be properly interpreted as there was no single application of *S. carpocapsae* (Choo *et al.* 2002). Ideally the concentration of the

agents when used alone should give < 50 % mortality for synergy to be detected. In a study conducted by Shapiro-Ilan *et al.* (2004) *B. bassiana* or *M. anisopliae* were combined with *H. indica* or *S. carpocapsae* against the pest *Curculio caryae* (the pecan weevil). The results recorded were mainly antagonistic with a few exceptions of additivity. It was suggested that the negative interactions occurred as a results of the interactions of the pathogens before or during infections, or that antagonistic toxins after the initial infection and hence there the bacteria from the nematodes was suppressing the growth of the fungus or vice versa (Shapiro-Ilan *et al.* 2004).

Ansari *et al.* (2008) conducted laboratory and field trials against *Otiorhynchus sulcatus* (the black vine weevil) using a combination of *M. anisopliae* and EPN. In laboratory conditions synergy occurred with either *H. bacteriophora* or *S. kraussei* and mortality with *S. feltiae* was recorded as additive. Ansari *et al.* (2008) also performed greenhouse trials with the combination of these agents applied at various concentrations. The combination of *M. anisopliae* and *H. bacteriophora* resulted in synergistic interactions in all treatments. The combinations of *M. anisopliae* and *S. feltiae* only resulted in synergy when a high concentration of fungus was used with a low concentration of nematodes; all other interactions were additive (Ansari *et al.* 2008). Both the field and laboratory results coincided in this study indicating that laboratory assays will give a good indication of what interactions may be found in field conditions. This was also the case for laboratory and field trials against *Hoplia philanthus* (the Welsh chafer) larvae, which were exposed to combinations of *M. anisopliae* and *H. bacteriophora.* All the interactions recorded with these two agents in both laboratory and field trials were synergistic (Ansari *et al.* 2006; Ansari *et al.* 2004).

Other factors that may influence the outcome of a trial (the assessment of whether a combination gives additive, synergistic or antagonistic effect) include time of assessment and the time between applications of the agents. *Coptognathus curtipennis* (the barley chafer) was exposed to *M. anisopliae* and *H. bacteriophora* in laboratory experiments. Initially the results were additive but when the larvae were assessed after five weeks the interaction became synergistic (Anbesse *et al.* 2008). Similar results in laboratory trials using *M. anisopliae* and nematodes against *Hoplia philanthus*. Larvae were exposed to *M. anisopliae* for 0, 1, 2, 3 or 4 weeks before the nematodes (*H. megidis* or *S. glaseri*) were added. Synergy was found in all combinations by the end of

each experiment, although weeks 1 – 3 after application of nematodes showed additive results (Ansari *et al.* 2004). However in field trials against the same pest, *H. bacteriophora* was applied four weeks after *M. anisopliae* and when the trial was assessed on week seven synergism was detected. When the trials were assessed a year after application the results showed additivity (Ansari *et al.* 2006). The reason for the lack of synergism after year one may have been that the individual treatments had already caused high mortality and there was not enough scope for synergy to occur (Ansari *et al.* 2006).

Metarhizium anisopliae and *H. bacteriophora* when applied simultaneously give a high level of control and consistent results when used against the black vine weevil and Welsh chafer in the above studies. However, this is not the case in all studies e.g. when Shapiro-Ilan *et al.* (2004) used the pecan weevil as host. In order to attain synergism pest resistance may need to be reduced by stress (Kaya & Gaugler 1993). If insects are exposed to EPF for a time period before adding nematodes it may stress the insects and enable the nematode to invade the insect easily and hence causes higher mortality (Ansari *et al.* 2008).

In several studies EPN are applied after application of EPF. *Spodoptera exigua* (the beet army worm) larvae were exposed to *B. bassiana* for 48 hr before being exposed to EPN in laboratory conditions. *Beauveria bassiana* and *H. bacteriophora* gave a higher mortality than when they were applied on their own but with *S. carpocapsae* there was no increase. However, much cannot be concluded about the time interval as there was no simultaneous application and it cannot be concluded if time delay had an effect on the interaction (Barbercheck & Kaya 1991). In laboratory experiments *H. philanthus* (the Welsh chafer) were exposed to *M. anisopliae* for 0, 1, 2, 3 or 4 weeks before the nematodes (*H. megidis* or *S. glaseri*) were added. A synergistic interaction was recorded in all experiments (Ansari *et al.* 2004). *Otiorhynchus sulcatus* was exposed to *M. anisopliae* on day zero and nematodes were added either 0, 1 or 2 weeks after the fungus. Synergism was recorded with *H. bacteriophora* for all time delays and for *S. feltiae* only when added after week 1 or 2*;* when *S. kraussei* was added on week 1 and 2 the mortality was additive, but it was synergistic when added on week zero (Ansari *et al.* 2008). These results show that the chances of attaining synergy may be increased in some cases when larvae are exposed to fungus for a time period before the nematodes,

but not in all e.g. *S. kraussei*. However, these synergistic interactions seem to be species specific and different interactions occur with different agents used (Ansari *et al.* 2008). *Coptognathus curtipennis* (the barley chafer) was exposed to *M. anisopliae* and *H. bacteriophora* either simultaneously or 2 or 3 weeks after the fungus was added. To achieve synergism nematodes had to be added 2 or 3 weeks after the fungus (Anbesse *et al.* 2008). These experiments reinforce the view that synergistic interactions can be increased if larvae are exposed to fungus before the nematodes, in some cases.

All of the aforementioned studies indicate that *M. anisopliae* can achieve synergy when applied with different nematode species against various insect species. *Beauveria bassiana* has yet to show any synergy with a nematode species. However, limited research has gone into the interactions of *B. bassiana* with nematodes against different hosts.

The virulence of the agents used in treatments can affect the outcome of the interaction. In a study conducted on *Diatraea saccharalis* (the sugarcane borer), larvae were exposed to two strains of *M. anisopliae* for 48 hr and before adding *H. bacteriophora*. It was found that the time of death was reduced when the larvae were exposed to a moderately virulent strain of *M. anisopliae* with *H. bacteriophora* but not when a more virulent strain of fungus was used (Acevedo *et al.* 2007).

When overwintering black vine weevils in greenhouses were exposed to *M. anisopliae* and *S. kraussei* the interaction recorded was synergistic in the first trial, but when the trial was repeated results were additive (Ansari *et al.* 2009). This shows that the combination of these pathogens may not be consistent. It was hypothesised that the inconsistent results may be due to the sensitivity of the fungus to temperature (Ansari *et al.* 2009).

Nematodes and fungi may infect their host in different areas of the insect's body during a dual infection (Tarasco *et al.* 2011). The efficiency of infection and development by both entomopathogens is influenced by the media, temperature, moisture, virulence of the agents and concentrations (Acevedo *et al.* 2007; Ansari *et al.* 2004; Barberchek & Kaya 1990; Jabbour *et al.* 2011; Tarasco *et al.* 2011). Barbercheck and Kaya (1990) found evidence of antagonism when *G. mellonella* larvae were dually infected with *B.*

bassiana and either *H. bacteriophora* or *S. feltiae.* Only ca. 1 % of cadavers produced progeny in dual infected insects. They found that *B. bassiana* development was better than that of the nematodes at lower temperatures (< 20˚) when combined together (Barberchek & Kaya 1990). Tarasco *et al.* (2011) also observed strong competition between EPF and EPN in dually infected *G. mellonella* larvae. This antagonistic interaction is probably due to the inhibitory effect that the bacteria contained in the nematodes has on the fungus, and direct competition between them (Tarasco *et al.* 2011), as symbiotic bacteria carried by both *Steinernema* spp. and *Heterorhabditis* spp. can inhibit EPF growth (Ansari *et al.* 2005; Tarasco *et al.* 2011). When nematodes were added simultaneously with the fungus or before the fungus, conidia production was significantly reduced and IJ were produced from the cadaver (Acevedo *et al.* 2007; Ansari *et al.* 2005). The production of IJ was significantly reduced in combined infections with a highly virulent fungus or when the fungus was added before the nematodes (Acevedo *et al.* 2007; Ansari *et al.* 2005; Barberchek & Kaya 1990).

When conditions are optimised, combinations of entomopathogens can be as effective in controlling pests as synthetic pesticides. Choo *et al.* (2002) showed that a combination of *S. carpocapsae* and *B. bassiana* gave significantly better control of the oriental beetle than the chemical pesticide, fenitrothion. Ansari *et al.* (2006) also showed that *M. anisopliae* with *H. bacteriophora* controlled the Welsh chafer better than the commercial product Dursban 5G (active ingredient: chlorpyrifos),

1.5 The Large Pine Weevil – a forest pest

The large pine weevil, *Hylobius abietis* (Coleoptera; Curculionidae), is a major pest of re-forested plantations across Europe. In reforestation mature trees are felled and after about two years saplings are planted close to the old stumps. Weevils oviposit in the soil around the roots of the stumps from felled trees (Nordlander *et al.* 1997). When the larvae hatch out they make their way to the stumps and feed under the bark; after 1 – 3 years they pupate and emerge as adults (Moore *et al.* 2004). When the adults emerge they feed on the saplings and can kill the sapling by removing the bark in a ring around the stem. If saplings are left untreated weevil populations can destroy 50 % of the crop (Heritage & Moore 2001). Larvae usually take between 18 and 36 months to develop before emerging as adults from stumps, hence timing of felling and planting of trees can reduce the amount of damage (Leather *et al.* 1994; Orlander & Nilsson 1999). In Ireland

the state-owned forestry company, Coillte, harvested 1.5 x 10^6 m^2 in 2009 (Coillte 2012). Clear felling provides an ideal habitat for the large pine weevil to inhabit and reproduce to high levels.

1.5.1 *Hylobius abietis* life cycle

Female weevils lay their eggs during May to August in the soil around roots of stumps or occasionally in the bark of the stumps if conditions are dry (Nordlander *et al.* 1997). A female can lay up to 70 eggs in the first season (Bylund *et al.* 2004). Temperature and moisture affect where the eggs are laid in the soil, if conditions are dry eggs will be laid lower down in the soil (Nordlander *et al.* 1997). Eggs usually hatch after about a month in field conditions and larvae will make their way to the bark of the stump to begin feeding (Nordlander *et al.* 1997). Larvae feed on the base and inside the bark and go through 4 – 5 instars before pupation occurs. Larvae usually enter a diapause during their last larval stage (during the winter) (Christiansen 1971). In Spring when temperatures get above 10˚C the larvae exit the diapause stage and begin their pupal stage, which last 1 – 4 weeks and they then emerge as adults. The life cycle of a pine weevil can take 1 – 4 years depending on temperature and host plant species (Christiansen 1971; Leather *et al.* 1999; Moore *et al.* 2004; Salisbury & Leather 1998). Once the adults emerge they begin feeding before they are sexually mature; the quantity they eat depends on the quality of the bark (Wainhouse *et al.* 2004). Once sexually mature after about 2 weeks the females lay their eggs during the Spring and Summer months. When the temperatures become cool in late autumn the adults can go into hibernation (Nordenhem 1989). Adult weevils can live for up to four years (Leather *et al.* 1999).

1.5.2 Synthetic control of *Hylobius abietis*

Initially to prevent damage, conifer seedlings were treated with DDT (dichlorodiphenyltrichloroethane) from the 1950s until its ban in the 1970s (Beard 2006; Langstrom *et al.* 2004). Lindane replaced DDT but again it proved to be hazardous to the environment and was withdrawn in 2000 from the Irish market (Department of Agriculture 2012). Pyrethroids such as alpha-cypermethrin are currently used for pine weevil control. The treatment involves dipping the seedling before planting and one spray post planting (Dillon *et al.* 2007; Leather *et al.* 1999; Thacker *et al.* 2003). Although it has a low acute toxicity it has been shown to have a chronic

toxicity towards some soil dwelling organisms and can affect reproduction (Yilmaz *et al.* 2004). Despite its highly hazardous qualities of this chemical permission for use has been granted against *H. abietis* in the U.K. and Ireland until June 2014 by the Forestry Stewardship Council (2008).

1.5.3 Biological control of the *Hylobius abietis*

Both EPN and EPF have been applied in the field to try to control weevil populations. The EPN families Steinernematidae and Heterorhabditidae have both been effective in parasitizing the large pine weevil. A preliminary field study in the UK in 1994 showed that *S. carpocapsae* reduced weevil populations by 70 %. But when the nematodes were applied to late larvae instar control was recorded at 50 %, when larvae had pupated control reach 96 % (Brixey 1997). *Steinernema carpocapsae, S. feltiae* and *H. megidis* were tested in field trials in 2000. The *Steinernema* spp. gave a control of only 53 – 56 % and *H. megidis* was significantly lower (Torr *et al.* 2006). A range of nematodes were tested against pine weevil larvae over a two year period. *Heterorhabditis downesi* was found to control numbers of pine weevils to an economically viable level (Dillon *et al.* 2006). Further research showed that the application of *H. downesi* on tree stumps does not affect the main parasitoid of the pine weevil, *Bracon hylobii,* and found that the interaction between the nematodes and the parasitoid were additive (Dillon *et al.* 2007; Dillon *et al.* 2008). A study investigating the potential of *B. hylobii* as a potential biocontrol agent determined it could have a significant contribution in helping suppress weevil populations (Henry & Day 2001). The use of *M. anisopliae* and *B. bassiana* has been recorded to give successful mortality of the pine weevil and their associated metabolites did not pose a threat to animal or human health nor cause environmental problems (Malinowski 2009). *Metarhizium anisopliae* and *B. bassiana* have been shown to be pathogenic to all life stages of *H. abietis* and could potentially be used as a biopesticide for this insect (Ansari & Butt 2012). Field trials using EPN and EPF separately and in combination are currently being conducted at NUI Maynooth, Ireland (Williams *et al.* unpublished).

1.6 The Black Vine Weevil – a horticultural pest

The black vine weevil, *Otiorhynchus sulcatus* (Coleoptera; Curculionidae), is a major horticultural pest and infests more than 70 plant species across Europe (Smith 1932). The eggs are laid in the soil and when larvae emerge they migrate towards the root

system, feed on the roots and can kill the plant (Cross & Burgess 1997). The adults also cause damage but not to the same extent as larvae; they emerge from the soil and begin to feed on the leaves and flowers causing cosmetic damage (Moorhouse *et al.* 1992). Adults are all parthenogenetic females and each can lay up to 600 eggs which can lead to huge economic loss of crops (Cross & Burgess 1997; Moorhouse *et al.* 1992). It has been suggested that the adult males died during the last ice age in Europe (Moorhouse *et al.* 1992).

1.6.1 *Otiorhynchus sulcatus* life cycle

Otiorhynchus sulcatus go through one generation a year (Moorhouse *et al.* 1992). A new generation of adults emerge from overwintering sites in summer. Once adults emerge they begin feeding on vegetation and feeding occurs at night (Moorhouse *et al.* 1992). When temperatures increase egg laying begins in July and can last until October, but it usually ends in September (Smith 1930). Hatching is dependent on temperature and humidity, eggs can take between 8 days and 56 days to hatch (Lola-Luz *et al.* 2005; Shanks & Finnigan 1973). Larvae initially feed on finer roots. As larvae grow bigger they feed on the larger roots systems and go through 6 or 7 instars (Smith 1930). *Otiorhynchus sulcatus* takes abound 3 or 7 months to grow from egg to late instar stage when reared indoors or outdoors, respectively (La Lone & Clarke 1981). Larvae pupate in the soil close to the roots if temperatures are suitable (Stenseth 1979). *Otiorhynchus sulcatus* overwinter either as pupae or larvae; survival is dependent on temperature and the stage to which they have developed before hibernating (Moorhouse *et al.* 1992). They emerge as adults in early summer and begin to feed on the new leaves and flowers.

1.6.2 Synthetic control of *Otiorhynchus sulcatus*

DDT was the insecticide used in the early 1950s to control *O. sulcatus* until it was taken off the market in the 1970s. Controlled released chlorpyrifos was found to give promising control when incorporated into module compost but results were poor when incorporated directly into soil (Cross & Burgess 1997). Carbosulfan was found to be an effective control for the 3rd instar larvae (Masaki *et al.* 1999). Other pesticides which are commonly used to control *O. sulcatus* include imidacloprid, chlorpyrifos and bifenthrin (Shah *et al.* 2007). However, these pesticides are not ideal as they pose a threat to

human health and the environment and pests can become tolerant of them (Van Emden & Service 2004).

1.6.3 Biological control of *Otiorhynchus sulcatus*

As described above *O. sulcatus* overwinter as larvae, and sometimes pupae. Pesticides are usually applied when soil temperatures are low hence the pesticide being applied needs to work efficiently at low temperatures (Shanks *et al.* 1990). The EPNs *S. kraussei* and *S. feltiae* can give effective control of *O. sulcatus* in experiments with low temperatures, which suggests they could be potentially used when weevils are overwintering (Long *et al.* 2000; Willmott *et al.* 2002). *S. carpocapsae* is not as effective in controlling *O. sulcatus* as *H. megidis, H. downesi* or *H, bacteriophora* (Willmott *et al.* 2002). *Heterorhabditis megidis* caused 93 % mortality of larvae in strawberry grow bags, while *H. downesi* only caused 51 % mortality (Lola-Luz & Downes 2007). *Heterorhabditis bacteriophora* showed a reduction of populations by 90 – 100 % when applied to weevils in pots (Gill *et al.* 2001). Both of the EPFs *B. bassiana* and *M. anisopliae* are pathogenic to *O. sulcatus* (Bruck 2004; Bruck & Donahue 2007; Bruck *et al.* 2005) and *M. anisopliae* is now commercially available to control the weevil. A combination of EPF and EPN has also been shown to be effective at controlling these weevils. Ansari *et al.* (2008, 2009) showed a combination of *M. anisopliae* with either *H. bacteriophora*, *S. feltiae* or *S. kraussei* gave a higher control rate then when the agents were applied individually.

1.7 Project Objectives

The potential interactions between EPN and EPF against *H. abietis* have not been previously studied, Ansari *et al*. (2004, 2008, 2009) have studied the interactions against *O. sulcatus* in both laboratory and greenhouse experiments. In this current study, this work was repeated to establish if the results could be replicated in laboratory experiments at NUI Maynooth, Ireland, and also to investigate potential novel interactions using *H. abetis* as host. The specific aims of the project are as follows:

- Investigate what interactions occur when either *S. feltiae* or *H. bacteriophora* are combined with *M. anisopliae* using the black vine weevil as host in laboratory conditions, revisiting previous work conducted by Ansari *et al.* (2008).

- Investigate what interactions occur when either *S. carpocapsae*, *H. bacteriophora* or *H. downesi* are combined with either *M. anisopliae* or *B. bassiana* using the large pine weevil as host in laboratory conditions.
- Investigate a proposed mechanism for synergy (Ansari *et al.* 2008) that EPN are more attracted to EPF infected insects.
- Determine which combination of EPN and EPF would give best synergistic results.
- Evaluate which pathogen causes the death of the insect during dual infections.
- Investigate the interactions and control of selected agents (based on laboratory experiments) against the large pine weevil in field conditions.

Chapter 2 – Methods and Materials

2.1 Source and culturing nematodes and fungi

2.1.1 Source of nematodes

Steinernema carpocapsae (Weiser) (Millenium), *Heterorhabditis bacteriophora* (Poinar) (Nemasys G) and *Steinernema feltiae* (Stanuszek) (Nemasys) were sourced from Becker-Underwood, Littlehampton, U.K. The *Heterorhabditis downesi* (Stock, Griffin and Burnell) strain used was K122, obtained from a stock culture at NUI Maynooth, originally isolated by Dr Christine Griffin from a sandy costal site in Co. Wexford, Ireland.

2.1.2 Culturing of nematodes

All nematode strains were cultured through *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae (section 2.2.1). The lid and base of a 9 cm diameter Petri dish (Greiner Bio-one) was lined with 9 cm filter paper (Fisherbrand). One thousand infective juveniles (IJ) were applied to both filter papers in 1 ml tap water and ten *G. mellonella* were added to the dish and incubated at 20˚C. *Steinernema* spp. were left for 7-9 days and *Heterorhabditis* spp. were left for 10-13 days before being put onto a White trap (Kaya & Stock 1997). A White trap consisted of a 15 cm diameter Petri dish with a lid of a 5 cm diameter Petri dish placed in the centre, this was covered by a 9 cm filter paper and tap water was added. When IJs emerged they were harvested every two days and washed three times in 500 ml of tap water. Suspensions were stored in 25 - 30 ml of tap water at a concentration of 1000 IJ/ml and stored at 9˚C. Nematodes were re-cultured every 8-12 weeks.

2.1.3 Source of fungi

Beauveria bassiana (Bals.) Vuill. (strains: BUEXP 1504, BUEXP 1694) and *Metarhizium anisopliae* (Metsch.) Sorokin. (stains BUEXP 1501, BUEXP 1502, BUEXP 1503) were sourced from Becker-Underwood, Littlehampton, U.K. A commercial product of *Metarhizium anisopliae* (F52), Met52, was obtained from National Agrochemical Distributors, Lusk, Dublin. Met52 is produced by Novozyme and spores are grown on rice grains, when Met52 was used in experiments the spores were sieved off the rice grains. Germination of fungus was tested by plating spores on

Sabouraud Dextrose agar (SDA) or Potato Dextrose agar (PDA). A low concentration of spores (about $1x10^1$–$1x10^2$ spores/ml) was made up in 0.03 % (v/v) Tween 80 (Sigma-Aldrich). The plates were then sealed with parafilm and left to germinate at 20˚C for 12-16 hr. Germination was determined by counting 200 spores on the plate and the percentage of spores with hyphal growth was calculated (Shah *et al.* 2009). If sporulation was below 85 % a new batch of fungus was obtained from the producer. Fungal spores were stored at 4˚C.

2.2 Source and culturing of insect larvae

2.2.1 *Galleria mellonella* (The greater wax moth)

Galleria mellonella larvae were obtained from Livefoods Direct Ltd., Sheffield, U.K. They were stored at 15˚C in a box of wood shavings (supplied by the producer) for a maximum of 4 weeks.

2.2.2 *Hylobius abietis* (Large pine weevil) L. (Coleoptera: Curculionidae)

Hylobius abietis larvae were collected from Scots pine (*Pinus sylvestris*) and Sitka spruce (*Picea sitchensis*) stumps in plantations which had been felled 12-18 months previously. The locations of the plantations were: Kilduff, Co. Westmeath; Hortland, Co. Kildare; Kildalkey Co. Meath; Rossnagad, Co. Laoise. The soil was removed from around the stump and the bark was removed using a chisel. The larvae were collected and put into 24-well cell culture plates (24-well plates) (Costar). The collected larvae were kept out of direct sun light. Lightly moistened paper towel was placed in the lid of the 24-well plate and the larvae were stored at 4˚C for a maximum of 4 weeks.

2.2.3 *Otiorhynchus sulcatus* (Black vine weevil) F. (Coleoptera: Curculionidae)

Otiorhynchus sulcatus larvae were reared in a greenhouse at Teagasc Research Centre, Kinsealy, Dublin. Adult *O. sulcatus* were kept in a box (25 cm x 17 cm x 8 cm) at room temperature. The lid contained air holes (5 cm diameter) for ventilation and the box was kept out of direct sunlight. A food source consisting of some small branches of yew (*Taxus baccata*) was replaced weekly and eggs were collected weekly. The eggs (90 eggs/plant) were added to a strawberry plant (bare rooted runners, flower pot size: 16

cm x 15 cm) and covered with a thin layer of compost. The plants were kept in a greenhouse for 12 weeks (August 2011- November 2011) and watered weekly until the larvae reached $6^{th}/7^{th}$ instar.

2.3 *Galleria mellonella* experiments

2.3.1 The effect of moisture content on nematode movement though compost to invade *Galleria mellonella*

A single *G. mellonella* larva was added to a 30 ml medicine cup (Sarstedt) and 15 ml Westland Garden Health multi-purpose compost was added on top. Fifty IJ of *S. carpocapsae* or *H. bacteriophora* were added to the compost in three different volumes: 0.5 ml, 1 ml and 2 ml. The moisture content of the compost was 63 %, 66 % and 73 % respectively. Control cups received 2 ml of tap water and its moisture content was 73 %. The cups were covered with a lid with two 6 mm diameter holes. They were then stored at 20˚C for four days. The *G. mellonella* were removed from the soil, washed with tap water and stored at 20˚C for two days in a 5 cm diameter Petri dish lined with filter paper. The larvae were dissected to count the number of nematodes that had invaded. There was a total of ten cups per treatment. The moisture content for each treatment was determined by adding 1 ml, 2 ml or 4 ml of tap water to 30 ml of compost and leaving it to dry in an oven at 100˚C until all the moisture had evaporated (24 hr). The compost was weighed before putting it into the oven (wet weight) and weighed at 8hr intervals until the compost had lost all its moisture (i.e. stopped losing weight). Moisture content was calculated using the formula:

$$\text{Moisture content \%} = \frac{\text{Wet weight of compost} - \text{Dry weight of compost}}{\text{Wet weight of compost}} \times 100$$

2.3.2 Effect of *Metarhizium anisopliae* infection on invasion of *Galleria mellonella* by nematodes 1-5 days after exposure to fungus

Galleria mellonella larvae were dipped in a spore suspension of *Metarhizium anisopliae* F52 ($2.6 - 4.7 \times 10^6$ spores) (Table 2.1) made up in 0.03 % (v/v) Tween 80; control larvae were dipped in 0.03 % (v/v) Tween 80 only. Five larvae were dipped together in

10 ml solution for 10 s, and then transferred to a Buchner funnel lined with filter paper to remove excess liquid by suction. Ten insects per dish were put into a 9 cm Petri dish lined with 9 cm filter paper and stored at 20˚C for 24 hr, 48 hr, 72 hr, 96 hr or 120 hr, to allow fungal infection and growth. After each fungal growth period ten larvae from the fungus and ten from the control treatment were removed and each larva was placed individually in a plastic mesh cage (1.5 cm x 1.5 cm). The cage was then inserted into the bottom of a 30 ml cup (diameter of base: 2.5 cm, diameter of top: 3.5 cm, length: 4 cm) and 15 ml of Westland Garden Health multi-purpose compost was added on top. *Steinernema carpocapsae* or *Heterorhabditis bacteriophora* were pipetted on top of the compost in 1 ml tap water at a concentration of 150 IJ/ml or 200 IJ/ml respectively. Lids were placed on top with one pinhole for air. The cups were placed in random order on a tray and incubated at 20˚C. After 22 hr the larvae were removed from the containers and washed with tap water. They were then individually put in a 5 cm Petri dish lined with a moistened 5 cm filter paper and incubated for 3 or 4 days at 20˚C. The larvae were dissected and the number of nematodes in each cadaver was counted. Ten larvae from the fungus treatment and ten from the control treatment were kept in individual 5 cm Petri dishes lined with moistened filter paper at 20˚C without exposure to nematodes and the day they died was recorded. There were four experiments; two were conducted using *H. bacteriophora* and *S. carpocapsae* and two were conducted with *S. carpocapsae*. In the first experiment (Experiment 1) IJ were added 72 hr and 120 hr after the larvae were dipped in *M. anisopliae* F52 or Tween 80. In the other three experiments (Experiment 2, Experiment 3, Experiment 4) IJ were added 24 hr, 48 hr or 72 hr after exposure to *M. anisopliae* or Tween 80 and Experiment 4 included a fourth day (Table 2.1). The moisture content of the compost was determined before the experiment as described in section 2.3.1.

Table 2.1 Details of four experiments conducted to investigate if *Galleria mellonella* are more attractive to nematode invasion after different time periods of *Metarhizium anisopliae* infection.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Date	10.6.2011	4.7.2011	29.8.2011	17.10.2011
Spores/ml	4.7×10^6	4.2×10^6	4.5×10^6	2.6×10^6
Nematode species	*Steinernema carpocapsae* *Heterorhabditis bacteriophora*	*Steinernema carpocapsae* *Heterorhabditis bacteriophora*	*Steinernema carpocapsae*	*Steinernema carpocapsae*
Day IJ added	3, 5	1,2,3	1,2,3	1,2,3,4
Fungus sporulation	89 %	89 %	89 %	89 %
Moisture content	70 %	68 %	69 %	66 %

Fungal spores were suspended in 0.03 % (v/v) of Tween 80. Infective juveniles (IJ) were suspended in tap water and added to the assay 1, 2, 3, 4 or 5 days after exposure to fungus. The larvae were exposed to IJ for 22 hr.

2.4 *Hylobius abietis* experiments

Assay design for *Hylobius abietis* was based on an assay designed by Ansari *et al.* (2008), for *Otiorhynchus sulcatus* larvae experiments.

2.4.1a Effect of sterile and non-sterile bark (Scots pine), a food source for *Hylobius abietis*, on larval mortality

One *H. abietis* larva was added to a 30 ml cup and exposed to six treatments (N = 40 larvae). Compost was added in a volume of 15 ml (Shamrock Irish moss peat) and a chunk of Scots pine bark (1 cm x 1 cm x 0.25 cm) weighed 0.5 g. The treatments were as follows:

- Six sheets of filter paper (diameter 2.5 cm)
- Compost only
- Fed with bark for one day before the trial and compost added on day of trail
- Bark and compost
- Pasteurised bark (70˚C for 30 min) and compost
- Autoclaved bark and compost

The pasteurised bark and autoclaved bark were left to cool before adding to the cups and lids of all cups had one pinhole for ventilation. The cups were placed in random order on a tray and incubated at 20˚C for 15 days. All larvae were collected from Kilduff, Co. Meath and stored for four days at 4˚C before use. Moisture content (71 %) was determined as described in section 2.3.1. The experiment was conducted for 15 days.

2.4.1b Effect of bark (Scots pine) structure and ventilation type on mortality of *Hylobius abietis* larvae

Assays were as described in Section 2.4.1a except for the changes described for each treatment (N = 20 larvae):

- Bark, compost and pinhole in the lid.
- Ground up bark, compost and pinhole in the lid
- Compost only and a pinhole in the lid
- Compost only and two 6 mm holes in the lid

The cups were placed in random order on a tray and incubated at 20˚C for 28 days. All larvae were collected from Hortland, Co Kildare and stored 7 days at 4˚C before use. Moisture content (72 %) was determined as described in section 2.3.1.

2.4.2 Dose response and interaction of nematodes and fungus using *Hylobius abietis* larvae as a host.

Late instar *Hylobius abietis* larvae were placed individually into a 30 ml cup. For fungus only and interaction assays the fungal spores were made up in a suspension using 0.03 % (v/v) Tween 80 solution. Fungal suspension (1 ml for fungus only and 0.5 ml for interaction) was added to 15 ml Shamrock Irish moss peat compost and mixed 8 times in a 30 ml cup before adding on top of the larvae. All fungal concentrations were made up using a series of tenfold dilution (Table 2.2, 2.4). Nematode concentrations for nematode only and interaction assays were made up in 1 ml (nematode only) or 0.5 ml (interaction study) of tap water and added on top of the compost (Table 2.3, 2.4). Cups with nematode only treatments contained compost with no spores. In interaction assays nematodes were added on days 0, 7 and 14 and if nematodes were not being added to a cup, water was added to maintain moisture conditions. Bark (0.5 g) from Scots pine

trees was used in some experiments (Table 2.2, 2.3, 2.4). Controls received tap water (nematode), 0.03 % (v/v) Tween 80 (fungus) or both (interaction) only. In interaction experiments compost in nematode only treatments received 0.5 ml of 0.03 % (v/v) Tween 80 and fungus only treatments received 0.5 ml tap water. Mortality was either checked every second day/ third day or weekly.

Assays were also conducted in a similar manner but using 24-well plates. Fungal treatments were added as dry spores into the compost. This was done by adding the dry spores to bulk compost and mixing it by moving the compost from one pile to another ensuring to take the compost from the bottom of the pile. This mixing was repeated ten times to give a fungal concentration per ml of compost (2.5 ml/well). Nematode concentrations were added in 100 µl of tap water; controls and fungus only treatments received 100 µl of tap water. In interaction assays nematodes were added on days 0, 7 and 14. If nematodes were not being added to a well 100 µl of tap water was added to maintain moisture conditions. Mortality was checked weekly.

In both assay types when a larva was found dead it was removed from the compost and put into a 5 cm Petri dish lined with moistened filter paper and incubated at 20˚C. Larvae from nematode only assays were dissected after 4-7 days to confirm the presence of nematodes. Larvae from control, fungus and interaction assays were left at 20˚C for 10-14 days to allow for any entomopathogenic fungus to sporulate. If fungal growth was not evident after 14 days the larvae were dissected to determine if they were killed by nematodes. If insects were not killed by entomopathogenic nematodes or fungi they were classified as being killed by other causes.

In the tables displayed below (Tables 2.2-2.4) the layout of experiments is indicated. Experiments with the same number indicate the dose response assay conducted for the interaction assay with the same number. The different letter indicates separate experiments.

Table 2.2 Details of three fungus dose response experiments against *H. abietis*.

	Experiment 1a	Experiment 2a	Experiment 5a
Date	1.12.2010	17.1.2011	17.5.2012
Weevil source[a]	Kilduff	Killduff	Kildalkey
Tree species[b]	Scots pine	Scots pine	Scots pine/Spruce
Head capsule size	N/A	N/A	3.10 mm
Container	30 ml cup	30 ml cup	24 well-plate
Fungus strain and concentration[c]	M.a 1501 ($4.7x10^3 – 10^6$) M.a 1502 ($2.7x10^3 – 10^6$) M.a 1503 ($4.7x10^3$- 10^6) B.b 1504 ($2.7x10^3 – 10^6$)	M.a 1503 ($1.4x10^3 – 10^8$) M.a 1504 ($1.7x10^3 – 10^8$)	Met52 ($1.4x10^1$- 10^6)
Bark	Yes	Yes	No
N	7	9	20
Mortality checked[d]	$2^{nd}/3^{rd}$ day	$2^{nd}/3^{rd}$ day	Weekly
Moisture content	74 %	71 %	69 %
Exposure time[e]	20 days	21 days	21 days

[a]Location where the larvae were collected from. [b]The tree species the larvae were collected from. [c]M.a, *Metarhizium anisopliae*; B.b, *Beauveria bassiana*. 1501 etc. indicate the BUEXP strain used. Concentration in spores/ ml compost. Fungus was added in tenfold serial dilution i.e. 10^3, 10^4, 10^5,$10^{6.}$ [d]Interval at which mortality of larvae was checked. [e]Length of the experiment.

Table 2.3 Details of five nematode dose response experiments against *H. abietis*.

	Experiment 1b	Experiment 2a	Experiment 3	Experiment 4a	Experiment 5b
Date	14.12.2010	17.1.2011	10.3.2012	27.3.2012	18.5.2012
Weevil source[a]	Kilduff	Kilduff	Kildalkey	Kildalkey	Rossnagad
Tree species[b]	Scots pine	Scots pine	Scots pine/Spruce	Scots pine/Spruce	Scots pine
Head capsule size	N/A	N/A	2.90 mm	3.05 mm	2.99 mm
Container	30ml cup	30ml cup	30ml cup	24 well-plate	24 well-plate
Nematode species[c]	S.c	S.c	S.c	S.c	H.d
	H.b	H.b	H.b	H.b	
IJ[d]	100, 500, 1000, 5000	50, 100, 250, 500, 1000	1000, 2500, 5000, 7500	5, 10, 25, 50, 100, 200	25, 50, 100, 250, 500, 1000
Bark	Yes	Yes	No	No	No
N	5	9	20	20	20
Mortality checked[e]	$2^{nd}/3^{rd}$ day	$2^{nd}/3^{rd}$ day	Weekly	Weekly	Weekly
Moisture content	75 %	71 %	68 %	66 %	69 %
Exposure time[f]	7	6	7	7	7

[a]Location where the larvae were collected from. [b]The tree species the larvae were collected from. [c]S.c, *Steinernema carpocapsae*; H.b, *Heterorhabditis bacteriophora*; H.d *Heterorhabditis downesi*. [d]Number of infective juveniles (IJ) added in tap water. [e]Interval at which mortality of larvae was checked. [f]Length of the experiment.

Table 2.4 Details of five nematode and fungus interaction experiments against *H. abietis*

	Experiment 1c	Experiment 2c	Experiment 4b	Experiment 4c	Experiment 5c
Date	17.1.2011	26.5.2011	17.4.2012	8.5.2012	8.6.2012
Weevil source[a]	Kilduff	Hortland	Kildalkey	Kildalkey	Rossnagad
Tree species[b]	Scots pine	Scots pine	Scots pine/spruce	Scots pine/spruce	Scots pine
Head capsule size	N/A	N/A	3.00 mm	3.02 mm	2.98 mm
Container	30ml cup	30ml cup	24 well-plate	24 well-plate	24 well-plate
Fungus strain and concentration[c]	M.a 1503 ($1.4x10^5$) B.b 1504 ($1.7x10^5$)	B.b 1504 ($1.3x10^5$)	B.b 1694 ($1.7x10^5$, 10^6)	B.b 1694 ($1.7x10^4$, $6.3x10^3$)	Met52 ($1.3x10^4$) BUEXP 1694 ($6.3x10^3$)
Nematode[d]	S.c H.b	H.b	H.b	S.c	H.d
IJ[e]	100	70	70, 200	50, 300	25, 100
Bark	Yes	No	No	No	No
N	9	30	20	20	20
Day ij added[f]	0	0,7,14	0,7	0,7,14	0,7
Mortality checked[g]	$2^{nd}/3^{rd}$ day	Weekly	Weekly	Weekly	Weekly
Moisture content[h]	71 %	67 %	67 %	73 %	74 %
Exposure time[i]	21	21	14	21	14

[a]Location where the larvae were collected from. [b]The tree species the larvae were collected from. [c]M.a, *Metarhizium anisopliae*; B.b, *Beauveria bassiana*. 1503 and 1504 indicate the BUEXP strain used. Concentration in spores/ ml compost. [d]S.c, *Steinernema carpocapsae*; H.b, *Heterorhabditis bacteriophora.* [e]Number of infective juveniles (IJ) added in tap water. [f]Day after exposure to fungus IJ were added. [g]Interval at which mortality of larvae was checked. [h]Length of the experiment.

2.4.3 Nematode and fungus single and combined application against *Hylobius abietis* at a field site, Rossnagad, Co. Laois.

Nematode and fungal treatments were applied to Scots pine stumps which had been felled 18 months previously. The field site was divided into 20 blocks with six treatments each applied to one stump in each block. The treatments were as follows: *H. downesi* $3.5x10^6$ IJ/stump (full dose), *H. downesi* $1.75x10^6$ IJ/stump (half dose), *B. bassiana* (BUEXP 1694) $1x10^9$ spores/stump (full dose), *B. bassiana* (BUEXP 1694) $5x10^8$ spores/stump (half dose), a combination of half dose of both *H. downesi* with *B. bassiana*, and a control with tap water. Stumps for each treatment were colour coded before application of treatments. Treatments were applied on 6.06.2012. Nematodes were applied in tap water and the fungus was applied in 0.03 % (v/v) Tween 80 suspension. Treatments were made up in 5 L volumes in the laboratory and transferred to the site ensuring the nematode suspensions were regularly mixed. A 500 ml aliquot was applied around the perimeter of the stump. Ten blocks were destructively assessed four weeks after application. This involved removing the bark off one quarter of the stumps diameter (including at least one root) and as deep as the last larva was found (max depth 20-25 cm). All weevils (dead or alive) were collected in 24 well-plates. The lid of a plate was lined with moistened tissue paper and plates were stored at 20˚C for two weeks. After two weeks any fungal growth or nematode infection was recorded.

The fifth week after the application of treatments emergence traps (Moore 2001) were erected around the other ten blocks to collect adults emerging from the stumps. Briefly, the traps consisted of four steel rods for support which were inserted into a plastic cap and positioned over the tree stump. A white plastic 1000 ml bottle with a funnel in the lid was placed underneath the plastic cap. This was then covered with a plastic mesh around the entire stump. The mesh was securely fastened to the plastic lid using a cable tie and soil was dug up and placed around the end of the mesh on the ground (Fig 2.1). Traps were emptied every three weeks.

Fig 2.1 Emergence traps used to collect emerging *Hylobius abietis* adults.

2.4.4 Identification of *Beauveria* spp. cultured from *Hylobius abietis*, collected at the Rossnagad field experiment

Fungus from any *H. abietis* larvae which had abundant fungal growth two weeks after recovering from stumps in the field trial were identified by extraction digest of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA)

Culturing fungi

All procedures were carried out using aseptic techniques. Fungi were cultured on 9 cm Potato Dextrose Agar (PDA) plates (Oxoid Ltd, England, U.K.). Cellophane discs (Caroline Packaging Ltd., England, U.K.) (cut to 9 cm diameter) were covered with a thin layer of distilled water and autoclaved. The cellophane discs were allowed to cool before being placed onto the PDA plates, so that they stuck to the agar (Reay *et al.* 2008). An insect with fungal growth was then placed in the centre of each cellophane disc. There were eleven samples in total taken from different stumps and one known *B. bassiana* sample as a control. The plates were sealed with parafilm and incubated at 20˚C for five days to allow hyphal growth.

PCR protocol

After sufficient hyphal growth had occurred (3 cm diameter around insect cadaver) it was peeled off the cellophane disc (avoiding any contaminated areas) and placed in a 1.5 ml eppendorf tube. To break up the fungal cell walls the samples were exposed to freeze and thaw action. The cells of the hyphae were broken down further using a plastic pestle (Sigma-Aldrich Co. LLC) (separate pestles were used for each sample). Qiagen DNEasy Mini Plant Kit and protocol was used to extract the DNA. The lysate of

the sample was collected in a 1.5 ml eppendorf and the amount of DNA collected was measured using The Nanodrop 1000 (Mason, Dublin, Ireland). The ITS region of the rDNA repeat unit was used for PCR reactions, as protocols and primers have been published for *Beauveria bassiana* (Glare *et al.* 2008) and *Beauveria bassiana* were determined at NUI Maynooth (C. Harvey, personal communication). A Promega GoTaq Polymerase DNA kit was used to amplify the ITS region in the fungal DNA. HinfI was used to detect a three base pair deletion in *B. caledonica* and compared it to *B. bassiana*. The restriction enzyme BanII was used to detect an eight base pair insertion in *B. caledonica* and this was compared with *B. bassiana* (Glare *et al.* 2008; C. Harvey, personal communication). The final volume for each reaction was 25 µl; each reaction received 22.5 µl of the master mix plus 2.5 µl of the DNA lysate (Table 2.7). The samples were then put through a PCR rotation cycle. The samples were exposed to a range of temperatures and cycles which are listed in Table 2.8. The lid was set to 105˚C.

Table 2.7 List of ingredients and quantities in DNA polymerisation.

Ingredients	µl/ sample	concentration/sample[e]
ddH_2O[a]	10.875	-
Buffer[b]	5	-
$MgCl_2$	2.5	3 mM
dNTP[c]	2	0.2 mM in each dNTP
ITS Primer F[d]	1	2 µM
ITS Primer R[d]	1	2 µM
Taq Polymerase	0.125	U/µl
Total	22.5	

[a]Double distilled water. [b]GoTaq reaction buffer. [c]Deoxyribonucleotide triphosphate – mix of 0.5 µl of each dATP, dCTP, dGTP, dTTP. [d]Internal Transcribed Spacer, F-forward primer, R – reverse primer. [e] mM – millimolar, µM – micromolar, U/µl – Unit/ microliter.

Table 2.8 PCR rotation cycle using Mastercycle Gardient

Step	**Action**	**Cycles**	**Temperature (˚C)**	**Duration (min)**
1	Denaturing	1	94	5
2[a]	Denaturing	40	94	0.5
3	Primer annealing		55	0.5
4	Elongation		72	1
5	Elongation	1	72	5
6	Cooling	1	4	Held until needed

[a]Steps 2 – 4 were repeated 40 times.

Gel electrophoresis of PCR samples

A 1 % (w/v) agarose gel was prepared by adding 1 g of agarose to 100 ml Tris-Acetate-EDTA (TAE) buffer in a 250 ml Duran bottle. The lid was screwed on loosely and the bottle was put into a microwave at a low power for seven minutes to melt the agarose, swirling occasionally. It was then allowed to cool on the bench for 5 – 10 min and 10 µl of SYBRsafe dye was added. The gel was poured onto the casting tray and allowed to cool for 20 min. The gel was covered with 1 x TAE buffer and 5 µl of DNA ladder (mi-100 bp + DNA Marker Go provided by Metabion, Martinsried, Germany) was loaded into the first and last well. The PCR samples, 5 µl, were mixed with 1 µl of 5x DNA loading buffer (Bioline; London, UK) and the samples were loaded in the wells between the ladders. The electrophoresis chamber was run at 100 volts for 30 min. Gels were illuminated and photographed using a Syngene G:Box (Mason).

Restriction Digest

Two restriction enzymes were used; *Ban II* and *Hinf I*. The ingredients for the restriction digest were as follows: 5 µl of PCR product, 2 µl buffer 4, 1 µl enzyme and 12 µl double distilled water (ddH_2O). Samples were vortexed and put through a PCR rotation cycle; 2 cycles at 37˚C for 8 hr to activate the enzyme and 1 cycle at 80 ˚C to deactivate the enzyme; the lid was set to 37˚C. The gel electrophoresis was conducted as described above, except a 4 % agarose gel was used and the electrophoresis chamber was run at 70 volts for 1.75 hr. The gel was viewed as described above.

2.5 *Otiorhynchus sulcatus* experiments

2.5.1 Dose response and interaction of nematodes and fungus using *Otiorhynchus sulcatus* as a host.

The assay design was based on experiments conducted by Ansari *et al.* (2008). Late instar *Otiorhynchus sulcatus* larvae were added to 30 ml cups with an organic carrot disk 1 cm x 1cm (replaced weekly) and 15 ml of compost (Westland garden health multi-purpose). Fungal spores were added to the compost in 0.5 ml 0.03 % (v/v) Tween 80 and mixed (Table 2.9, 2.10). Fungus only treatments and control treatments also received 0.5 ml of tap water. Control and nematode only treatments received 0.5 ml

0.03 % (v/v) Tween mixed into the compost (before nematodes were added). Nematodes were added on top of the compost in 0.5 ml of tap water on days 0, 7 and 14; tap water was added to the cups which did not receive nematode (Table 2.5.1.1 – 2.5.1.2). A lid with two 6 mm diameter holes was placed on top and the holes were cover with a plastic mesh (2 cm x 1 cm). Mortality was checked weekly, dead larvae were removed from the assay and cause of death was determined as described in section 2.4.2.

In the tables below (Tables 2.0 – 2.10) the layout of experiments is indicated. Experiments with the same number indicate the dose response assay conducted from the interaction assay with the same number. The different letter indicates separate experiments.

Table 2.9 Details of one fungus and two nematode dose response experiments using *O. sulcatus* as host.

	Experiment 1a	Experiment 2a	Experiment 2b
Date	26.11.2011	26.11.2011	11.1.2012
Instar	5^{th}	5^{th}	6^{th}
Fungus strain (spores/ml)[a]		M.a ($1x10^4$ - 10^8)	
Nematode (IJ)[b]	S.f (30,60,120)		S.f (30,100) H.b (30,100,250)
N	20	20	10
Exposure time[c]	7	21	7

[a]M.a, *Metarhizium anisopliae* F52. Fungus was made up in tenfold serial dilutions i.e. 10^4, 10^5, 10^6,10^7, 10^8. [b]S.f, *Steinernema feltiae*, H.b, *Heterorhabditis bacteriophora*; IJ Number of infective juveniles added in tap water. [d]Length of the experiment.

Table 2.10 Details of three nematode dose response experiments using *Otiorhynchus sulcatus* as a host

	Experiment 1a	Experiment 2c	Experiment 2d
Date	26.11.2011	20.1.2012	8.3.2012
Instar	6^{th}	6^{th}	$6^{th}/7^{th}$
Fungus strain (spores/ml)[a]	M.a ($1x10^6$)	M.a ($1.7x10^5$)	M.a (1.7×10^5)
Nematode (IJ)[b]	S.f (10)	S.f (100) H.b (10)	S.f (100) H.b (10)
Day IJ added[c]	0,7,14	0,7,14	0,7,14
N	20	20	20
Exposure time[d]	21	21	21

[a]M.a, *Metarhizium anisopliae* F52. [b]S.f, *Steinernema feltiae*, H.b, *Heterorhabditis bacteriophora*; IJ Number of infective juveniles added in tap water. [c]Number of days after exposure to fungus nematodes were added. [d]Length of the experiment.

2.6 Statistics

All statistics were carried out on the statistical software package Minitab 16®. Binomial data were analysed using the chi-squared test. Continuous measure data sets were tested for normality using Kolmogorov – Smirnov. If data were not normal by distribution they were transformed by the square root. Data were tested for equal variance using Levene's test. Data with more than two test groups were tested using a One-way ANOVA or Two-way ANOVA; where $P < 0.05$. Tukeys test was performed to find where the significant differences occurred. When data were not normal treatment medians were compared with a Kruskal-Wallis test, followed by multiple pairwise comparisons using a Mann-Whitney test using Bonferroni 95 % confidence intervals, obtained by calculating $(1 - \alpha / g)$ where α is the level of significance (i.e. $P = 0.05$) and g is the number of pairwise comparisons. Lethal concentrations were calculated using Probit analysis. Tests were significant when $P < 0.05$.

In the interaction experiments when mortality of insects in the untreated controls was 20 % or less, mortality was corrected using Abbot's formula (Abbott 1925) and interactions between agents were calculated using the formula: $M_E = M_{Nem} + M_{Fun}(1 - M_{Nem})$, where M_E is the expected mortality of the combined agents, M_{Nem} is the mortality of the nematode only application and M_{Fun} is the mortality of the fungus only application. A chi-squared test was applied to the expected and the observed with one degree of freedom: $\chi^2 = (M_{Obs} - M_E)^2 / M_E$, where M_{Obs} is the mortality of the nematode and fungus combined treatment. If $\chi^2 < 3.84$ the mortality was additive but if $\chi^2 > 3.84$ there was a significant interaction, either antagonistic or synergistic (Ansari *et al.* 2008). If the observed mortality (M_{Obs}) was less than the expected mortality (M_E) the interaction was antagonistic, but if the observed mortality was greater the interaction was synergistic.

If the control mortality for interaction assays was above 20% the following formula was used: $P_E = P_0 + (1 - P_0)(P_{Nem}) + (1 - P_0)(1 - P_{Nem})(P_{Fun})$, where P_E is the expected combined mortality of the agents, P_0 is the control mortality, P_{Nem} is the mortality of the nematode only application and P_{Fun} is the mortality of the fungus only application. A chi-squared test was applied to the expected and the observed: $\chi^2 = (L_{Obs} - L_E)^2 + (D_{Obs} - D_E)^2$, where L_{Obs} is the number of living larvae observed, L_E is the number of living

larvae expected, D_{Obs} is number of dead larvae observed and D_E is the number of dead larvae expected (Shapiro-Ilan *et al.* 2004).

Chapter 3 - Results

3.1 The effect of moisture content on nematode movement though compost to invade *Galleria mellonella* larvae

The number of *Steinernema carpocapsae* IJ invading *Galleria mellonella* larvae in compost with 63 % moisture content was significantly lower than the numbers invading larvae in compost with moisture contents of 66 % and 73 % (One-way ANOVA: F = 7.41, DF = 2, P = 0.003. Tukeys test $\alpha = 0.05$) (Fig 3.1A). There was no significant difference between the numbers of *Heterorhabditis bacteriophora* IJ invading the *G. mellonella* larvae after moving through compost with moisture contents of 63%, 66% and 73% (One-way ANOVA: F = 2.70, DF = 2, P = 0.085) (Fig 3.1B). However, the trend shown by *H. bacteriophora* is similar to the trend shown by *S. carpocapsae*. There was only one control (with no nematodes) for this experiment and moisture content was 73 %, the mortality in the control was 10 % by the end of the experiment.

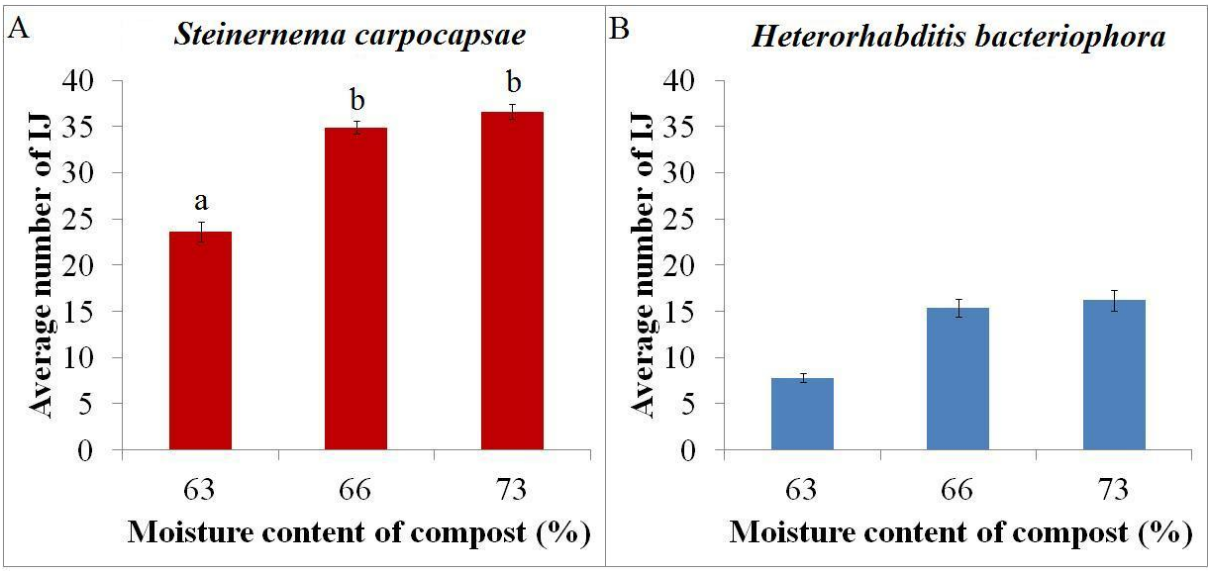

Fig 3.1 Average number of nematodes (50 IJ applied for both species) invading *Galleria mellonella* (N =10) larvae after 4 days in peat moss compost with different soil moisture contents. Error bar represent standard error. Bars with the same letter or no letters are not significantly different from each other (Tukeys test: $\alpha = 0.05$).

3.2 Effects of *Metarhizium anisopliae* infection on invasion of *Galleria mellonella* by nematodes 1-5 days after exposure to fungus

Galleria mellonella larvae were exposed to *Metarhizium anisopliae* or 0.03 % (v/v) Tween 80 for 72 hr and 120 hr in Experiment 1 and for 24 hr, 48 hr and 72 hr in

Experiment 2. They were then exposed to *Heterorhabditis bacteriophora* for 22 hr. In Experiment 1 there was a significant difference between the numbers of IJs infecting larvae exposed to Tween control or fungus after 72 hr (One-way ANOVA: F = 6.61, DF = 1, P = 0.019) and 120 hr (Mann-Whitney test: W = 151, P = 0.0006). More IJs were found to invade the Tween 80 dipped larvae than the *M. anisopliae* dipped larvae (Fig. 3.2 A). All the control (no nematodes added) *M. anisopliae* dipped larvae died by this fungus after twelve days. One of the 0.03 % (v/v) Tween 80 controls (no nematodes added) died during the experiment. In Experiment 2 no significant difference was found in the number of *H. bacteriophora* infecting the larvae between treatment or exposure time (Two-way ANOVA: Exposure time: DF = 2, F = 2.82, P = 0.063; Treatment: DF = 1, F = 3.44, P = 0.069; Interaction: DF = 2, F = 0.963, P = 0.963) (Fig. 3.2b). The trend in Experiment 2 does not follow that in Experiment 1; more IJs invaded the *M. anisopliae* exposed larvae in Experiment 2. All dipped *M. anisopliae* control (no nematodes added) larvae died by this fungus after ten days and all 0.03 % (v/v) Tween 80 control (no nematodes added) larvae survived the duration of the experiment.

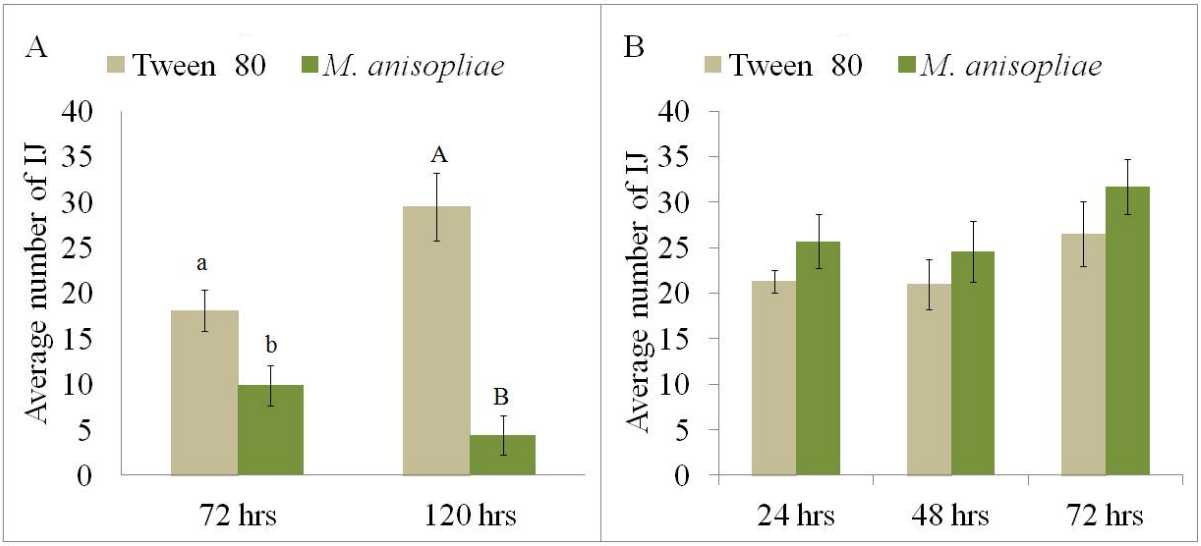

Fig. 3.2 Average number of *Heterorhabditis bacteriophora* (out of 200 IJ applied) invading *Galleria mellonella* (N = 10) after exposure to either 0.03 % (v/v) Tween 80 or *Metarhizium anisopliae.* **A:** Experiment 1: $4.7x10^6$ spores/ml 0.03 % (v/v) Tween 80, **B:** Experiment 2: $4.2\ x10^6$ spores/ml 0.03 % (v/v) Tween 80. Error bar represent standard error. Bars with the same letter or no letter are not significantly different from each other.

Larvae were also exposed to *S. carpocapsae* for 22 hr in Experiments 1 and 2 and two additional experiments were also conducted (Experiment 3 was a repeat of Experiment 2; Experiment 4 larvae were exposed to an additional exposure time 96 hr) (Fig 3.3, 3.4). Exposure time was significant in all experiments (Table 3.1). Although the time

which nematodes were applied is significant there is not a consistent trend across treatments. Only one experiment showed a significant difference between treatments (Experiment 2), and there was also a significant interaction between time and treatment (Table 3.1). More nematodes invaded the *M. anisopliae* dipped larvae than the Tween dipped ones after 48 hr but not after 24 hr or 72 hr (Fig 3.3 B). Control mortality for experiments 1 and 2 are as described in the previous paragraph. In experiments 3 and 4 all control (no nematodes added) *M. anisopliae* larvae died at 9 or 12 days, respectively, after being dipped and all the 0.03 % (v/v) Tween 80 control (no nematodes added) survived the duration of the experiment.

Table 3.1 Two-way ANOVA results for the number of *S. carpocapsae* invading *G. mellonella* exposed to *M. anisopliae* or Tween 80 after different time periods.

		DF	F	P
S.c Exp 1	Exposure time	1	6.58	**0.015**
	Treatment	1	2.28	0.140
	Interaction	1	0.00	0.962
S.c Exp 2	Exposure time	2	20.75	**0.000**
	Treatment	1	6.75	**0.017**
	Interaction	2	5.25	**0.009**
S.c Exp 3	Exposure time	2	13.94	**0.000**
	Treatment	1	0.66	**0.419**
	Interaction	2	0.77	0.469
S.c Exp 4	Exposure time	3	18.85	**0.000**
	Treatment	1	1.98	0.163
	Interaction	3	1.94	0.131

S.c = *Steinernema carpocapsae*. Exp = Experiment.

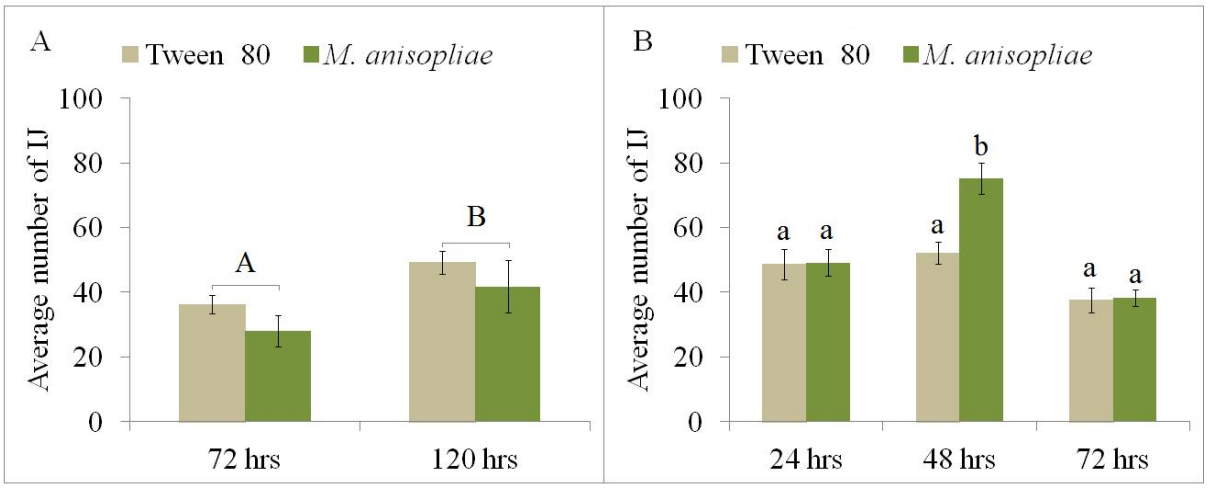

Fig 3.3 Average number of *Steinernema carpocapsae* (out of 150 IJ applied) invading *Galleria mellonella* (N = 10) after exposure to either 0.03 % (v/v) Tween or *Metarhizium anisopliae.* **A:** Experiment 1: 4.7×10^6 spores/ml 0.03 % (v/v) Tween 80, **B:** Experiment 2: 4.2×10^6 spores/ml 0.03 % (v/v) Tween 80. Error bar represent

standard error. Bars and groups with the same letter are not significantly different from each other.

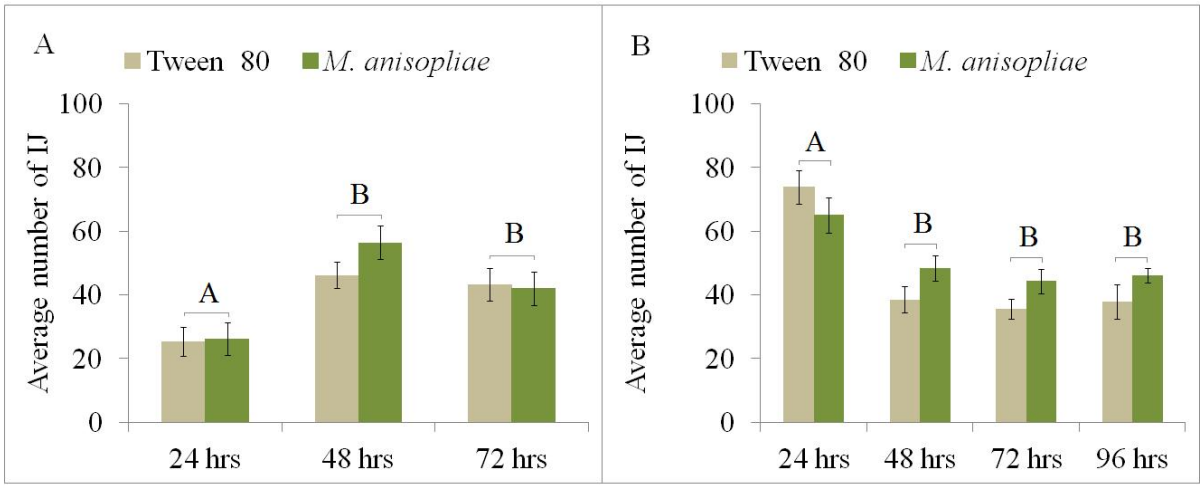

Fig 3.4 Average number of *Steinernema carpocapsae* (out of 150 IJ applied) invading *Galleria mellonella* (N = 10) after exposure to either 0.03 % (v/v) Tween or *Metarhizium anisopliae.* **A:** Experiment 3: $4.5x10^6$ spores/ml 0.03 % (v/v) Tween 80, **B:** Experiment 4: $2.6x10^6$ spores/ml 0.03 % (v/v) Tween 80. Error bar represent standard error. Bars and groups with the same letter are not significantly different from each other.

3.3a Effect of sterile and of non-sterile bark (Scots pine), a food source for *Hylobius abietis,* on mortality (larvae from Kilduff, Co. Offaly)

Hylobius abietis larvae were exposed to different treatments in order to determine if food source was affecting natural mortality. The larvae were exposed to six different treatments in 30 ml cups for 15 days and percentage mortality was recorded (Table 3.2). A chi-squared test showed no significant difference between treatments (Chi-sq = 5.059, DF = 5, P = 0.409). When larvae were exposed to filter paper only the lowest mortality, 25 %, was recorded. The highest mortality, 42.5 %, was recorded when larvae were fed 24 hr before the trial and exposed to compost during the trial. Larvae exposed to compost with pasteurised or autoclaved bark both resulted in 27.5 % mortality. The compost only treatment had a mortality of 30 % and the treatment with compost and bark recorded a mortality of 35 %.

Table 3.2 *Hylobius abietis* larvae exposed to different treatments and mortality recorded after 15 days.

Treatment	Filter paper[a]	Fed before trial[b]	Compost[c]	Bark[d]	No. dead	Mortality %
1	✓	-	-	-	10	25
2	-	✓	✓	-	17	42.5
3	-	-	✓	-	12	30
4	-	-	✓	✓	14	35
5	-	-	✓	Pasteurised	11	27.5
6	-	-	✓	Autoclaved	11	27.5

[a]Six 2.5 cm filter paper were added to a 30 ml cup. [b]Larvae were fed for 24 hr before the trial. [c]Compost was added to a 30 ml cup. [d]0.5 g of bark (ca. 1 cm x 1 cm) was added to a 30 ml cup; in treatment 5 the bark was pasteurised and in treatment 6 it was autoclaved. The tick marks indicate it was added to the cup. N = 40.

3.3b Effect of bark (Scots pine) presence and structure and of ventilation type on mortality of *Hylobius abietis* larvae from Hortland, Co. Kildare

Larvae were exposed to four different treatments in 30 ml cups for 22 days to determine if bark or moisture content had an effect on mortality (Table 3.3). A chi-squared test showed the difference between treatments was close to significant (Chi-squared test: Chi-sq = 7.386, DF = 3, P = 0.061). When the lowest mortality (treatment three), 10 %, was compared to the highest mortality (treatment four), 45 %, a significant difference was found (Chi-squared test: Chi-sq = 6.144, DF = 1, P = 0.013). The number of larvae dead in treatments one and two resulted in the same mortality of 20 %.

Table 3.3 *Hylobius abietis* larvae exposed to different treatment and mortality recorded after 22 days

Treatment	Compost[a]	Bark[b]	Ventilation[c]	No. dead	% Mortality
1	✓	Ground	Pin hole	4	20
2	✓	Chunk	Pin hole	4	20
3	✓	-	2x6mm holes	2	10
4	✓	-	Pin hole	9	45

[a]Tick indicate compost was added to the cups. [b]Ground indicates 0.5 g of bark was ground up in a coffee grinder and added to the cups, Chunk indicates 0.5g of a 1 cm x 1 cm chunk of bark was added. [c]The type of hole that was added to the lid for ventilation.

3.4.1 Dose response and interaction of nematodes and fungus using *Hylobius abietis* larvae as a host

The concentrations of entomopathogens to use in interaction experiments were determined from dose response experiments. In the first fungus dose response experiment, Experiment 1a, larvae were exposed to three strains of *M. anisopliae* and

one strain of *B. bassiana* for 20 days. *M. anisopliae* 1503 gave a clear dose response allowing a LC_{50} to be calculated, $2.6x10^6$ spores/ml compost. The other strains of *M. anisopliae* did not follow an expected dose response pattern and an LC_{50} could not be calculated (Fig 3.5 A). The three highest concentrations for *M. anisopliae* 1501 killed fewer larvae than the lowest concentration. The lowest concentration of *M. anisopliae* 1502 killed more larvae than the second lowest concentration. Therefore *M. anisopliae* 1503 was used in the interaction experiments. *B. bassiana* also did not show a definite dose response; three of the concentrations killed 29 % and the second highest concentration had the lowest mortality. However as it was the only *B. bassiana* strain it was used in the interaction experiment; the highest concentration which had a mortality of less than 50 %, $2.7x10^6$ spores/ml compost, was used for the interaction assay (Fig 3.5 A). In all the *M. anisopliae* treatments a number of the larvae were killed by a *Beauveria* sp.; 1/7 larvae in each of six treatments and 2/7 larvae in each of three treatments. In the four *B. bassiana* treatments all four had 1/7 larvae killed by a *Beauveria* sp. and three had an additional 1/7 killed by other causes. The untreated control had 1/7 larvae killed by a *Metarhizium* sp. (Fig 3.5 A).

The larvae in Experiment 1b were exposed to *S. carpocapsae* and *H. bacteriophora* for seven days. The nematode concentrations for both species were too high to estimate an LC_{50}. *Steinernema carpocapsae* at a concentration of 1000 IJ/30 ml cup resulted in a mortality of 80 %, while 5000 and 500 resulted in 100 % mortality. All concentrations above 500 IJ/30 ml cup in the *H. bacteriophora* treatments resulted in 100 % mortality. Therefore the concentration which resulted in a mortality of less than 50 % was used: 100 IJ/30 ml cup, for both *S. carpocapsae* and *H. bacteriophora* (Fig 3.5 B). The untreated control mortality was 0 %. Dead insects in all treatments were killed by the nematodes applied (Fig 3.5 B).

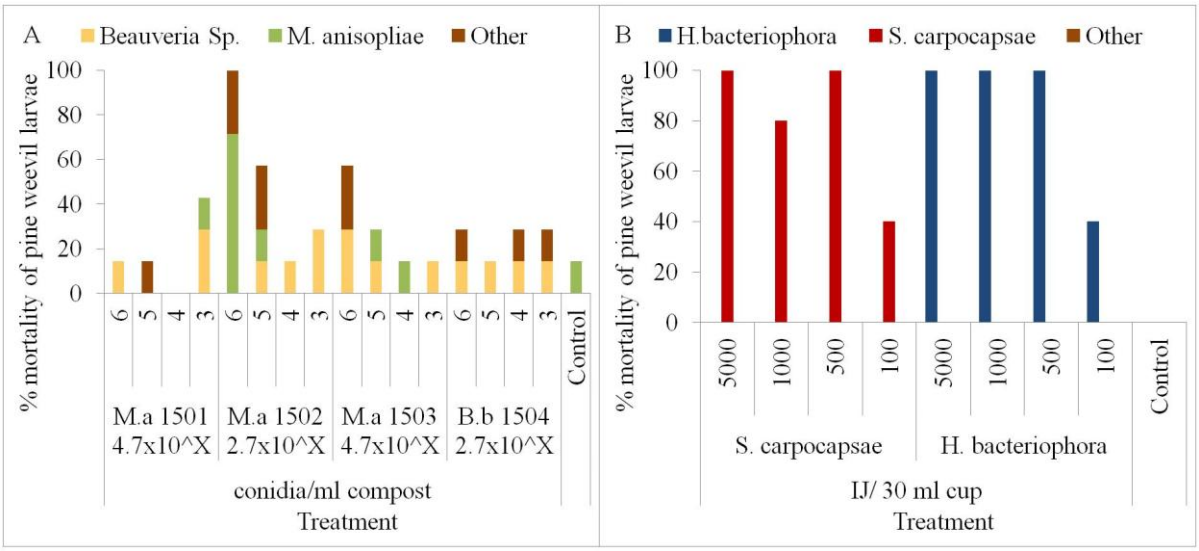

Fig 3.5 Cause of *H. abietis* larvae mortality. M.a, *Metarhizium anisopliae*; B.b, *Beauveria bassiana*. **A:** Cause of death after being exposed to three strains of *M. anisopliae* and one strain of *B. bassiana* for 20 days at different concentrations in a 30 ml cup. The number 1501 etc. indicate the BUEXP strain of the fungus used. The figures on the x-axis indicate the power the dose is raised to i.e. $4.7x10^{6}$etc. N = 7. **B:** Cause of death after being exposed to *S. carpocapsae* and *H. bacteriophora* for 7 days at different concentrations in a 30 ml cup. N = 7.

In the interaction experiment, Experiment 1c, larvae were exposed to single and combined treatments of EPF and EPN for 17 days. Synergy was recorded between *M. anisopliae* and *H. bacteriophora* (Table 3.4). The combination of *B. bassiana* with *S. carpocapsae* and *M. anisopliae* with *S. carpocapsae* gave no interaction while a combination of *B. bassiana* and *H. bacteriophora* gave antagonistic results (Table 3.4, Fig 3.6). In the nematode only treatments all larvae were killed by the applied nematodes. Death in the fungus only treatments was mainly caused by the applied EPF: *B. bassiana* 3/9 insects, *M. anisopliae* 1/9 insects. In the combined treatments the majority of kill was caused by the applied nematodes. Only 1/9 insects were killed by a

Table 3.2 Interactions observed when combining EPN with EPF using *Hylobius abietis* as host.

Exp[a]	Fungus Species[b]	spores/ml compost	Nem Species[c]	IJ/insect	Interval (day)	O M[d]	E M[e]	χ^2	Interaction[f]	Duration (day)
1c	B.b	1.7x10^5	S.c	100	0	67	79	31.89	Antagonistic	17
1c	B.b	1.7x10^5	H.b	100	0	78	84	2.68	Additive	17
1c	M.a	1.4x10^5	S.c	100	0	67	67	0	Additive	17
1c	M.a	1.4x10^5	H.b	100	0	100	74	35.14	Synergistic	17
2c	B.b	1.3x10^5	H.b	70	0	70	79	4.88	Antagonistic	9
2c	B.b	1.3x10^5	H.b	70	7	40	54	7.89	Antagonistic	9
4b	B.b	1.7x10^5	H.b	70	0	35	5	20	Synergistic	7
4b	B.b	1.7x10^5	H.b	200	0	15	25	4	Synergistic	7
4b	B.b	1.7x10^6	H.b	70	0	0	15	14.5	Antagonistic	7
4b	B.b	1.7x10^6	H.b	200	0	20	32	32.5	Antagonistic	7
4c	B.b	5.2x10^3	S.c	300	0	75	54	8.64	Synergistic	14
4c	B.b	5.2x10^3	S.c	300	7	44	54	1.69	Additive	14
4c	B.b	5.2x10^3	S.c	50	0	44	50	0.67	Additive	14
4c	B.b	5.2x10^3	S.c	50	7	31	42	2.75	Additive	14
4c	B.b	2.5x10^3	S.c	300	0	44	44	0	Additive	14
4c	B.b	2.5x10^3	S.c	300	7	69	44	14.57	Synergistic	14
4c	B.b	2.5x10^3	S.c	50	0	13	39	17.56	Antagonistic	14
4c	B.b	2.5x10^3	S.c	50	7	25	30	0.69	Additive	14
5c	B.b	2.3x10^3	Hd	100	0	61	32	25.45	Synergistic	7
5c	B.b	2.3x10^3	Hd	25	0	28	22	1.65	Additive	7
5c	M.a	1.8x10^3	Hd	100	0	17	36	9.97	Antagonistic	7
5c	M.a	1.8x10^3	Hd	25	0	22	26	0.65	Additive	7

[a]Exp, Experiment. [b]B.b, *Beauveria bassiana*; M.a, *Metarhizium anisopliae*. [c]S.c, *Steinernema carpocapsae*; H.b, *Heterorhabditis bacteriophora*, H.d, *Heterorhabditisdownesi.* [d]O.M, Observed Mortality (%) of twenty replicates in one trial. [e]E.M, Expected Mortality (%) = $P_0 + (1 - P_0)(P_1) + (1 - P_0)(1 - P_1)(P_2)$ where P_1 is the mortality of the EPF applied alone, P_2 is the mortality of the EPN applied alone and P_0 is control mortality (Shapiro-Ilan *et al.* 2004) (Experiments 1c and 2c). Experiments 4b-5c were calculated using: Expected Mortality (%) =Mn + Mf(1-Mn) where Mn is the mortality of the nematodes and Mf is the mortality of the fungus. All values were corrected using Abbots formula (Ansari *et al.* 2008). [f]Interactions are based on the χ^2 ratio between the expected and observed mortality.

Beauveria spp. in the combined treatment of *H. bacteriophora* and *B. bassiana.* The mortality recorded in the untreated control was due to other causes (Fig 3.6).

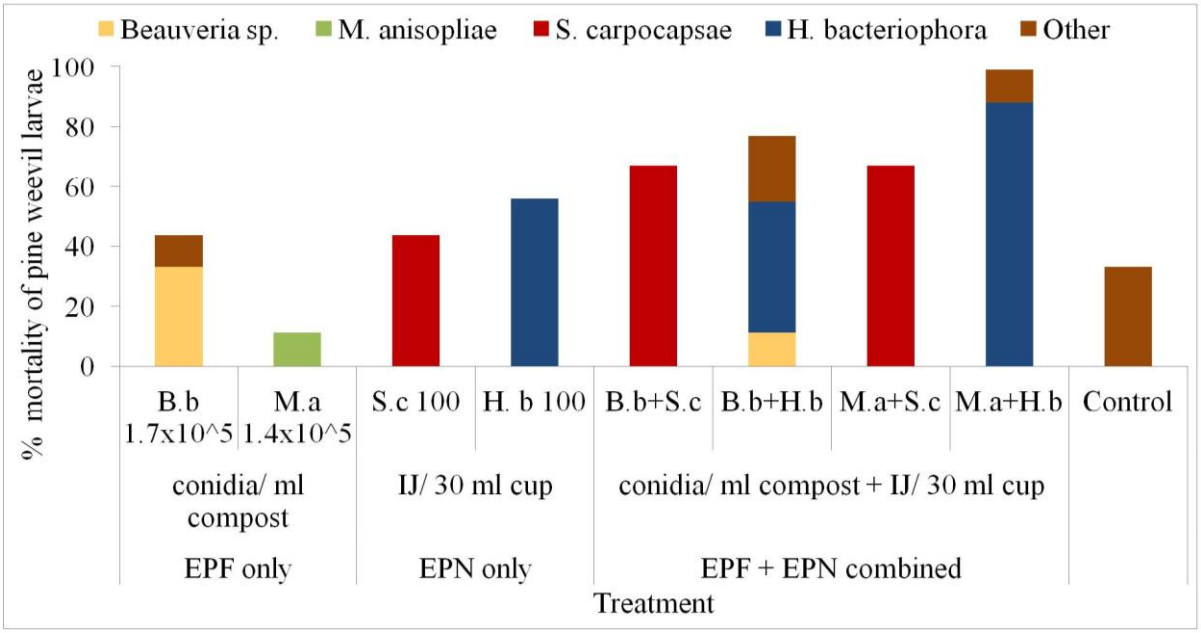

Fig 3.6 Experiment 1c cause of *H. abietis* larvae mortality after 17 days exposure to single and combined concentrations of EPF and EPN in a 30 ml cup. M.a, *Metarhizium anisopliae*; B.b, *Beauveria bassiana;* S.c, *Steinernema carpocapsae*; H.b, *Heterorhabditis bacteriophora.* N = 9.

Experiment 2 consisted of dose response assays with *M. anisopliae* BUEXP 1503 and *B. bassiana* BUEXP 1504 (Experiment 2a) and *S. carpocapsae* and *H. bacteriophora* (Experiment 2b) and an interaction assay with *B. bassiana* and *H. bacteriophora* (Experiment 2c). The LC_{50} for *M. anisopliae* and *B. bassiana* were $8.2x10^5$ and $1.9x10^5$ spores/ml compost respectively (Fig 3.7 A). The mortality of the nematodes did not follow a clear dose response and a dose which gave less than 50 % was chosen based on the results in Fig 3.7 B; *H. bacteriophora* 70 IJ/30 ml cup. The cause of death in the *M. anisopliae* treatments was mainly caused by this applied EPF, expect one insect killed by a *Beauveria* sp. and one killed from other causes. The *B. bassiana* treated larvae were mainly killed by a *Beauveria* sp. except 1/9 larvae were killed by other causes in each of five treatments. The mortality recorded in the untreated control for Experiment 2a was a result of other causes, 4/9 insects (Fig 3.7 A). The larvae which were exposed to *S. carpocapsae* were all killed by this EPN. Most of the larvae exposed to *H. bacteriophora* were killed by this EPN except in two treatments (500 IJ and 50 IJ) where 1/9 larvae in each treatment was killed by other causes. The untreated control mortality in Experiment 2b was a result of other causes, 1/9 insects (Fig 3.7 B).

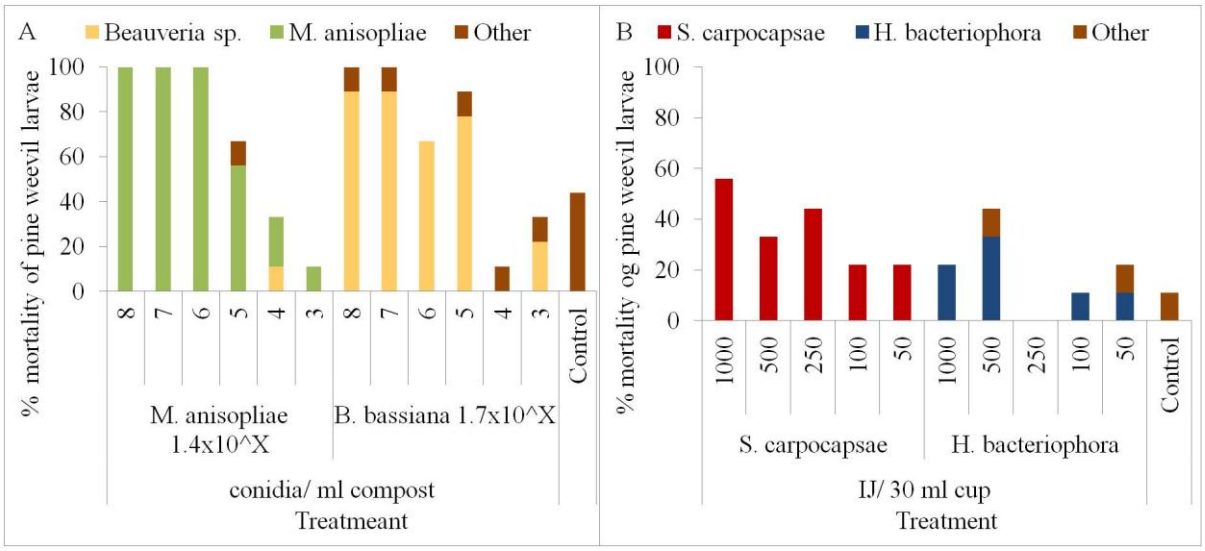

Fig 3.7 Cause of *H. abietis* larvae mortality. **A:** Experiment 2a the cause of death after being exposed to *M. anisopliae* and *B. bassiana* for 21 days at different concentrations in a 30 ml cup. The figures on the x-axis indicate the power the dose is raised to i.e. $4.7x10^6$etc. N = 9. **B:** Experiment 2b the cause of death after being exposed to *S. carpocapsae* and *H. bacteriophora* for 7 days at different concentrations in a 30 ml cup. N = 9.

In the interaction experiment (Experiment 2c) *B. bassiana* and *H. bacteriophora* were used for the interaction. These two entomopathogens were chosen as *Beauveria caledonica* has been found to be a native pathogen of *H. abietis* in Ireland (Glare *et al.* 2008), the EPF possess a similar life cycle to *B. bassiana* and *H. bacteriophora* combined with EPN has already been shown to give synergistic interactions against the vine weevil (Ansari *et al.* 2008). *Beauveria bassiana* was added to the assays on day 0 and *H. bacteriophora* was added on day 0 and day 7. The interaction which was recorded was antagonistic after nine days exposure for both time points when the nematodes were added (Table 3.2, Fig 3.8). In the nematode only treatments and the day zero combination treatment mortality was caused mainly by the applied nematode. In the day zero nematode only treatment one insect was killed by a *Metarhizium* sp. and in the day zero combination treatment 5/30 insects were killed by a *Beauveria* spp. In the fungus only treatment and the day seven combination treatment larval mortality was mainly caused by a *Beauveria* spp. Control mortality was due to other causes (Fig 3.8).

Experiment 3 was an EPN dose response assay exposing larvae to *S. carpocapsae* and *H. bacteriophora* for 7 days. In this assay the larvae were exceptionally resilient to nematode infection even when exposed to high concentrations of nematodes (Fig 3.9). Larvae exposed to 7500 IJ of *S. carpocapsae* and *H. bacteriophora* reached 50 % and 55 % mortality respectively. All larval mortality in the treatments was caused by the

applied EPN. The mortality in the control treatment was a result of other causes. As the larval mortality only reached 50 % when exposed to extremely high doses of IJ the assay style was changed from 30 ml cups to a close contact assay in 24-well plates.

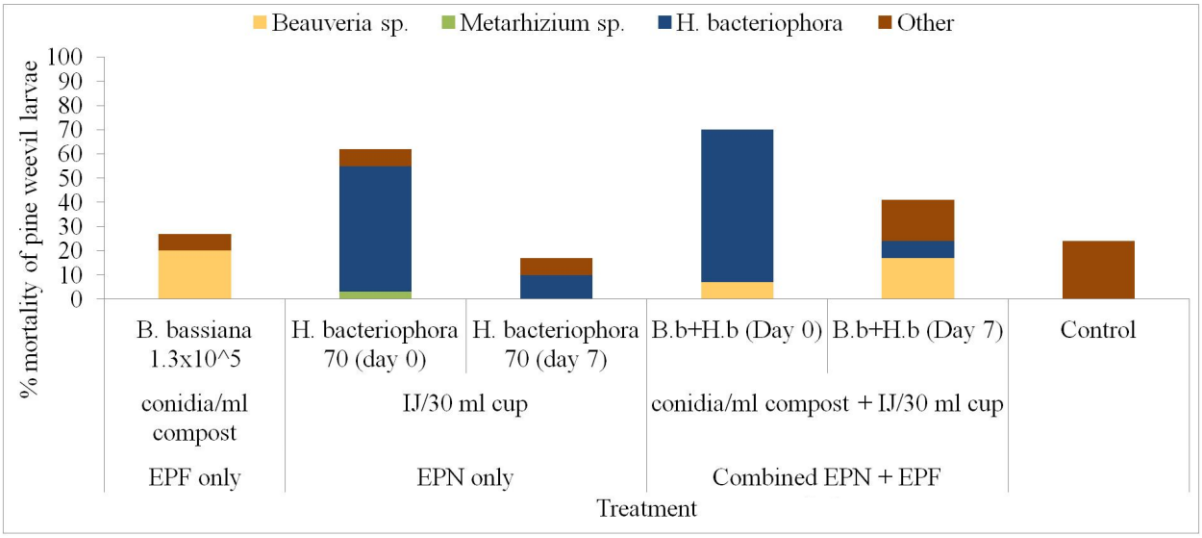

Fig 3.8 Cause of *H. abietis* larvae mortality after 9 days exposure to single and combined concentrations of *Beauveria bassiana* and *Heterorhabditis bacteriophora* in a 30 ml cup. B.b, *Beauveria bassiana;* H.b, *Heterorhabditis bacteriophora.* The day in the brackets indicate the day the IJ were added. N = 30.

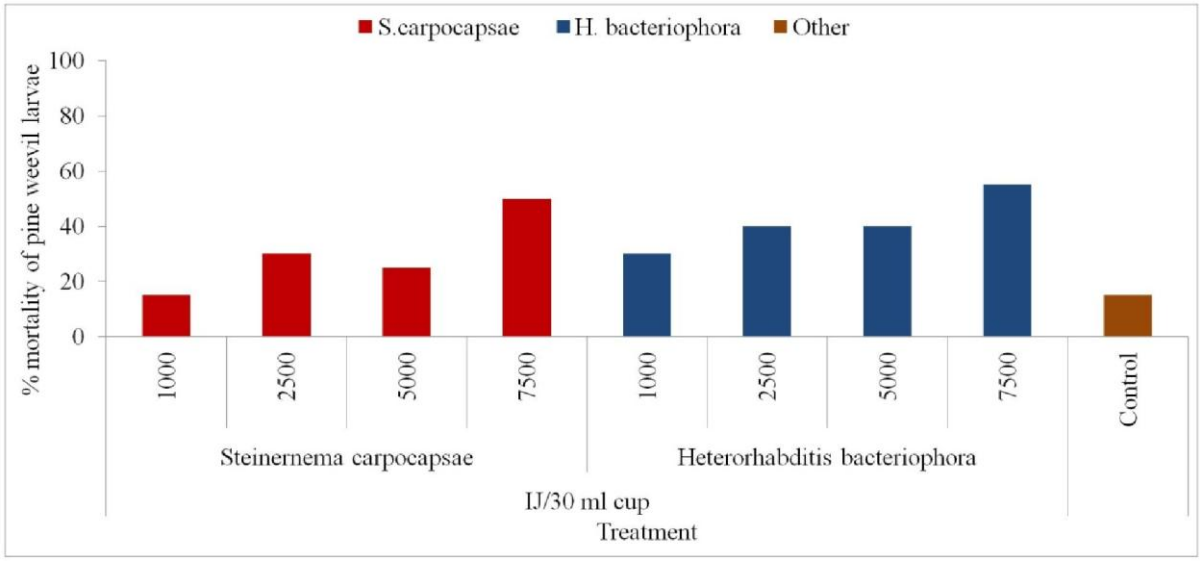

Fig 3.9 Experiment 3 the cause of *H. abietis* larvae mortality after being exposed to *S. carpocapsae* and *H. bacteriophora* for 7 days at different concentrations in a 30 ml cup. N = 20.

Experiment 4a was a dose response assay using a 24-well plate exposing larvae to *S. carpocapsae* and *H. bacteriophora*. There was no clear dose response for either nematode species, although mortality did increase slightly with an increase in IJ concentration (Fig 3.10). The highest concentration for both species resulted in less than

50 % mortality; 200 *S. carpocapsae* IJ killed 20 % and 200 *H. bacteriophora* IJ killed 30 % of insect larvae. The nematode concentrations for the interaction assays (Experiment 4b and 4c) were based on these values; Experiment 4b: *H. bacteriophora* 70 IJ/well and 200 IJ/well, Experiment 4c: *S. carpocapsae* 50 IJ/well and 300 IJ/well. The cause of death in the *S. carpocapsae* treatments was mainly due to the applied EPN except in the 50 IJ treatment where 2/20 insects were killed by a *Beauveria* sp. and 1/20 insects were killed by other causes. In the *H. bacteriophora* treatments most of the mortality was caused by the applied EPN. There was no mortality recorded in the untreated control (Fig 3.10).

The fungus which was used in the interaction experiments was *B. bassiana* BUEXP 1694 and the concentration was determined from previous experiments; Experiment 4b: $1.7x10^5$ and $1.7x10^6$ spores/ml compost, Experiment 4c: $1.3x10^4$ and $6.5x10^3$ spores/ml compost.

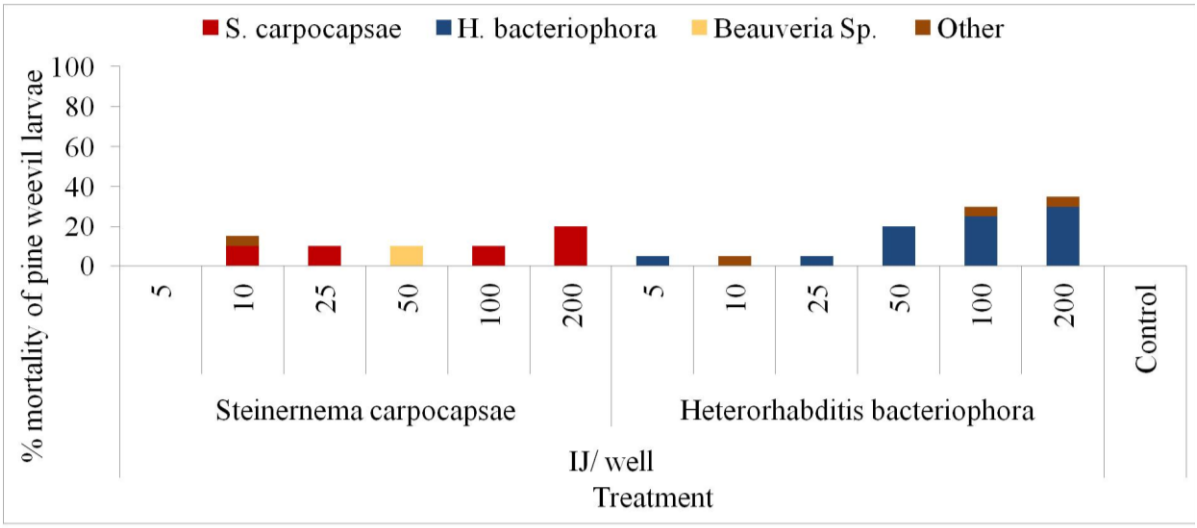

Fig 3.10 Experiment 4a the cause of *H. abietis* larvae mortality. Cause of death after being exposed to *S. carpocapsae* or *H. bacteriophora* for 7 days at different concentrations in a 24-well plate. N = 20.

Experiment 4b was an interaction experiment using two concentrations of *B. bassiana* with two concentrations of *H. bacteriophora*; both pathogens were added on the same day, day 0. The application of *B. bassiana* at the low dose and *H. bacteriophora* gave synergistic results at both concentrations (Table 3.2). However, when the higher concentration of *B. bassiana* was combined with both concentrations of *H. bacteriophora* antagonism was found (Table 3.2). The mortality in the three treatments with a high concentration of nematodes was mainly caused by the applied nematode. A *Beauveria* sp. caused the mortality in the high fungal treatment, 1/20 insects, the low

fungus treatment, 2/20 insects, and the combined treatment with low concentrations of fungus and nematodes, 1/20 insects. There was no larval death in the following treatments: Low fungus concentration, the combination of low nematode and high fungus concentration and the untreated control (Fig 3.11).

Experiment 4c was an interaction experiment using a combination of two concentrations of *B. bassiana* and two concentrations of *S. carpocapsae*; the fungus was added on day 0 and the nematodes were added on days 0 and 7. The high concentrations of both *B. bassiana* and *S. carpocapsae* gave synergistic results when added together on day zero (Table 3.2). When larvae were exposed to the low dose of *B. bassiana* for seven days before adding the high dose of *S. carpocapsae,* synergism was found (Table 3.2). The low dose of both *B. bassiana* and *S. carpocapsae* added on day zero gave antagonistic results (Table 3.2). All other combinations gave additive effects (Table 3.2). In nine out of twelve of the treatments where *S. carpocapsae* was applied the majority of the mortality was caused by this EPN. There was mortality caused by a *Beauveria* sp. in all treatments except the low concentration of *S. carpocapsae* added on day zero. Mortality as a result of other causes was recorded in all treatments except three *S. carpocapsae* single applications (Fig 3.12).

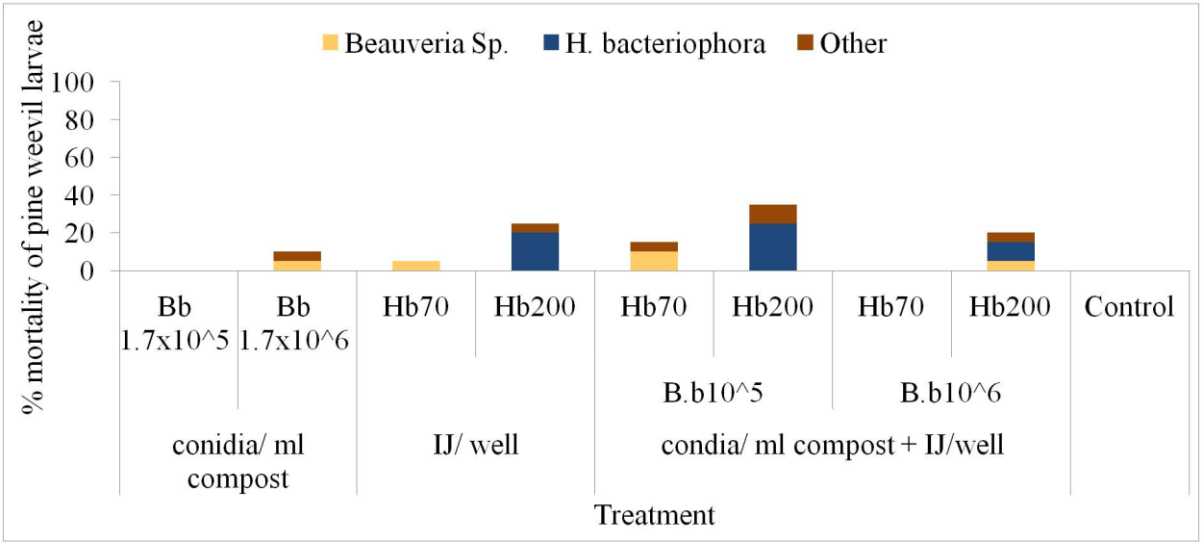

Fig 3.11 Experiment 4b the cause of death of *Hylobius abietis* larvae exposed to single and combined doses of *Heterorhabditis bacteriophora* (H.b) and *Beauveria bassiana* (B.b) for 7 days in a 24-well plate. Nematodes were added to the assay on day 0 along with the fungus. N= 20.

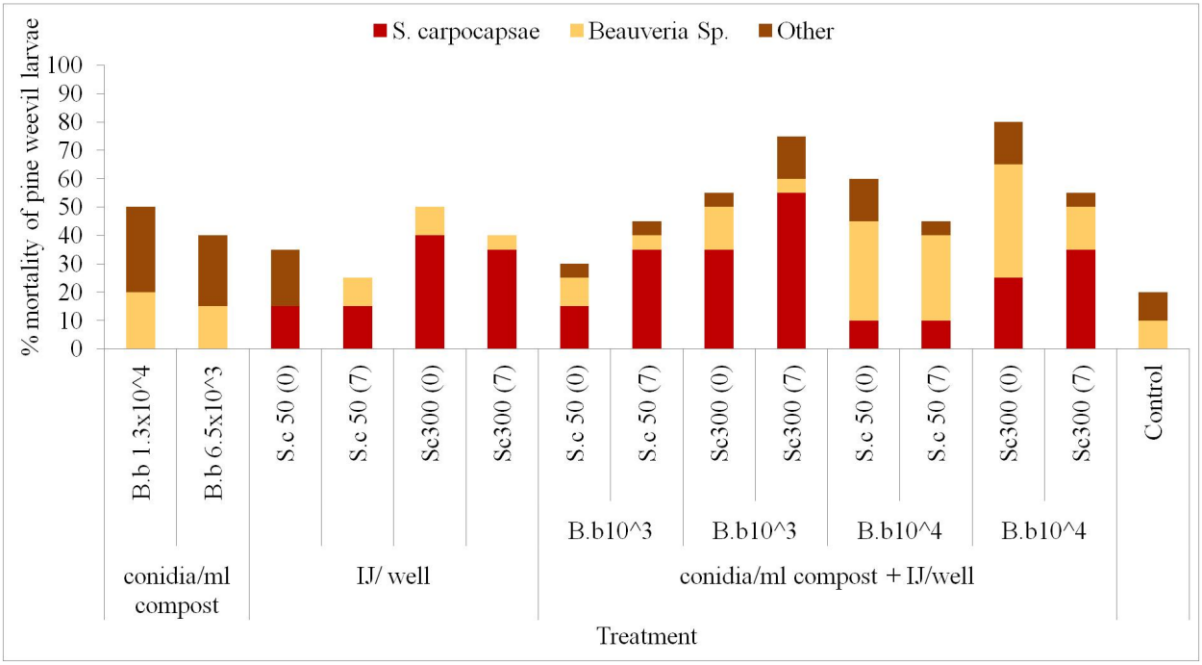

Fig 3.12 Experiment 4c the cause of death of *Hylobius abietis* larvae exposed to single and combined doses of *Steinernema carpocapsae* (S.c) and *Beauveria bassiana* (B.b) for 14 days in a 24-well plate. The figure in brackets indicates the day that the nematodes were added to the assay. All fungus was added on day 0. N= 20.

Experiment 5a was a dose response assay using a commercial strain of *M. anisopliae*, Met52, in 24-well plates. The LC_{50} for *M. anisopliae* was $2.3x10^3$ spores/ml compost (Fig 3.13 A). Larval mortality was mainly due to *M. anisopliae* except for the three lower concentrations where 3/20 insects were killed by a *Beauveria* sp. and in the second highest value it killed 1/20 insects. A *Beauveria* sp. also killed 2/20 insects in the control treatment. Other causes resulted in death of 1/20 insects in five of the treatments. In Experiment 5b a dose response for *H. downesi* was conducted in 24 well-plates. The LC_{50} calculated for *H. downesi* was 251 IJ/well (Fig 3.13 B). All mortality was caused by the applied EPN and there was no control mortality. The concentration for *B. bassiana* used in the interaction experiment (Experiment 5c) was determined from previous experiments.

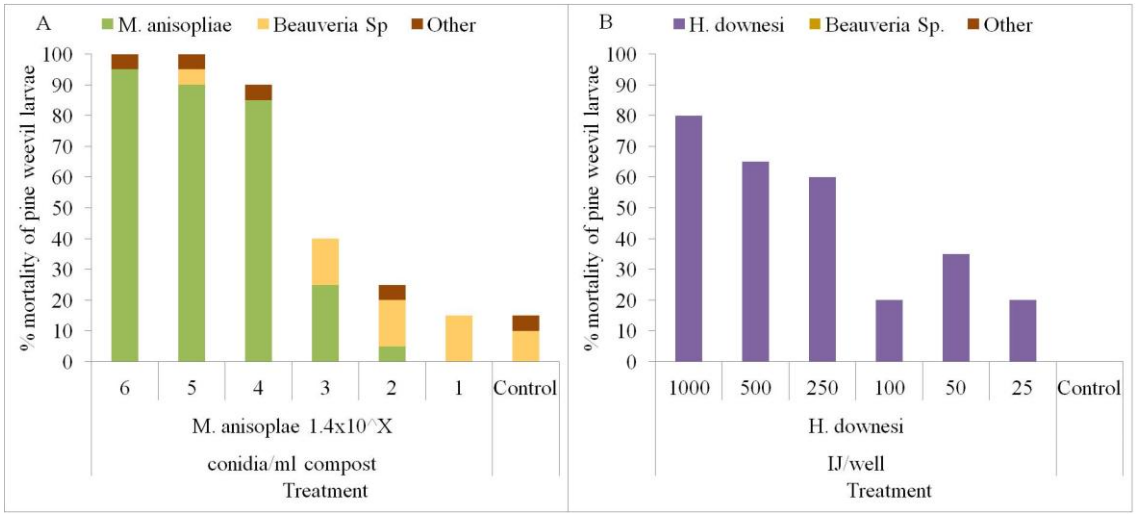

Fig 3.13 Cause of *Hylobius abietis* larvae mortality. **A:** Experiment 5a the mortality of larvae after exposed to different concentrations of *Metarhizium anisopliae* (Met52) for 14 days in 24-well plates. The figures on the x-axis indicate the power the dose is raised to i.e. $1.4x10^{6}$etc. spores/ml compost. N = 20. **B:** Experiment 5b the mortality of larvae after exposure to *Heterorhabditis downesi* for 7 days in 24-well plates. N = 20.

In Experiment 5c larvae were exposed to single or combined (EPN with EPF) applications of two concentrations of *H. downesi* and single concentrations of *M. anisopliae* and *B. bassiana* (Fig 3.14). The combination of *B. bassiana* ($2.3x10^{3}$ spores/ml compost) and a high dose of *H. downesi* (100 IJ/well) gave a synergistic interaction (Table 3.2). *M. anisopliae* ($1.8x10^{3}$ spores/ml compost) combined with the high dose of *H. downesi* (100 IJ/well) showed an antagonistic interaction (Table 3.2). When a low dose of *H. downesi* (25 IJ/well) was combined with either *B. bassiana* or *M. anisopliae* additive results were recorded (Table 3.2). In all treatments where *H. downesi* was applied the majority of the mortality was caused by this EPN. Some mortality in the *H. downesi* mortality was a result of other causes; 1/20 insects in both the *H. downesi* single applications, 2/20 insects in the *B.* bassiana and low concentration of *H.* downesi and 1/20 insects in the combination of *M. anisopliae* with the low concentration of *H. downesi.* All mortalities that occurred in the fungal only applications were not a result of the experimentally applied pathogen; 3/20 for *B. bassiana* and 4/20 for *M. anisopliae* (Fig 3.14). The untreated control had 2/20 insects killed by other causes (Fig 3.14).

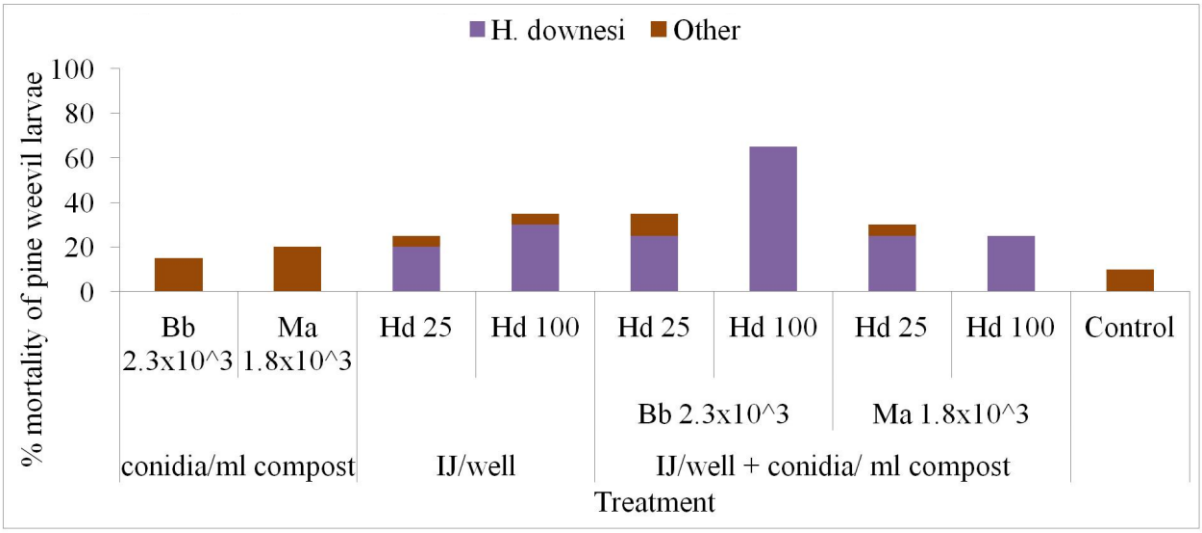

Fig 3.12 Experiment 5a the cause of death of *Hylobius abietis* larvae exposed to single and combined doses of *Steinernema carpocapsae* (S.c), *Beauveria bassiana* (B.b) and *Metarhizium anisopliae* (M.a) for 7 days in a 24-well plate. Nematodes were added to the assay on day 0 along with the fungus. N= 20.

3.4.2 Nematode and fungus single and combined application against *Hylobius abietis* at a field site, Rossnagad, Co. Laois

Mortality of developing weevils in stumps

Pine stumps were treated with single and combined applications of *B. bassiana* BUEXP 1694 and *H. downesi*. All larvae found were collected and it was recorded if they were alive, killed by an entomopathogen or killed by other causes (Table 3.3). There was a significant difference of the number of alive insects amongst the different treatments (Chi-squared test: χ^2 = 228.478, DF = 5, P = 0.000) but no significant difference was found among the total number of insects found dead between blocks (Kruskal-Wallis test: P = 0.658) however there was a significant difference in the numbers dead between treatments (Kruskal-Wallis test: P = 0.000). The average number of dead weevils in the treatments which contained *H. downesi* was significantly different from the fungus only treatments and the untreated control. More dead weevils were recovered from the *H. downesi* treated stumps. The fungus only treatments were also significantly different from the untreated control; the fungus only treatments recorded more dead insects. The average number of dead weevils in the *H. downesi* only treatments (full dose and half dose) and the combination of *H. downesi* with *B. bassiana* were significantly different to the mortality in control (Fig 3.13). There was a significant difference in the number of dead larvae found in the half *H.* downesi concentration compared to the full *B.*

bassiana concentration (Fig. 3.13). The mortality recorded in the combined half concentrations of *H. downesi* and *B. bassiana* (BUEXP 1964) was found to be additive (Observed Mortality: 58 %, Expected Mortality: 60%, $\chi^2 = 0.07$).

Table 3.3 Total numbers of *Hylobius abietis* larvae alive and cause of death, collected from ten pine stumps treated with H.d, *Heterorhabditis downesi*, B.b *Beauveria bassiana* or untreated control. Full and half concentrations see Fig 3.13 for explanation.

Cause of death	Control	1/2 H.d	Full H.d	1/2 B.b	Full B.b	1/2 H.d+1/2 B.b
H. downesi	0	65	67	0	0	54
Beauveria sp.	4	8	5	21	16	2
Hd+*Beauveria* sp.	0	0	0	0	0	2
Other cause	2	17	5	10	10	9
Total dead	6	90	77	31	26	67
Total alive	143	71	48	164	151	49
Total collected	149	161	125	195	177	116
% Dead	4	56	62	16	15	58

Table 3.4 Treatments which are significantly different from each other using a Mann-Whitney test with Bonferroni 95% confidence intervals $(1 - \alpha / g)$.

	Half Hd	Full Hd	Half Bb	Full Bb	1/2 Hd + 1/2 Bb
Control	W = 58 **P = 0.0004**	W = 63 **P = 0.0017**	W = 65 P = 0.0028	W = 83 P = 0.1041	W = 66 **P = 0.0036**
Half Hd	-	W = 111.5 P = 0.6501	W = 138.5 **P = 0.0126**	W = 139 **P = 0.0113**	W = 115 P = 0.4727
Full Hd	-	-	W = 129.5 P = 0.696	W = 132.5 P = 0.413	W = 109 P = 0.7913
Half Bb	-	-	-	W = 116.5 P = 0.4057	W = 84 P = 0.1212
Full Bb	-	-	-	-	W = 80.5 P = 0.0696

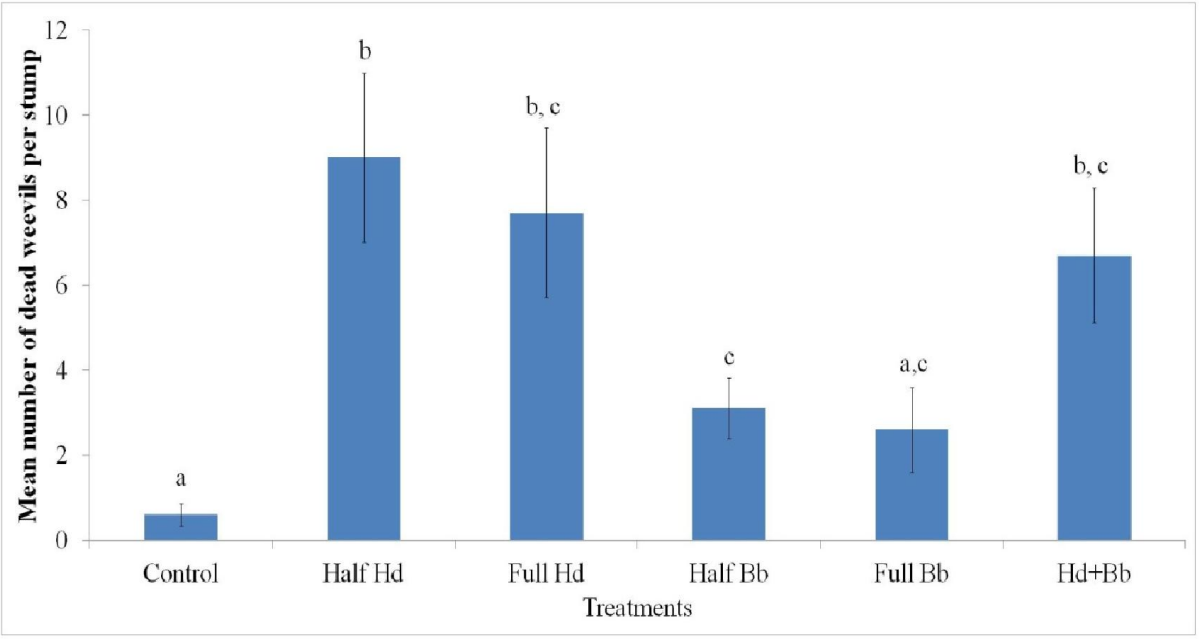

Fig 3.13 *Hylobius abietis* mean numbers dead per stump after 28 days exposure to *Heterorhabditis downesi* (H.d) and/or *Beauveria bassiana* (B.b), in pine stumps (N = 10) in randomised blocks. Half H.d = 1.7 $x10^6$ IJ/stump, Full H.d = $3.5x10^6$ IJ/stump, Half B.b = $5x10^8$, Full B.b = $1x10^9$. Error bars indicate the standard error. Bars with different letters are significantly different. Mann-Whitney test with Bonferroni 95% confidence intervals ($1 - \alpha / g$).

Emergence trap

Emergence traps were erected around Scots pine stumps in ten blocks treated with single and combined applications of *B. bassiana* BUEXP 1694 and *H. downesi*. Adults were collected every two weeks for eight weeks after putting up the emergence tents. The number of adults emerging from the different treatments were not normally distributed, therefore the data were transformed by the square root. The numbers of adult *H. abeitis* emerging from stumps were not significantly different amongst blocks (Two-way ANOVA: DF = 9, F = 1.69, P = 0.120). There was a significant difference found in the number of adults emerging from the different treatments (Two-way ANOVA: DF = 5, F = 7.96, P = 0.001). There were significantly fewer adult weevils emerging from the stumps treated with the full concentration of *H. downesi* (mean: 3 weevils/stump) (Fig 3.14) when compared to the control (mean: 31 weevils/stump) and single applications of *B. bassiana* (mean full *B. bassiana*: 48 weevils/stump, mean half *B. bassiana*: 29 weevils/stump). The numbers emerging from the half dose *H. downesi* and the combined dose of *H. downesi* and *B. bassiana* were similar, 10 weevils/stump and 12 weevils/stump respectively. The full treatment of *H. downesi* was significantly different from both of the single treatments of *B. bassiana*. The half treatment of *H.*

downesi and he combination treatment were significantly different from the full *B. bassiana* treatment. There was no significant difference between the control treatment and either of the single *B. bassiana* treatments (Fig. 3.14).

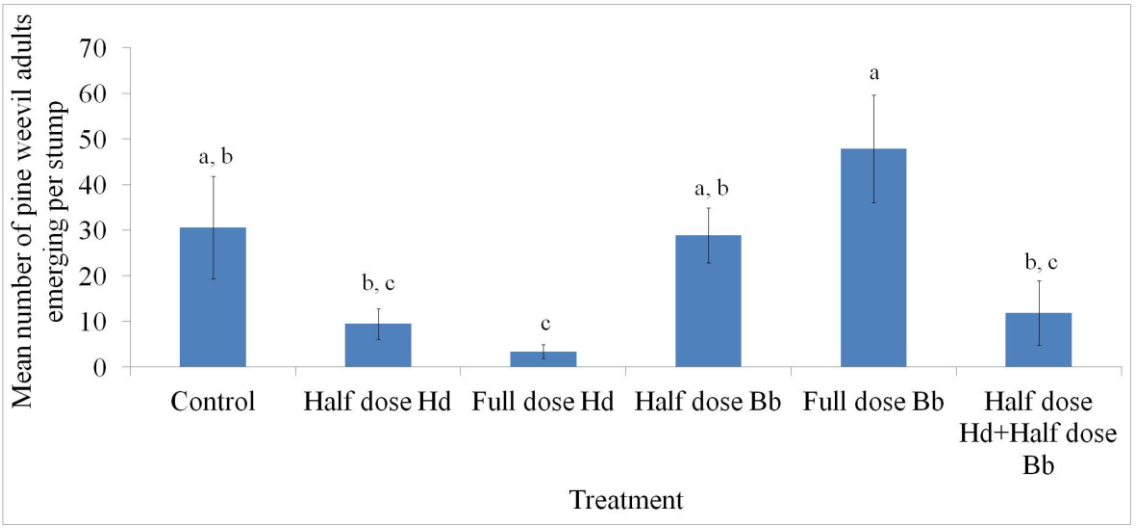

Fig 3.14 Average of the square root of the number of *Hylobius abietis* adults emerging from Scots pine tree stumps eight weeks after application. Error bars indicate standard error. Bars with different letters indicate a significant difference (Tukeys test: $\alpha = 5$).

3.4.3 Identification of *Beauveria* sp. cultured from *Hylobius abietis* larvae, collected at Rossnagad, Co. Laois

The DNA of eleven fungal samples from stumps in the field trial were extracted and compared with the applied *B. bassiana* BUEXP 1694 strain (sample 12). DNA from the ITS region was digested using *Ban II* and *Hinf I* restriction enzymes (Fig 3.15 and Fig 3.16). Only sample 1 had a similar band pattern to *B. bassiana* BUEXP 1694. Samples 3, 6 and 8 were identified as *B. caledonica* (Harvey *et al.* unpublished) which is a native fungus to Ireland (Glare *et al.* 2008). Samples 5, 10 and 11 were not *Beauveria* sp. Samples 2, 4, 7 and 9 had an unusual DNA banding result. This was interpreted as having a combination of both *B. bassiana* and *B. caledonica* banding patterns.

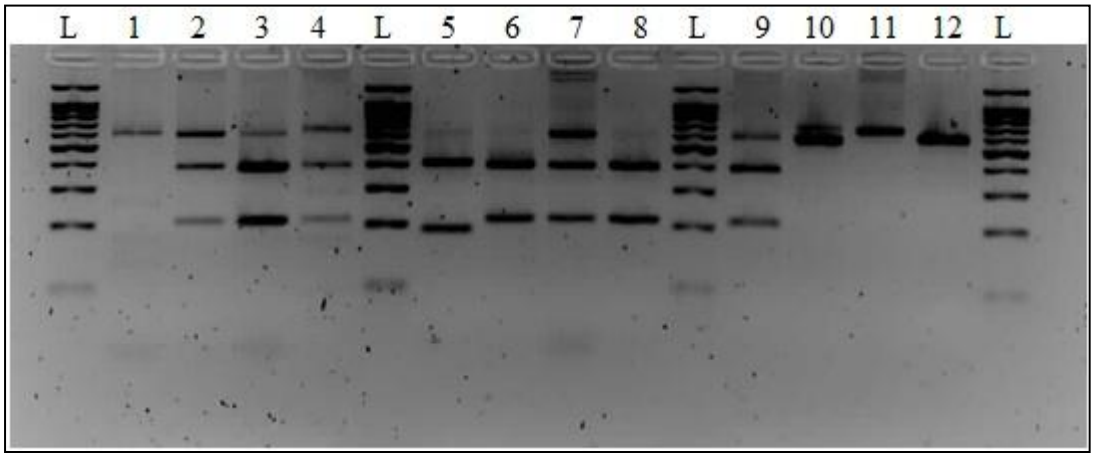

Fig 3.15 *BanII* restriction digest of unknown fungus samples 1 – 11 (see Table 3.6) and known sample 12, *Beauveria bassiana* BUEXP 1694. L indicates the ladder.

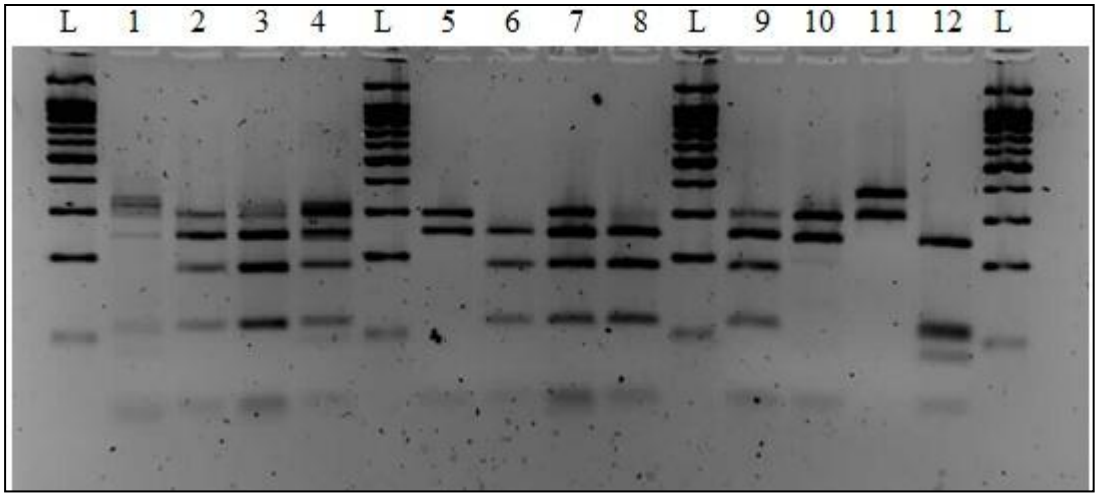

Fig 3.16 *HinfI* restriction digest of unknown fungus samples 1 – 11 (see Table 3.6) and known sample 12, *Beauveria bassiana* (BUEXP 1694). L indicates the ladder.

Table 3.6 Blocks and treatments from which fungal samples were taken with identity based on Fig's. 3.15 and 3.16.

			Identity		
Sample Number	Block number	Treatment	B.b	B.c	Other
1	13	Control	✓		
2	17	½ *B. bassiana* + ½ *S. carpocapsae*	✓	✓	
3	13	½ *B. bassiana*		✓	
4	13	½ *B. bassiana*	✓	✓	
5	12	Full *B. bassiana*			✓
6	17	½ *B. bassiana*		✓	
7	13	½ *B. bassiana*	✓	✓	
8	17	Full *B. bassiana*		✓	
9	13	½ *B. bassiana*	✓	✓	
10	18	½ *B. bassiana*			✓
11	18	½ *B. bassiana*			✓
12[a]	-	-	✓		

[a]Sample 12 is the known *B. bassiana* species that was applied in the field

3.5 Dose response and interaction of nematodes and fungus using *Otiorhynchus sulcatus* as a host

Otiorhynchus sulcatus larvae were exposed to *S. feltiae* and *M. anisopliae* (Met52) in an interaction assay using 30 ml cups. Concentrations which were used in the first interaction assay (Experiment 1) were based on results in Ansari *et al*. (2008). Fungus was added to the assay on day zero and the nematodes were added on days zero, seven or fourteen. The combined application of *S. feltiae* (10 IJ) and *M. anisopliae* (1x10^6 spores) was found to be synergistic when both entomopathogens were added together on day zero (Table 3.4). Antagonism was recorded when the IJ were added seven days after the larvae had being exposed to the fungus and the interaction was additive when the nematodes were applied 14 days after exposure to the fungal (Table 3.4). The cause of death by *M. anisopliae* ranged from 35 % - 50 % in all treatments it was applied (Fig. 3.17). In the combined application when the nematodes were added 14 days after the fungus 15 % mortality was caused by *S. feltiae*. When *S. feltiae* was applied on its own to assays the nematode killed 5 % (1/20) when added on day seven and 35 % when added on day 14. The kill recorded when the nematode was applied alone on day zero was due to other causes, 5 %. Nematodes only caused kill, 15 %, when applied 14 days after the fungus. The larval mortality in the untreated control was by other causes (Fig 3.17).

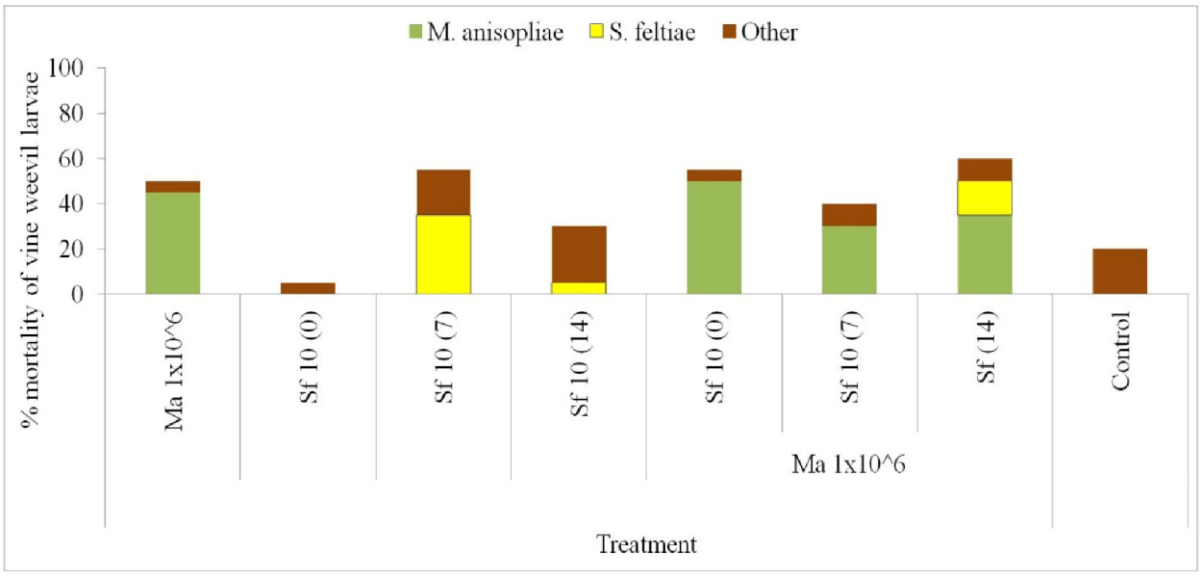

Fig 3.17 Experiment 1 *Otiorhynchus sulcatus* larvae mortality after 21 days exposure to single and combined doses of *Steinernema feltiae* (S.f) and *Metarhizium anisopliae* (M.a). The figure in brackets indicates the day that the nematodes were added to the assay. All fungus was added on day zero. N = 20.

A dose response assay (Experiment 2a) was prepared with *M. anisopliae* and *S. feltiae*. The calculated LC_{50} for *M. anisopliae* was $2.3x10^5$ spores/ml compost after 21 days and the calculated LC_{50} for *S. feltiae* was 132 IJ/cup after 7 days (Fig 3.18 A-B). Death was caused by the entomopathogen applied. In the *M. anisopliae* assays larvae were killed by other causes in all treatments, ranging from 5 % - 20 % (Fig 3.18 A). In the *S. feltiae* assays only one *S. feltiae* treatment had mortality by other causes which was 5 %. The untreated control treatment had 20 % mortality by other causes (Fig 3.18 B). A separate dose response assay was conducted with *H. bacteriophora* (Experiment 2b). The value for an LC_{50} could not be calculated as the lowest concentration (30 IJ/30 ml cup) resulted in a mortality of 80 %. The concentration to use was estimated from the results: 10 IJ/30 ml cup. All mortality in this experiment was caused by *H. bacteriophora* and there was no control mortality (Fig 3.19).

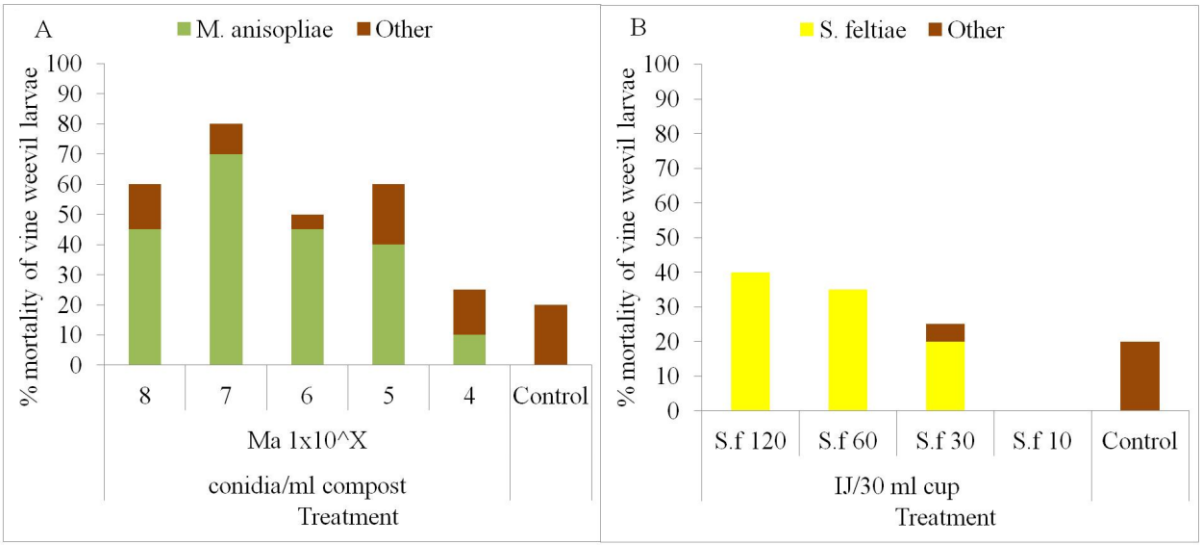

Fig 3.18 Cause of death of *Otiorhynchus sulcatus* larvae. **A:** Experiment 2a the cause of death after being exposed to *Metarhizium anisopliae* (M.a) for 21 days at different concentrations in a 30 ml cup. The figures on the x-axis indicate the power the fungal dose is raised to i.e. $1x10^6$etc. N = 20. **B:** Experiment 2a the cause of death after being exposed to *S. feltiae* (S.f) for 7 days at different concentrations in a 30 ml cup. N = 20.

Table 3.4 Interactions observed when combining EPN with EPF using *Otiorhynchus sulcatus* as host.

Exp[a]	Fungus Species[b]	spores/ml compost	Nem Species[c]	IJ/cup	Interval (day)	O M[d]	E M[e]	χ^2	Interaction[f]	Duration (day)
1	M.a	1x10^6	S.f	10	0	44	26	12.06	Synergistic	21
1	M.a	1x10^6	S.f	10	7	25	65	24.85	Antagonistic	21
1	M.a	1x10^6	S.f	10	14	50	46	0.34	Additive	21
2c	M.a	1.7x10^5	S.f	100	0	20	51	19.05	Antagonistic	21
2c	M.a	1.7x10^5	S.f	100	7	50	36	5.22	Synergistic	21
2c	M.a	1.7x10^5	S.f	100	14	35	44	1.75	Additive	21
2c	M.a	1.7x10^5	H.b	10	0	20	85	49.71	Antagonistic	21
2c	M.a	1.7x10^5	H.b	10	7	80	85	0.29	Additive	21
2c	M.a	1.7x10^5	H.b	10	14	65	74	1.04	Additive	21
2d	M.a	1.7x10^5	S.f	100	0	26	44	7.06	Antagonistic	21
2d	M.a	1.7x10^5	S.f	100	7	58	30	26.79	Synergistic	21
2d	M.a	1.7x10^5	S.f	100	14	42	16	46	Synergistic	21
2d	M.a	1.7x10^5	H.b	10	0	100	96	0.21	Additive	21
2d	M.a	1.7x10^5	H.b	10	7	89	62	11.51	Synergistic	21
2d	M.a	1.7x10^5	H.b	10	14	47	44	0.27	Additive	21

[a]Exp, Experiment. [b]M.a, *Metarhizium anisopliae*. [c]S.f, *Steinernema feltiae*; H.b, *Heterorhabditis bacteriophora*. [d]O.M, Observed Mortality (%) of twenty insects per treatment. [e]E.M, Expected Mortality (%) =Mn + Mf(1-Mn) where Mn is the mortality of the nematodes and Mf is the mortality of the fungus. All values were corrected using Abbots formula ((Ansari *et al.* 2008). [f]Interactions are based on the χ^2 ratio between the expected and observed mortality.

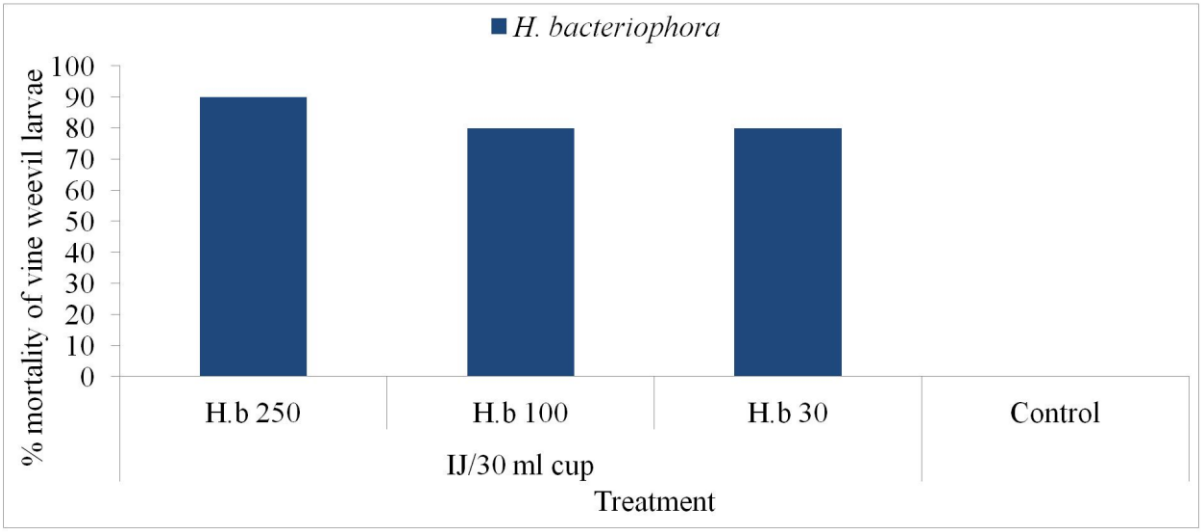

Fig 3.19 Experiment 2b the cause of death of *Otiorhynchus sulcatus* larvae after 7 days exposure to *Heterorhabditis bacteriophora* (H.b) at different concentrations. N =10.

Experiment 2c (designed to investigate the potential interaction between pathogens) consisted of single and combined concentrations of *M. anisopliae, S. feltiae* and *H. bacteriophora*; fungus was added on day zero and the nematodes were added on days zero, seven or fourteen (Fig 3.20). Synergism was found when *S. feltiae* (100 IJ/cup) was added to the assay seven days after exposure to *M. anisopliae* ($1.7x10^5$ spores/ml compost). Antagonism was found when *S. feltiae* and *H. bacteriophora* were added on the same day as the fungus. All other combinations resulted in additive interactions (Table 3.4). Mortality was mainly caused by the entomopathogens applied. In the combined application both *M. anisopliae* and *S. feltiae* caused mortality. Mortality which was caused by other causes was recorded in all treatments except for three and ranged from 5 % to 15 %. There was no control mortality (Fig. 3.20).

In Experiment 2d synergism was found when *S. feltiae* (100 IJ/cup) was added seven and 14 days after the larvae were exposed to *M. anisopliae* ($1.7x10^5$ spores/ml compost). Synergism was also found when *H. bacteriophora* (10 IJ/cup) was added seven days after the fungus. Antagonism was found when *S. feltiae* was added on the same day as the fungus and additive results were found when *H. bacteriophora* was added zero and 14 days after the fungus (Table 3.4). The majority of the mortality was caused by the entomopathogen applied to the assay and there was mortality caused by both the nematodes and fungus in the combined treatments (Fig 3.21). Mortality was induced by other causes in all treatments except two *S. feltiae* treatments, ranging from 5 % - 20 %. Control mortality was 5 %, as a result of other causes.

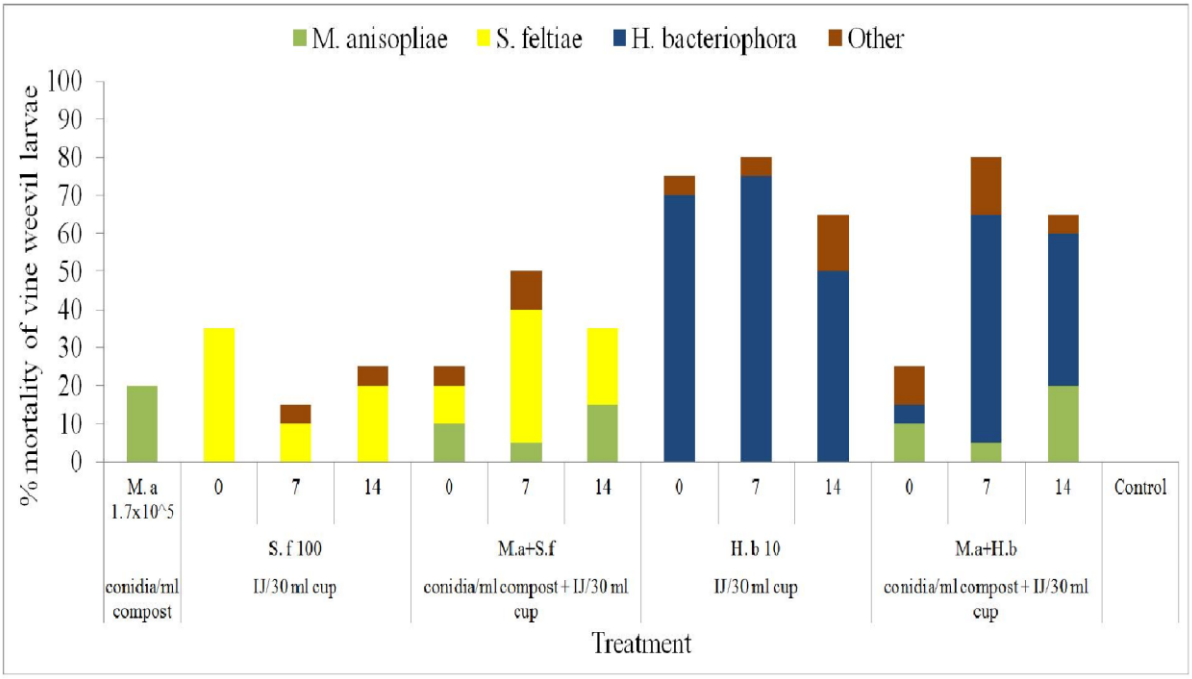

Fig 3.20 Experiment 2c the cause of *Otiorhynchus sulcatus* larvae mortality after 21 days exposure to single and combined doses of *Steinernema feltiae* (S.f), *H. bacteriophora* (H.b) and *Metarhizium anisopliae* (M.a). The figure in brackets indicates the day that the nematodes were added to the assay. All fungus was added on day zero. N = 20.

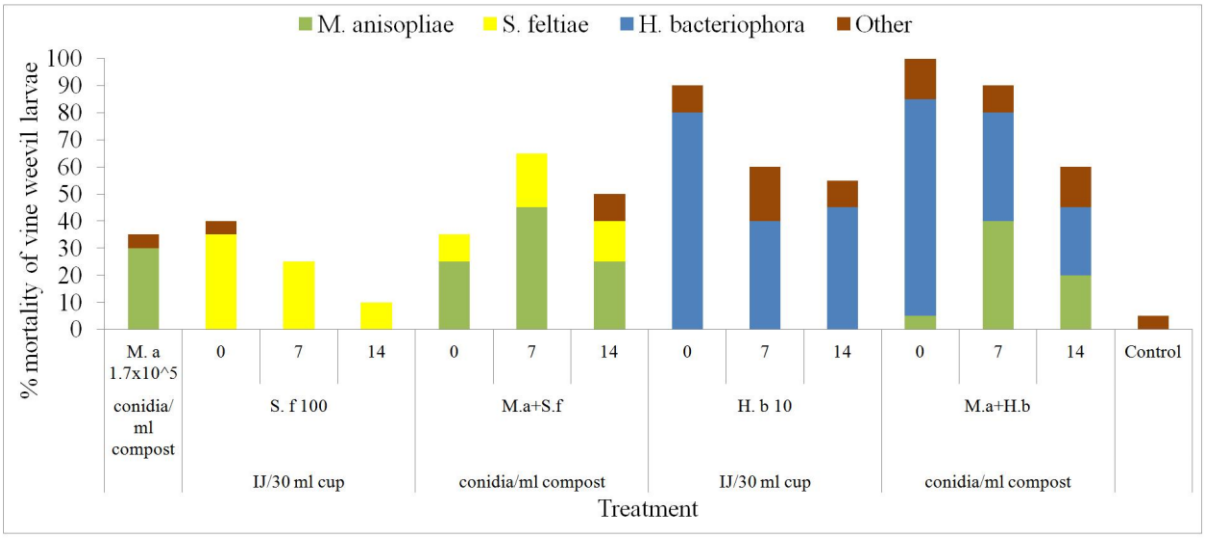

Fig 3.21 Experiment 2d the cause of *Otiorhynchus sulcatus* larvae mortality after 21 days exposure to single and combined doses of *Steinernema feltiae* (S.f), *H. bacteriophora* (H.b) and *Metarhizium anisopliae* (M.a). The figure in brackets indicates the day that the nematodes were added to the assay. All fungus was added on day zero. N = 20.

Chapter 4 – Discussion

Nematode movement through soil is affected by moisture content (Gustin & Schumacher 1989; Kung *et al.* 1991). In this study the number of *S. carpocapsae* IJ invading *G. mellonella* larvae was reduced when moisture content was at 63 % when compared to 66 % and 73 %. With *H. bacteriophora* the number invading did not differ significantly between the different moisture contents of the compost, although it did follow a similar pattern to *S. carpocapsae* i.e. fewer IJ invaded larvae in the bioassay where moisture content was 63 %. These results indicate that moisture content should not be less than 66 % in order to maintain optimum mobility for both of these nematode species in compost; the lowest moisture content which was used in this study was 66 %. More *S. carpocapsae* IJ invaded the *G. mellonella* hosts then *H. bacteriophora* indicating that *S. carpocapsae* performs better in compost than *H. bacteriophora*. This was also found in the experiments conducted in section 3.2 where more *S. carpocapsae* were recorded in *G. mellonella* infected with EPF or a Tween control when compare to *H. bacteriophora*. *Steinernema carpocapsae* is classified as an ambusher, which means that it tends to remain at the surface, detects its host by volatiles at short range and is generally associated with pests that are active at the soil surface (Kruitbos *et al.* 2010). However it has been shown to move efficiently in organic soils (Kruitbos *et al.* 2010) and indicates it may have cruise forager (associated with deep soil dwelling insects) qualities (Wilson *et al.* 2012). However, there was no difference between these species in the number of insects (pine weevil larvae) killed in the other experiments, both species killing similar numbers, suggesting the relative success of the two nematode species may be dependent on the host.

Bioassays for *H. abietis* and *O. sulcatus* include interaction experiments based on bioassays designed by Ansari *et al.* (2008). In initial experiments using *H. abietis* as host the mortality of insects with no entomopathogen applied was very high, making interpretation of results difficult. It is important when doing laboratory bioassay that they are correctly designed for identifying the parameters of the agent being tested (Butti & Goettelz 2000). In the initial experiments Scots pine bark was added as a food source and a pin hole in the lid of the assay was used for ventilation. In an attempt to reduce control mortality it was investigated if the addition of a food source or if the method of ventilation was affecting mortality. The addition of sterile, non-sterile or no bark to bioassays did not affect mortality levels. In a separate experiment the bioassay which had two 6 mm diameter holes for ventilation had lower

mortality than the bioassay which had only a pin hole, indicating that the degree of ventilation may affect mortality in the bioassay. In the bioassay with a pin hole for ventilation condensation was found to build up on the inside of the cup. The build up of humidity in an assay can lead to an increase of fungal growth (Doberski 1981). Insects require sufficient clean air through all of their morphological stages to remain healthy (Chown *et al.* 2006). Based on the results of the two aforementioned experiments it was decided that no bark would be added to the bioassays, as this was the simpler procedure, and two ventilation holes 6 mm in diameter would be used in the rest of the experiments.

In this study there were 22 interaction experiments carried out against *H. abietis* larvae; six of these were recorded as synergistic. Only one of the six interaction experiment carried out in a 30 ml cup (with *M. anisopliae* and *H. bacteriophora*) was synergistic. The other five synergisms were recorded in close contact assays in 24 well-plates with the combinations of *B. bassiana* with *H. bacteriophora, S. carpocapsae* or *H. downesi*. When the experiments were repeated the results were not consistent, synergy was not repeatable for any experiment under the same conditions. In only one of five experiments with simultaneous and delayed application of nematodes where *H. abietis* larvae were exposed to *B. bassiana* before *H. bacteriophora* was synergy recorded. This doesn't support the hypothesis that if *H. abietis* larvae are exposed to fungus first synergism is more likely to occur. Ansari *et al.* (2008) found that the probability of synergy increased when black vine weevil larvae were exposed to fungus for one or two weeks before adding *S. feltiae*, but results with *H. bacteriophora* were always synergistic whether the agents were applied together or with a delay. A study against the Welsh chafer also showed synergy when EPN and EPF were applied simultaneously or when the EPF was added first (Ansari *et al.* 2004). Shapiro-Ilan *et al.* (2003) found no synergistic interaction with any combination of EPN and EPF that was tested against the pecan weevil. In order to obtain synergy various environmental conditions must be taken into account e.g. temperature, medium, virulence of entomopathogen, assay type etc. In this study mortality of pine weevil using a combination of EPN and EPF was not consistently better when based on application of agents alone. EPN in all experiments gave higher mortality than the EPF at the range of concentrations tested. In a study by Dillon *et al.* (2006) *H. downesi* in field trials resulted in significant mortality of *H. abietis* larvae.

There was high mortality of *H. abietis* larvae by a *Beauveria* spp. recorded in the untreated controls by days 14 and 21. A native fungus *B. caledonica* was isolated from insects in one of

the field sites the larvae were collected from and it is postulated that the larvae in the untreated controls were killed by this EPF. When larvae were collected and brought back to the lab they were stored at 4˚C until needed for experiments. When insects are stressed they can become more susceptible to infections (Kaya & Gaugler 1993; Thurston *et al.* 1993). The change of environment, from a chamber underneath bark to a 24 well-plate with no food, would have left the larvae less able to fight infection. If larvae were already infected with an EPF the change in temperature from 4˚C to 20˚C (temperature at which experiments were conducted) would have accelerated the EPF activity on the larvae. Recommendations for avoiding this would be to establish a laboratory culture of *H. abietis* and rear larvae from eggs, however this would be very labour intensive.

In the second year of this investigation *H. abietis* larvae were less susceptible to *S. carpocapsae* and *H. bacteriophora* when compared to year one. In experiment 4a (year 2) when larvae were exposed to 7500 IJ/30 ml cup, *S. carpocapsae* killed 50 % and *H. bacteriophora* killed 55 %, while in experiment 1b (year 1) 500 IJ/30 ml cup killed 100% for both species. This decrease in susceptibility may be because the larvae were collected from different sites. However, this is unlikely as in the first year larvae were collected from two different sites and larvae collected in year two were also collected from two different sites. Another reason could be the loss of virulence of the nematodes used over time (attenuation). Re-culturing of nematodes through insects can change the biological attributes of nematodes (Stuart & Gaugler 1996). The *S. carpocapsae* used were re-cultured through *G. mellonella* only twice before the experiments and *H. bacteriophora* were re-cultured four times. Attenuation does not seem to be the likely cause as the nematodes used were only re-cultured once or twice in the laboratory before use in experiments. At the beginning of experiments in year two there was a problem with the *H. bacteriophora* as they did not produce any luminescence when the larvae were killed, which were used for LC_{50} experiments but results are not shown. A new batch of *H. bacteriophora* was obtained for the close contact assays but larvae were still less susceptible to the nematodes. However, the batch of nematodes used in each year may not have been the best quality, with reduced virulence. Larval age can also affect susceptibility to entomopathogens, older insect larvae can develop resistance to nematode infection (Shapiro *et al.* 1999). The larval head capsule size was not measured in year one so the exact instar of the larvae was not determined. These larvae were collected from December until May and the larvae in year two were collected from March until May.

The larvae in year two may have been a little older and more resistant to nematodes, head capsule size was measured in year two.

In year two field trials were conducted against *H. abietis* using *B. bassiana* and *H. downesi*. The results from the destructive sampling of tree stumps showed that the mortality due to the combination of *B. bassiana* and *H. downesi* was additive and *H. downesi* only applications gave the best control. The results from the emergence traps also showed that *H. downesi* only applications reduced the numbers of weevils emerging better than the combined treatment and the fungus only treatments. There was no significant difference between the half dose of nematodes and full dose of nematodes indicating that a low dose of *H. downesi* would be the best option from these results to use as a biopesticide against *H. abietis* in field conditions. Similarly Dillon *et al.* (2007) found that a half dose of *H. downesi* was almost as good as a full dose in reducing the numbers of adult *H. abietis* emerging from stumps. Any white fluffy fungus which was found on larvae collected from the field (during destructive sampling) was identified in the laboratory. Only one *B. bassiana* sample was found and it was collected from an untreated control stump. This may have occurred by the dispersal of spores by animals, human trampling or wind or perhaps is a native strain. Three samples were recorded as *B. caledonica* and four samples were recorded as having a combination of both *B. bassiana* and *B. caledonica*. The high occurrence of *B. caledonica* found on weevils (compared to the applied *B. bassiana*) suggests it may have potential as a biopesicide. The dual infection recorded may indicate that both of these EPF can both occupy an insect simultaneously or a hybrid of the two species may have occurred. The latter is unlikely as the time scale was not long enough for this to happen (Holliday, 1968).

No consistent synergistic interaction was recorded against *H. abietis* larvae. It was then investigated if the methodology being used was suitable for detecting synergy. Ansari *et al.* (2008) found synergistic interactions using *M. anisopliae* and EPN against *O. sulcatus* using a similar protocol. These experiments were repeated with *O. sulcatus* in order to compare results. They found *M. anisopliae* combined with *H. bacteriophora* recorded synergy in simultaneous and time delayed treatments, while in this investigation synergy with this combination only gave synergy in the second experiment when the EPN was added seven days after the EPF (Table 4.1). This may have been due to a batch of *H. bacteriophora* which did not kill larvae as efficiently as was expected. Ansari *et al.* (2008) recorded no interaction when *M. anisopliae* and *S. feltiae* were applied together and synergism when the nematodes

were added after the fungus. Only the third experiment conducted in this study showed similar results with this combination (Table 4.1). The difference in results in this study may be due to the age of the larvae. In experiment larvae were 6th and 7th instar while the larvae used in Ansari *et al.* (2008) were 3rd instar. From these results it can be seen that synergy can be achieved using this protocol to control *O. sulcatus* larvae, but my experiments did not give the same results as Ansari *et al.* (2008). This may be due to the aforementioned reasons but it does show that in order to attain synergy conditions must be correct; application time and larval stage may be key criteria.

Table 4.1 Comparison of results with previous study, Ansari *et al.* (2008) using a combination *M. anisopliae* with EPN.

		Ansari *et al.* 2008	Exp 1	Exp 2c	Exp 2d
	Week IJ added	Interaction	Interaction	Interaction	Interaction
S. feltiae	0	Additive	Synergy	Antagonism	Antagonism
	7	Synergy	Antagonism	Synergy	Synergy
	14	Synergy	Additive	Additive	Synergy
H. bacteriophora	0	Synergy	-	Antagonism	Additive
	7	Synergy	-	Additive	Synergy
	14	Synergy	-	Additive	Additive

It has been suggested that (Kaya & Gaugler 1993) one of the mechanisms responsible for synergy is that infection of insects with sub lethal doses of EPF makes them more susceptible to EPN. In this study *G. mellonella* larvae were exposed to *M. anisopliae* or no fungus and then exposed to *H. bacteriophora* or *S. carpocapsae*. Two experiments were conducted with *H. bacteriophora.* In the first experiment more nematodes were found to invade the uninfected cadavers, while in the second experiment there was no significant difference in the number of IJ invading infected and uninfected cadavers. These results were inconclusive and do not support the hypothesis that more *H. bacteriophora* IJ will invade an already fungus infected host. Four experiments were conducted with *S. carpocapsae* and *M. anisopliae*. Only one experiment showed more IJ invading an infected cadaver after 48 hr. However, when this time interaction was repeated twice, the number of IJ invading an infected and uninfected cadaver was not significantly different, though slightly more were found in fungus infected cadavers. This suggests that *S. carpocapsae* infection also is not strongly affected by fungal infection. The variable results here again show that interactions between EPN and EPF are complex and difficult to predict.

The inconsistent results in this study may be attributed to a number of factors including insects collected from different sites, age of insects, handling and storage of the insects, reliance of the insect on the complex ecosystem they inhabit and/or the response of their immune system to stresses (Schmid-Hempel 2005; Swanson *et al.* 1994). These stresses can cause a reduction in the defences of the insect immune system making them more susceptible to infection (DeBlock & Stoks 2008). The complex relationships that the insects in this study have with their natural microenvironment are relatively understudied (Leather *et al* 1999; Moorhouse *et al.* 2008). The quality of the EPN and EPF applied may have been inconsistent throughout the investigation. EPN and EPF applied were not stored for the same period before application in assays and their efficiency may have been reduced over time (Gaugler & Kaya 1993). In the experiments conducted on *H. abietis* the exact conditions were rarely repeated as the control mortality was quite high before the completion of the experiment and this resulted in modification of the assay. The repeated experiments conducted on *O. sulcatus* consisted of the same bioassay but the age of the insect differed among the three assays which may account for the difference in the results.

Due to new legislation chemical pesticides are often discontinued and as a result the need for safe biological control agents increases. EPN and EPF have shown to target insects effectively and are promising agents to use as biological control agents. However, a lot more research is needed to investigate how these agents interact with the environment in which they are applied. This study has investigated the possibility of combining EPN and EPF against *H. abietis* and *O. sulcatus*. The results were not encouraging as significant control was not achieved. The mechanisms which occur within the insect immune system against EPN and EPF could be investigated against these target pests in order to get a better understanding of how they cause the death of the host. In this study the results did not show a clear synergistic effect however it did occur in some cases. It has been shown that a combination of EPN and EPF can achieve synergy against other insects (Ansari *et al.* 2008; Ansari *et al.* 2004). A different combination of EPNs and EPFs may produce a clearer synergistic result. The results from the field trials showed the *H. downesi* only application reduced the numbers of *H. abietis* emerging from the stumps when compared to *B. bassiana.* The ability of the applied EPF to reach the target host needs to be investigated as not many insects from the hacked stumps from field trials were found to have infection from the applied *B. bassiana.* This may due to the fact that the spores are not reaching the larvae which live deep down in the bark of

the tree. More field trials should be conducted in order to determine which agents work best in field conditions. Other species of EPN and EPF also need to be investigated as a number of insects from field trials were found to have been killed by the native EPF *B. caledonica*; perhaps native entomopathogens would be more effective against these pests (Williams *et al.* unpublished). However, a range of interactions of the applied biocontrol agent and the environment it is applied to need to be further researched e.g. responses to vibrations and volatiles, hunting strategies of nematodes, ability to infect the host, competition (Barbercheck *et al.* 1991; Cambell & Gaugler 1993; Gotwald & Tedders 1982; Koppenhofer *et al.* 1995; Lewis *et al.* 1993).

References

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

Acevedo, J. P. M., Samuels, R. I., Ribeiro, M. I. & Dolinski, C. 2007 Interactions between isolates of the entomopathogenic fungus *Metarhizium anisopliae* and the entomopathogenic nematode *Heterorhabditis bacteriophora* JPM4 during infection of the sugar cane borer *Diatraea saccharalis* (Lepidoptera: Pyralidae). *Journal of Invertebrate Pathology* 96, 187-192.

Akhurst, R. J., Bedding, R., Akhurst, R. & Kaya, H. 1993 Bacterial symbionts of entomopathogenic nematodes: The power behind the throne. *Nematodes and biological control of insect pests*. CSIRO publications, Melbourne, Australia, pp. 127-135.

Anbesse, S. A., Adge, B. J. & Gebru, W. M. 2008 Laboratory screening for virulent entomopathogenic nematodes (*Heterorhabditis bacteriophora* and *Steinernema yirgalemense*) and fungi (*Metarhizium anisopliae* and *Beauveria bassiana*) and assessment of possible synergistic effects of combined use against grubs of the barley chafer *Coptognathus curtipennis*. *Nematology* 10, 701-709.

Ansari, M. A., Tirry, L. & Moens, M. 2004 Interaction between *Metarhizium anisopliae* CLO 53 and entomopathogenic nematodes for the control of *Hoplia philanthus*. *Biological Control* 31, 172-180.

Ansari, M., Tirry, L. & Moens, M. 2005 Antagonism between entomopathogenic fungi and bacterial symbionts of entomopathogenic nematodes. *BioControl* 50, 465-475.

Ansari, M. A., Shah, F. A., Tirry, L. & Moens, M. 2006 Field trials against *Hoplia philanthus* (Coleoptera: Scarabaeidae) with a combination of an entomopathogenic nematode and the fungus *Metarhizium anisopliae* CLO 53. *Biological Control* 39, 453-459.

Ansari, M. A., Shah, F. A. & Butt, T. M. 2008 Combined use of entomopathogenic nematodes and *Metarhizium anisopliae* as a new approach for black vine weevil, Otiorhynchus sulcatus, control. *Entomologia Experimentalis et Applicata* 129, 340-347.

Ansari, M. A., Shah, F. A. & Butt, T. M. 2009 The entomopathogenic nematode *Steinernema kraussei* and *Metarhizium anisopliae* work synergistically in controlling overwintering larvae of the black vine weevil, *Otiorhynchus sulcatus*, in strawberry growbags. *Biocontrol Science and Technology* 20, 99-105.

Ansari, M. A. & Butt, T. M. 2012 Susceptibility of different developmental stages of large pine weevil *Hylobius abietis* (Coleoptera: Curculionidae) to entomopathogenic fungi and effect of fungal infection to adult weevils by formulation and application methods. *Journal of Invertebrate Pathology* 111, 33-40.

Atakan, E., Elekçioğlu, I. H., Gözel, U., Güneş, Ç. & Yüksel, O. 2009 First report of *Heterorhabditis bacteriophora* (Poinar, 1975) (Nematoda: Heterorhabditidae) isolated from the red palm weevil, *Rhynchophorus ferrugineus* (Oliver, 1790) (Coleoptera: Curculionidae) in Turkey. *EPPO Bulletin* 39, 189-193.

Barberchek, M. E. & Kaya, H. K. 1990 Interactions between *Beauveria bassiana* and the entomogenous nematodes, *Steinernema feltiae* and *Heterorhabditis heliothidis*. *Journal of Invertebrate Pathology* 55, 225-234.

Barbercheck, M. E. & Kaya, H. K. 1991 Competitive interactions between entomopathogenic nematodes and *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) in soilborne larvae of *Spodoptera exigua* (Lepidoptera: Noctuidae). *Environmental Entomology* 20, 707-712.

Barnett, H. L. & Hunter, B. B. 1972 Illustrated Genera of Imperfect Fungi; 3rd ed., Macmillan New York.

Beard, J. 2006 DDT and human health. *Science of The Total Environment* 355, 78-89.

Berenbaum, M. C. 1978 A Method for Testing for Synergy with Any Number of Agents. *Journal of Infectious Diseases* 137, 122-130.

Blackshaw, R. P. 1988 A survey of insect parasitic nematodes in Northern Ireland. *Annals of Applied Biology* 113, 561-565.

Boemare, N., Laumond, C. & Mauleon, H. 1996 The Entomopathogenic Nematode-Bacterium Complex: Biology, Life Cycle and Vertebrate Safety. *Biocontrol Science and Technology* 6, 333-346.

British Mycological Society. 1970 Fungal Records Database of Britain and Ireland. Accessed 1 October 2012, <http://www.fieldmycology.net>

Brixey, J. 1997 The potential for biological control to reduce *Hylobius abietis* damage. *Forestry Commission Research Information Note,* 273.

Bruck, D. J. 2004 Natural Occurrence of Entomopathogens in Pacific Northwest Nursery Soils and Their Virulence to the Black Vine Weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae). *Environmental Entomology* 33, 1335-1343.

Bruck, D. J., Snelling, J. E., Dreves, A. J. & Jaronski, S. T. 2005 Laboratory bioassays of entomopathogenic fungi for control of *Delia radicum* (L.) larvae. *Journal of Invertebrate Pathology* 89, 179-183.

Bruck, D. J. & Donahue, K. M. 2007 Persistence of *Metarhizium anisopliae* incorporated into soilless potting media for control of the black vine weevil, *Otiorhynchus sulcatus* in container-grown ornamentals. *Journal of Invertebrate Pathology* 95, 146-150.

Burnell, A. M. & Stock, S. P. 2000 *Heterorhabditis, Steinernema* and their bacterial symbionts lethal pathogens of insects. *Nematology* 2, 31-42.

Butt, T. M. & Goettelz, M. S. 2000 Bioassays of entomogenous fungi. *Bioassays of entomopathogenic microbes and nematodes*, 141.

Butt, T. M., Jackson, C. W., Magan, N. & Jackson, C. 2001 Introduction-fungal biological control agents: progress, problems and potential, p. 1-8. In T. M. Butt, C. Jackson, and N. Morgan (ed.), Fungal biocontrol agents: progress, problems and potential. CAB International, Wallingford, United Kingdom.

Bylund, H., Nordlander, G. & Nordenhem, H. 2004 Feeding and oviposition rates in the pine weevil *Hylobius abietis* (Coleoptera: Curculionidae). *Bulletin of Entomological Research* 94.

Campbell, J. F., & Gaugler, R. 1993. Nictation behaviour and its ecological implications in the host search strategies of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae). *Behaviour*, 155-169.

Chandler, D., D. Hay & A. P. Reid. "Sampling and occurrence of entomopathogenic fungi and nematodes in UK soils." *Applied Soil Ecology* 5.2 (1997): 133-141.

Choo, H., Kaya, H., Huh, J., Lee, D., Kim, H., Lee, S. & Choo, Y. 2002 Entomopathogenic nematodes (*Steinernema* spp. and *Heterorhabditis bacteriophora*) and a fungus *Beauveria brongniartii*; for biological control of the white grubs, *Ectinohoplia rufipes* and *Exomala orientalis*, in Korean golf courses. *BioControl* 47, 177-192.

Chown, S. L., Gibbs, A. G., Hetz, S. K., Klok, C. J., Lighton, J. R. B. & Marais, E. 2006 Discontinuous Gas Exchange in Insects: A Clarification of Hypotheses and Approaches. *Physiological and Biochemical Zoology* 79, 333-343.

Christiansen, E. 1971 Laboratory study on factors influencing pre-imaginal development in Hylobius abietis L. (Col., Curculionidae). *Norsk Entomologisk Tidsskrift* 18.

Coillte 2012 Coillte log sales. Accessed on 27 September 2012. <http://www.coillte.ie/coillteforest/log_sales/.>

Cross, J. V. & Burgess, C. M. 1997 Localised insecticide treatment for the control of vine weevil larvae (*Otiorhynchus sulcatus*) on field-grown strawberry. *Crop Protection* 16, 565-574.

Daniel, C. & Wyss, E. 2010 Field applications of *Beauveria bassiana* to control the European cherry fruit fly *Rhagoletis cerasi*. *Journal of Applied Entomology* 134, 675-681.

Daoust, R. A. & Roberts, D. W. 1983 Studies on the prolonged storage of *Metarhizium anisopliae* conidia: Effect of temperature and relative humidity on conidial viability and virulence against mosquitoes. *Journal of Invertebrate Pathology* 41, 143-150.

DeBach, P. & Rosen, D. 1991 *Biological control by natural enemies*: London Cambridge university press.

DeBLOCK, M. & Stoks, R. 2008. Short-term larval food stress and associated compensatory growth reduce adult immune function in a damselfly. *Ecological entomology*, 33, 796-801.

Department of Agriculture, Food and Marine, 2012 Pesticide Control Service. Update Biological Product Registers. Accessed on 20 September 2012 < http://www.pcs.agriculture.gov.ie/>

Dillon, A. B., Ward, D., Downes, M. J. & Griffin, C. T. 2006 Suppression of the large pine weevil *Hylobius abietis* (L.) (Coleoptera: Curculionidae) in pine stumps by entomopathogenic nematodes with different foraging strategies. *Biological Control* 38, 217-226.

Dillon, A. B., Downes, M. J., Ward, D. & Griffin, C. T. 2007 Optimizing application of entomopathogenic nematodes to manage large pine weevil, *Hylobius abietis* L. (Coleoptera:Curculionidae) populations developing in pine stumps, *Pinus sylvestris*. *Biological Control* 40, 253-263.

Dillon, A. B., Rolston, A. N., Meade, C. V., Downes, M. J. & Griffin, C. T. 2008 Establishment, persistence, and introgression of entomopathogenic nematodes in a forest ecosystem. *Ecological Applications* 18, 735-747.

Dix, I., Burnell, A. M., Griffin, C. T., Joyce, S. A., Nugent, M. J. & Downes, M. J. 1992 The identification of biological species in the genus Heterorhabditis (Nematoda: Heterorhabditidae) by cross-breeding second-generation amphimictic adults. *Parasitology* 104, 509-518.

Doberski, J. W. 1981 Comparative laboratory studies on three fungal pathogens of the elm bark beetle *Scolytus scolytus*: Effect of temperature and humidity on infection by

Beauveria bassiana, Metarhizium anisopliae, and *Paecilomyces farinosus*. *Journal of Invertebrate Pathology* 37, 195-200.

Ennis, D. E., Dillon, A. B. & Griffin, C. T. 2010 Pine weevils modulate defensive behaviour in response to parasites of differing virulence. *Animal Behaviour* 80, 283-288.

European Union. Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 establishing a framework for Community action to achieve the sustainable use of pesticides. *Official Journal of the European Union L* 309, 16.

Fantke, P., Friedrich, R. & Jolliet, O. 2012 Health impact and damage cost assessment of pesticides in Europe. *Environment International* 49, 9-17.

Fargues, J., Goettel, M. S., Smits, N., Ouedraogo, A. & Rougier, M. 1997 Effect of temperature on vegetative Growth of *Beauveria bassiana* isolates from different origins. *Mycologia* 89, 383-392.

Feng, M. G., Poprawski, T. J. & Khachatourians, G. G. 1994 Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control: current status. *Biocontrol Science and Technology* 4, 3-34.

Feng, M.-G., Pu, X.-Y., Ying, S.-H. & Wang, Y.-G. 2004 Field trials of an oil-based emulsifiable formulation of *Beauveria bassiana* conidia and low application rates of imidacloprid for control of false-eye leafhopper *Empoasca vitis* on tea in southern China. *Crop Protection* 23, 489-496.

Forst, S. & Dowds, B. 1997 Xenorhabdus and Photorhabdus spp.: Bugs that kill bugs. *Annual Review of Microbiology* 51, 47.

Forestry Stewardship Council 2008. FSC pesticides policy guidance addendum: List of approved derogations for use of highly hazardous pesticides. Forest Stewardship Council, Canada. <http://www.fsccanada.org/docs/fsc-gui-30-001a_v10_en_approved_derogations_for_use_of_pesticides_2008-10-21_01.pdf>.

Gaugler, R. 2002 *Entomopathogenic Nematology*. Cambridge, MA, USA: CABI Publishing.

Georgis, R., A. M. Koppenhöfer, L. A. Lacey, G. Bélair, L. W. Duncan, P. S. Grewal, M. Samish, L. Tan, P. Torr, and R. W. H. M. Van Tol 2006 "Successes and failures in the use of parasitic nematodes for pest control." *Biological Control*, 38, 103-123.

Gill, S., Lutz, J., Shrewsbury, P. & Raupp, M. 2001 Evaluation of biological and chemical control methods for black vine weevil, *Otiorhynchus sulcatus* (Fabricius)(Coleoptera: Curculionidae), in container grown perennials. *Journal of Environmental Horticulture* 19, 166-170.

Gillespie, A. T. & Claydon, N. 1989 The use of entomogenous fungi for pest control and the role of toxins in pathogenesis. *Pesticide Science* 27, 203-215.

Glare, T. R., Reay, S. D., Nelson, T. L. & Moore, R. 2008 *Beauveria caledonica* is a naturally occurring pathogen of forest beetles. *Mycological Research* 112, 352-360.

Glare, T., Caradus, J., Gelernter, W., Jackson, T., Keyhani, N., Kohl, J., Marrone, P., Morin, L. & Stewart, A. 2012 Have biopesticides come of age? *Trends in Biotechnology* 30, 250-258.

Godonou, I., Green, K. R., Oduro, K. A., Lomer, C. J. & Afreh-Nuamah, K. 2000 Field Evaluation of selected formulations of *Beauveria bassiana* for the management of the banana weevil (*Cosmopolites sordidus*) on plantain (Musa spp., AAB Group). *Biocontrol Science and Technology* 10, 779-788.

Gottwald, T. R., & Tedders, W. L. 1982. Studies on conidia release by the entomogenous fungi *Beauveria bassiana* and *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) from adult pecan weevil (Coleoptera: Curculionidae) cadavers. *Environmental Entomology*, *11*(6), 1274-1279.

Grewal, P. S., Ehlers, R. & Shapiro-Ilan, D. I. 2006 *Nematodes as biocontrol agents*: CABI.

Griffin, C. T., Moore, J. F. & Downes, M. J. 1991 Occurrence of Insect-Parasitic Nematodes (Steinernematidae, Heterorhabditidae) in the Republic of Ireland. *Nematologica* 37, 92-100.

Griffin, C. T., O'Callaghan, K. M. & Dix, I. 2001 A self-fertile species of *Steinernema* from Indonesia: further evidence of convergent evolution amongst entomopathogenic nematodes? *Parasitology* 122, 181-186.

Gustin, R. D. & Schumacher, T. E. 1989 Relationship of some soil pore parameters to movement of first-instar western corn rootworm (Coleoptera: Chrysomelidae). *Environmental Entomology* 18, 343-346.

Hajek, A. E. 1997 Ecology of terrestrial fungal entomopathogens. *Advances in microbial ecology* 15, 193-249.

Hallsworth, J. E. & Magan, N. 1999 Water and temperature relations of growth of the entomogenous fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces farinosus*. *Journal of Invertebrate Pathology* 74, 261-266.

Haukeland, S. & Lola-Luz, T. 2010 Efficacy of the entomopathogenic nematodes *Steinernema kraussei* and *Heterorhabditis megidis* against the black vine weevil *Otiorhynchus sulcatus* in open field-grown strawberry plants. *Agricultural and Forest Entomology* 12, 363-369.

Henry, C. J. & Day, K. R. 2001 Egg allocation by Bracon hylobii Ratz., the principal parasitoid of the large pine weevil (*Hylobius abietis* L.), and implications for host suppression. *Agricultural and Forest Entomology* 3, 11-18.

Heritage, S., & Moore, R. 2000. The assessment of site characteristics as part of a management strategy to reduce damage by Hylobius. *Information Note-Forestry Commission*, (38).

Holliday, R. 1968. Genetic recombination in fungi. *Replication and Recombination of Genetic Material*. W.J. Peacock and R.D. Brock, editors. Australian Academy of Science, Canberra. 157–174.

Inglis, G. D., Goettel, M. S., Butt, T. M., Strasser, H., Jackson, C. & Magan, N. 2001 Use of hyphomycetous fungi for managing insect pests. *Fungi as biocontrol agents: Progress, problems and potential*, 23-69. CABI.

Jabbour, R., Crowder, D. W., Aultman, E. A. & Snyder, W. E. 2011 Entomopathogen biodiversity increases host mortality. *Biological Control* 59, 277-283.

James, R. R., Croft, B. A., Shaffer, B. T. & Lighthart, B. 1998 Impact of temperature and humidity on host pathogen interactions between *Beauveria bassiana* and a Coccinellid. *Environmental Entomology* 27, 1506-1513.

Kaya, H. K. & Gaugler, R. 1993 Entomopathogenic nematodes. *Annual review of entomology* 38, 181-206.

Kaya, H. K. & Stock, S. P. 1997 Techniques in insect nematology. In *Manual of Techniques in Insect Pathology* (ed. L. Lacey), pp. 281-324: Academic Press.

Kaya, G. P. & Hassan, S. 2000 Entomogenous fungi as promising biopesticides for tick control. *Experimental and Applied Acarology* 24, 913-926.

Kershaw, M. J., Moorhouse, E. R., Bateman, R., Reynolds, S. E. & Charnley, A. K. 1999 The Role of Destruxins in the Pathogenicity of Metarhizium anisopliae for Three Species of Insect. *Journal of Invertebrate Pathology* 74, 213-223.

Koppenhofer, A. M. & Fuzy, E. M. 2008 Effect of the anthranilic diamide insecticide, chlorantraniliprole, on *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) efficacy against white grubs (Coleoptera: Scarabaeidae). *Biological Control* 45, 93-102.

Koppenhöfer, A. M., Kaya, H. K., & Taormino, S. P. 1995. Infectivity of entomopathogenic nematodes (Rhabditida: Steinernematidae) at different soil depths and moistures. *Journal of Invertebrate Pathology*, 65, 193-199.

Kruitbos, L. M., Heritage, S., Hapca, S. & Wilson, M. J. 2010 The influence of habitat quality on the foraging strategies of the entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis megidis*. *Parasitology* 137, 303.

Kung, S. P., Gaugler, R. & Kaya, H. K. 1991 Effects of soil temperature, moisture, and relative humidity on entomopathogenic nematode persistence. *Journal of Invertebrate Pathology* 57, 242-249.

La Lone, R. S. & Clarke, R. G. 1981 Larval Development of Otiorhynchus sulcatus (Coleoptera: Curculionidae) and Effects of Larval Density on Larval Mortality and Injury to Rhododendron. *Environmental Entomology* 10, 190-191.

Lacey, L. A. 1997 Manual of Techniques in Insect Pathology. Academic Press, New York.

Lacey, L. A., Frutos, R., Kaya, H. K. & Vail, P. 2001 Insect Pathogens as Biological Control Agents: Do They Have a Future? *Biological Control* 21, 230-248.

Langstrom, B., Day, K. R., Lieutier, F., Day, K. R., Battisti, A., Grégoire, J.-C. & Evans, H. F. 2004 Damage, Control and management of weevil pests, especially *Hylobius Abietis*, bark and wood boring insects in living trees in Europe, a synthesis, pp. 415-444: Springer Netherlands.

Leather, S. R., Ahmed, S. I. & Hogan, L. 1994 Adult feeding preferences of the large pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae). *European Journal of Entomology* 91, 385-385.

Leather, S. R., Day, K. R. & Salisbury, A. N. 1999 The biology and ecology of the large pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae): a problem of dispersal? *Bulletin of Entomological Research* 89, 3-16.

Lewis, E. E., Gaugler, R., & Harrison, R. 1993. Response of cruiser and ambusher entomopathogenic nematodes (Steinernematidae) to host volatile cues. *Canadian Journal of Zoology*, 71, 765-769.

Lewis, E. E., Campbell, J., Griffin, C., Kaya, H. & Peters, A. 2006a Behavioral ecology of entomopathogenic nematodes. *Biological Control* 38, 66-79.

Lewis, E. E., Campbell , J., Griffin, C. T., Kaya, H. & Peters, A. 2006b Behavioral ecology of entomopathogenic nematodes. *Biological Control* 38, 66-79.

Lezama-Gutierrez, R., la Luz, A. T.-d., Molina-Ochoa, J., Rebolledo-Dominguez, O., Pescador, A. R., Lapez-Edwards, M. & Aluja, M. 2000 Virulence of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) on *Anastrepha ludens* (Diptera: Tephritidae): Laboratory and Field Trials. *Journal of Economic Entomology* 93, 1080-1084.

Lola-Luz, T., Downes, M. & Dunne, R. 2005 Control of black vine weevil larvae *Otiorhynchus sulcatus* (Fabricius)(Coleoptera: Curculionidae) in grow bags outdoors with nematodes. *Agricultural and Forest Entomology* 7, 121-126.

Lola-Luz, T. & Downes, M. 2007 Biological control of black vine weevil *Otiorhynchus sulcatus* in Ireland using *Heterorhabditis megidis*. *Biological Control* 40, 314-319.

Long, S. J., Richardson, P. N. & Fenlon, J. S. 2000 Influence of temperature on the infectivity of entomopathogenic nematodes (Steinernema and Heterorhabditis spp.) to larvae and pupae of the vine weevil *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). *Nematology* 2, 309-317.

Malinowski, H. 2009 Entomopathogenic fungi as insecticides in forest protection. *Progress in Plant Protection* 49, 865-873.

Mannion, C. M. & Jansson, R. K. 1992 Comparison of ten entomopathogenic nematodes for control of sweet potato weevil (Coleoptera: Apionidae). *Journal of Economic Entomology* 85, 1642-1650.

Masaki, M., Tsuchiya, T. & Nakahara, S. 1999 Effect of 3% carbosulfan granule to the black vine weevil, *Otiorhynchus sulcatus* (F) (Coleoptera; Curculionidae). *Research Bulletin of the Plant Protection Service Japan*, 61-64.

Meyling, N. V. & Eilenberg, J. r. 2007 Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control. *Biological Control* 43, 145-155.

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

Moore, R. 2001 Emergence trap developed to capture adult large pine weevil *Hylobius abietis* (Coleoptera: Curculionidae) and its parasite *Bracon hylobii* (Hymenoptera: Braconidae). *Bulletin of Entomological Research* 91, 109-115.

Moore, R., Brixey, J. M. & Milner, A. D. 2004 Effect of time of year on the development of immature stages of the Large Pine Weevil (*Hylobius abietis* L.) in stumps of Sitka spruce (*Picea sitchensis* Carr.) and influence of felling date on their growth, density and distribution. *Journal of Applied Entomology* 128, 167-176.

Moorhouse, E. R., Charnley, A. K., & Gillespie, A. T. 2008. A review of the biology and control of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). *Annals of Applied Biology*, 121, 431-454.

Moorhouse, E. R., Charnley, A. K. & Gillespie, A. T. 1992 A review of the biology and control of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). *Annals of Applied Biology* 121, 431-454.

Morgan, J. A. W., Kuntzelmann, V., Tavernor, S., Ousley, M. A. & Winstanley, C. 1997 Survival of Xenorhabdus nematophilus and Photorhabdus luminescens in water and soil. *Journal of Applied Microbiology* 83, 665-670.

Morley-Davies, J., Moore, D. & Prior, C. 1996 Screening of *Metarhizium* and *Beauveria* spp. conidia with exposure to simulated sunlight and a range of temperatures. *Mycological Research* 100, 31-38.

Nguyen, K. B. & Smart Jr, G. C. 1994 Neosteinernema longicurvicauda n. gen., n. sp.(Rhabditida: Steinernematidae), a parasite of the termite *Reticuldermes flavipes* (Koller). *Journal of nematology* 26, 162.

Nordenhem, H. 1989 Age, sexual development, and seasonal occurrence of the pine weevil *Hylobius abietis* (L.). *Journal of Applied Entomology* 108, 260-270.

Nordlander, G., Nordenhem, H. & Bylund, H. 1997 Oviposition patterns of the pine weevil *Hylobius abietis*. *Entomologia Experimentalis et Applicata* 85, 1-9.

Orlander, G. & Nilsson, U. 1999 Effect of reforestation methods on pine weevil (*Hylobius abietis*) damage and seedling survival. *Scandinavian Journal of Forest Research* 14, 341-354.

Ormond, E. L., Thomas, A. P. M., Pugh, P. J. A., Pell, J. K. & Roy, H. E. 2010 A fungal pathogen in time and space: the population dynamics of Beauveria bassiana in a conifer forest. *FEMS Microbiology Ecology* 74, 146-154.

Ouedraogo, A., Fargues, J., Goettel, M. S. & Lomer, C. J. 1997 Effect of temperature on vegetative growth among isolates of *Metarhizium anisopliae* and *M. flavoviride Mycopathologia* 137, 37-43.

Peng, G., Wang, Z., Yin, Y., Zeng, D. & Xia, Y. 2008 Field trials of *Metarhizium anisopliae* var. acridum (Ascomycota: Hypocreales) against oriental migratory locusts, *Locusta migratoria manilensis* (Meyen) in Northern China. *Crop Protection* 27, 1244-1250.

Reay, S. D., Brownbridge, M., Cummings, N. J., Nelson, T. L., Souffre, B., Lignon, C. & Glare, T. R. 2008 Isolation and characterization of *Beauveria* spp. associated with exotic bark beetles in New Zealand Pinus radiata plantation forests. *Biological Control* 46, 484-494.

Schmid-Hempel, Paul 2005. "Evolutionary ecology of insect immune defenses."*Annual Review Entomology* 50, 529-551.

Salisbury, A. N. & Leather, S. R. 1998 Migration of larvae of the large Pine Weevil, *Hylobius abietis* L. (Col., Curculionidae): Possible predation a lesser risk than death by starvation? *Journal of Applied Entomology* 122, 295-299.

Samson, R. A., Evans, H. C. & Latg J. P. 1988 *Atlas of entomopathogenic fungi*: Springer-Verlag GmbH & Co. KG.

Scholte, E. J., Njiru, B. N., Smallegange, R. C., Takken, W. & Knols, B. G. J. 2003 Infection of malaria (*Anopheles gambiae* ss) and filariasis (*Culex quinquefasciatus*) vectors with the entomopathogenic fungus *Metarhizium anisopliae*. *Malaria Journal* 2, 29.

Shah, P. A. & Pell, J. K. 2003 Entomopathogenic fungi as biological control agents. *Applied Microbiology and Biotechnology* 61, 413-423.

Shah, F. A., Ansari, M. A., Prasad, M. & Butt, T. M. 2007 Evaluation of black vine weevil (*Otiorhynchus sulcatus*) control strategies using *Metarhizium anisopliae* with sublethal doses of insecticides in disparate horticultural growing media. *Biological Control* 40, 246-252.

Shah, F. A., Gaffney, M., Ansari, M. A., Prasad, M. & Butt, T. M. 2008 Neem seed cake enhances the efficacy of the insect pathogenic fungus *Metarhizium anisopliae* for the control of black vine weevil, *Otiorhynuchs sulcatus* (Coleoptera: Curculionidae). *Biological Control* 44, 111-115.

Shah, F. A., Ansari, M. A., Watkins, J., Phelps, Z., Cross, J. & Butt, T. M. 2009 Influence of commercial fungicides on the germination, growth and virulence of four species of entomopathogenic fungi. *Biocontrol Science and Technology* 19, 743 - 753.

Shanks, C. H. & Finnigan, B. F. 1973 Temperature and Relative Humidity Effects on Eggs and First-Stage Larvae of the Black Vine Weevil, *Otiorhynchus sulcatus. Environmental Entomology* 2, 855-858.

Shanks, J. R., Carl, H. & Agudelo-Silva, F. 1990 Field pathogenicity and persistence of heterorhabditid and steinernematid nematodes (Nematoda) infecting black vine weevil larvae (Coleoptera: Curculionidae) in cranberry bogs. *Journal of Economic Entomology* 83, 107-110.

Shapiro-Ilan, D. I., Cate, J. R., Pena, J., Hunsberger, A. & McCoy, C. W. 1999 Effects of temperature and host age on suppression of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) by entomopathogenic nematodes. *Journal of Economic Entomology* 92, 1086-1092.

Shapiro-Ilan, D. I., Gouge, D. H. & Koppenhofer, A. M. 2002 16 Factors Affecting Commercial Success: Case Studies in Cotton, Turf and Citrus. *Entomopathogenic nematology*, 333. CABI.

Shapiro-Ilan, D. I., Jackson, M., Reilly, C. C. & Hotchkiss, M. W. 2004 Effects of combining an entomopathogenic fungi or bacterium with entomopathogenic nematodes on mortality of *Curculio caryae* (Coleoptera: Curculionidae). *Biological Control* 30, 119-126.

Shapiro-Ilan, D. I., Gouge, D. H., Piggott, S. J. & Fife, J. P. 2006 Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. *Biological Control* 38, 124-133.

Sifakis, S., Mparmpas, M., Soldin, O. P., & Tsatsakis, A. 2011. Pesticide Exposure and Health Related Issues in Male and Female Reproductive System. *Pesticides-Formulations, Effects, Fate*, 495-526.

Smith, F. F. 1930 Studies of the Black Vine Weevil. *Journal of the Washington Academy of Sciences* 20, 185-188.

Smith, F. F. 1932 *Biology and control of the black vine weevil*: US Department of Agriculture. USDA Technical Bulletin 325.

Stenseth, C. 1979 Effects of temperature on development of *Otiorrhynchus sulcatus* (Coleoptera: Curculionidae). *Annals of Applied Biology* 91, 179-185.

Stock, S., Griffin, C. & Burnell, A. 2002 Morphological characterisation of three isolates of *Heterorhabditis* Poinar, 1976 from the `Irish group' (Nematoda: Rhabditida: Heterorhabditidae) and additional evidence supporting their recognition as a distinct species, *H. downesi*; n. sp. *Systematic Parasitology* 51, 95-106.

Stuart, R. J. & Gaugler, R. 1996 Genetic adaptation and founder effect in laboratory populations of the entomopathogenic nematode *Steinernema glaseri*. *Canadian Journal of Zoology* 74, 164-170.

Sulistyanto, D. & Ehlers, R.-U. 1996 Efficacy of the Entomopathogenic Nematodes *Heterorhabditis megidis* and *Heterorhabditis bacteriophora* for the control of grubs (Phyllopertha horticola and Aphodius contaminatus) in Golf Course Turf. *Biocontrol Science and Technology* 6, 247-250.

Susurluk, I. A., Kumral, N. A., Bilgili, U. & Acıkgoz, E. 2011 Control of a new turf pest, *Dorcadion pseudopreissi* (Coleoptera: Cerambycidae), with the entomopathogenic nematode *Heterorhabditis bacteriophora*. *Journal of Pest Science* 84, 321-326.

Swanson, F. J., Jones, J. A., Wallin, D. O., & Cissel, J. H. 1994. Natural variability—implications for ecosystem management. U.S. Department of Agriculture, Forest Service. *ME Jensen and PS Bourgeron, editors*, 2, 80-94.

Tarasco, E., Santiago Alvarez, C. N., Triggiani, O. & Quesada Moraga, E. 2011 Laboratory studies on the competition for insect haemocoel between *Beauveria bassiana* and *Steinernema ichnusae* recovered in the same ecological niche. *Biocontrol Science and Technology* 21, 693-704.

Thacker, J. R. M., Bryan, W. J., McGinley, C., Heritage, S. & Strang, R. H. C. 2003 Field and laboratory studies on the effects of neem (*Azadirachta indica*) oil on the feeding activity of the large pine weevil (*Hylobius abietis* L.) and implications for pest control in commercial conifer plantations. *Crop Protection* 22, 753-760.

Thakore, Y. 2006 The Biopesticide Market for Global Agricultural use: BBC Research. Accessed on 10 October 2012 < http://www.bccresearch.com/report/biopesticides-market-chm029c.html>.

Thurston, G. S., Kaya, H. K., Burlando, T. M. & Harrison, R. E. 1993 Milky disease bacterium as a stressor to increase susceptibility of Scarabaeid larvae to an entomopathogenic nematode. *Journal of Invertebrate Pathology* 61, 167-172.

Torr, P., Wilson, M. J. & Heritage, S. 2006 15 Forestry Applications. *Nematodes As Biocontrol Agents*, 281. CABI.

Unruh, T. R. & Lacey, L. A. 2001 Control of codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae), with *Steinernema carpocapsae*: effects of supplemental wetting and pupation site on infection rate. *Biological Control* 20, 48-56.

Van Emden, H. F. & Service, M. W. 2004 *Pest and Vector Control*. West Nyack, NY, USA: Cambridge University Press.

Vega, F. E., Posada, F., Catherine Aime, M., Pava-Ripoll, M., Infante, F. & Rehner, S. A. 2008 Entomopathogenic fungal endophytes. *Biological Control* 46, 72-82.

Vey, A., Hoaglandz, R. E. & Butt, T. M. 2001 12 Toxic Metabolites of Fungal Biocontrol Agents. *Fungi as biocontrol agents: Progress, problems and potential*, 311. CABI

Wainhouse, D., Boswell, R. & Ashburner, R. 2004 Maturation feeding and reproductive development in adult pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae). *Bulletin of Entomological Research* 94, 81-87.

Wang, Y., Campbell, J. F. & Gaugler, R. 1995 Infection of entomopathogenic nematodes *Steinernema glaseri* and *Heterorhabditis bacteriophora* against *Popillia japonica* (Coleoptera: Scarabaeidae) larvae. *Journal of Invertebrate Pathology* 66, 178-184.

Weeden, C. R. 2003 *Biological control: a guide to natural enemies in North America*. New York: Cornell University.

Whipps, J. M., Lumsden, R. D., Butt, T. M., Jackson, C. & Magan, N. 2001 Commercial use of fungi as plant disease biological control agents: status and prospects. *Fungi as biocontrol agents: Progress, problems and potential*, 9-22. CABI.

Williams, C. D., Dillion, A. B., Harvey, C. D., Hennessy, R., Mc Namara L. & Griffin, C. T., Unpublished. Control of a major pest of forestry, *Hylobius abietis*, with entomopathogenic nematodes and fungi using eradicant and prophylactic strategies.

Willmott, D. M., Hart, A. J., Long, S. J., Edmondson, R. N. & Richardson, P. N. 2002 Use of a cold-active entomopathogenic nematode *Steinernema kraussei* to control overwintering larvae of the black vine weevil *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) in outdoor strawberry plants. *Nematology* 4, 925-932.

Wilson, M. J., Ehlers, R. U. & Glazer, I. 2012 Entomopathogenic nematode foraging strategies-is *Steinernema carpocapsae* really an ambush forager? *Nematology* 14, 389-394.

World Health Organisation. 2006 *Pesticide Residues in Food - 2004 : Evaluations 2004, Part II - Toxicological Pesticide Residues in Food, No 20*. Albany, NY, USA: World Health Organization.

Wright, R. J., Witkowski, J. F., Echtenkamp, G. & Georgis, R. 1993 Efficacy and persistence of *Steinernema carpocapsae* (Rhabditida: Steinemematidae) applied through a center-Pivot irrigation system against larval com rootworms (Coleoptera: Chrysomelidae). *Journal of Economic Entomology* 86, 1348-1354.

Wraight, S. P., Carruthers, R. I., Jaronski, S. T., Bradley, C. A., Garza, C. J. & Galaini-Wraight, S. 2000 Evaluation of the entomopathogenic fungi *Beauveria bassiana* and *Paecilomyces fumosoroseus* for Microbial Control of the Silverleaf Whitefly, *Bemisia argentifolii*. *Biological Control* 17, 203-217.

Wright, M. S., Raina, A. K. & Lax, A. R. 2005 A strain of the fungus *Metarhizium anisopliae* for controlling subterranean termites. *Journal of Economic Entomology* 98, 1451-1458.

Wraight, S. P., Inglis, G. D. & Goettelz, M. S. 2007 Fungi. In *Field manual of techniques in invertebrate pathology: application and evaluation of pathogens for control of insects and other invertebrate pests* (ed. L. A. Lacey & H. K. Kaya): Springer.

Yilmaz, M., Gul, A. & Erbasli, K. 2004 Acute toxicity of alpha-cypermethrin to guppy (Poecilia reticulata, Pallas, 1859). *Chemosphere* 56, 381-385.

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