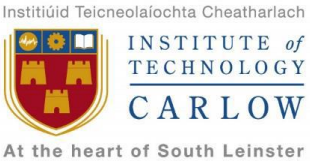
An investigation on the interactions between entomopathogenic nematodes and plant growth promoting bacteria



By

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Abstract

Increasing demands on global food production have resulted in the overuse of chemical fertilisers and pesticides, leading to an increase in environmental pollution and pest resistance. Traditional fertiliser applications are carried out to supplement soil with a bioavailable form of nutrients essential for plant growth, and pesticides are applied to prevent crop damage from economically detrimental pests. The adverse impacts of these conventional agricultural practices include water, air and soil pollution in addition to direct negative effects on human health. There is growing public and political concern on these issues and if current intensive practices are continued, natural resources including clean water, fertile soil, and biodiversity, in flora and fauna, are under severe threat for future generations. In order to facilitate the transition away from the use of conventional agri-chemicals and prevent further environmental pollution, research, and resulting application, must focus on the use of naturally occurring biofertilisers and the biological control of economically important pests. This study was the first of its kind and examined the basic fundamental interactions between entomopathogenic nematodes (EPN; Rhabditida: *Heterorhabditis* and *Steinernema*) and plant growth promoting (PGP) bacteria in order to provide a framework for the development of an environmentally sustainable, reliable, cost-effective, multi-function product for biocontrol and PGP. A comprehensive examination on the effects of PGP bacteria on EPN showed that these naturally occurring soil organisms did not significantly affect EPN survival, infectivity, virulence, attraction to their host or reproduction. Major findings of this work include Greenhouse results which revealed that a combination of *Heterorhabditis bacteriophora* and the PGP bacteria *Pseudomonas fluorescens* F113 *gfp* and *P. fluorescens* L321 *gfp* individually contributed significantly to an increase in oilseed rape (*Brassica napus*) fresh weight. Laboratory-based bioassay results showed that *P. fluorescens* F113 *gfp* successfully colonised infective juveniles (IJ) of *Steinernema feltiae* SB 12(1) and *H. bacteriophora*. Moreover, there appears to be some level of interaction between *P. fluorescens* F113 *gfp* and EPN and their associated endosymbionts (*Xenorhabdus bovienii* and *Photorhabdus luminescens*) as *P. fluorescens* F113 *gfp* survived inside *Galleria mellonella* following nematode infection and insect mortality. Interactions between EPN and bacterial species and strains varied. However, the overall results are positive and indicate that the EPN and PGP bacteria examined here are compatible and show promising potential to be developed and formulated as a combined agricultural product, for insect control and increased soil and plant health.

Scientific contributions

Results from this study have been disseminated at the following national and international conferences:

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Hurley. M J., Brazil. D. and Kakouli-Duarte, T. (2014). The effects of endophytic bacteria isolated from bioenergy crops on the biology and behaviour of entomopathogenic nematodes, unpublished paper presented at: *Environmental Sciences Association of Ireland, Environ*. Trinity College, Dublin, 26-28 February, 2014.

Hurley. M J., Brazil. D. and Kakouli-Duarte, T. (2014). Entomopathogenic nematode biology and behaviour: An investigation on nematode infectivity and fecundity following endophytic bacterial exposure, unpublished poster presented at: *Institute of Technology Carlow Research symposium*. Institute of Technology, Carlow, 2 May 2014.

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Table of Figures

Table of Tables

Table of Contents

List of symbols and abbreviations

Symbols	Description
%	Percent
±	Plus or minus
° C	Degree Celsius
ACC	1-Aminocyclopropane-1-carboxylic Acid
ANOVA	Analysis of Variance
C. elegans	*Caenorhabditis elegans*
cDNA	Complimentary Deoxyribonucleic Acid
CFU	Colony Forming Units
cm	Centimetre
CN	Genome copy number
Cq	Quantitation Cycle
d(d)H2O	Deionised (double) water
DAPG	2,4-diacetylphloroglucinol
EDTA	Ethylenediaminetetraacetic
EPA	Environmental Protection Agency
EPN	Entomopathogenic Nematode
g	Gram
G. mellonella	*Galleria mellonella*
gfp	Green fluorescent protein
GMO	Genetically Modified Organism
H. bacteriophora	*Heterorhabditis bacteriophora*
H_2O	Water
$HgCl_2$	Mercury Chloride
hr	Hour
IJ	Infective juvenile
Kan	Kanamycin
kb	Kilobase pair
LB	Luria-Bertani
LC50	Lethal Concentration 50
m	Metre
Mg	Magnesium
mg	Milligram
min	Minute (s)
mm	Millimetre
MM	Master mix
NA	Nutrient agar
nm	Nano metres
OD	Optical density
PGP(B)	Plant growth promotion (bacteria)

qPCR	Quantative polymerase chain reaction
rif	Rifampicin
RPM	Revolutions Per Minute
S. carpocapsae	*Steinernema carpocapsae*
S. feltiae	*Steinernema feltiae*
SE	Standard error
SGA	Sucrose glutaminc acid agar
Sp.	Species
Tm	Melting temperature
TSA	Tryptic Soya agar
µg	Microgram
µl	Microlitre
NCBI	National centre for Biotechnology Information
OSR	Oilseed rape
psi	Pounds per square inch
NBTA	Nutrient agar with bromothymol blue triphenyltetrazolium chloride
BLAST	Basic Local Alignment Search
RNA	Ribonucleic acid

Chapter 1 General Introduction

1.1 Introduction

Increasing demands on food production globally have resulted in the widespread application of chemical fertilisers and pesticides. These demands show no signs of slowing down with the current world population set to increase from 7.3 billion to 9.7 billion by 2050 (UN DESA report, 'World Population Prospects'). Traditional fertiliser applications are carried out to supplement soil with a bioavailable form of nutrients essential for plant growth and pesticides are applied to prevent crop damage from economically detrimental pests. The adverse impacts of these conventional agricultural practices include water, air and soil pollution in addition to direct negative effects on human health (Guleria & Tiku, 2009; Kim *et al.*, 2017; Moss, 2008).

Chemical fertilisers provide nutrients essential for plant growth and increased yields however long term use can significantly affect soil fertility (van der Bom *et al.*, 2018; Zhang *et al.*, 2018). Overuse of nitrogen fertilisers can reduce soil microbial diversity and function due to reduction in organic matter, soil acidification and an overall depletion of nutrients (Han *et al.*, 2015; Wang *et al.*, 2018). Reductions in soil pH due to nitrification of ammonium not only affects microbial community structure but also affects plant growth (van der Bom *et al.*, 2018). Moreover, contamination of both ground and

surface waters due to the leaching of nitrate has been directly linked to aquatic ecosystem eutrophication and negative impacts on human health (Billen *et al.*, 2009; Forman *et al.*, 1985). There is growing public and political concern on these issues and if current intensive practices are continued, natural resources including clean water, fertile soil, and biodiversity in flora and fauna are under severe threat for future generations.

On a governmental level, a number of measures have been put in place to tackle these issues. The European Water Framework Directive (Directive 2000/60/EC) is one such measure and it aims to restore and protect the ecological quality of water bodies across Europe. The Nitrates Directive (91/676/EEC) is an integral part of the Water Framework Directive. It is of particular interest here, as it focuses on promoting the use of good farming practices for the prevention of nitrate pollution of ground and surface waters from an agricultural source. Another key measure introduced was the EU Directive 2009/128/EC governing and regulating the sustainable use of pesticides and promoting Integrated Pest Management (IPM) in order to reduce the risks and impacts of pesticide use on human health and the environment. Moreover, to protect pollinators, and in particular honey bees, further restrictions on plant protection products containing neonicotinoids (including clothianidin, imidacloprid and thiamethoxam) have been applied, restricting their use to greenhouses only (EU Directive 2009/128/EC; EU Report 2011/2108[INI]).

Sustainable agriculture includes a collection of processes that when applied will ensure that current food and economic needs are met without compromising resources for future generations. Focusing on promoting and restoring soil fertility, reducing water use and increasing its quality and lower pollution levels and fertiliser inputs will contribute to the process of sustainability. However, in order to farm in a sustainable manner, processes

must be based on a fundamental understanding of the collective ecosystem services by examining the relationships between organisms and the environment they inhabit.

The ecology and application of two environmentally and agriculturally important groups, entomopathogenic nematodes (EPN) and endophytic bacteria, will be discussed in this Chapter, with a particular focus on agricultural practice. Moreover, the interactions between the two will be examined throughout this study in order to determine compatibility for future use as an environmentally friendly and dual biofertiliser and biocontrol product.

1.2 Nematode ecology and diversity

Nematodes belong to the phylum Nematoda and are colourless, invertebrate roundworms. Nematodes vary in size, from 0.3mm-8m in length, with the majority being microscopic and averaging less than a millimetre in length (Yeats, *et al*., 2009). Nematodes are known to inhabit environments with various climatic conditions, from pristine to extremely polluted (Bongers & Ferris, 1999). Up to 50% of nematode species live in the marine environment, with the remainder inhabiting freshwater and soil ecosystems. A total of about 40,000 free-living, invertebrate and plant-associated nematode species have been described and accepted, however, it has been suggested that the actual number of species could be anything up to 10,000,000, as many species are undiscovered (Blaxter, 2011; Lambshead & Boucher, 2003; Yeats *et al.*, 2009).

There are many species of parasitic nematodes that cause disease in plants, livestock and humans, and there are also approximately 30 families with close associations to insects (Campbell *et al.*, 2003). There are a number of approaches for the characterisation of nematodes, including highly specialised morphological identification methods, molecular identification techniques, and characterisation based on differing life strategies and feeding groups. Historically, the phylum Nematoda was thought to be comprised of two main classes: the Secernentea and Adenophorea (Chitwood, 1958). The Secernentea were those nematodes that mainly inhabit terrestrial environments, with some species isolated in marine and freshwater environments. The Adenophorea were thought to inhabit marine, freshwater and soil environments (Dorris *et al.*, 1999). More recent advances in DNA sequencing technologies allowed for a new classification for the

phylum. Blaxter *et al.*, (1998) reported five clades within the phylum Nematoda, these were the Dorylaimia (clade I), Enoplia (clade II), Spirurina (clade III), Tylenchina (clade IV) and Rhabditina (clade V). These clades are represented by the Roman numerals below in Figure 1.2.1. De Ley & Blaxter (2004) further classified the phylum into three major branches Dorylaimia, Enoplia and Chromadoria (highlighted in red, purple and blue in Figure 1.2.1) with three suborders of Spirurina, Tylenchina and Rhabditina. Faster and more cost-effective sequencing technologies have provoked a great increase in nematode genome projects, which will ultimately lead to increased knowledge on many aspects of nematode evolutionary history and biology.

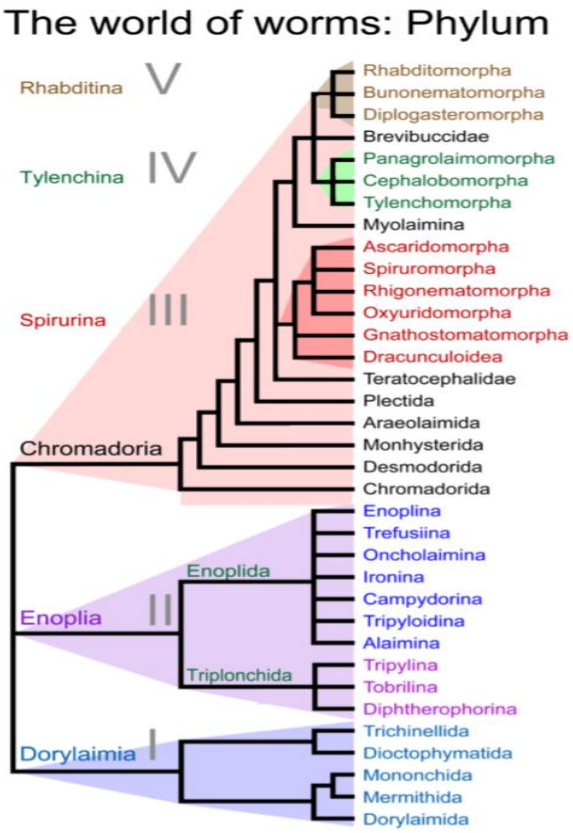

Figure 1.2.1 Classification of the Phylum Nematoda based on molecular phylogenetic analyses of the small subunit ribosomal RNA gene adopted from Blaxter (2011). Entomopathogenic nematodes from the families Steinernematidae and Heterorhabditidae form part of the Chromadoria branch.

1.3 Entomopathogenic nematodes (EPN)

Entomopathogenic nematodes (EPN) are soil-inhabiting, insect parasites that have widespread uses in the biological control of a number of important lepidopteran, dipteran and coleopteran pests (Burnell & Stock, 2000; Shapiro-Ilan *et al.*, 2006; Smart, 1995; Stuart *et al.*, 2006). The third stage infective juveniles (IJ) are the only free-living stage of the EPN from the families Steinernematidae and Heterorhabditidae (Secernentea, Rhabditida) (Kaya & Gaugler, 1993; Lewis *et al.*, 2006). EPN IJ seek, infect and kill their insect host with the aid of mutualistic bacteria carried in the nematode's gut. Members of Steinernematidae carry bacteria from the genus *Xenorhabdus* sp., whereas the members of Heterorhabditidae harbour various *Photorhabdus* sp. (Ferreira & Malan, 2014; Shapiro-Ilan *et al.*, 2006).

Evolutionary changes in the intestine of the Steinernematidae and Heterorhabditidae allowed for the development of a bacterial chamber, from which their symbiotic bacteria are secreted. Once inside the host haemolymph the IJ releases its symbiotic bacterium which then overrun the insect's defence systems and begin to multiply, killing the insect generally within 48 hr (Georgis *et al.*, 2006; Smart, 1995). Once mortality occurs the insect cadaver becomes red if the insects are killed by heterorhabditids and black/brown if killed by steinernematids (Kaya & Gaugler, 1993); the difference in the insect cadaver colour is due to pigment production by the mutualistic bacteria.

Table 1.3.1 Classification within the phylum Nematoda of species examined in this thesis

Phylum	**Nematoda**	**Nematoda**
Class	Chromadorea	Chromadorea
Order	Rhabditida	Rhabditida
Family	Steinernematidae	Heterorhabditidae
Genus	*Steinernema*	*Heterorhabditis*
Species	*feltiae; carpocapsae*	*bacteriophora*

EPN have a moderately narrow host range thus they are unlikely to cause unselective mortality (Smart, 1995). Moreover, this narrow host range means that the selection of an appropriate nematode is crucial for the control of a particular insect, as would be the case in the selection of a chemical insecticide.

1.3.1 EPN life cycle

Most of the life cycle of EPN takes place inside the host cadaver over a 12-16 day period, from IJ insect penetration to IJ emergence (generalised life-cycle shown in Figure 1.3.1). The IJ enter the insect through the mouth, anus, spiracles or by direct penetration. If nematode penetration is by mouth or anus, the nematode enters the insect hemocoel through the gut wall (Smart, 1995). However, if nematode entry is via the spiracles penetration occurs through the tracheal wall. The progeny nematodes develop through four juvenile stages before becoming adults. This starts with the IJ that develop through the third juvenile stage (J3) to the fourth-stage juveniles (J4), following feeding on their

bacterium and metabolic by-products. J4 then develop into adults of the first generation (Smart, 2005).

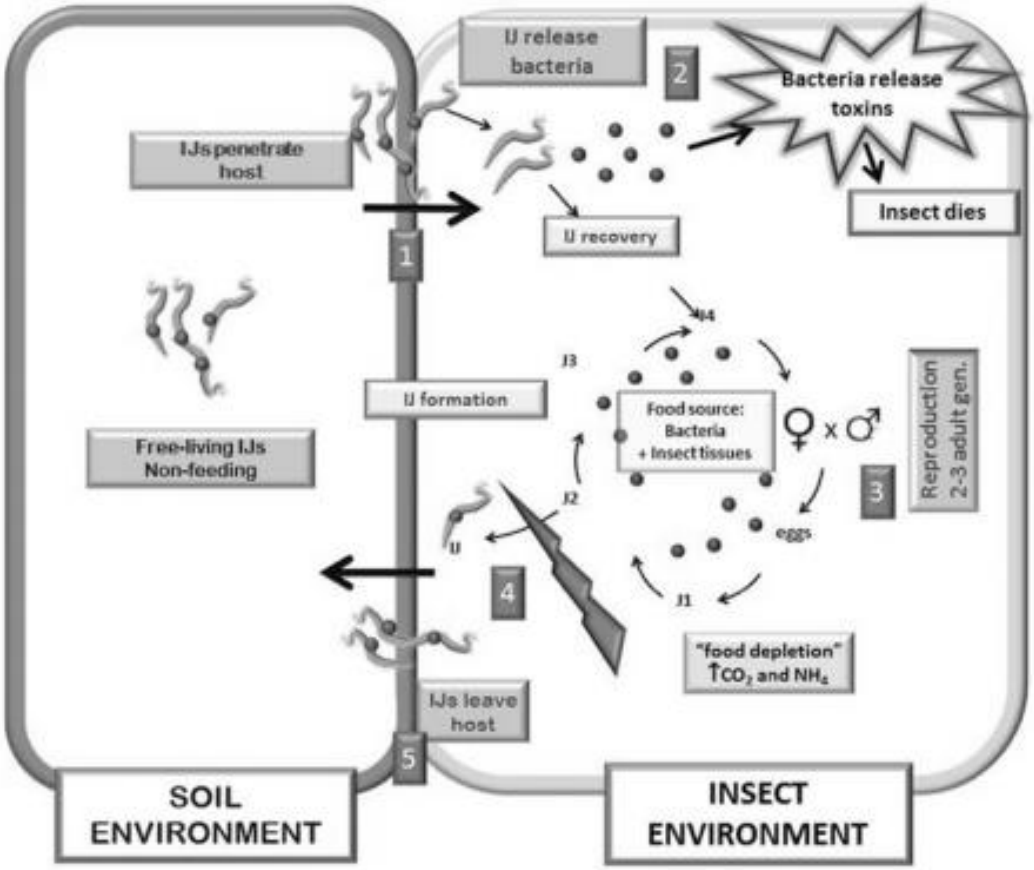

Figure 1.3.1 Generalised life cycle of EPN (*Steinernema* sp. and *Heterorhabditis* sp.) from Campos-Herrera, (2015).

Nematode reproduction and development differ between members of Heterorhabditidae and Steinernematidae. First generation Heterorhabditid adults become both hermaphroditic and amphimictic. If conditions remain favourable further generations will develop predominately as males and females. Contrastingly, steinernematids are amphimictic, with all generations producing both males and females (Grewal *et al.*, 2005). After mating the females lay eggs that hatch as first-stage juveniles (J1), these, in

turn, moult successively to second stage juveniles (J2), J3 and J4 and then to males and females of the second generation. The number of generations is dependent on the availability of resources within the host cadaver (Kaya & Gaugler, 1993). When conditions become unfavourable the late J2 cease feeding, incorporate a pellet of their bacterium and moult to the J3 retaining their cuticle as a sheath and leave the cadaver in search of new hosts.

1.3.2 EPN symbiotic bacteria

Bacteria of the genera *Photorhabdus* and *Xenorhabdus* belong to the family Enterobacteriaceae and are Gram-negative, motile insect parasites (Burnell & Stock, 2000) that are mutually associated with EPN of the families Steinernematidae and Heterorhabditidae, respectively (Ehlers, 2001). *Photorhabdus* and *Xenorhabdus* can be cultured on growth media under laboratory conditions, but phase variations often occur (Akhurst, 1993; Forst *et al.*, 1997). The phenotypic variations are known as Phase I and Phase II variants, with phase I better adapted to conditions inside both the nematode and insect (Smigielski *et al.*, 1994). Phase II variants occur during prolonged incubation and may be better adapted to environmental conditions outside the nematode; motility and antibiotic metabolite production are reduced in these variants (Smigielski *et al.*, 1994; Somvanshi *et al.*, 2010). Some species of *Photorhabdus* emit luminescence from the cadaver of the infected insect, but this does not occur with *Steinernema* infected insects. Another difference is the location of bacterial symbionts inside the nematode as seen in Figure 1.3.2. In *Steinernema* sp. *Xenorhabdus* bacteria are kept in a special vesicle in the nematode 's intestine, whereas in *Heterorhabditis* sp. *Photorhabdus* is found in the anterior part of the intestine (Forst *et al.*, 1997; Goodrich-Blair & Clarke, 2007).

Natural insect infection by *Photorhabdus* or *Xenorhabdus* starts inside the host's body cavity. In order to support successful nematode development, the bacteria must carry out a number of functions. Firstly, the bacteria must overcome the insect's natural defences and cause insect mortality. Secondly, it must facilitate nematode development by multiplying rapidly and producing nutrients from the insect cadaver. Thirdly, nematode symbiotic bacteria protect the nematodes by producing metabolites that prevent other soil organisms from entering the host. Finally, the bacteria must colonise the EPN IJ before the nematodes leave the insect in search of a new host (Georgis *et al.*, 2006; Goodrich-Blair & Clarke, 2007; Smart, 1995). The nematodes also provide protection for the bacteria, as bacterial survival without their nematode symbiont in the environment is limited. Morgan *et al.* (1997) examined the survival of *X. nematophilus* and *P. luminescens* in water and soil and noted that cells declined to undetectable levels in river water after six days and soil after seven days.

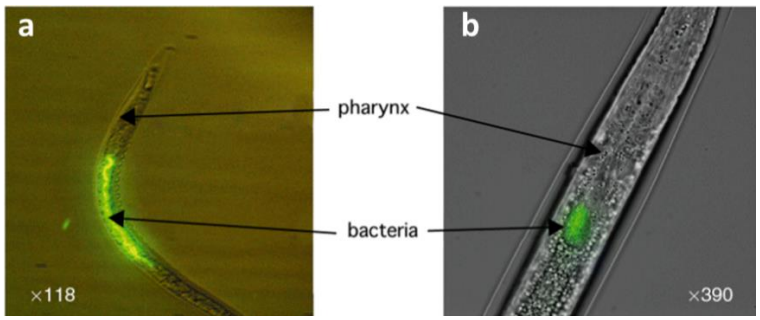

Figure 1.3.2 Variation in the location of EPN symbiotic bacteria. a) *Photorhabdus* sp. bacterium from *Heterorhabditis* sp. occupying a large portion of the lumen of the nematode gut and b) *Xenorhabdus* bacterium from *Steinernema* sp. localised within a vesicle in the posterior of the pharynx. Image from Goodrich-Blair & Clarke (2007).

Representative species from both bacterial genera have been shown to produce insecticidal toxins, enzymes that overcome the insects' immune system and antibiotics that can suppress competition from other soil microorganisms (Akhurst, 1993; Ffrench-

Constant *et al.*, 2007; Forst *et al.*, 1997). Nematodes act as vectors transporting the symbiotic bacteria inside the insect, thus they rarely have contact with the insect cuticle. Despite this, genes associated with oral insecticidal activity, including the toxin complexes (Tc's), '*Photorhabdus* insect-related' (Pir) toxins and the Makes caterpillars floppy (Mcf), have been identified in the genomes of EPN from the families Steinernematidae and Heterorhabditidae. Once ingested/injected these toxins cause damage to the insect gut epithelium (Ffrench-Constant *et al.*, 2007). Particular attention has been paid to these large orally active toxins due to their potential uses in agriculture. These toxins have activity against species of mosquitoes and caterpillars (Daborn *et al.*, 2002; Dowling *et al.*, 2004) and at least one component has been expressed in *Arabidopsis* to confer insect resistance.

The symbiotic bacteria have adapted to overcome or suppress the insect defence mechanisms. Chitin is found in the exoskeleton of insects, vertebrates, fungi and yeast. Production of hydrolases, such as chitinase, glucanase and proteases, can reduce the impact of pathogens as they have the ability to degrade cell wall structures (Javed *et al.*, 2013). *Xenorhabdus* and *Photorhabdus* can secrete proteins such as proteases, lipases, hemolysins and chitinases (Forst *et al.*, 1997; Richards & Goodrich-Blair, 2009). In pseudomonads, the chitinase encoding gene *chiA* is involved in bacterial defence as it is associated with fungal cell wall degradation, providing defence against fungi with bactericidal activity (Javed *et al.*, 2013). Chitinase proteins form a significant part of Tc in *Photorhabdus* and *Xenorhabdus* and it has been proposed that such chitinase associated toxins may contribute to the degradation of the insect cadaver in late infection (Nielsen-LeRoux *et al.*, 2012).

Induction of proteases such as *apr*E, produced by *Pseudomonas fluorescens* CY09 (Liao & McCallus, 1998), is involved in the biocontrol of nematodes. A metalloprotease from *B. thuringiensis* InhA1 has been linked to inhibition in the production and activity of antimicrobial peptides (Dalhammar & Steiner, 1984). A similar metalloprotease PrtA, secreted during the growth of *Photorhabdus* sp. strain Az29, inhibits antimicrobial activity of the synthetic antibacterial factors cecropin A and B and also inhibits activity in *Galleria mellonella* haemolymph (Cabral *et al.*, 2004). Park *et al.* (2007) report that *X. nematophila* could counteract antimicrobial peptides by suppressing their production in *Manduca sexta*, in the presence and absence of a known transcript inducer, *Salmonella enterica*. Additional antimicrobial molecules produced by *Photorhabdus* include hydroxy-stilbene, and by *Xenorhabdus* include benzylidene-acetone and xenorhabdicin. These compounds induce antibiotic activity against both Gram-negative and positive bacteria (Akhurst, 1993; Hall *et al.*, 1995; Morales-Soto & Forst, 2011; Williams *et al.*, 2005). As a result of the insufficient knowledge on (a) the specific role of the toxins in infection (Ffrench-Constant *et al.*, 2003) and (b) the poor bacterial survival and proliferation in the environment outside the nematode host (Morgan *et al.*, 1997), the biocontrol potential of these symbiotic bacteria remains uncertain.

1.3.3 Foraging strategies

There are two different perspectives that can be used to describe how organisms forage for resources. The first is based on the organism's behavioural responses to environmental stimuli. Here, the host's suitability, habitat, chemical cues and location (Laing, 1937) are all considered. The second perspective is based on information relating to how organisms move through their environment (Grewal *et al.*, 2009). When examining behavioural responses, foraging strategies can be further divided into cruise (active searchers) and

ambush (sit and wait) approaches (McLaughlin, 1989). Classification of organisms as either cruisers or ambushers can be based on differences in the time allocated to motionless scanning (ambushers) versus when moving through the environment (cruisers) (O'Brien *et al.*, 1990). These models were not originally described for EPN, but they can be applied to a number of taxa including EPN (Lewis *et al.*, 2006).

According to Grewal *et al.* (1994), foraging strategies for a number of EPN species can be predicted through analysis of responses to host volatile cues and dispersal behaviour on smooth and nictation substrates, in the form of agar and agar overlaid with sand. Foraging strategies adopted by EPN are not species specific. *H. bacteriophora*, *Heterorhabditis megidis*, *Steinernema arenarium*, and *Steinernema glaseri* show a positive directional response to host volatile chemicals and travel similar distances on both substrates, indicating a cruising approach to finding hosts. Contrastingly, *Steinernema carpocapsae* and *Steinernema scapterisci* travel less distance on the nictation substrate than on smooth agar and do not show any directional response, suggesting an ambushing mode of foraging. The allocation of ambusher or cruiser strategies is more of a spectrum rather than in definitive categories. This is evident for *S. feltiae* and *Steinernema thilisiensis*, as these species travel less distance on nictation substrate than on smooth agar and also respond directionally to host volatiles, showing characteristics of both strategies and holding an intermediary position (Campbell & Gaugler, 1997; Grewal *et al.*, 2009; Lortkipanidze *et al.*, 2016).

In practice, EPN adopting the ambusher strategy generally remain in the same location for prolonged periods of time, don't rely on host chemical cues and may nictate or jump to catch high mobility prey that passes by (Campbell *et al.*, 2003; Campbell & Kaya, 2000; Gaugler & Campbell, 1993) generally acting nearer to the soil surface. On the

other hand, cruisers are better suited to sedentary hosts as IJ will continually move throughout the soil profile, using chemical cues such as carbon dioxide, to search out hosts deeper in the soil profile.

A foraging strategy adopted by an EPN species will influence their distribution throughout the soil profile and the distance travelled by IJ and subsequently the hosts they are likely to encounter (Millar & Barbercheck, 2002). Therefore, host susceptibility and suitability (in terms of location in the soil) must be considered before applying EPN for biocontrol. Moreover, when applying EPN the agricultural practices must also be considered. Millar & Barbercheck (2002) carried out field trials to examine the effects of tillage practices on EPN distribution and showed that as a result of occupying different locations in the soil profile, *S. carpocapsae,* an ambush forager located closer to the soil surface, was significantly affected by tillage practices. On the other hand, *H. bacteriophora,* a cruise forager, was not significantly affected by tillage practices.

Host-associated chemical cues are of particular importance for cruise foragers and can act as an attractant (Ansari & Butt, 2011) or repellent (Kunkel *et al.*, 2006) for IJ allowing them to discriminate between suitable and unsuitable hosts. Ambush foragers also respond to volatile cues, but host contact is first required (Lewis *et al.*, 1995). In addition to host-associated cues, insect-induced plant signals emitted from roots damaged by feeding insects have been shown to attract EPN (Rasmann *et al.*, 2005; van Tol *et al.*, 2001). One such chemical identified is (E)-β-caryophyllene (EβC) produced by damaged roots following feeding by rootworm (Chiriboga M. *et al.*, 2018; Rasmann *et al.*, 2005).

Rasmann *et al.* (2005) recorded a fivefold increase in nematode infection of the Western Corn rootworm (*Diabrotica virgifera virgifera*) in maize varieties releasing EβC compared to non-EβC production varieties. Similarly, feeding by black vine weevil

larvae (*Otiorhynchus sulcatus*) on the roots of the Northern white-cedar (*Thuja occidentalis*) stimulated the release of chemical odours that attracted the EPN *H. megidis* (van Tol *et al.*, 2001). Root-associated bacteria must also be considered when examining the effects of herbivore feeding belowground. Chiriboga *et al.* (2018) described an increase in production of EβC and enhanced expression of the EβC synthase gene (tps23) after rootworm (*Diabrotica balteata*) feeding on maize roots colonised by *Pseudomonas protegens* CHA0. The complexity of the belowground interactions and the subsequent cascading effects on the soil ecosystem is evident here. Examining the interactions between plants, their associated bacteria, herbivores and entomopathogens will provide new insights into these multitrophic interactions and potentially increase efficiency in biocontrol and PGP.

1.3.4 Factors influencing EPN distribution and proliferation in soil

There are a number of factors that have been found to influence the distribution of EPN in soil (Stuart *et al.*, 2006). Taking into consideration factors including host presence, susceptibility and suitability, soil abiotic and biotic factors, competition and EPN natural enemies, is essential when considering utilising EPN as biological control agents to suppress economically important insect pests (Smart, 1995).

1.3.4.1 EPN competition and displacement

Differences in behavioural characteristics and responses to environmental factors allow a diverse range EPN species to co-exist in the soil environment (Koppenhöfer & Kaya, 1996; Millar & Barbercheck, 2002; Stuart *et al.*, 2006; Stuart & Gaugler, 1994), however competition between and within species still occurs. Examining intra and/or interspecific EPN competition is essential as it has the potential to negatively impact the population

dynamics, persistence and even the establishment of introduced EPN. Intraspecific competition between nematodes of the same species for host resources has been reported for a number of Steinernematidae and Heterorhabditidae species (Koppenhöfer *et al.*, 1995; O'Callaghan *et al.*, 2014; Zervos *et al.*, 1991) with males of a number of steinernematids killing other males and females of the same species (O'Callaghan *et al.*, 2014). Nematode interspecific competition can be both direct and indirect within a host. Direct competition involves interferences between different species, and indirect refers to effects resulting from changes in environmental conditions or the presence of other species (Stuart, 2005). Although different EPN species can co-exist, one species tends to dominate another. Alatorre-Rosas & Kaya (1990) reported interspecific interactions between *H. heliothidis* and *S. feltiae, S. bibionis*, or *S. glaseri*. When horizontal dispersal was examined, all the *Steinernema* sp. outcompeted *H. heliothidis* for hosts located up to 30 cm away from the nematode application point, with co-infection of insects also occurring. Direct aggression between *Steinernema* sp. has been reported with varying degrees of mortality. O'Callaghan *et al.* (2014) reported that *S. longicaudum* was the most efficient killer followed by *S. carpocapsae, S. kraussei* and *S. feltia*e. In order to eliminate or minimise the possibility of displacing any non-target natural enemies, it is necessary to increase the understanding of EPN competitive abilities, as this could aid the evaluation of particular EPN species for biological control.

1.3.4.2 Effects of abiotic factors on EPN survival and distribution

EPN distribution, abundance and survival are highly mediated by the diversity in the soil ecosystem (Stuart, 2005). In terms of abiotic factors, there are a number of environmental factors (UV, moisture and temperature), agricultural practices (addition of pesticides,

tillage practices) and soil characteristics (pH, texture) that must be considered (Gaugler & Kaya, 1990; Stuart *et al.*, 2006).

The negative effect of UV light on EPN survival has been reported (Kaya, 1990) and must be considered when applying EPN in agriculture. Adequate soil moisture levels are required to allow for movement, but too much moisture leading to oxygen depletion can be harmful to EPN. The optimum temperature of a particular EPN species must also be investigated as reproduction and host infection tend to be to temperature dependent. Optimum temperatures vary between EPN species and strains. For example, *S. glaseri* is relatively heat tolerant and can maintain efficacy above 29 ºC, whereas *S. feltiae* is cold tolerant maintaining efficacy at and below 15ºC (Stuart, 2005; Grewal, *et al.*, 1994). These environmental requirements can be met by applying EPN in the evening, and the right time of the year, with conventional insecticide spraying or drip systems.

Soil productivity is influenced by the type of management practices applied. Disturbances can be the result of physical (tillage), chemical (fertiliser application) or biological (overgrazing) activities and can result in a reduction of plant growth, disruption of microbial communities, increased runoff (Nawaz *et al.*, 2013; Pereira *et al.*, 2018). The effects of tillage practices were discussed briefly above and have been shown to affect the survival and distribution of some EPN species in soil (Millar & Barbercheck, 2002). Soil pH maintained between the values of 4 and 8 is required for EPN survival, with detrimental effects recorded for pH 10 (Kung 1990). Application of chemical fertilisers, including lime, can greatly alter the pH of the soil. Moreover, the effect of such fertilisers and chemical pesticides can vary on EPN and, depend on the concentrations and types used, these can have either positive, neutral or negative effects (Shapiro, 1996).

1.3.4.3 Natural enemies of EPN

Soil biotic factors are likely to influence EPN distribution and abundance, as soil contains diverse communities of flora and fauna that are all interconnected in the soil food web (Bongers, 1999). EPN interactions with soil microbes and microarthropods may be positive or negative (Kaya and Koppenhöfer 1996; Wilson and Gaugler 2004; Greenwood 2011). EPN natural enemies include bacteria, fungi, mites, predatory nematodes, along with other soil nematodes, all of which have the ability to reduce EPN populations in the soil (Shapiro-Ilan, *et al.*, 2005; Kaya, 2002). In laboratory-based bioassays, studies suggest that the presence of nematophagous fungi, bacteria, nematodes, or mites, have a considerable impact on EPN, but the correlations in nature remain understudied (Kaya, 2002). The presence of a suitable host is one of the primary influencing factors in the survival of EPN. Hosts provide a location for EPN reproduction, although an increase in insect density will, in turn, lead to an increase in the attraction of predators, parasites and pathogens (Stuart, 2005). The application of EPN-infected cadavers has been examined to increase efficiency in biocontrol (Koppenhöfer & Kaya, 1996; Shapiro-Ilan *et al.*, 2006; Shapiro *et al.*, 1996; Shapiro & Lewis, 1999). Thus, the response of the soil microbiota, and in particular microarthropods, to EPN-infected cadavers has been increasingly examined, with results varying from negative to stimulatory (Jabbour & Barbercheck, 2011). Greenwood *et al.* (2011) found that invertebrate taxonomic richness was greater following application of *S. carpocapsae*-infected cadavers than with *H. bacteriophora*-infected cadavers. Moreover, application of steinernematids increased the attraction of two mite taxa, *Galumnidae* and *Scheloribates* sp., whereas *Histiostomatidae, Scheloribates* sp., *Eupodes* sp. mite taxa were repelled. Many species of mites have been

shown to be nematophagous (Cakmak *et al.*, 2010; Ekmen *et al.*, 2010; Poinar, G. O., 1979) negatively affecting biological pest control by feeding on purposely released, or naturally occurring, EPN. In addition to mites, ants have been shown to have increased attraction to and increased feeding on, EPN-killed insects, with significant effects on EPN development (Baur *et al.*, 1998; Zhou *et al.*, 2002). Susceptibility between EPN families varied, with ant workers scavenging more on steinernematid-killed (*S. carpocapsae, S. glaseri, S. feltiae*) insects than on heterorhabditid-killed (*H. bacteriophora, H. megidis, H. marelatus*) insects (Baur *et al.*, 1998).

Both EPN and plant-associated bacteria have widespread applications in agriculture. The use of both taxa independently is well documented and current applications include increasing soil health, plant growth promotion (PGP), biological control and improving crop health. The application of both types of these soil organisms for biocontrol and increased plant growth would result in a reduction of chemical insecticide and fertiliser inputs, leading to more sustainable practices for agriculture and food security. However, there is little knowledge on the interactions between these two agriculturally important soil organisms.

1.4 Plant-associated bacteria

The soil ecosystem harbours large communities of microbial life including bacteria, archaea, fungi, algae and protozoa, and forms the basis for the higher food webs as it is the location for many of the biochemical reactions that contribute to the decomposition and cycling of organic matter, disintegration of rock and redistribution of nutrients for plants. Microorganisms located in the plant rhizosphere (the zone that consists of the below-ground root zone) may be neutral, stimulatory or deleterious to plant growth

(Raaijmakers *et al.*, 2009). In both agricultural and natural ecosystems, these beneficial plant-associated bacteria play a key role in supporting or enhancing plant health and growth. Both the above and below-ground plant parts harbour a diverse range of microbial communities. The majority of plant-associated bacteria originates from the soil environment (Compant *et al.*, 2010) and have the potential to colonise the rhizoplane (root surface) and internal plant roots (Hallmann *et al.*, 1997; Mwajita *et al.*, 2013).

Plant-associated bacteria including rhizobacteria (bacteria that occupy the immediate region around the root zone and have the ability to colonise plant tissues) and bacterial endophytes (bacteria that colonise internal plant sections) have many applications in agriculture and the environment. In agriculture, they have been shown to promote plant growth and health as well as improve soil health (Lee, *et al.*, 2000; Azevedo, *et al.*, 2000) and in the environment, they are involved in phyto and rhizoremediation.

Endophytic bacteria can be neutral or beneficial to the host plant (Backman & Sikora, 2008; Hardoim *et al.*, 2008). Bacterial plant colonisation can protect the plant from environmental stresses and contribute to pest management by acting as biological control agents protecting host plants against pathogens and predators (Azevedo *et al.*, 2000; Bacilio-Jiménez *et al.*, 2001). Biocontrol capabilities of bacterial endophytes will be discussed further in Section 1.5.1. Once plant roots are colonised, bacterial strains can relocate to the aerial plant parts, however, the bacterial density in the phyllosphere may be reduced in comparison to rhizosphere or root density (Compant *et al.*, 2010).

Endophytes can originate from the soil, seed or phyllosphere and are thought to be biphasic in nature moving between plants and the soil environment (Hardoim *et al.*, 2008). If they are not vertically transmitted using the seed as a vessel, they can enter plant tissues through the natural openings of the roots, flowers, stems or stoma. The

production of pectinases and cellulases also allow for direct penetration of plant tissues. Once inside the plant, endophytes can enter the vascular system and spread throughout the host plant's tissues occupying the stoma, emerging lateral roots, germinating radicals, and nodules (Germaine *et al.*, 2004; Hallmann *et al.*, 1997). Plants exist in a diverse and competitive environment and as a result, have adapted specialised mechanisms to thrive. This includes bacterial endophytes that can successfully colonise the plant and infer a beneficial trait, such as production of antimicrobial compounds. In order to be successful, endophytic bacteria must possess the mechanisms necessary to exploit and persist in the nutrient-rich environment between the plant and bulk soil.

There are many different bacterial species that are endophytic in nature, examples include *Acetobacter, Arthrobacter, Bacillus, Enterobacter, Erwinia, Pseudomonas, Microbacterium, Azospirillium, Staphylococcus* and *Rhizobium* (Germaine *et al*., 2004; McInroy & Kloepper, 1995; Moore *et al.*, 2006). The microbial community associated with, and colonising a plant can be very diverse as a single host can facilitate numerous bacterial species with varying specificity. Moreover, geographical distribution, plant age and colonising tissue type also play a role in microbial diversity (Kobayashi & Palumbo, 2000).

To date endophytes have been isolated from an array of plant species of agricultural importance including rice, corn, alfalfa, cotton, potatoes, willow, poplar, wheat, soybean, perennial grasses and legumes (Barraquio *et al.*, 1997; Culhane, 2016; Moore *et al.*, 2006; Otieno, 2014; Zinniel *et al.*, 2002). *Pseudomonas fluorescens* endophytic bacterial strains L321, L111 and L228 used in this study, were isolated from *Miscantus* (Keogh, 2009) and been shown to successfully colonise plants including *Lolium perenne* and *Brassica napus* (Culhane, 2016; Lally, 2016; Otieno, 2014). Genome sequencing of these three *P.*

fluorescens strains show that they contain genes associated with plant colonisation (Lally, 2016). Pectin and cellulose are structural plant components, all three strains also contain the cellulose synthase operon and both L321 and L111 contain a gene encoding a pectin lyase enzyme. Bacterial motility has also been described as a vital mechanism utilised in plant colonisation by *P. fluorescens* strains and it is responsible for that flagellar driven chemotaxis that guides bacteria towards plant root exudates (de Weert *et al.*, 2002). Three of the strains examined in this study (L321, L111 and L228) contain the genes for flagella pilus assembly and motility, moreover, there are at two chemotaxis mechanisms in L321 and L111 and one present in L228 (Lally, 2016).

In order for microbes to confer plant beneficial effects (PGP or biocontrol), efficient colonisation of the plant environment is essential. One of the primary aims of this study is to identify compatible nematode and bacterial strains for a dual biocontrol and biofertiliser product. Thus, these plant colonisation traits coupled with the proven colonisation capabilities across a number of different plant species makes these isolates of particular relevance for this research.

1.4.1 Agricultural applications of plant-associated bacteria

The use of plant-associated bacteria contributes towards the development of sustainable agriculture and offers a more environmentally friendly alternative to chemical pesticides and fertilisers. Plant-associated bacteria can improve soil health through the remediation of contaminated land and increase plant growth through the suppression of plant pathogens or the acquisition of essential plant nutrients.

1.4.1.1 Plant growth promoting bacteria

Plant microbes can cause disease and inhibit plant growth, while others can actively or passively promote growth through a variety of mechanisms (Ma *et al.*, 2011). There are a number of mechanisms by which plant-associated bacteria can promote plant growth and acquire nutrients under adverse conditions, these include nitrogen fixation, solubilisation of phosphate, production of phytohormones such as indole acetic acid (IAA) and siderophore production (Ma *et al.*, 2011; Puente *et al.*, 2009; Ryan *et al.*, 2008; Zhang *et al.*, 2011).

The overuse of chemical fertilisers, including nitrogen (N), over the past decades has contributed to groundwater contamination and an increase in greenhouse gas emission (Bhattacharjee *et al.*, 2008). Fertilisers help ensure food security and productivity but excess or inappropriate use can have serious impacts on environmental health, negatively affecting the quality of air, soil and water. Soil microbial diversity can also be affected by fertiliser applications, with consistent decreases in functional diversity of both soil microflora and nematode populations as a result of N application (Sarathchandra *et al.*, 2001). The International Nitrogen Initiative was set up in 2003 in order to optimise the beneficial role of N in sustainable food production while also minimising the negative effects on the environment as a result of food and energy production. The overall aims include providing coordinated scientific support for international nitrogen management policy (Brownlie *et al.*, 2015).

N_2 makes up approximately 80 % of the atmosphere and is essential for plant growth, however, plants cannot directly access this dinitrogen form. To reduce the reliance and

use of chemical fertilisers, an alternative approach of supplying bioavailable N for plants is the use of nitrogen-fixing bacteria. A number of free-living bacteria including, *Azospirillum, Burkholderia*, *Rhizobium*, *Azoarcus* and *Pseudomonas*, have the ability to fix nitrogen. Biological Nitrogen Fixation is a process, catalysed by the enzyme nitrogenase (present in some bacteria), by which atmospheric N is converted from an un-reactive state or non-bioavailable form (N_2) into the bioavailable form ammonia (NH_3), (Bhattacharjee *et al.*, 2008). Bacteria capable of biological N fixation have been found to be associated with a number of globally important crops including, forage grasses, sugarcane and rice. Plants absorb bioavailable N through their roots. The dependence of crops and the limited bio-availability of N in soil has resulted in the widespread application of N based fertiliser worldwide (Emerich *et al.*, 2009). Yanni *et al.*, (1997) examined the effects of a reduction in conventional nitrogen application on rice grain yield when plants were inoculated with *Rhizobium trifolii*, here there were no differences in grain yield, indicating that the bacterial inoculant may have partially substituted N. A more recent study carried out by Yanni *et al.*, (2011) indicated that Rice plants inoculated two IAA producing, diazotrophic *Rhizobium* species *(R. leguminosarum* bv. trifolii E11 and *Rhizobium*. sp. IRBG74) stimulated rice growth and increased grain yields. Moreover, Hungria *et al.*, (2010) reported that four strains of *Azospirillum brasilense* were capable of increasing the yield of Maize when plants were grown under reduced fertiliser applications.

Phosphorus (P), along with N is a major essential macronutrient for biological growth and development. P is often a limiting mineral nutrient in natural ecosystems and can only be taken up in its monobasic or diabasic soluble forms. Insufficient levels of P stunts plant growth leading to reduced crop yields. Soluble P is highly reactive with other

elements, such as heavy metals, and as a result, levels are often low, particularly in stressed soil.

Many plant-associated bacteria, including *Pseudomonas, Bacillus* and *Rhizobium*, have been identified as phosphate solubilisers. Insoluble P can be converted into the bioavailable form by these microbes by acidification, chelation, exchange reactions, the release of organic acids or extracellular phosphatase production (Anandham *et al.*, 2008). A laboratory-based study carried out by Menton, (2010), reported that a number of endophytes isolated from *Miscanthus* were capable of solubilising a fixed form of phosphorous (tricalcium diortho phosphate). Inoculation of plants with plant growth promoting bacteria can improve plant development, nutrient levels and soil enzyme activity within the plant environment (Madhaiyan *et al.*, 2010) and co-inoculation of plants with bacteria carrying out various functions offer a promising approach in improving plant and soil health. Madhaiyan *et al.*, (2010) examined the effects of tomato, red pepper and rice nutrient uptake and growth following inoculation with a bacterial consortium comprising, a methylotrophic *Methylobacterium oryzae* CBMB20, an N-fixing *Azospirillum brasilense* CW903 and a P solubilising bacterium *Burkholderia pyrrocinia* CBPB-HOD. Increased shoot or root length were recorded in addition to significant increases in leaf P and Zn levels, and increases in the crop yield. A number of endophytic bacterial isolates used in this study have previously been shown to solubilise P (Lally, 2016; Menton, 2010; Otieno, 2014) and in some cases, increase plant growth.

Iron (Fe) is an essential nutrient and a necessary co-factor for many bacterial enzymatic reactions. Fe exists predominantly in its ferric state (Fe^{3+}), this reacts to form insoluble hydroxides that are unavailable to plants and microorganisms (Ma, *et al.*, 2010). In order

to uptake sufficient Fe, bacteria must produce siderophores, these are produced by various bacteria as a secondary metabolite and are ferric ion specific chelators secreted under iron stressed conditions (Khan *et al.*, 2018). Siderophore producing rhizobacteria can chelate Fe^{3+}, this resulting siderophore-Fe complex can then be taken up by the roots. Production of siderophores can compensate for any biological changes that may occur in a plant due to environmental stresses, that would result in poor iron uptake. Moreover, siderophore-producing bacteria can act as bio-control agents due to their high Fe affinity (Schippers *et al.*, 1987), resulting low levels of Fe will prevent fungal pathogens from proliferating in the plant rhizosphere (O'Sullivan & O'Gara, 1992).

Phytohormones produced by plant-associated bacteria include ethylene (C_2H_4), indole-3-acetic acid (IAA), gibberellins and cytokines and have been associated with germination, growth, and reproduction and protect plants against environmental stresses (Ma *et al.*, 2009). Ethylene has many beneficial properties including disease resistance, increased biotic and abiotic stress tolerance and inducing physiological changes in the plant at the molecular level. The level of ethylene must be strictly regulated as overproduction in plants can result in inhibition of root growth and root hair formation.

Ethylene is a plant hormone secreted to induce various types of growth regulation in plants. This hormone also induces environmental stress responses (Wang et al., 2002) and overproduction has been associated with the growth restriction as a result of flooding, drought, cold temperatures, and exposure to pathogens (Abeles *et al.*, 1992; Glick *et al.*, 2007). 1-aminocyclopropane-1-carboxylate (ACC) is a precursor of ethylene in plants and the enzyme ACC deaminase can metabolise ACC into α-ketobutyric acid and ammonia (Zhang, *et al.*, 2011). Microorganisms that produce ACC deaminase decreases the level of ethylene in the plant, lowering the effects of plant stress responses and

promote plant growth by metabolising ACC (Arshad *et al.*, 2007; Glick *et al.*, 2007; Penrose & Glick, 2001). As a result of decreasing ethylene levels plant productivity is increased and stresses are alleviated. The ACC deaminase enzyme is coded by the gene 1-aminocyclopropane-1-carboxylate deaminase acdS. Two of the *P. fluorescens* strains examined in this project, L321 and L111, have the acdS gene present in their genome and shown ACC deaminase activity *in-vitro* (Lally, 2016; Otieno, 2014).

IAA is produced in plant seeds, roots, stems and leaves and is a product of the Tryptophan metabolic pathway. The production of IAA by bacteria can have major influences on plant growth regulation and beneficial for bacterial-plant signalling (Ali *et al.*, 2009; Naveed *et al.*, 2015; Spaepen *et al.*, 2007). IAA producing bacteria include *Aeromonas veronii, Azospirillum brasilense, Agrobacterium* sp., *Bradyrhizobium* sp. and *Enterobacter* sp. There are multiple metabolic pathways that can be utilised by microorganisms to produce IAA, the main precursor of the biosynthetic pathway is tryptophan this can be converted to IAA by indole-3-acetonitrile, indole-3-acetamide or tryptamine.

A number of endophytic bacteria examined in this study have been shown to possess a variety of PGP traits including N fixation, plant home production, P solubilisation and siderophore production (Lally, 2016; Menton, 2010; Otieno, 2014). These traits played a key role in selecting isolates for nematode compatibility screening in this study. PGP is of particular interest in this study. However, plant-associated bacteria also play an essential role in improving plant growth in stressed environments and removing toxic compounds from contaminated environments through the processes of bio and phytoremediation.

1.4.1.2 Remediation potential of plant-associated bacteria

The widespread use of synthetic hydrocarbons and petroleum products has led to the release of metals and xenobiotics resulting in high levels of environmental pollution (Don & Pemberton, 1981; Top *et al.*, 2002). These harmful chemicals can have significant negative effects on natural soil organisms. Moreover, the bioaccumulation of toxic chemicals can lead to serious health problems and/or genetic disorders in human if they were to enter the food chain (Keogh, 2009; Timmis & Pieper, 1999). Environmental applications of plant-associated bacteria include technologies such as bioremediation, phytoremediation and improved soil health (Furini *et al.*, 2015; Glick, 2010; Lisitskaya & Trosheva, 2013; Wubs *et al.*, 2016). Over the last two decades, bioremediation has emerged as a tool to clean up heavy metal contaminated or polluted environments (Ma *et al.*, 2011). Bioremediation of oil-contaminated soil by a number of bacterial endophytes used in this study has been documented previously (Culhane, 2016).

Bioremediation is a term used to refer to any process that uses microorganisms to remove contaminants from the environment, returning it to its natural state (Heitzer & Sayler, 1993). This is a slower process than traditional engineering methods, however, it can be applied *in situ* without the removal of polluted soil (Timmis & Pieper, 1999; Top *et al.*, 2002) and offers a more environmentally sustainable cost-effective cleanup method. Globally, the cost of carrying out these traditional remediation processes have been estimated at $25-50 billion (Tsao, 2003), this often results in polluted areas being abandoned rather the cleaned up.

A study carried out by Keogh, (2009) demonstrates that endophytes, used in this current study can be used to bioaugment plants for phytoremediation. Phytoremediation is the plants to remove environmental pollution and has gained significant attention (Azevedo *et al.*, 2000; Doty, 2008). Phytoremediation takes advantage of the natural ability of plants to extract chemicals from water, soil and air to degrade or eliminate metals, pesticides or solvents (Doty, 2008; Glick, 2003). Sites such as mining sites, industrial ground and landfills are particularly suited to phytoremediation as there are high levels of contamination and little development potential.

Naturally occurring bacteria that have adapted to survive under stressed conditions and in some cases become capable of using the pollutant as a substrate or a nutrient source allowing them to proliferate (Liu *et al.*, 1993) and degrade the contaminating substances. Microbes have been shown to degrade benzene, toluene and ethylene. Although this process has been proven effective it generally occurs over a long time period and may take decades to complete. Zhang *et al.*, (2011) examined Copper (Cu) remediation in oilseed rape by metal tolerant, P solubilising, siderophore and ACC producing microbes. Pot experiments showed that co-inoculation of plants with the strains, *Ralstonia* sp. J1-22-2, *P. agglomerans*, and *Pseudomonas thivervalensis* Y1-3-9 resulted in increases in biomass and Cu accumulation in plant tissues, with maximum Cu uptake by *P. agglomerans* Jp3-3. Sriprapat & Thiravetyan, (2016) examined to evaluate the benzene uptake rates in *Syngonium podophyllum*, *Sansevieria trifasciata*, *Euphorbia milii*, *Chlorophytum comosum*, *Epipremnum aureum*, *Dracaena sanderiana*, *Hedera helix*, and *Clitoria* ternatea and found that the removal rate of benzene by sterile plants was less than non-sterilised plants. Moreover, a number of benzene-resistant bacteria isolated from *C. comosum* were successful at removing benzene, with one endophytic *Enterobacter* EN2 strain showing a high rate of IAA production, benzene tolerance and subsequent removal.

A combination of contaminant-resistant and plant-associated bacteria play an important role in bacterial-assisted phytoremediation of stressed arable land.

There have been a number of studies demonstrating the successful use of biopiling to remediate contaminated soil. Germaine *et al.*, (2015) used 'Ecopiling' to remediate petroleum impacted industrial soil, this is a process that involves biostimulation of naturally occurring hydrocarbon degraders, bio-augmentation through inoculation with a known PAH-degrading consortia and phytoremediation, through the effect of root growth and penetration throughout the soil and the resulting stimulation of microbial activity in the rhizosphere. A complete reduction in the total petroleum hydrocarbons (TPHs) (initial concentration of 1613mg TPHs kg^{-1} soil) was recorded after two years, using a site-specific TPH degrading bacterial consortia a biopile system and a phytocap comprising a mixture of perennial ryegrass (*L. perenne)* and white clover (*Trifolium repens*). The effectiveness of PAH phytoremediation in contaminated soil can also be enhanced through the introduction of PAH-degrading microbes. Liu *et al.*, (2016) investigated the use of ecopiling for the bioremediation of creosote-contaminated soil/sediment. Here, a site-specific PAH-degrading bacterial microbiome was applied to ecopiles in the form of encapsulated calcium alginate beads and a 94 - 95 % reduction in PAH concentrations was recorded after 730 days.

Genome sequencing of three of the *P. fluorescens* strains L321, L111 and L228 used throughout this study show a number of agriculturally important traits. The three strains have genes associated with effluxing heavy metals (Lally, 2016). L228 can also utilise benzoate *in vitro* and possesses the operons corresponding to the *ben*, *cat* and *pca* genes (Culhane, 2016; Lally, 2016) that are necessary for the full conversion of benzoate to Krebs Cycle intermediates via the catechol branch. These traits could play protective

roles for microbes existing in toxic concentrations of heavy metals, indicating their potential use in bioremediation. Moreover, the combination of these remediation capabilities and PGP traits (discussed in Section 1.4.1.1) demonstrate the potential use of such bacterial isolates as microbial inoculants in stressed arable land and as part of a bioremediation or phytoremediation process. Another mechanism for promoting soil health and plant growth is the reduction in traditional chemical inputs and the introduction of biological fertiliser and insect control products. These activities can be implemented through an integrated pest management (IPM) system.

1.5 Integrated pest management (IPM)

IPM can be defined as the ecological approach to maintaining pests at a tolerable level (Prokopy & Kogan, 2009), and has been accepted as the main strategy for managing agriculturally damaging pests (Peshin *et al.*, 2014). Such pests can include vertebrates, invertebrates, weeds and pathogens (Karuppuchamy & Venugopal, 2016). IPM is regulated at a government level with the introduction of EU directive 2009/128/EC that encourages EU member states to promote IPM and sustainable pesticide use. IPM is not simply the elimination of chemical pesticides, and in fact, many approaches include some pesticide use (Zalom, 2010). Pests of economic importance are managed by a framework that incorporates environmental, health, social and economic factors (Karuppuchamy & Venugopal, 2016; Zalom, 2010).

Pest management is not the complete elimination of all pests, rather it is the reduction of pests below the economic threshold for the grower. Ramsden *et al.* (2017) described the economic threshold in arable crops as the pest population density at which pest control

measures should be first implemented to prevent economic damage. IPM actions eliminate the need for the environmentally harmful practice of spraying chemicals on crops, irrespective of actual pest damage or abundance. IPM as a process, requires continuous observations, inspections and detailed and accurate record keeping to successfully evaluate the process and make future recommendations (Gibb & Gibb, 2015).

The IPM process is based on a hierarchical pyramid consisting of seven key areas for consideration (Figure 1.5.1). Starting at the base with various actions that can be carried out both prior to planting and up until harvest. The circular tier in the centre outlines the ecological aspects for consideration, with the top tier consisting of chemical pesticide application, should all other considerations fail (Stenberg, 2017). Biological control (biocontrol) is a non-chemical control method and can be defined as the use of living organisms to reduce the impact and/or population of economically important pests (Eilenberg *et al.*, 2001; Smith, 1919). Biocontrol is one of the more researched areas in the IPM process.

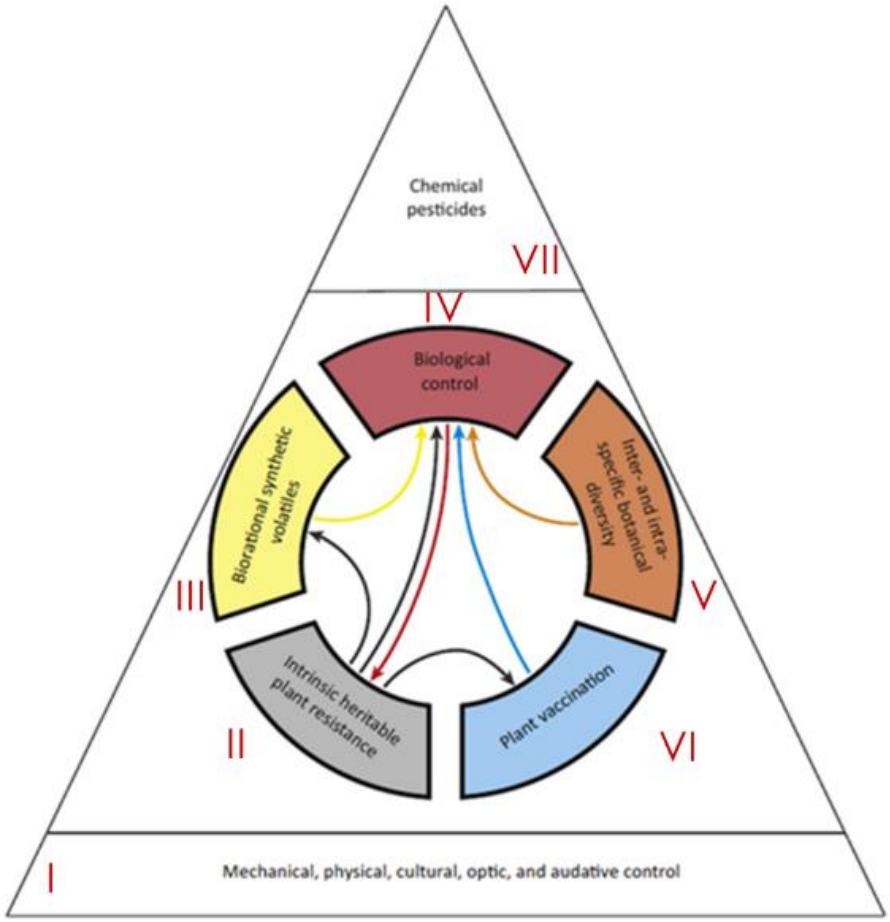

Figure 1.5.1 Both EPN and bacterial endophytes play an integral role in integrated pest management. This pyramid shows the most important IPM processes (from Stenberg [2017]).

EPN are suitable for the IPM concept because they are relatively specific to their target pest(s), can be applied with standard pesticide equipment, have a large group of EPN species, and are easy to culture and maintain (Lewis *et al.*, 2006; Shapiro-Ilan *et al.*, 2006). EPN are not just beneficial in terms of pest control, but they are safe for the end user, there is no need for personal protective equipment and have been exempted from the U.S. Environmental Protection Agency (EPA) pesticide registration legislation. Moreover, insect resistance problems are unlikely. Similarly, the naturally occurring plant-associated microbes are ideal for the IPM process. Like EPN, endophytes can target

specific pests, can produce toxins for biocontrol and can also be incorporated at the start of the IPM process by means of seed and soil priming. Stenberg (2017) outlined the need for targeted defences against multiple pests and also improved consistency response to priming.

1.5.1 Biocontrol activities of endophytic bacteria

The overuse of pesticides has been directly related to the intensification of agriculture over previous decades. Although pathogenic microorganisms are a predominant threat to food production worldwide, the overuse of chemical pesticides has led to an increase in environmental pollution and pathogen resistance (Compant *et al.*, 2005). Endophytic bacteria can promote plant growth through the suppression of plant diseases (van Loon, 2007) and have been proven commercially as biopesticides. Inoculation of plants with plant growth promoting bacteria can reduce fungal, bacterial and viral diseases as well as damage caused by insects and nematodes (Kerry, 2000; Ryan *et al.*, 2008). Bacteria from genera such as *Bacillus*, *Pseudomonas* and *Streptomyces* are examples of such endophytes.

The production of compounds such as enzymes and secondary metabolites are essential for the success of endophytes as biocontrol agents (Compant *et al.*, 2005). The mechanism that has become the focal point is the production and release of molecules that either kill target pathogens or inhibit their growth; this process is known as "Antibiosis" (Weyens *et al.*, 2009). Numerous biological control bacteria have been studied and a large variety of compounds involved in antibiosis have been identified. Examples of these compounds include oligomycin A and xanthobaccin, which are produced by *Streptomyces* and *Stenotrophomonas* (Kim *et al.*, 1999; Nakayama *et al.*, 1999). In addition, hydrogen cyanide, phenazine, 2.4-diacetylphloroglucinol (DAPG)

and pyoluteorin are produced by pseudomonads (Défago, 1993; Haas & Défago, 2005; Souza & Raaijmakers, 2003). Predators exert a major pressure on free-living bacteria. Jousset *et al.* (2009) indicate that the production of secondary metabolites, such as DAPG and pyoluteorin by endophytes, can significantly reduce the fitness of predators thus act as a defence mechanism.

Plant protection against root-knot nematodes has become increasingly important due to their negative effect on plant growth and subsequent crop yields (Siddiqui & Mahmood, 1999). Tomato plants grown in a greenhouse and inoculated with the plant-associated bacteria, *Bacillus cereus*, *Bacillus subtilis*, *Pseudomonas putida*, *P. fluorescens* and *Serratia proteamaculans* had a reduction in gall formation and *Meloidogyne incognita* juveniles in the soil (Zhao *et al.*, 2018). These rhizosphere microbes also increased plant biomass and *M. incognita* biocontrol (Zhao *et al.*, 2018).

The production of insecticidal toxins by microbes has focused predominately on that derived from *Bacillus thuringiensis*, more commonly known as the Bt toxin, used commercially for the control of lepidopteran pests (Oestergaard *et al.*, 2006). Pests ingest the *B. thuringiensis* Crystal (Cry) protein, which is in turn converted to an activated toxin that results in intestinal damage (Masson *et al.*, 1995). The development of genetically modified Bt crops (crops engineered to express the Cry protein) resulted in reduced environmental impact from pesticides. However, there are currently concerns about growing resistance to the Bt toxin (Tabashnik *et al.*, 2013, 2014; Welch *et al.*, 2015). Insecticidal toxin production by a number of *P. protegens* CHAO (previously *P. fluorescens* CHAO [Ramette *et al.*, 2011]) and *P. protegens* Pf-5 isolates have been documented, as bacteria can produce the Fluorescens Insect Toxin (FIT; Péchy-Tarr *et al.*, 2008). *Pseudomonas* species that produce this toxin have been found to induce lethal

effects to *Drosophila melanogaster, G. mellonella* and *M. sexta* (Masson *et al.*, 1995; Olcott *et al.*, 2010; Péchy-Tarr *et al.*, 2008). A similar insecticidal toxin called Makes caterpillars floppy (Mcf) toxin is produced by the EPN symbiotic bacterium associated with the families Steinernematidae and Heterorhabditidae, *Xenorhabdus* sp. and *Photorhabdus* sp. toxin (Daborn *et al.*, 2002; Dowling *et al.*, 2004; Ffrench-Constant *et al.*, 2007). Mcf results in the shedding of the insect midgut epithelium and potential apoptosis. The survival and proliferation of EPN symbionts, outside the nematode host, has not been well documented. However, an examination of the compatibility of EPN and bacterial endophytes would, indicate their potential role in the future of IPM.

1.5.2 Biocontrol activities of EPN

EPN have widespread uses in the biological control of a number of important lepidopteran, dipteran and coleopteran pests (Burnell & Stock, 2000; Shapiro-Ilan *et al.*, 2006; Smart, 1995; Stuart *et al.*, 2006). Companies, such as e-nema (Germany), BioNema (UK), Supernemos (Ireland) are selling EPN for the biocontrol of plant pests but large-scale field applications are limited in part due to unpredictable EPN field performance. Nematode virulence varies for different insect pests thus the species of EPN applied must be selected carefully based on each specific target pest. EPN from the genera *Heterorhabditis* and *Steinernema* have been employed internationally as biocontrol agents for insect pests (Ehlers, 2001; Ffrench-Constant *et al.*, 2007). The EPN *Steinernema feltiae* has been used to control a number of Sciaridae and Bibionoidea larvae and the lettuce pest *Liriomyza huidobrensis*. *S. feltiae* has also been extensively studied for the biocontrol of dipteran pests in mushrooms, glasshouses and nurseries (Cuthbertson *et al.*, 2003). *S. carpocapsae* has been used against cutworms (*Agrotis* sp.) (Kunkel &

Grewal, 2003) and the Potato tuber moth (*Phthorimaea operculella*) (Eivazian Kary *et al.,* 2018). *Heterorhabditis* sp. are commonly used to control the black vine weevil (*Otiorhynchus sulcatus)* (Westerman & van der Werf, 1998; Ansari & Butt, 2011) and the Japanese beetle (*Popillia japonica*) (Grewal *et al*., 1995).

Despite the limited interactions between plant-parasitic and entomopathogenic nematodes, potential antagonistic effects between the two nematode groups have received notable focus with studies showing varying results. The effects of individual EPN species, a combination of species, their symbiotic bacterium, numerous application methods and differing PPN species may account for this variation in results. Root-knot nematode, *Meloidogyne incognita* and *M. arenaria,* infection in tomato plants was reduced and plant yields increased, when plants were treated with *S. feltiae* IJ, infected insect cadavers and cell-free cultures of its associated symbiotic bacterium, *X. bovienii* (Kepenekci *et al.*, 2018). Moreover, EPN application methods significantly affected biocontrol efficiency, with *S. feltiae* IJ showing a higher reduction in gall formation, followed by *X. bovienii* supernatant with seed coating and topical application, and finally infected cadaver treatments (Kepenekci *et al.*, 2018). A combination of EPN species (*S. carpocapsae, S. feltiae, S. glaseri and H. bacteriophora*) treatment has also been shown to reduce root-knot nematode egg masses, while also increasing plant height and biomass (Kepenekci *et al.*, 2016). However, here, the most effective application method for *M. incognita* and *M. arenaria* control was recorded in plants dipped in *X. bovienii* (Kepenekci *et al.*, 2016). In contrast, greenhouse trials carried out by Grewal *et al.* (1999), reported that nematodes (*S. carpocapsae*, *S. feltiae*, and *S. riobrave*) applied as infected insects were all capable of reducing *M. incognita* tomato root penetration but

control treatments of direct IJ application failed to reduce root penetration. Pérez & Lewis (2002) observed that *M. incognita* infected tomato seedlings treated with two *Steinernema* sp. (*S. feltiae and S. riobrave)* had fewer juveniles infecting their roots and a subsequent reduction in egg formation. Similarily, Fallon *et al.* (2002) reported that *S. feltiae* showed reduction in tomatoes and soybean root penetration but *S. riobrave* and *Heterorhabditis indica* did not.

Although these findings are largely positive, using EPN as an antagonist for PPN becomes far more complex and unpredictable when moving from a highly controlled greenhouse setting to a more dynamic field environment. As in the case with EPN species selection for insect biocontrol, certain EPN or combinations of EPN may be more or less effective against particular PPN species and their associated plants. Thus, individual interactions must be comprehensively investigated before large scale application. Despite the widespread applications of EPN and endophytes individually as biological control agents and biofertilisers, respectively, the potential of synergism between EPN and bacterial endophytes has yet to be explored. Commercial exploitation of endophyte-EPN interactions could play a significant role in sustainable, environmentally friendly agriculture applications for both food and non-food crops. The nematodes used throughout this study are known insect biocontrol agents and the bacterial endophytes examined were previously characterised and shown to display traits associated with PGP. Endophytes selection based on these characteristics were important to meet the future aims of developing a combined biocontrol and biofertiliser product.

1.6 Aims and objectives of this work

Both EPN and endophytic bacteria possess a number of beneficial attributes making them suitable candidates for sustainable agriculture. These are their position in the food web, and the fact that they are ubiquitous, relatively easy to sample, robust enough to be resistant to low levels of pollution and pathogens, and widely studied by nematologists and microbiologists. Both EPN and endophytic bacteria are used commercially in agriculture. EPN are used for biocontrol and, endophytic bacteria are utilised for PGP, phytoremediation and phytostabilisation. The aim of this project was to explore the interactions between EPN and bacterial endophytes in order to provide fundamental knowledge on the compatibility of these two beneficial soil organisms for use as a combined biofertiliser and biocontrol product.

Three *P. fluorescens* F113 strains were used interchangeably throughout the project. These were *Pseudomonas fluorescens* F113 PCB*rif*, *Pseudomonas fluorescens* F113 wt, *Pseudomonas fluorescens* F113 *gfp*. *P. fluorescens* F113 is a Rhizospheric bacteria, in the *Pseudomonas corrugata* phylogroup of the *P. fluorescens* complex (Garrido-Sanz *et al.*, 2017). It was used as it is a known coloniser of plant roots, has plant beneficial traits (Barahona *et al.*, 2016; Redondo-Nieto *et al.*, 2013) and has been shown to negatively affect a number of bacterial and plant parasitic nematodes (Cronin *et al.*, 1997). The remaining bacterial endophytes used throughout this project were isolated by previous IT Carlow research students and have been well characterised in terms of their bioremediation, biocontrol and PGP capabilities(Culhane, 2016; Lally, 2016; Menton, 2010; Otieno, 2014), and in the case of *P. fluorescens* L321, L228 and L111 detailed genome information are available (Lally, 2016). Additional strain characterisation and information is detailed in Chapter 2.

The objectives specific to each chapter are outlined below:

Chapter 2 - Investigation of the basic interactions between entomopathogenic nematodes and bacterial endophytes

The objectives of the study presented in Chapter 2 were to determine the susceptibility of the EPN *S. feltiae* SB 12(1), *S. feltiae (*e-nema), *S. carpocapsae* and *H. bacteriophora*, to endophytic bacterial isolates (*Pseudomonas fluorescens* F113 PCB*rif*, S19, S118, S222, L23, L228, L330, L313, L321, L324, R114, and R225) and examine the antimicrobial capabilities of endophytic bacteria towards EPN symbiotic bacteria (*Xenorhabdus* and *Phorothabdus* sp.). Bioassays were employed to examine the toxic effects of DAPG-producing and DAPG-negative endophytic bacterial isolates, as well as chemically produced synthetic DAPG to EPN. The symbiotic bacteria associated with EPN are located in the nematode's intestine and it is reasonable to postulate that the presence of these bacteria may protect the nematodes against endophytic bacterial pathogenicity. The antimicrobial activity of endophytic bacterial isolates against bacterial symbionts from *S. feltiae* (*Xenorhabdus bovienii)* and *H. bacteriophora (Photorhabdus luminescens)* was investigated.

Chapter 3 – Determination of the effects of endophytic bacteria on entomopathogenic nematode behaviour and biology

The objectives of the study presented in Chapter 3 were to investigate the effect of exposure to endophytic bacteria, and in some cases the bacterial antibiotic DAPG, on the pathogenicity of EPN for the control of the black vine weevil, *O. sulcatus* (Coleoptera: Curculionidae) and the wax moth, *G. mellonella* (Lepidotera: Pyralidae). These effects were assessed in terms of nematode infectivity in laboratory and greenhouse experiments,

nematode virulence in nematode dose-response experiments, nematode attraction to their host insect and EPN development in *G. mellonella*.

Chapter 4 – Determination of the effects of nematodes on bacterial survival, colonisation of plants and plant growth promoting properties

The objectives of the study presented in Chapter 4 were to investigate the effects of nematodes on (a) endophytic bacterial growth on solid media and (b) bacterial plant colonisation and PGP, as well as (c) endophytic bacterial colonisation of EPN IJ and (d) survival of bacteria post-IJ insect infection. Moreover, the presence of insecticidal genes, associated with nematode symbiotic bacteria, in the bacterial genomes of *P. fluorescens* F113, L321, L228 and L111 was examined. The effects of *S. feltiae* SB 12(1) and *H. bacteriophora*, and a combination of the two EPN species, on the colonisation of oilseed rape by *P. fluorescens* F113 and L321, was examined in-plant trials. qPCR was used to examine bacterial colonisation of EPN IJ over increasing exposure times. In addition, the persistence of *P. fluorescens* F113 after insect infection was examined to determine the survival of the endophytic bacteria following direct exposure to the EPN symbiotic bacteria *in vivo*.

Chapter 5 - General discussion and future work

This chapter presents a general discussion assessing the compatibility of EPN and bacterial endophytes for use as a biocontrol and biofertiliser product. Moreover, future work tasks aimed at moving forward in the development of such a product are also outlined.

Chapter 2 Investigation of the basic interactions between entomopathogenic nematodes and bacterial endophytes

2.1 Introduction

The success of endophytic bacteria to act as biological control agents is dependent on their mode of action against plants or their pathogens. Such natural bacterial defence systems include siderophore production, excretion of cell wall hydrolases (Cherin & Chet, 2002) as well as the production of antimicrobial compounds (Haas & Défago, 2005). One such mechanism being investigated in this Chapter is antibiosis. This is the production and release of molecules that either kill target pathogens or inhibit their growth (Weyens *et al.*, 2009). Numerous bacterial species have been studied for their biological control potential and a large variety of compounds involved in antibiosis have been identified. Examples of these compounds include oligomycin A and xanthobaccin, these are produced by *Streptomyces* and *Stenotrophomonas* (Kim *et al.*, 1999; Nakayama *et al.*, 1999). In addition, hydrogen cyanide, phenazine, 2.4-diacetylphloroglucinol (DAPG) and pyoluteorin are produced by pseudomonads (Défago, 1993; Souza & Raaijmakers, 2003).

Studies carried out on various *Pseudomonas* strains have demonstrated that antibiotics produced as secondary metabolites are beneficial biological control agents, and have a broad-spectrum of activity (Raaijmakers *et al.*, 2002). The primary bacterial antibiotic being investigated in this chapter is DAPG. DAPG is produced by a number of *Pseudomonas fluorescens* strains and has been investigated for the biocontrol of the potato pest *Globodera rostochiensis* (potato cyst nematode). Cronin *et al.*, (1997) directly related the production the DAPG to an increase in the ability of *G. rostochiensis* eggs to hatch, with the resulting juveniles showing a loss of mobility.

Entomopathogenic nematode (EPN) virulence is dependent on the success of their mutualistic bacterium to kill an insect. Many bacterial groups have been shown to be nematophagous, including parasitic bacteria, rhizobacteria, endophytic bacteria and also EPN symbiotic bacteria (Cronin *et al.*, 1997; Meyer *et al.*, 2009; Siddiqui *et al.*, 2005). The level of pathogenicity of these bacterial species varies as does their mode of action, which includes the production of toxins and in nematode intestinal damage. In the case of *Pseudomonas aeruginosa* PAO1, bacterial cells become established in the intestine of *Caenorhabditis elegans* causing infection, which is followed by locomotion problems, distension of the intestine, cell lysis and ultimately death (Gallagher & Manoil, 2001). The endophytic bacteria *P. fluorescens* CHAO have also been shown to be toxic to juveniles and inhibit egg hatch in root-knot nematodes (Norabadi *et al.*, 2014). Similar findings have not been recorded in EPN.

The objectives of the study presented in Chapter 2 were to determine the susceptibility of the EPN *S. feltiae* SB 12(1) and of the commercial strains of *S. feltiae, S. carpocapsae* and *H. bacteriophora* (e-nema), to endophytic bacterial isolates and examine the

antimicrobial capabilities of endophytic bacteria. These effects were assessed in terms of:

- EPN susceptibility to endophytic bacteria
- DAPG toxicity to EPN
- Antimicrobial effects of endophytic bacteria on EPN symbiotic bacteria.

Susceptibility and toxicity assays are often used to determine sensitivities for various toxicants against invertebrates, by means of lethal or sub-lethal endpoints. These assays can be used to provide both qualitative and quantitative data on any adverse effects on an organism by a toxicant. Bioassays were employed to examine the toxic effects of DAPG-producing and DAPG-negative endophytic bacterial isolates, in addition to chemically synthesised synthetic DAPG on EPN. The symbiotic bacteria associated with EPN are located in the nematode's intestine and it is reasonable to postulate that the presence of these bacteria may protect the nematodes against endophytic bacterial pathogenicity. Bacterial symbionts from *S. feltiae* (*Xenorhabdus bovienii)* and *H. bacteriophora (Photorhabdus luminescens)* were isolated from nematode-infected *Galleria mellonella* cadavers and cultured according to (Akhurst, 1980). Experiments were subsequently carried out in order to investigate the antimicrobial activity of endophytic bacterial isolates against the two symbiotic bacterial genera.

2.2 Materials and Methods

Experiments were carried out with appropriate replications and controls, between 21-23°C unless otherwise stated. The number of replicates and types of control treatments varied between experiments are detailed in each section. Endophytic bacterial isolates were cultured at 30°C for 24 hr and EPN symbiotic bacteria were cultured at 27-28°C for 36-48 hr, unless otherwise stated. All bacterial isolates were cultured and maintained using aseptic technique. EPN culturing was carried out at 21-23°C and were not maintained under sterile conditions but care was taken to ensure strains and species were not cross contaminated.

Statistical analyses were carried out using SPSS 23 (IBM SPSS Statistics for Windows). Where the homogeneity of variances (Levene's test; $p < .05$) and assumption of normality (Shapiro-Wilk test; $p < .05$) were violated, the non-parametric alternative was applied. Non-parametric tests were applied as they do not make the same assumptions about normality. *Post hoc* analysis was carried out using a Bonferroni correction unless otherwise stated. Values marked with an asterisk (*) indicate significant differences ($p < .05$) were detected following *Post hoc* analysis.

2.2.1 Culture and maintenance of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae)

Four EPN species and strains in the families Steinernematidae and Heterorhabditidae were cultured and maintained during this project and species and sources are outlined in Table 2.2.1. EPN were reared in parallel in the larvae of the wax moth, *Galleria mellonella* (Lepidotera: Pyralidae). *G. mellonella* were sourced commercially from Live Foods Direct (Sheffield, UK). Standard size Petri dishes (92 X 16 mm) were inverted

and the lids were lined with Whattman filter paper. Approximately 1-1.5 ml of a dense IJ suspension was applied to the filter paper until it was moist. Five *G. mellonella* larvae were placed onto the filter paper and the base was placed on top and lightly sealed to prevent the filter paper drying out. Plates were then incubated for 3-10 days until insect mortality occurred.

Once insect mortality had occurred nematode infective juveniles (IJ) were recovered according to White, (1927). Briefly, a Petri dish (35 x 10 mm) was inverted and the lid placed on the base. A platform was created by placing filter paper over the dish. This was placed in a transparent container and enough sterile deionised H_2O (dH_2O) was added to submerge the edges of the filter paper. The infected insects were placed onto the platform and incubated for up to 14 days. After emergence, IJ were harvested by decanting the water into an empty transparent dish. The dish containing the infected hosts was replenished with sterile dH_2O and incubated for a further 1-3 days. This process was carried out until the desired amount of nematodes were harvested. Nematode stocks were cleaned by letting them settle at the bottom of the dish forming a loose pellet, before removing the supernatant with a Pasteur pipette and adding fresh sterile dH_2O. This was repeated three times. Nematodes were then stored at 4ºC until required and were used for experimentation, no more than two weeks post-emergence.

The three commercial EPN species (*S. carpocapsae, H. bacteriophora, and S. feltiae)* were cultured as outlined above. These species were obtained from e-nema, in a fine soil sample. To extract the nematodes half of the sample was mixed in 75 ml of sterile dH_2O and allowed to settle. 1 ml of this suspension was checked under a stereoscope for the presence of live IJ. Nematodes were cultured in *G. mellonella* as outlined above.

Table 2.2.1 Entomopathogenic nematode species and their origin used in this project.

Nematode	Abbreviation	Symbiotic bacterium	Source
Steinernema feltiae SB 12(1)	*S. feltiae* SB 12(1)	*Xenorhabdus bovienii*	Boyle, (2007)Institute of Technology Carlow
Steinernema feltiae	*S. feltiae* e-nema	*Xenorhabdus bovienii*	e-nema, (Schwentinental, Germany)
Steinernema carpocapsae	*S. carpocapsae*	*Xenorhabdus nematophilus*	e-nema, (Schwentinental, Germany)
Heterorhabditis bacteriophora	*H. bacteriophora*	*Photorhabdus luminescens*	e-nema, (Schwentinental, Germany)

2.2.2 Culture and maintenance of endophytic bacterial isolates

The bacterial endophytes utilised during this project are from the IT Carlow stock endophyte collection and were isolated from tissues from a range of plants, including *Miscanthus* sp. and oilseed rape (OSR; *Brassica napus*), during previous research projects. The following endophytic bacterial strains *Pseudomonas fluorescens* F113 pcb*rif*, *P. fluorescens* F113 wt, *P. fluorescens* F113 *gfp* S19, S118, S222, L23, L111, L124 L228, L330, L313, L321 wt, L321 *gfp*, L324, R114, and R225 were examined during the course of this project. The labels represent the plant part where they were isolated (L= leaf; S=shoot; R=rhizome), the first number represents the plant sample number, followed by the replicate colony. For example, L321 is leaf 3 colony 21. These endophytes were selected as they include those with important biocontrol and plant growth promoting (PGP) properties, as outlined in Table 2.2.2 and Table 2.2.2.

Stock bacterial endophytes were stored at -70 ˚C in glycerol. 10 µl of each stock bacterial endophyte strain was inoculated into a McCartney bottle containing 10 ml of sterile nutrient broth (LAB M) and incubated at 30˚C for 24 hr on a shaker (100 rpm). 10 µl of the culture was streaked onto nutrient agar (LAB M) plates, using the quadrant streak method, and incubated at 30ºC for 24 hr. To ensure that pure isolates were obtained,

single colonies from each plate were re-streaked onto nutrient agar a total of three times. A single colony that matched the known bacterial morphological and biochemical characteristics for each strain, was picked from the nutrient agar and re-inoculated into 10 ml nutrient broth, and incubated as outlined previously. Morphological and biochemical bacterial endophyte characteristics are presented in Appendix 1.

Replicated bacterial stocks suitable for long-term storage were prepared from these purified cultures and subsequently used throughout this study. This was carried out by aseptically adding 750 µl of the pure endophyte nutrient broth culture to a sterile 1.5 ml centrifuge tube containing 250 µl of a 40% glycerol solution. Tubes were clearly labelled and numbered sequentially and vigorously vortexed to mix, before storing at -70ºC until required.

2.2.3 Endophytic bacterial standard curves

A bacterial standard curve establishes the relationship between the concentration of a bacterial culture and the signal measured by an instrument. Plate counts are also used as they give a more accurate reading of the number of live cells. Here, standard curves were constructed to determine the bacterial concentration, in colony forming units per volume unit (CFU/ml). Sterile $^{1}/_{4}$ ringers was used to dilute 24hr bacterial cultures from OD 0.9-0.1 and total viable bacterial colonies were determined by serial dilutions (10^{-1} to 10^{-8}) and plate counts on nutrient agar. Cultures were incubated as described in section 2.2.2 and subsequently the CFU/ml was determined. The CFU/ml was plotted against the bacterial OD to generate standard curves and these are presented in the Appendix 2.

Table 2.2.2 Biological control characteristics of the endophytic bacterial isolates used throughout this project. The biocontrol capabilities of bacterial endophytes and plant parasitic nematodes were investigated by previous IT Carlow research students. Endophytic bacterial antimicrobial activity against two species of fungus (*Fusarium sp., Pythium sp.*) and the plant pathogenic bacterial species *Bacillus subtilis* was examined on solid media containing either high or low concentrations of Iron. This was carried out to determine if the production of siderophores was responsible for their antimicrobial effects. Biological control against the plant-parasitic nematodes was determined by susceptibility testing in 96 well multiwell plates. The presence of the *phl*D gene as an indication of DAPG production was determined via PCR using *phl*D specific primers (Menton, 2010).

Endophyte label	**Strain ID**	**Biological control capabilities**									**Reference**
		***Fusarium* sp.**		***Pythium* sp.**		***Bacillus subtilis***		**Plant parasitic nematodes**			
		High	Low	High	Low	High	Low	*Globodera pallida*	*Melloidogyne javanica*	*Phl*d	
F113 wt	*Pseudomonas fluorescens*	+	+	+	+	ND	ND	+	+	+	Otieno (2015); Egan 2016 (pers comm)
S19	*Pseudomonas trivialis*	-ve	+	-ve	-ve	-ve	-ve	ND	ND		Menton (2010)
S118	*Pseudomonas fluorescens*	+	+	+	+	+	-ve	+	+	+	Menton (2010); Egan 2016 (pers comm)
S222	*Pantoea agglomerans*	+	+	+	+	+	-ve	+	+		Menton (2010); Egan 2016 (pers comm)
L23	*Pseudomonas veronii*	+	-ve	+	+	+	+	ND	ND		Menton (2010)
L111	*Pseudomonas fluorescens*	ND	ND	ND	ND	ND	ND	ND	ND		Keogh (2009)
L228	*Pseudomonas fluorescens*	+	+	+	+	+	-ve	+	+		Menton (2010); Egan 2016 (pers comm)
L313	*Pseudomonas fluorescens*	+	-ve	-ve	ve	+	-ve	ND	ND		Menton (2010)
L321 wt	*Pseudomonas fluorescens*	+	+	+	+	ND	ND	+	+		Otieno (2015); Egan 2016 (pers comm)
L324	*Pseudomonas fluorescens*	+	-ve	+	+	+	+	ND	ND		Menton (2010);
L330	*Xanthomonas sp*	+	-ve	+	-ve	-ve	+	ND	ND		Menton (2010);
R114	*Pantoea agglomerans*	-ve	-ve	-ve	-ve	+	-ve	ND	ND		Menton (2010);
R225	*Pantoea amnigenus*	-ve	-ve	+	-ve	+	+	ND	ND		Menton (2010);

ND: Not determined -ve: No biocontrol capabilities detected + : biocontrol capabilities detected

Table 2.2.3 Biochemical and plant beneficial characteristics of the bacterial strains used throughout this project.

Endophyte label	Strain ID	Gram	Oxidase	Catalase	Siderophore	Cellulase	Nitrate reduction	Phosphate solubilisation	Motility	Reference
F113	*Pseudomonas fluorescens*	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	Menton (2010); Lally (2016)
S19	*Pseudomonas trivialis*	-ve	+ve	+ve	-ve	+ve	-ve	+ve	ND	Menton (2010)
S118	*Pseudomonas fluorescens*	-ve	+ve	+ve	+ve	+ve	-ve	+ve	ND	Menton (2010)
S222	*Pantoea agglomerans*	-ve	-ve	+ve	-ve	+ve	+ve	+ve	ND	Menton (2010)
L23	*Pseudomonas veronii*	-ve	+ve	+ve	+ve	+ve	-ve	+ve	ND	Menton (2010)
L124	*Pseudomonas fluorescens*	-ve	+ve	+ve	+ve	-ve	+ve	+ve	ND	Menton (2010)
L228	*Pseudomonas fluorescens*	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	Menton (2010); Lally (2016)
L313	*Pseudomonas fluorescens*	-ve	+ve	+ve	+ve	+ve	-ve	+ve	ND	Menton (2010)
L321	*Pseudomonas fluorescens*	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	Lally (2016)
L324	*Pseudomonas fluorescens*	-ve	+ve	+ve	+ve	-ve	-ve	+ve	ND	Menton (2010)
L330	*Xanthomonas* sp.	-ve	+ve	+ve	-ve	+ve	-ve	+ve	ND	Menton (2010)
R114	*Pantoea agglomerans*	-ve	-ve	+ve	-ve	+ve	-ve	+ve	ND	Menton (2010)
R225	*Pantoea amnigenus*	-ve	-ve	+ve	-ve	+ve	-ve	+ve	ND	Menton (2010)

ND: Not determined -ve: Negative reaction +ve : Postitive reaction

Table 2.2.4 Various *P. fluorescens* F113 and L321 isolates used throughout this project

Endophyte label	Strain ID	Transposon	Genes	Thesis section	Reference
F113 *gfp*	*Pseudomonas fluorescens*	mini-Tn5	gfp;*kan*	4.2.3, 4.2.4	(Otieno, 2014)
F113 pcb*rif*	*Pseudomonas fluorescens*	TnPCB	bph;*rif*	2.3.1, 3.2.3	(Brazil *et al.*, 1995)
F113 wt	*Pseudomonas fluorescens*	NA	No genetic modification	All additional experiments	(Shanahan *et al.*, 1992)
L321 *gfp*	*Pseudomonas fluorescens*	mini-Tn5	gfp;*kan*	4.2.3	(Otieno, 2014)
L321 wt	*Pseudomonas fluorescens*	NA	No genetic modification	All additional experiments	(Keogh, 2009)

2.2.4 Determination of the susceptibility of *S. feltiae* SB 12(1), *S. feltiae* (e-nema), *S. carpocapsae* and *H. bacteriophora* to endophytic bacterial isolates

EPN susceptibility was assessed in terms of IJ mortality following exposure to bacterial endophytes for 24 and 48 hr. Bioassays were carried out in 96-well multiwell plates. 100 IJ in 50 µl sterile dH_2O were exposed to a 10^8 CFU/ml (concentration determined *via* standard curves and plates counts) endophytic bacterial culture for 24 and 48 hr. Following incubation, at room temperature in the dark, nematodes were moved to a counting dish and a stereoscope and blunt probe were used to determine if IJ were dead or alive. Replication was six-fold per nematode and bacterial combination, with independent bioassay plates for each time point. IJ in the control treatments received sterile dH_2O only.

2.2.4.1 Statistical analysis

A one-way multivariate analysis of variance (MANOVA) was conducted to determine the effect of bacterial exposure on nematode mortality. Infective juvenile mortality was measured independently after 24 and 48 hr in the presence and absence of bacterial

endophytes. Wilk's Lambda (Λ) was interpreted for multivariate tests, where significance was determined between control and bacterial treatments, pairwise comparisons were calculated using a Bonferroni correction.

2.2.5 Investigation on the toxicity of the synthetic bacterial antibiotic DAPG to the entomopathogenic nematodes *S. feltiae* SB 12(1), *S. feltiae* (e-nema), *S. carpocapsae* and *H. bacteriophora*

To determine the nematicidal capabilities of 2.4-diacetylphloroglucinol (DAPG) against the EPN, a laboratory-based bioassay was carried out using 96 well multiwell plates as the experimental arena.

2.2.5.1 Preparation of DAPG stock and working solutions

Chemically produced synthetic DAPG was sourced from University College Cork, Ireland. DAPG did not fully dissolve in dH_2O thus, it was dissolved in methanol. A 1000 µg/ml stock solution of DAPG was prepared by dissolving 0.1g DAPG in 100ml of 2.5 % Methanol. The stock solution was gently heated for 15minutes and left overnight to dissolve. Once fully dissolved the DAPG was filter sterilised before preparing 25, 50, 100 and 200µg/ml working solutions.

2.2.5.2 DAPG toxicity to entomopathogenic nematodes (Adopted from Meyer *et al.*, 2009)

Nematodes were exposed to increasing concentrations of synthetic DAPG from 0, 25, 50, 100 and 200 µg/ml (final concentrations) in 96 well multiwell plates. Approximately 100 IJ (suspended in 50 µl sterile dH_2O) were exposed to each concentration of DAPG, replication was ten-fold per nematode and DAPG treatment. DAPG toxicity to

nematodes was assessed as outlined in section 2.2.4, following 24, 48, 72 and 96 hr exposure. There were two controls used, control one was sterile dH_2O only and control two was 2.5 % methanol.

2.2.5.2.1 Statistical analysis

An ANOVA with repeated measures was used to determine if there were statistically significant differences between the mean number of dead IJ after 24, 48, 72 and 96 hr (within-subjects factor) following exposure to DAPG. Mauchly's test ($p < .05$), indicated that the assumption of sphericity was violated. Thus, epsilon (ε) was calculated with a Greenhouse & Geisser correction (Greenhouse & Geisser, 1959). This was carried out due to the sensitivity of the one-way repeated measures ANOVA to departures from sphericity.

2.2.6 Isolation and confirmation of entomopathogenic nematode symbiotic bacteria (*Xenorhabdus* sp. and *Photorhabdus* sp.)

To isolate *X. bovienii* and *P. luminescens*, five *G. mellonella* larvae were baited with *S. feltiae* SB 12(1) and *H. bacteriophora*, respectively. Once insect mortality occurred (in approximately 48 hr), the five cadavers were dissected in 10 ml sterile Ringer's solution. The insect carcasses were removed and the remaining haemolymph was serially diluted (10^{-1} to 10^{-5}) and plated out onto nutrient agar with bromothymol blue and 2,3,5-triphenyltetrazolium chloride (NBTA). *X. bovienii* colonies absorb the bromothymol blue dye resulting in blue colonies while Phase I *P. luminescens* have a dark red colour. Single colonies from each isolate were inoculated into tryptone soy broth and incubated at 27°C for 48 hr (100 rpm). A loop full from each 48 hr culture was streaked onto tryptone soy

agar (TSA) to obtain single colonies and incubated as described previously. This was repeated twice to obtain pure cultures of each isolate. Potential *X. bovienii* and *P. luminescens* isolates were streaked onto NBTA, MacConkey, and TSA and incubated as outlined above. Gram staining, oxidase, and catalase testing were carried out on the isolates. Isolates that matched the criteria outlined in Table 2.2.5 were presumed to be *X. bovienii* or *P. luminescens* and were stored at -70°C in nutrient broth containing 40% glycerol.

Table 2.2.5 Morphological and phenotypic characteristics of *Xenorhabdus bovienii* and *Photorhabdus luminescens* (Akhurst, 1980, 1993; Akhurst & Boemare, 1988; Smigielski *et al.*, 1994)

		X. bovienii	***P. luminescens***
Growth on:	NBTA	Blue colonies	Dark red colonies
	MacConkey	Red colonies	Red colonies*
	Tryptone Soy Agar	Yellow colonies	Yellow colonies
Reaction to:	Oxidase	Negative	Negative
	Catalase	Negative	Positive
	Gram	Negative	Negative

* *P. luminescens* produces yellow pigmentation when grown on MacConkey agar, but does not ferment lactose

2.2.6.1 Preparation of nutrient agar with Bromothymol blue and 2,3,5-Triphenyltetrazolium chloride (NBTA; Akhurst, 1980)

To prepare 1L of NBTA agar the following components were dissolved in sterile dH_2O and autoclaved for 15 minutes at 15psi and 121ºC.

12 g L^{-1} Nutrient agar

0.025 g L^{-1} Bromothymol blue

0.04 g L^{-1} 2,3,5-Triphenyltetrazolium chloride

After autoclaving, the agar was allowed to cool to approximately 60^0C before aseptically dispensing into Petri dishes.

2.2.6.2 Preparation of solid and liquid media

Nutrient agar, MacConkey agar, and tryptone Soy agar were prepared as per manufactures instructions. Nutrient, tryptone soy, and Luria-Bertani (LB) broth were dissolved as per the manufacture instructions in dH_2O and dispensed into McCartney bottles before sterilisation by autoclaving for 15 minutes at 15psi and 121ºC. Solid media was dissolved in an appropriate volume of dH_2O, autoclaved for 15 minutes at 15psi and 121ºC and subsequently dispensed into Petri dishes.

2.2.6.3 Biochemical tests and Gram staining

In addition to growth on differential media, biochemical tests and Gram stains were employed to confirm that the isolates matched the known reactions for *X. bovienii* and *P. luminescens*.

2.2.6.3.1 Gram Stain

The standard Gram staining procedure using crystal violet, iodine, ethanol and the counter stain safranin was carried out to confirm that bacterial isolates were Gram-negative. Following staining, the bacterial cells were visualised under 1000X magnification lens of an Optika microscope and the cell colour and shape was noted.

2.2.6.3.2 Oxidase test

Overnight cultures of *X. bovienii* and *P. luminescens* on nutrient agar were tested for the presence of the enzyme cytochrome-oxidase using 1% tetramethyl-p-phenylenediamine dihydrochloride. Using a sterile loop, a single isolated colony from a 24 hr culture was transferred to filter paper that had been pre-moistened with several drops of the reagent. The development of a dark blue/purple colour within 10 seconds was recorded as a positive result. *P. fluorescens* L321 was used as a positive control and *Escherichia coli* OP50 was used as a negative control.

2.2.6.3.3 Catalase test

For the detection of catalase, a single colony from a 24 hr *X. bovienii* and *P. luminescens* culture were placed onto a clean glass slide before a drop of fresh 3% hydrogen peroxide (H_2O_2) was applied. Immediate effervescence (within 10 seconds) was noted as a positive result, with no bubbling an indication of no activity. Here, *P. fluorescens* L321 was used as a positive control and a negative reaction for *X. bovienii* was deemed a sufficient negative control.

2.2.6.4 Bacterial growth curves for *X. bovienii* and *P. luminescens*

10 ml of each culture was grown to an OD_{600} of 1.0 in Luria-Bertani broth. Bacterial cells were pelleted by centrifugation at 10,000 rpm for 10 minutes. The pellet was re-suspended in 1 ml sterile Ringer's solution. 20 µl aliquots of this bacterial suspension were used to inoculate 180 µl of Luria-Bertani broth. 96 well multiwell plates were used and replication was six-fold per isolate. The 96 well plates were incubated in the Tecan GENios bioplate reader at 28°C at 100 rpm for 48 hr. The instrument was programmed

to record the O.D every hr at 600nm for 48 hr. A growth curve displaying the bacterial growth was computed using Microsoft Excel and are presented in Appendix 3.

2.2.7 Evaluation of endophytic bacterial antimicrobial activity against *X. bovienii* and *P. luminescens*

An agar well diffusion protocol was used to determine the endophytic bacterial antimicrobial activity against *X. bovienii* and *P. luminescens.* Endophytic bacterial isolates investigated were F113, L321, L124, and S222. An *X. bovienii* or *P. luminescens* bacterial lawn was prepared by pipetting 100 µl of a 10^8CFU/ml 24 hr culture onto 25 ml of half-strength TSA in Petri dishes (92 x 16mm). Both of the nematode symbiotic bacterial isolates investigated produce yellow-pigmented colonies on TSA. Thus, half strength TSA was used to allow for adequate visualisation of a zone of inhibition. The bacterial lawn was air dried in a laminar air flow cabinet for 15 minutes.

Three 1cm plugs were removed from the media using a sterile borer and discarded. 30 µl of 10^8CFU/ml endophyte culture (re-suspended in sterile dH_2O) was inoculated into each well, as seen Figure 2.2.1. Negative control treatments received sterile dH_2O only. A number of endophyte strains have shown biocontrol capabilities against *Bacillus subtilis.* Therefore, positive control plates were prepared with a *B. subtilis* bacterial lawn in the presence and absence of endophytes.

Petri dishes were incubated upright for 24 hr and antimicrobial activity was determined by agar well diffusion. The diameter of the zone of inhibition was measured using a Vernier calipers (mm) between the wells and the edge of the bacterial lawn.

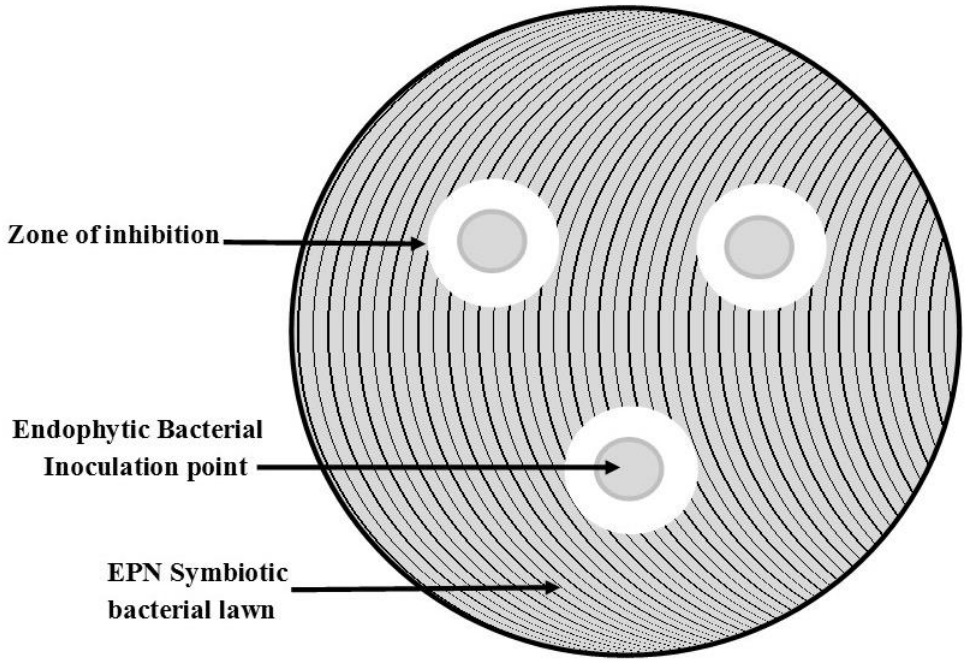

Figure 2.2.1 Bioassay plate setup to investigate the antimicrobial activity of endophytic bacteria F113 and L321 against *P. luminescens* and *X. bovienii*

2.2.7.1 Statistical analysis

An ANOVA was conducted to determine the significant differences in the diameter (mm) of the zone of inhibition in the control treatment wells with sterile dH_2O only, and the bacterial treatments that received 30 µl re-suspended culture. *Post hoc* analysis was carried out using a Dunnett t-test, to compare the control against the bacterial treatments.

2.3 Results

2.3.1 Determination of the susceptibility of *S. feltiae* SB 12(1), *S. feltiae* (e-nema), *S. carpocapsae* and *H. bacteriophora* to endophytic bacterial isolates

Nematode susceptibility tests were conducted in 96 well multiwell plates to assess the acute toxicity of the bacterial endophytes, F113 pcb*rif*, S19, S118, S222, L23, L228, L330, L313, L321, L324, R114 and R225 against IJ of the nematodes *S. feltiae* SB 12(1), *S. feltiae* (e-nema), *S. carpocapsae* and *H. bacteriophora* following 24 and 48 hr exposure. Figure 2.3.1 to Figure 2.3.4 display the Mean % IJ mortality for each nematode species following 24 and 48 hr exposure to a 10^8CFU/ml each bacterial strain.

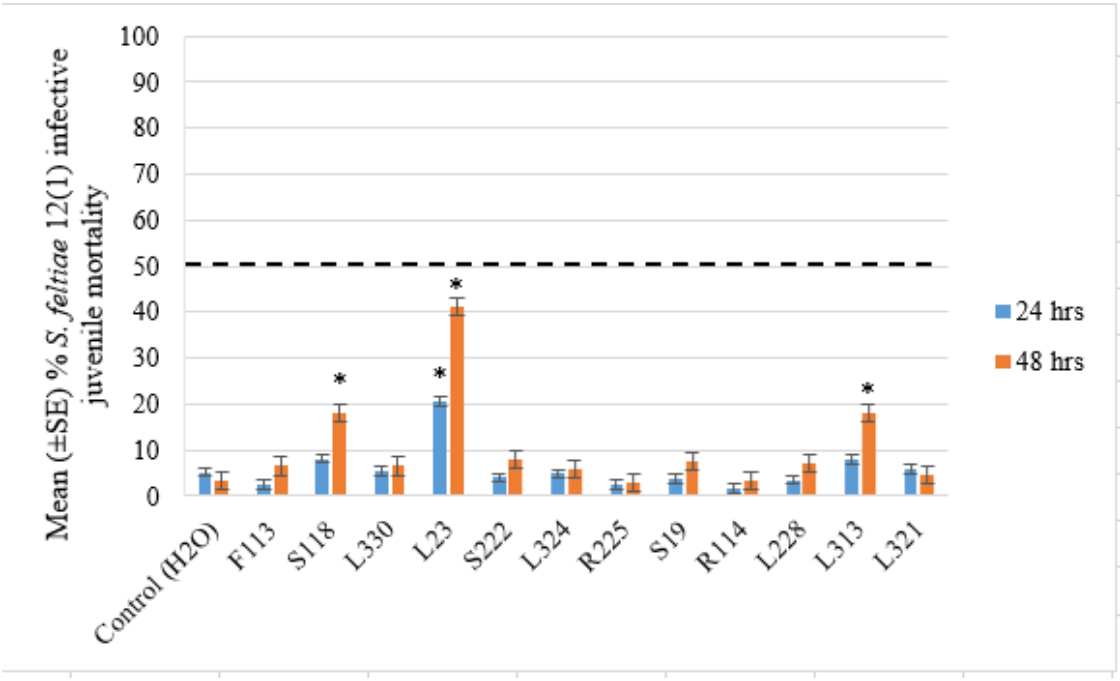

Figure 2.3.1 *S. feltiae* SB 12(1) infective juvenile mortality following exposure to endophytic bacteria (10^8CFU/ml) for 24 and 48 hr (±SE), n = 6 per bacterial and nematode combination with 100 infective juveniles per replicate. The black dotted line indicates 50% nematode mortality. Asterisk (*) indicates significant differences between control and bacterial treatment at the respective time points (24 and 48 hr).

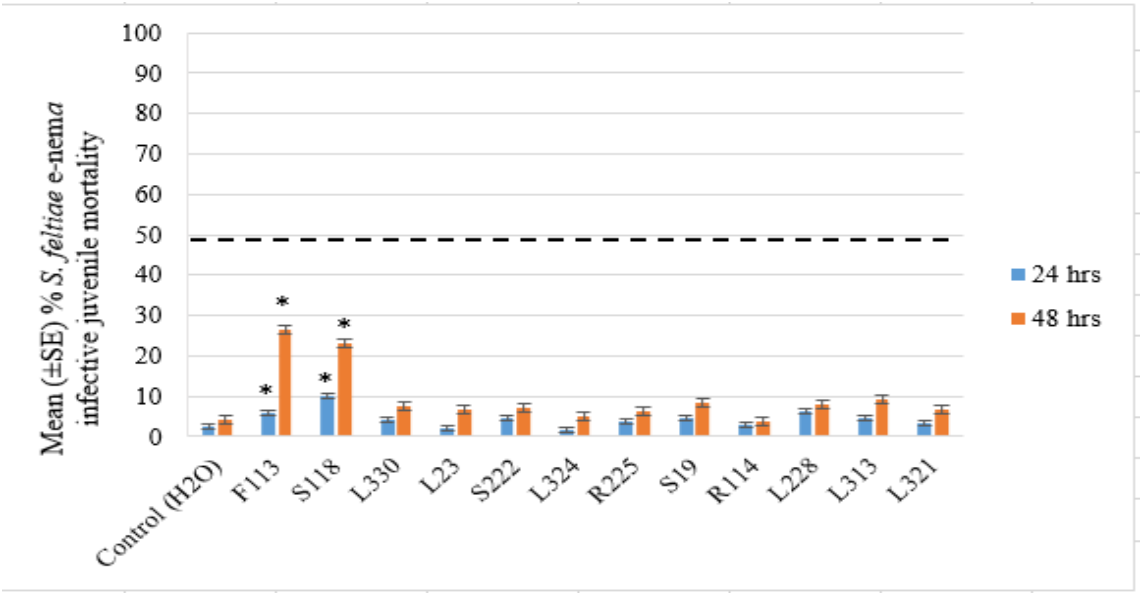

Figure 2.3.2 *S. feltiae* (e-nema) infective juvenile mortality following exposure to endophytic bacteria (10^8CFU/ml) for 24 and 48 hr (±SE) n = 6 per bacterial and nematode combination with 100 infective juveniles per replicate. The black dotted line indicates 50% nematode mortality. Asterisk (*) indicates significant differences between control and bacterial treatment.

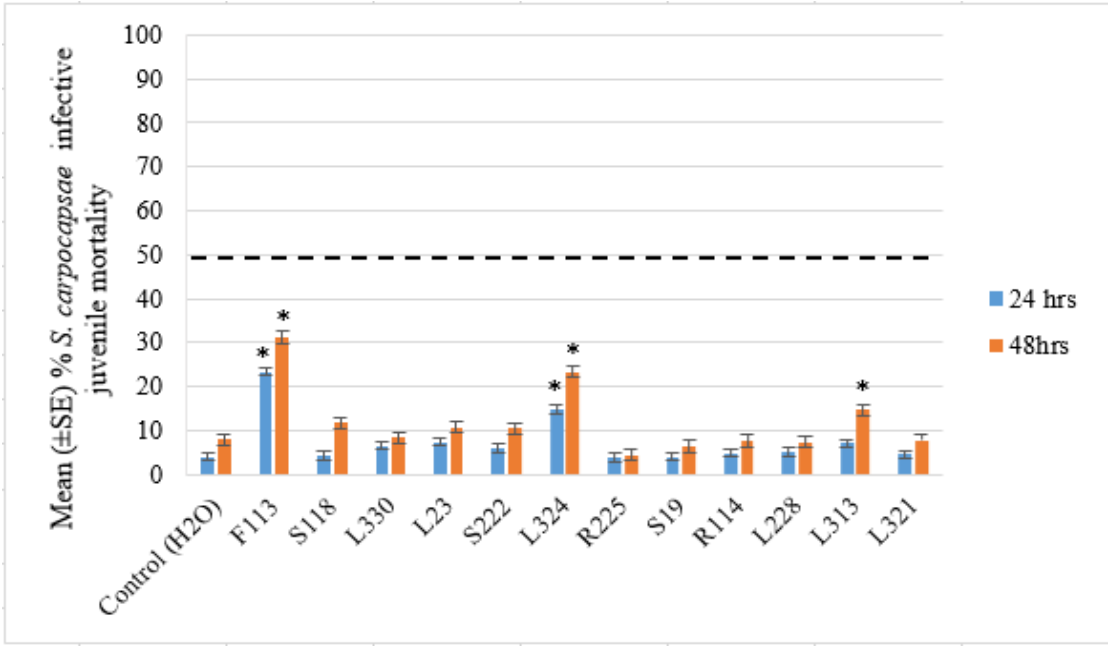

Figure 2.3.3 *S. carpocapsae* infective juvenile mortality following exposure to endophytic bacteria (10^8CFU/ml) for 24 and 48 hr (±SE) n = 6 per bacterial and nematode combination with 100 infective juveniles per replicate. The black dotted line indicates 50% nematode mortality. Asterisk (*) indicates significant differences between control and bacterial treatment.

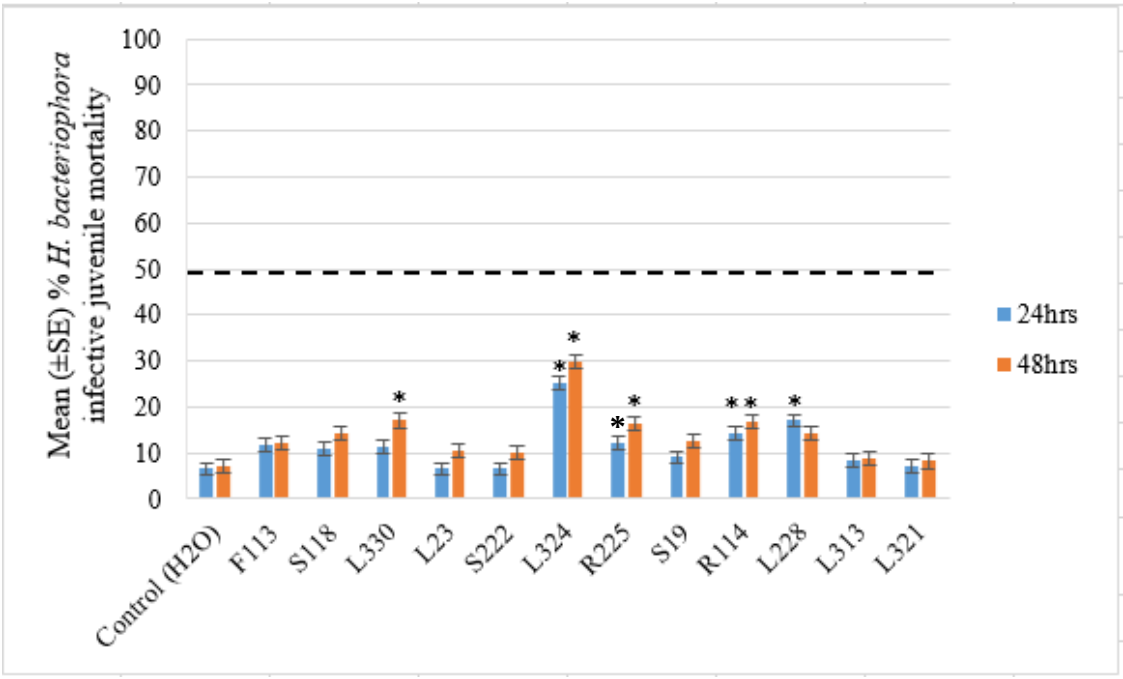

Figure 2.3.4 *H. bacteriophora* infective juvenile mortality following exposure to endophytic bacteria (10^8CFU/ml) for 24 and 48 hr (±SE) n = 6 per bacterial and nematode combination with 100 infective juveniles per replicate. The black dotted line indicates 50% nematode mortality. Asterisk (*) indicates significant differences between control and bacterial treatment.

A one-way multivariate analysis of variance (MANOVA) was conducted to determine the effect of bacterial exposure on nematode mortality. Infective juvenile mortality was measured independently after 24 and 48 hr in the presence and absence of bacterial endophytes. The differences between the treatments on the combined IJ mortality (for 24 and 48 hr exposure) was statistically significant for *S. feltiae* SB 12(1) [$F(22,118) = 35.129$, $p = .000$; Wilks' $\Lambda = .018$; partial $\eta^2 = .868$] ; *S. feltiae* e-nema [$F(22,118) = 19.150$, $p = .000$; Wilks' $\Lambda = .048$; partial $\eta^2 = .781$] ; *S.carpocapsae* [$F(22,118) = 16.283$, $p = .000$; Wilks' $\Lambda = .061$; partial $\eta^2 = .752$] and *H. bacteriophora* [$F(22,118) = 8.446$, $p = .000$; Wilks' $\Lambda = .151$ partial $\eta^2 = .612$]. Subsequent univariate analysis showed that IJ mortality in the control and treatment samples was significantly different ($p < .05$) for both exposure times. Pairwise comparisons were carried out using a Bonferroni correction and only those treatments where significant differences were detected are presented in Table 2.3.1.

Table 2.3.1 Pairwise comparisons between the control treatment and the bacterial treatments. All treatments where significant differences ($p < .05$) in the Mean percentage infective juvenile mortality were detected are presented.

			Mean % IJ Mortality	SE	Sig
***S. feltiae* SB 12(1)**	**24hr**	Control	5.167	1.035	-
		L23	20.667	1.035	0.000
	48hr	Control	3.500	2.040	-
		S118	18.000	2.040	0.000
		L313	18.667	2.040	0.000
		L23	41.333	2.040	0.000
***S. feltiae* e-nema**	**24hr**	Control	2.333	.645	-
		F113	5.667	.645	0.038
		S118	10.167	.645	0.000
	48hr	Control	4.333	1.067	-
		F113	26.333	1.067	0.000
		S118	23.000	1.067	0.000
S. carpocapsae	**24hr**	Control	4.000	.996	-
		F113	23.333	.996	0.000
		L324	14.833	.996	0.000
	48hr	Control	7.833	1.337	-
		F113	31.167	1.337	0.000
		L324	23.333	1.337	0.000
		L313	8.333	1.337	0.036
H. bacteriophora	**24hr**	Control	6.500	1.483	-
		L324	25.167	1.483	0.000
		R114	14.333	1.483	0.022
		R225	17.000	1.483	0.000
	48hr	Control	7.167	1.541	-
		L330			0.001
		L324	29.833	1.541	0.000
		R225	16.333	1.541	0.004
		R114	16.667	1.541	0.002

LC50 values were not determined as no bacterial isolates caused > 50% IJ mortality. The maximum percentage IJ mortality was recorded for *S. feltiae* SB 12(1) following exposure to bacterial isolate L23 (41.3 %) after 48 hr bacterial exposure. The mean percentage of dead IJ following exposure to bacterial isolate S118 was significantly different than the respective control treatment for both *S. feltiae* strains. However, the level of mortality between the nematode strains varied, with 18% mortality for *S. feltiae* SB 12(1) IJ, compared to the control treatment with 5.16%. There was 23% mortality for *S. feltiae* e-nema IJ when exposed to S118 for 48 hr, compared to 2.3% in the control treatment. *P. fluorescens* F113 was used as a positive control as it has been shown to negatively affect numerous plant-parasitic and bacterial feeding nematodes (Cronin *et al*., 1997; Khan *et al*., 2000 and Khan *et al*., 2016). After 48 hr exposure, 26% *S. feltiae* e-nema IJ were dead in the bacterial treatment F113 compared to 4.3% in the control treatment. Similarly, 31% *S. carpocapsae* IJ were dead in the bacterial treatment F113 compared to 7% in the control treatment. F113 did not significantly affect *S. feltiae* SB 12(1) or *H. bacteriophora* after 24 or 48 hr exposure. The bacterial isolates L228, L321, S19, and S222 did not differ significantly from the control treatments for any of the nematode species or strains examined.

2.3.2 Investigation on the toxicity of the synthetic bacterial antibiotic DAPG to the entomopathogenic nematodes *S. feltiae* 12(1), *S. feltiae* (e-nema), *S. carpocapsae* and *H. bacteriophora*

The toxic effects of increasing concentrations of DAPG to EPN IJ were assessed in 96 well multiwell plates. IJ mortality was examined following 24, 48, 72 and 96 hr exposure and results are presented in Figure 2.3.5 to Figure 2.3.8.

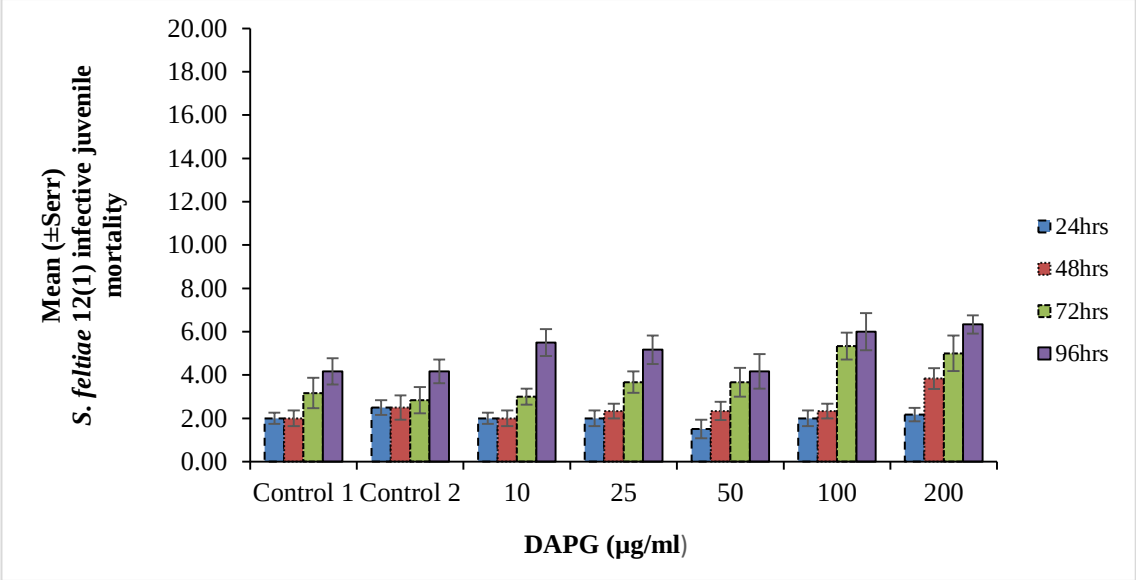

Figure 2.3.5 Mean % (±SE) *S. feltiae* SB 12(1) IJ mortality following 24, 48, 72 and 96 hr exposure to increasing concentrations of DAPG in a 96 well multiwell plate. Controls were EPN exposed to H_2O (control one) and EPN exposed to 2.5% Methanol (control two). Replication was tenfold per DAPG treatment.

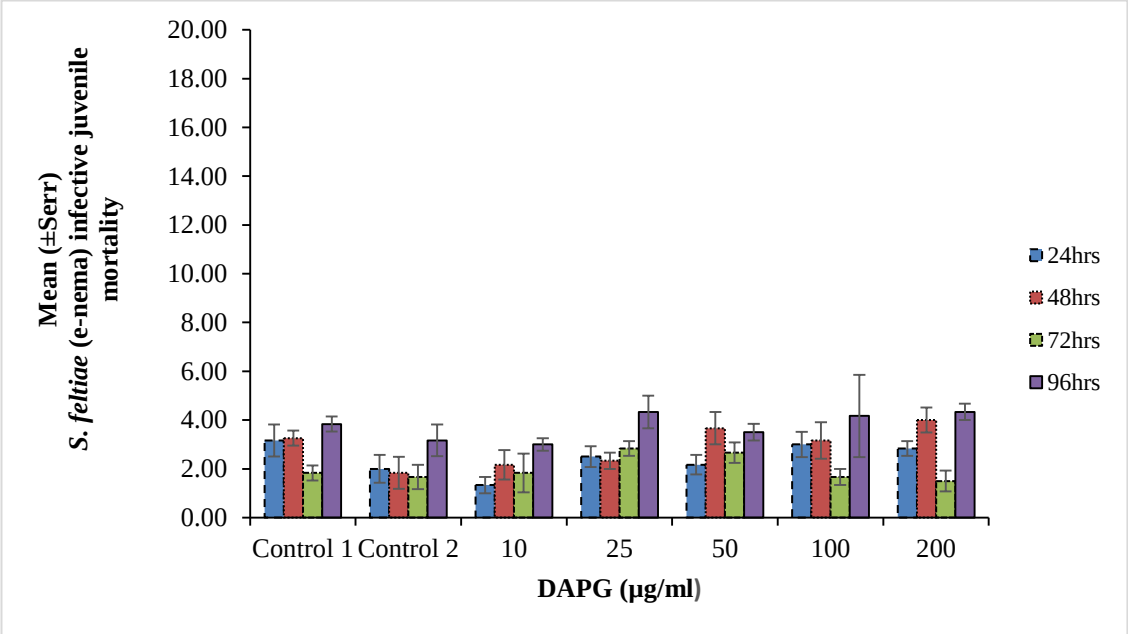

Figure 2.3.6 Mean % (±SE) *S. feltiae* (e-nema) IJ mortality following 24, 48, 72 and 96 hr exposure to increasing concentrations of DAPG in a 96 well micro-titre plate. Controls were EPN exposed to H_2O (control one) and EPN exposed to 2.5% Methanol (control two). Replication was tenfold per DAPG treatment.

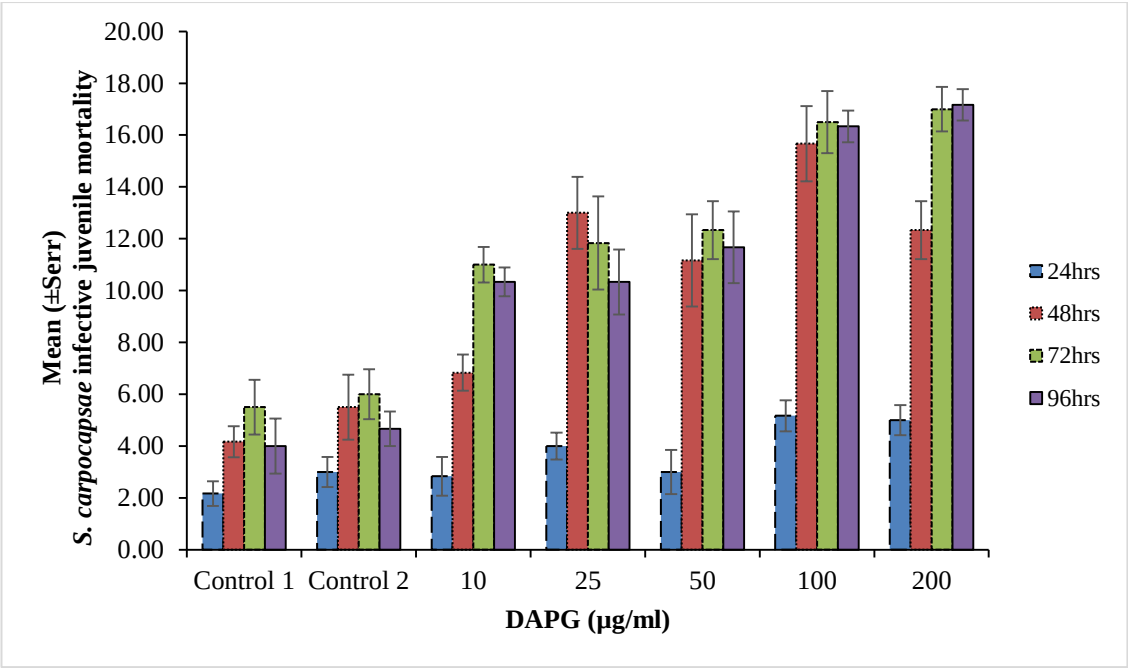

Figure 2.3.7 Mean % (±SE) *S. carpocapsae* IJ mortality following 24, 48, 72 and 96 hr exposure to increasing concentrations of DAPG in a 96 well multiwell plate. Controls were EPN exposed to H_2O (control one) and EPN exposed to 2.5% Methanol (control two). Replication was tenfold per DAPG treatment.

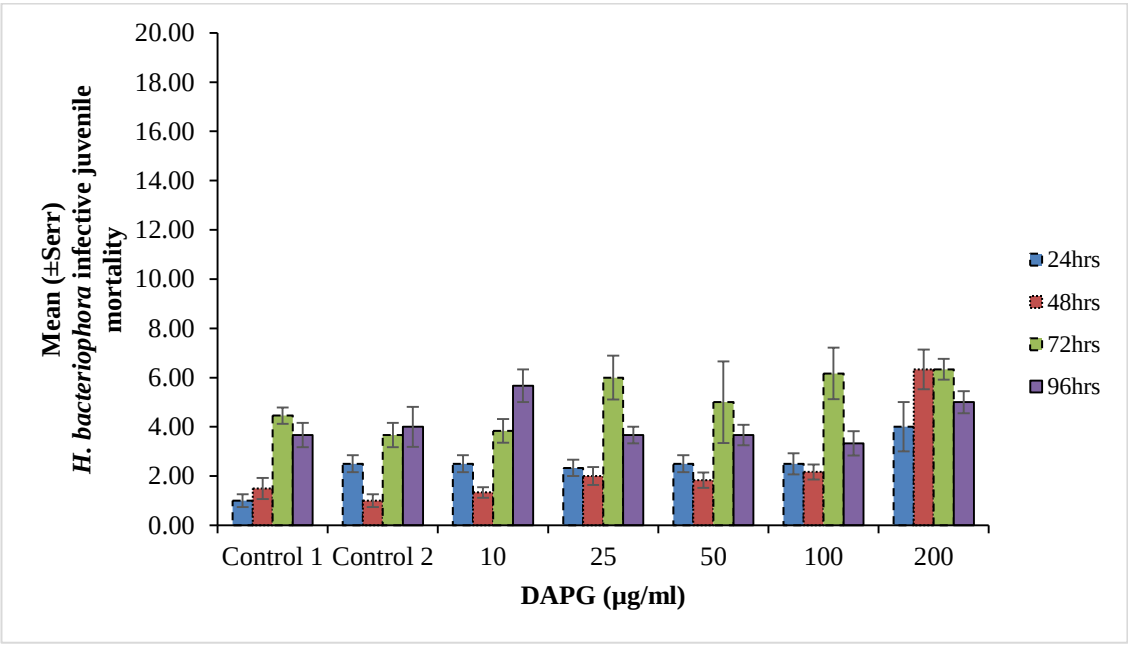

Figure 2.3.8 Mean % (±SE) *H. bacteriophora* IJ mortality following 24, 48, 72 and 96 hr exposure to increasing concentrations of DAPG in a 96 well multiwell plate. Controls were EPN exposed to H_2O (control one) and EPN exposed to 2.5% Methanol (control two). Replication was tenfold per DAPG treatment.

An ANOVA with repeated measures was conducted to examine the effect of DAPG and exposure time on IJ mortality. Mauchly's test of sphericity was violated ($p < .05$), for *S. feltiae* (e-nema) χ^2 (5) = 16.076, $p = .007$, *S. carpocapsae* χ^2 (5) = 18.868, $p = .002$, and *H. bacteriophora* χ^2 (5) = 21.684, $p = .001$. But was not violated for *S. feltiae* SB 12(1) χ^2 (5) = 9.759, $p = .082$. However epsilon (ε) was calculated similarly to the other nematode strains due to the sensitivity of the ANOVA to departures from sphericity.

Significant ***interactions between DAPG and exposure time*** were recorded for *S. carpocapsae, F* (13.357, 77.914) = 4.444, $p = .000$ and *H. bacteriophora F* (12.652, 73.801) = 2.405, $p = .010$. However, there were no such interaction effects determined for either *S. feltiae* SB 12 (1), *F* (15.347, 89.524) = 1.235, $p = .260$ or *S. feltiae* (e-nema), *F* (13.874, 80.933) = 0.916, $p = .544$. Thus, *S. carpocapsae* and *H. bacteriophora* IJ mortality increases with increasing DAPG concentrations when exposed for prolonged periods of time (up to 96 hr).

The *within subjects effects* test examines differences in IJ mortality across the four exposure times. Results indicate that ***exposure time*** had a significant effect on IJ mortality for *S. feltiae* SB 12 (1), *F* (2.5587, 89.524) = 48.382, $p = .000$, partial eta squared = .580; *S. feltiae* (e-nema), *F* (2.312, 80.933) = 12.339, $p = .000$, partial eta squared = .261; *S. carpocapsae, F* (2.226, 77.914) = 91.724, $p = .000$, partial eta squared = .724 and *H. bacteriophora F* (2.109, 73.801) = 29.305, $p = .000$, partial eta squared = .456. Pairwise comparisons are presented in Table 2.3.2. The most notably effect of increasing exposure time was recorded for *S. carpocapsae* as IJ mortality after 48, 72 and 96 hr was significantly greater than after 24 hr.

Table 2.3.2 Pairwise comparisons for DAPG induced nematode infective juvenile mortality at various ***exposure times***

Nematode	Exposure Time a	Exposure Time b	Mean Difference	Std. Error	Sig. (Bonferroni correction)	95% Confidence Interval for Difference[b] Lower Bound	95% Confidence Interval for Difference[b] Upper Bound
S. feltiae SB 12(1)	24hr	48hr	-.452	.200	.180	-1.012	.107
		72hr	-1.786*	.286	.000	-2.586	-.985
		96hr	-3.048*	.267	.000	-3.794	-2.301
	48hr	24hr	.452	.200	.180	-.107	1.012
		72hr	-1.333*	.297	.000	-2.164	-.503
		96hr	-2.595*	.269	.000	-3.347	-1.844
	72hr	24hr	1.786*	.286	.000	.985	2.586
		48hr	1.333*	.297	.000	.503	2.164
		96hr	-1.262*	.343	.005	-2.220	-.304
	96hr	24hr	3.048*	.267	.000	2.301	3.794
		48hr	2.595*	.269	.000	1.844	3.347
		72hr	1.262*	.343	.005	.304	2.220
S. feltiae (e-nema)	24hr	48hr	-.571	.289	.337	-1.380	.238
		72hr	.429	.250	.570	-.270	1.127
		96hr	-1.333*	.330	.002	-2.256	-.411
	48hr	24hr	.571	.289	.337	-.238	1.380
		72hr	1.000*	.210	.000	.413	1.587
		96hr	-.762	.371	.285	-1.799	.276
	72hr	24hr	-.429	.250	.570	-1.127	.270
		48hr	-1.000*	.210	.000	-1.587	-.413
		96hr	-1.762*	.359	.000	-2.765	-.759
	96hr	24hr	1.333*	.330	.002	.411	2.256
		48hr	.762	.371	.285	-.276	1.799
		72hr	1.762*	.359	.000	.759	2.765
S. carpocapsae	24hr	48hr	-6.214*	.487	.000	-7.577	-4.851
		72hr	-7.857*	.484	.000	-9.209	-6.505
		96hr	-7.048*	.354	.000	-8.037	-6.058
	48hr	24hr	6.214*	.487	.000	4.851	7.577
		72hr	-1.643	.708	.158	-3.624	.338
		96hr	-.833	.557	.862	-2.391	.725
	72hr	24hr	7.857*	.484	.000	6.505	9.209
		48hr	1.643	.708	.158	-.338	3.624
		96hr	.810	.521	.774	-.647	2.266
	96hr	24hr	7.048*	.354	.000	6.058	8.037
		48hr	.833	.557	.862	-.725	2.391
		72hr	-.810	.521	.774	-2.266	.647

H. bacteriophora	24hr	48hr	.167	.289	1.000	-.643	.976
		72hr	-2.429*	.341	.000	-3.382	-1.475
		96hr	-1.667*	.284	.000	-2.461	-.872
	48hr	24hr	-.167	.289	1.000	-.976	.643
		72hr	-2.595*	.396	.000	-3.703	-1.488
		96hr	-1.833*	.213	.000	-2.428	-1.239
	72hr	24hr	2.429*	.341	.000	1.475	3.382
		48hr	2.595*	.396	.000	1.488	3.703
		96hr	.762	.422	.479	-.420	1.943
	96hr	24hr	1.667*	.284	.000	.872	2.461
		48hr	1.833*	.213	.000	1.239	2.428
		72hr	-.762	.422	.479	-1.943	.420

Based on estimated marginal means *. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

An ANOVA examining the *between subjects effects* indicated that at least one concentration of ***DAPG*** significantly affected IJ mortality in *S. feltiae* SB 12 (1), F (6, 35) = 4.560, p = .000, partial eta squared = .439; *S. feltiae* (e-nema), F (6, 35) = 2.523, p = .039, partial eta squared = .302; *S. carpocapsae*, F (6, 35) = 44.363, p = .000, partial eta squared = .884 and *H. bacteriophora* F (6, 35) = 10.970, p = .000, partial eta squared = .653. DAPG was dissolved in 2.5% methanol, therefore two control treatments were used; control treatment (1) where nematodes were exposed to sterile dH_2O only and control treatment (2) where nematodes were exposed to 2.5% methanol only. For each nematode species and strain examined, the mean difference in IJ mortality between the two control treatments were not significant. This indicates that the methanol solution did not affect nematode mortality.

Table 2.3.3 Pairwise comparisons for infective juvenile mortality at increasing ***DAPG*** concentrations

Nematode	Treatment a	Treatment b	Mean Difference (a-b)	Std. Error	Sig. (Bonferroni correction)	95% Confidence Interval for Difference Lower Bound	95% Confidence Interval for Difference Upper Bound
S. feltiae SB 12(1)	Control 1 (H2O)	Control 2	-0.167	0.375	1.000	-1.394	1.060
		10µg/ml	-0.292	0.375	1.000	-1.519	0.935
		25µg/ml	-0.458	0.375	1.000	-1.685	0.769
		50µg/ml	-0.083	0.375	1.000	-1.310	1.144
		100µg/ml	-1.083	0.375	0.137	-2.310	0.144
		200µg/ml	-1.5000*	0.375	0.006	-2.727	-0.273
S. feltiae (e-nema)	Control 1 (H2O)	Control 2	1.000	0.415	0.449	-0.359	2.359
		10µg/ml	1.083	0.415	0.278	-0.276	2.443
		25µg/ml	0.167	0.415	1.000	-1.193	1.526
		50µg/ml	0.167	0.415	1.000	-1.193	1.526
		100µg/ml	0.167	0.415	1.000	-1.193	1.526
		200µg/ml	0.000	0.415	1.000	-1.359	1.359
S. carpocapsae	Control 1 (H2O)	Control 2	-0.833	0.775	1.000	-3.373	1.707
		10µg/ml	-3.7917*	0.775	0.000	-6.332	-1.252
		25µg/ml	-5.8333*	0.775	0.000	-8.373	-3.293
		50µg/ml	-5.5833*	0.775	0.000	-8.123	-3.043
		100µg/ml	-9.4583*	0.775	0.000	-11.998	-6.918
		200µg/ml	-8.9167*	0.775	0.000	-11.457	-6.377
H. bacteriophora	Control 1 (H2O)	Control 2	-0.417	0.410	1.000	-1.758	0.925
		10µg/ml	-0.958	0.410	0.528	-2.300	0.383
		25µg/ml	-1.125	0.410	0.198	-2.467	0.217
		50µg/ml	-0.875	0.410	0.834	-2.217	0.467
		100µg/ml	-1.167	0.410	0.153	-2.508	0.175
		200µg/ml	-3.0417*	0.410	0.000	-4.383	-1.700

* The mean difference is significant at the .05 level.

Post hoc analysis showed that *S. feltiae* (e-nema) IJ mortality was not affected by DAPG at any of the concentrations examined. There were significant differences ($p < .05$) in the mean difference (MD) in IJ mortality between Control treatment one and the DAPG 200 µg/ml treatment in *S. feltiae* SB 12 (1) (MD = -1.5000) and *H. bacteriophora* (MD = -3.0417). The most notable effect was recorded in *S. carpocapsae,* as the number of dead IJ in all the DAPG treatments were significantly higher than those in the control treatments. Mean differences, standard error and significance values are presented in Table 2.3.3. The ANOVA strongly confirms that the *DAPG dose* and *exposure time*

factors all have significant main effects on IJ mortality. In addition, the interactions between the two factors were also significant in *S. carpocapsae* and *H. bacteriophora*.

2.3.3 Isolation and confirmation of entomopathogenic nematode symbiotic bacteria (*Xenorhabdus* sp. and *Photorhabdus* sp.)

The entomopathogenic symbiotic bacteria *X. bovienii* and *P. luminescens* were isolated from *G. mellonella* cadavers infected by *S. feltiae* SB 12(1) and *H. bacteriophora*, respectively.

2.3.3.1 Morphology on TSA, NBTA and MacConkey agar

Following serial dilution and incubation of the insect haemolymph, single colonies were continually enriched onto TSA, for a total of three enrichments, to obtain pure cultures of each isolate. A single colony from each isolate was inoculated into LB broth and incubated at 27 ºC for 36 hr before being streaked onto TSA, NBTA, and MacConkey agar to examine morphology. Isolates that showed the morphological growth characteristics corresponding to those outlined in Table 2.2.5 are shown in Figure2.3.9 A-C. Three isolates for each bacterial species were selected for further testing.

2.3.3.2 Biochemical tests and Gram staining

Three potential isolates for each symbiotic bacterial species were Gram stained and examined for their reaction to oxidase and catalase reagents. All isolates were derived from *S. feltiae* SB 12(1) or *H. bacteriophora* (e-nema) infected insect haemolymph and subsequently selected from NBTA and purified on TSA. Results are presented in Table 2.3.4. Isolates showed characteristic morphological growth and biochemical reactions

relative to *X. bovienii* and *P. luminescens* (Akhurst & Boemare, 1988; Smigielski *et al.*, 1994). For this reason, isolates were identified as *X. bovienii* and *P. luminescens*. Further experimentation focused on *P. luminescens* EN200 and *X. bovienii* SB100 and these isolates were denoted as *P. luminescens* and *X. bovienii* throughout the remainder of this study. However it is important to note that these isolates were not confirmed at a molecular levels (16S identification) as *Xenorhabdus* and *Photorhabdus* sp.

Table 2.3.4 Entomopathogenic nematode symbiotic bacterial morphological and biochemical characteristics

Isolate ID	Nematode	Gram	Oxidase	Catalase	NBTA	MacConkey	Tryptone Soy
X. bovienii SB100	*S. feltiae* SB 12(1)	-ve	-ve	-ve	Blue	Red	Yellow
X. bovienii SB101	*S. feltiae* SB 12(1)	-ve	-ve	-ve	Blue	Red	Yellow
X. bovienii SB102	*S. feltiae* SB 12(1)	-ve	-ve	-ve	Blue	Red	Yellow
P. luminescens EN200	H. bacteriophora (e-nema)	-ve	-ve	+ve	Dark red	Red *	Yellow
P. luminescens EN201	H. bacteriophora (e-nema)	-ve	-ve	+ve	Dark red	Red *	Yellow
P. luminescens EN202	H. bacteriophora (e-nema)	-ve	-ve	+ve	Dark red	Red *	Yellow

* *P. luminescens* produced a yellow pigmentation in the MacConkey agar

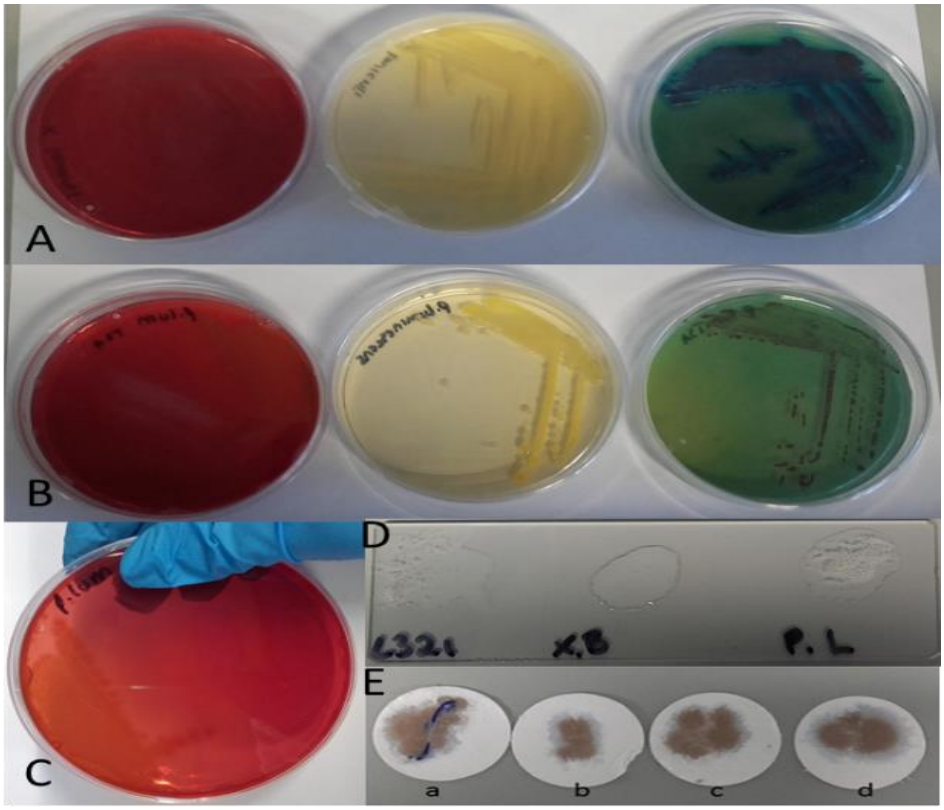

Figure 2.3.9 **(A)** *X. bovienii* growth on MacConkey, TSA and NBTA media; **(B)** *P. luminescens* growth on MacConkey, TSA and NBTA media; **(C)** *P. luminescens* growth on MacConkey agar, showing yellow pigmentation, but without lactose fermentation; **(D)** Catalase test results; formation of bubbles within 10 seconds indicates a positive result. *P. fluorescens* L321 (+ control) and *Photorhabdus luminescens* showed a positive reaction, whereas *Xenorhabdus bovienii* was negative for catalase activity; **(E)** Oxidase testing results; development of a purple colour for a: L321 (+ control) and negative reaction for b: *e- coli* (- control); c: *X. bovienii* and d: *P. luminescens.*

2.3.4 Evaluation of endophytic bacterial antimicrobial activity against *X. bovienii* and *P. luminescens*

A disk diffusion method was used to determine the endophytic bacterial (*P. fluorescens* F113 and *P. fluorescens* L321 and L124 and *P. agglomerans* S222) antimicrobial activity against *X. bovienii* and *P. luminescens.* These endophytic bacterial isolates were selected as they have known biocontrol capabilities and plant growth promotion traits. Three 1 cm plugs of agar were removed from the symbiotic bacterial lawns, using a sterile borer and 30 µl of a re-suspended endophyte culture was inoculated into each well. Control

wells were inoculated with sterile dH_2O only. Petri dishes were incubated for 24 hr and subsequently examined for the presence of a zone of inhibition around the disks. The diameter of the zone of inhibition was measured (mm). *B. subtilis* was used as a positive control as antimicrobial activity by as a number of the endophytes examined was previously shown. Results for Mean zone of inhibition (±SE) are presented in Table 2.3.5 and graphically in Figure 2.3.10.

Table 2.3.5 Antimicrobial activity of the endophytic strains F113, L321, S222 and L124 against *X. bovienii, P. luminescens and B. subtilis* as determined by a disk diffusion protocol.

Label	**ID**	***P. luminescens***		***X. bovienii***		***B. subtilis***	
		Mean Zone of Inhibition (mm)	**±SE**	**Mean Zone of Inhibition (mm)**	**±SE**	**Mean Zone of Inhibition (mm)**	**±SE**
Control		0.11	0.069	0.06	0.052	0.11	0.069
F113	*Pseudomonas fluorescens*	24.67	0.451	20.44	0.266	27.28	0.431
L321	*Pseudomonas fluorescens*	25.67	0.192	21.39	0.331	27.83	0.643
S222	*Pantoea agglomerans*	0.00	0.000	0.00	0.000	27.22	0.676
L124	*Pseudomonas fluorescens*	0.00	0.000	0.11	0.069	23.94	0.426

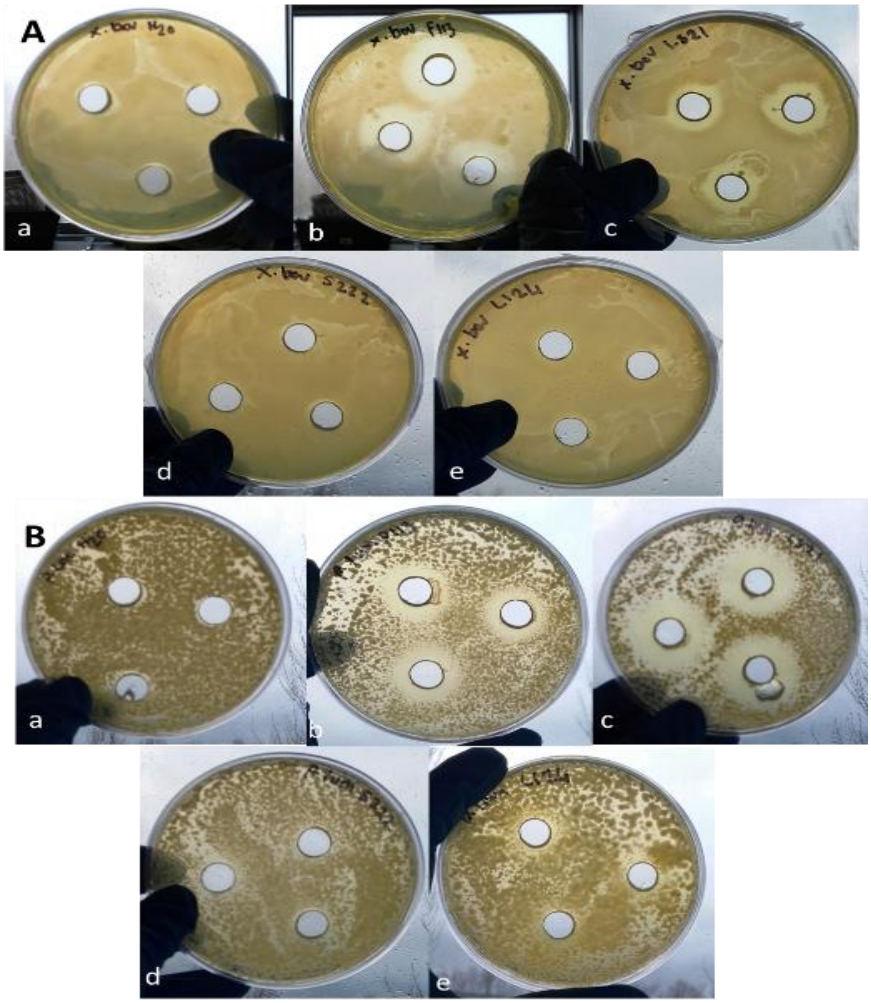

Figure 2.3.10 Plant growth promoting bacterial (*Pseudomonas fluorescens* F113, L321, L124 and *Pantoea agglomerans* S222) antimicrobial activity against A; *X. bovienii* and B; *P. luminescens*

Menton (2011) assessed the biocontrol capabilities of a number of endophytic isolates against *B. subtilis* and observed that S222 showed biocontrol activity under iron-limited conditions, whereas L124 showed biocontrol under high iron conditions. Endophytic bacterial isolates F113 and L321 were not examined by Menton, (2010).

Table 2.3.6 Pairwise comparisons using a Dunnett's t-test to compare the diameter (mm) of the zone of inhibition between the control treatments with no bacteria and the bacterial endophyte treatments.

Bacterial Lawn		Treatment a	Treatment b	Mean Difference (a-b)	±SE	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
P. luminescens	Dunnett t (2-sided)a	F113	Control (H2O)	24.55*	0.336	0.000	23.70	25.41
		L321	Control (H2O)	25.55*	0.336	0.000	24.70	26.41
		S222	Control (H2O)	-0.055	0.336	0.999	-0.91	0.79
		L124	Control (H2O)	-.055	0.336	0.999	-0.91	0.79
X. bovienii	Dunnett t (2-sided)a	F113	Control (H2O)	20.38*	0.294	0.000	19.64	21.13
		L321	Control (H2O)	21.33*	0.294	0.000	20.58	22.08
		S222	Control (H2O)	0.00	0.294	1.000	-0.75	0.74
		L124	Control (H2O)	0.11	0.294	0.987	-0.64	0.85
B. subtilis	Dunnett t (2-sided)a	F113	Control (H2O)	27.27*	0.748	0.000	25.26	29.07
		L321	Control (H2O)	27.72*	0.748	0.000	25.82	29.62
		S222	Control (H2O)	27.11*	0.748	0.000	25.21	29.01
		L124	Control (H2O)	23.83*	0.748	0.000	21.93	25.74

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests; all endophytic bacterial treatment compared to the control with no bacteria

An ANOVA was conducted to determine the significant differences in the diameter (mm) of the zone of inhibition in the control treatment wells with no endophytic bacterial inoculant and the bacterial treatments that received 30 µl of endophytic bacteria, into each well. *Post hoc* analysis was carried out using a Dunnett t-test. Results indicate that there was significant differences for *P. luminescens* [$F(4,40) = 3346.530$, $p = 0.000$], *X. bovienii* [$F(4,40) = 3346.530$, $p = 0.000$] and *B. subtilis* [$F(4,40) = 3346.530$, $p = 0.000$]. Pairwise comparisons are presented in Table 2.3.6. Results presented above indicate that all

endophytes examined have antimicrobial activity against *B. subtilis*. F113 and L321 significantly negative affect on the growth of both *P. luminescens* and *X. bovienii*.

2.4 Discussion

Examining toxic or inhibitory effects are essential when evaluating the compatibility of two organisms. The experiments carried out in Chapter 2 aim to investigate the compatibility of bacterial endophytes from the genera *Pseudomon*as, *Pantoea* and *Xanthomonas,* with the EPN from the families Heterorhabditidae and Steinernematidae. Firstly, compatibility was assessed in terms of EPN susceptibility to bacterial endophytes and the antimicrobial compound DAPG.

The effects of bacterial endophytes on IJ mortality were assessed in 96 well multiwell plates. No bacterial strain significantly and consistently affected all nematode species and strains examined. Similar bacterial susceptibility assays were conducted against the plant parasitic nematodes *Globodera pallida and Meloidogyne javanica* (Egan, 2018; personal communication). Results recorded during that study indicate that the bacterial isolates F113, S118, S222, L228 and L321 all significantly affected IJ mortality, with up to 100% juvenile mortality recorded, following exposure to a 10^8CFU/ml bacterial culture. This would indicate that plant-parasitic nematodes are much more susceptible to these bacterial isolates than EPN, as the mean IJ mortality recorded during this study, was approximately 10%, with a maximum mortality level of 41% recorded for *S. feltiae* SB12(1) following exposure to bacterial isolate *Pseudomonas veronii* L23. This is as expected as the toxic and/or inhibitory effects of rhizobacteria on plant parasitic nematodes (PPN) have been widely reported (Burkett-Cadena *et al.*, 2008; Cronin *et al.*, 1997; Khan *et al.*, 2008). Moreover, there is a great deal of potential for the use of such rhizobacteria for the biocontrol of these detrimental PPN.

The toxic and inhibitory effects of DAPG, and DAPG producing pseudomonads, to PPN, has also been documented. Cronin *et al*., (1997) indicated that DAPG may be responsible for the biocontrol capabilities of pseudomonads, as both DAPG and DAPG producing pseudomonads reduced mobility of *Globodera rostochiensis* juveniles, whereas no such effects were observed with a DAPG negative bacterial isolate. Meyer *et al.*, (2009) report that DAPG, at a concentration of 8.3 µg/ml, was toxic to adults of the PPN *Xiphinema americanum,* while also decreasing *Meloidogyne incognita* egg hatch, with no effects recorded for the second juvenile stage. When tested against the non-target bacterial feeding nematodes *C. elegans, Pristionchus pacificus* and *Rhabditis rainai*, DAPG stimulated early *C. elegans* egg hatch. However, no other nematode or nematode stage was affected (Meyer et *al*., 2009). In Chapter 2 a laboratory assay was conducted to determine the toxicity effects of DAPG on the beneficial EPN IJ.

S. feltiae (e-nema) IJ mortality was not affected by DAPG at any of the concentrations examined, whereas increased mortality levels were recorded for *S. feltiae* SB 12 (1) and *H. bacteriophora* following exposure to 200 µg/ml DAPG as seen in Figure 2.3.5 and Figure 2.3.8 and Table 2.3.3. The most notable effect was recorded for *S. carpocapsae,* as the number of dead IJ in all the DAPG treatments were significantly higher than the control treatments as shown in Figure 2.3.7. At 200 µg/ml DAPG dose, *S. carpocapsae* IJ mortality reached 17% following 96 hr exposure to DAPG compared to the 5% mortality in the control treatment. Direct IJ exposure to DAPG in multiwell plates creates a very extreme setting and is unlikely to replicate effects in a more natural environment. Thus, care must be taken to evaluate the overall effects of DAPG on additional aspects of EPN biology and behaviour. For this reason, the effects of DAPG on nematode insect infectivity was investigated in Chapter 3.

EPN are closely associated with their symbiotic bacteria and any factor affecting these symbionts will directly affect the EPN' ability to cause insect mortality. The production of antimicrobial compounds by bacteria is well documented and one such compound DAPG was examined for toxic effects in this chapter. *P. fluorescens* F113 and L321 significantly affected the growth of *P. luminescens* and *X. bovienii*. These results were not observed for isolates *P. fluorescens* L228 and *P. agglomerans* S222. Neither of these isolates has the operon for DAPG, however, they have shown biocontrol capabilities against *B. subtilis*, *Pythium* sp. and *Fusarium* sp. A combination of EPN and bacterial endophytes offers threefold biocontrol application potential; against PPN, herbivorous insects and plant pathogens. Such an approach has the additional benefits of promoting soil ecosystem health, due to a reduction of chemical insecticide input and that of chemical fertilisers use. It also helps increase crop yield and healthy plant growth due to the addition of endophytic bacteria with PGP traits. In order to fully exploit these benefits, one must examine first basic interactions between these two groups of organisms. Therefore the next step in this thesis is to investigate the effects of bacterial endophytes on EPN biology and behaviour.

Chapter 3 Determination of the effects of endophytic bacteria on entomopathogenic nematodes behaviour and biology

3.1 Introduction

Control of economically important agricultural pests has relied heavily on chemical pesticides. The overuse of pesticides has been directly related to the intensification of agriculture over previous decades, leading to an increase in environmental pollution and pest resistance (Compant *et al.*, 2005). Pressures on successful agricultural practices are ever increasing. A study published by the United Nations Department of Economic and Social Affairs predicts that the world population will increase from 7.2 billion in 2013 to 9.6 billion in 2050 (UN DESA; ST/ESA/SER.A/377, 2015), adding to already existing pressures on food production.

In order to facilitate the move away from the use of conventional chemical pesticides and prevent further environmental pollution, research must focus on the biological control of economically important pests. Sustainable biocontrol practices, such as the utilisation of a combination of entomopathogenic nematodes (EPN) (Rhabditida: Heterorhabditis and Steinernema) and endophytic bacteria, may offer one potential solution to the overuse of pesticides.

EPN, coupled with their entomopathogenic symbiotic bacteria (*Xenorhabdus* sp. and *Photorhabdus* sp.) infect an array of insects. EPN are ideal candidates for sustainable biocontrol as they are less environmentally detrimental than chemical alternatives, can be mass produced in liquid media, reproduce in high numbers inside the target insect and subsequently persist in the soil, and finally are non-toxic for both the user and the

environment. However, these advantages coupled with promising laboratory experiments cannot be fully exploited commercially unless insect control is predictable, consistent and comparable with conventional chemical pesticides. A greater understanding of EPN ecology and how to manipulate this will increase predictability and improve the chance for success of these nematodes in such a dynamic environment, as the soil.

Abiotic factors, such as inadequate soil texture and moisture levels, exposure to UV and the presence of chemical insecticides, can influence the survival, persistence, infectivity and efficacy of EPN in the soil (Hoctor *et al.*, 2012; Lacey *et al.*, 2005). The effect of naturally occurring biotic factors within the soil must also be considered when evaluating the potential use of EPN for biocontrol. Interactions between various nematode species and beneficial plant and soil microbes have received some attention recently with varying results. Plant microbes have been linked to the control of detrimental plant-parasitic nematodes (Cronin *et al.*, 1997; Meyer *et al.*, 2009) and also have been shown to increase the susceptibility of insects to EPN (Grewal *et al.*, 1997). On the other hand, insects feeding on plants colonised by endophytes have shown increased resistance to EPN (Kunkel *et al.*, 2004; Kunkel & Grewal, 2003). Both EPN and endophytic bacteria naturally function in their common soil habitat and have widespread applications in sustainable agriculture. However, the potential of synergism between the widely used EPN genera of *Steinernema* and *Heterorhabditis* with endophytic bacteria has yet to be fully explored. Understanding the effects of these biotic factors on EPN behaviour and evaluating their competitive abilities is essential to successfully develop EPN and bacterial endophytes commercially, as a combined biocontrol-plant growth promoting product.

The objectives of the study presented in Chapter 3 were to investigate the effect of exposure to endophytic bacteria and in some cases the bacterial antibiotic DAPG on the pathogenicity of EPN for the control of black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) and *Galleria mellonella* (Lepidotera: Pyralidae). These effects were assessed in terms of:

- Nematode infectivity in laboratory and greenhouse experiments
- Nematode virulence in nematode dose-response experiments
- Nematode attraction to their host insect

Nematode virulence and infectivity were evaluated in a laboratory experiment, using one laboratory-reared EPN species (*S. feltiae* 12[1]) and three commercial species (*S. feltiae, S. carpocapsae* and *H. bacteriophora* [e-nema]). Experiments employed to examine nematode infectivity against *G. mellonella* focused on *S. feltiae* SB 12(1) and *H. bacteriophora* (e-nema). For plant trials, 'Tamarin', a spring variety of oilseed rape (OSR; *Brassica napus*) and 'Temptation', a variety of strawberry (*Fragaria* x *ananassa*), were utilised to investigate nematode infectivity on *G. mellonella* and *O. sulcatus*, respectively. Two separate bioassays were employed to investigate EPN attraction to their host in sand columns and on the agar surface. The EPN species and bacterial strains investigated varied between experiments. However, *S. feltiae* SB 12(1) and *Pseudomonas fluorescens* F113 were consistently used in all experiments. *P. fluorescens* was used as this strain is well characterised and understood (Redondo-Nieto *et al.*, 2013).

3.2 Materials and Methods

Experiments were carried out with appropriate replications and controls, between 21-23°C unless otherwise stated. Endophytic bacterial isolates were cultured at 30°C for 24 hr and 10^8CFU/ml bacterial cultures were used for experimentation. All bacterial isolates were cultured and maintained using aseptic technique, EPN cultures were not maintained under sterile conditions, however, care was taken to ensure strains and species were not cross contaminated.

Statistical analysis was carried out using SPSS 22 (IBM SPSS Statistics for Windows). Where the homogeneity of variances (Levene's test; $p < 0.05$) and assumption of normality (Shapiro-Wilk test; $p < 0.05$) was violated, the non-parametric alternative was applied. Non-parametric tests were applied as they do not make the same assumptions about normality as parametric testing. *Post hoc* analysis was carried out using a Bonferroni correction unless otherwise stated. Values marked with an asterisk (*) indicate significant differences ($p < 0.05$) were detected following *post hoc* analysis.

3.2.1 Nematode, bacterial and insect cultures

Nematode and endophytic bacterial species, strains, sources and assays in which they were used are listed in Chapter 2. Nematodes were reared in parallel in the larvae of the late instar wax moth, *Galleria mellonella*. These insects were sourced commercially from Live Foods Direct (Sheffield, UK). IJ were recovered as they emerged from dead insects using White traps (White, 1927). Two weeks following emergence, IJ were washed using sterile dH_2O, pooled and stored at 10ºC until required for bioassays. For these bioassays, IJ that had emerged less than 14 days prior to experimentation were used.

The endophytic bacterial strains investigated were isolated from tissues from a range of bioenergy crops and are maintained as part of the Institute of Technology Carlow stock endophyte collection. Bacterial strains were aseptically cultured in nutrient broth at 30°C and maintained on nutrient agar, 24 hr bacterial cultures (10^8CFU/ml) were used for experimentation.

3.2.2 Examination of the effects of endophytic bacteria on nematode ability to cause insect host mortality in a laboratory bioassay

The effects of endophytic bacteria (F113wt, S19, S118, S222, L23, L228, L313, L330, L324, R114 and R225) on the ability of four EPN strains (*S. feltiae* SB 12(1), *S. feltiae* (e-nema), *S. carpocapsae* and *H. bacteriophora*) to cause insect host mortality was investigated. Experimental units consisted of standard (92 X 16mm) Petri dishes containing 50g of sand (Figure 3.2.1 [a]). Non-toxic sand was sourced commercially and autoclaved at 121ºC and 15psi, for 15 minutes. The sand was placed into sterile beakers, covered with sterile aluminium foil and dried at 60ºC for 48 hr to remove excess moisture. 400 IJ (contained in 200µl dH_2O) and 400µl 10^8CFU/ml bacterial culture were added to each experimental unit for 24 hr prior to adding insects, to allow the bacteria and nematodes to interact. The final moisture content of the sand was adjusted to 10%w/v.

Twenty *G. mellonella* larvae were added to each experimental unit and incubated at 21ºC in the presence of the bacteria and nematodes. Controls in the experiment consisted of *G. mellonella* incubated in the presence of nematodes and bacteria separately, and also in the absence of both bacteria and nematodes. *G. mellonella* were scored for mortality after five days, by probing with a blunt instrument and a colour change appropriate to each

nematode species. Replication was three-fold for each nematode species and bacterial strain combination. The experiment was run in three independent trials, with a total of nine replicates for each nematode and bacterial combination.

3.2.2.1 Statistical analysis

The homogeneity of variances and assumption of normality were violated so a Kruskal-Wallis H test using SPSS 22 was conducted to explore the individual impacts of the bacterial isolates on the susceptibility of *G. mellonella* to the EPN *S. feltiae* SB 12(1), *S. feltiae, S. carpocapsae* and *H. bacteriophora* (e-nema). Where significant differences ($p<0.05$) were identified, pairwise comparisons were calculated using Dunn's (1964) procedures with a Bonferroni adjustment. The whole data set was incorporated when making a specific pairwise comparison, in a similar manner to *post hoc* tests following a one-way ANOVA.

Firstly, a Kruskal-Wallis H test was run to determine if there were significant differences ($p<0.05$) in insect mortality between the three independent experimental trials. The trials were divided into three groups as follows, group 1: Trial 1, group 2: Trial 2 and group 3: Trial 3. There were no significant differences in the mean rank (distributions) between the twelve dependent variables (bacterial treatments and control with no bacteria) for the three trials, thus the insect mortality results were analysed together for each EPN species. A second Kruskal-Wallis H test was run to determine if there were significant differences in the mean rank of each of the four nematode species investigated and the control with no EPN. The groups were divided based on the treatment applied, as follows, group 1: Control no bacteria (H_2O), group 2: F113, group 3: S19, group 4: S118, group 5: S222, group 6: L23, group 7: L228, group 8: L313, group 9: L330, group 10: L324, group 11: R114 and group 12: R225. Finally, a Kruskal-Wallis H test was run to determine if there

were significant differences in mean rank of insect mortality for the bacterial treatments (dependent variable) between the five nematode groups (independent variable; group 1: *S. feltiae* [SB 12(1)], group 2: *S. feltiae* [e-nema], group 3: *S. carpocapsae*, group 4: *H. bacteriop*hora and group 5: control with no EPN).

3.2.3 Investigation of the effects of endophytic bacteria on *S. feltiae* SB 12(1) and *H. bacteriophora* infectivity in oilseed rape (OSR)

OSR seeds were treated with the bacterial strains *P. fluorescens* F113 PCB*rif* and S118, in addition to the synthetic DAPG (100µg/ml) and sterile dH_2O (control) using the sodium alginate bead technique as outlined in section 3.2.3.1. Coated seeds were germinated in a Petri dish for 48 hr before planting in sterile soil. Replication was three fold with three seeds per pot (experimental unit). Plants were grown in a greenhouse, maintained at a constant temperature (20ºC, ±3ºC) for 12 weeks until they became established (Figure 3.2.1 [b]). At this point, two nematode treatments (5,000 and 20,000 IJ) were added to the respective pots. One week post nematode application the root system of the plants were exposed by lightly removing soil from the surface and 15 *G. mellonella* were added to each plant. Plants were further incubated in the greenhouse, in the presence of nematodes and insects for 3 days, after which insects were removed and scored for mortality via colour change and probing with a blunt instrument. Controls consisted of plants inoculated with nematodes and insects (no bacterial endophytes and no DAPG), plants inoculated with insects and DAPG (no nematodes and no endophytes) and plants with insects only (no nematodes, no endophytes and No DAPG).

3.2.3.1 Sodium alginate bead preparation (Bashan *et al.*, 2002)

Stock solutions of sodium alginate, calcium chloride ($CaCl_2$) and powered skimmed milk were prepared as outlined in Table 3.2.1.

Table 3.2.1 Stock and working solutions for the preparation of alginate beads

Stock Solutions			**Working Solution**	
Component	**Concentration**	**Final volume**	**Components**	**volume**
Sodium alginate	2%w/v	250ml	Skimmed milk	5ml
$CaCl_2$	2%w/v	250ml	Sodium alginate	15ml
Powdered skimmed milk	10%w/v	50ml	Bacterial inoculum(10^7CFU/ml)	5ml

All components of the stock solution were autoclaved separately for 15 minutes and 15psi, with the exception of the skimmed milk; this was autoclaved for 10 minutes only. The components of the working solution were poured into a sterile Petri dish and mixed with a sterile rod. The alginate mixture and OSR seeds were added to a sterile 20 ml syringe that had the spout sealed with Parafilm. The Parafilm was removed and a sterile needle was used to ensure the coated seeds dropped out individually into a beaker containing $CaCl_2$ on a magnetic stirring plate. The beads were hardened in the $CaCl_2$ for 15 minutes and subsequently washed three times with sterile ddH_2O and stored at 4°C in a sealed plastic bag until required (no more than one week).

3.2.3.2 Statistical analysis

A Kruskal-Wallis H test using SPSS 22 was conducted to explore the individual impacts of the bacterial isolates F113wt and S118 and the synthetic antibiotic DAPG on EPN

infectivity on *G. mellonella* in pots containing sterile soil and OSR plants. Two nematode doses (5,000 and 20,000 IJ) for each nematode species, *S. feltiae* SB 12(1) and *H. bacteriophora*, were investigated separately against all bacterial treatments. The treatments were divided into four groups (group 1: control with no bacteria or DAPG (H_2O), group 2: F113, group 3: S118 and group 4: DAPG [100 µg/ml]). Where significant differences (p <0.05) were identified pairwise comparisons were calculated using Dunn's (1964) procedures with a Bonferroni adjustment.

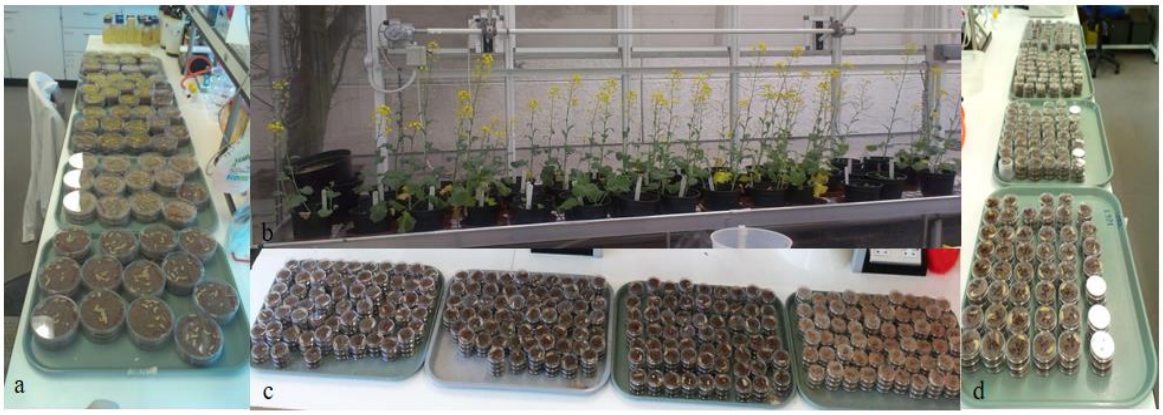

Figure 3.2.1 Experimental setups to investigate nematode infectivity and virulence. Figure a: trial 1 laboratory bioassay investigating the effect of bacterial endophytes insect host mortality. Figure b: greenhouse experiment to determine the effects of bacterial endophytes on the infectivity in OSR. Figure c&d: bioassay set up to investigate the effects of endophytic bacteria on nematode virulence.

3.2.4 Examination of the effects of bacterial endophytes on EPN virulence (Fan & Hominick, 1991)

EPN and bacterial endophytes were reared and maintained as outlined in sections 2.2.1 and 2.2.2. The experimental unit consisted of Petri dishes (35x10mm) filled with 10g of sterile dried sand. This experiment was set up as shown in Figure 3.2.1(c), in the presence and absence of bacterial endophytes. Where no bacterial endophytes were present, sterile dH_2O was used as a control. Five nematode doses (0, 25, 50, 100 and 200 IJ) were

incubated in the presence of a 10^8CFU/ml bacterial culture for 24 hr at 21ºC before adding *G. mellonella* hosts (final moisture content 10%). The experiment continued for a further 48 hr at 21ºC, when it was terminated by freezing the insects at -20ºC to halt further nematode development. The hosts were washed in sterile dH_2O prior to dissection in 2ml sterile ringers. The nematodes present in each insect were counted. Replication was tenfold with one *G. mellonella* host per replicate. The results were expressed by plotting the mean number of nematodes invading per host against the respective nematode doses.

3.2.4.1 Statistical analysis

Data were not normally distributed therefore a Spearman's rank-order correlation was conducted to assess the relationship between the numbers of IJ applied to the number of IJ recovered from each insect. In addition, a Kruskal-Wallis H test was conducted to explore the effect of individual bacterial isolates on nematode virulence at each IJ dose applied. Where significant differences (p <0.05) were identified, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons.

3.2.5 Investigation on the effect of endophytic bacteria and DAPG on fecundity of *S. feltiae* SB 12(1) and *H. bacteriophora* (e-nema) in *G. mellonella*

The following bioassay was employed to determine the effect of bacterial endophyte and DAPG exposure to nematode progeny production. Three *G. mellonella* larvae were added to a Petri dish (35 x 10 mm) containing 9 g of sterile non-toxic sand, and exposed to 100 IJ each, in the presence and absence of the endophytic bacterial strains F113 and S118, and the DAPG. Control treatments (no bacterial endophytes and no DAPG) received sterile dH_2O only. An equal volume of IJ suspension (containing 100 IJ/larva) and a 24

hr 10^8 CFU/ml (final bacterial concentration 10^4 CFU/ml) bacterial culture, or 200 µg/ml DAPG stock solution (final concentration 100 µg/ml), was added to each Petri dish to adjust the final moisture content to 10% w/v. Replication was three fold with three insects per replicate.

To examine IJ emergence, insect cadavers were removed from the sand 72 hr after nematode application. Cadavers for each nematode species and bacterial treatment were pooled together (n=9), washed with sterile dH_2O and individually placed onto White traps (White, 1927) at 23^0C. The traps were examined daily for nematode emergence and larvae were removed and the resulting IJ counted two days after initial emergence was noted. This assay was carried out a total of three times, with at least two weeks between trials to allow for culture of fresh nematodes.

3.2.5.1 Statistical analysis

A Kruskal-Wallis H test using SPSS 22 was conducted to explore the individual impacts of the bacterial isolates F113 and S118 and the synthetic bacterial antibiotic DAPG on nematode progeny production in *G. mellonella*. Firstly, a Kruskal-Wallis H test was run to determine if there were significant differences ($p<0.05$) in the number of emerged IJ between the three independent experimental trials. As there were significant differences between the trials, each experimental trial was examined independently. A second Kruskal-Wallis H test was run to determine if there were significant differences in nematode progeny production between the treatments (control, F113, S118 and DAPG) for each nematode species and experimental trial independently. Where significant differences ($p<0.05$) were identified, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons.

3.2.6 Determining the effect of bacterial endophytes on the attraction of *S. feltiae* SB 12(1), *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* (e-nema) to *G. mellonella*

Two separate bioassays were carried out to investigate the effect of endophytic bacteria on nematode host seeking. Firstly, nematode attraction to *G. mellonella* in the presence and absence of bacterial endophytes was investigated. Secondly, the ability of EPN to infect and kill *G. mellonella* in sand columns was determined.

3.2.6.1 Nematode movement on agar

The endophytic bacterial strains used were *Pseudomonas fluorescens* F113, L321 and L228. 9cm Petri dishes were graduated according to Nicholas & Andrew (1976), with concentric rings marked at an interval of 1 cm on the bottom of each dish as shown in Figure 3.2.2. Sterile SGA agar was prepared as outlined in section 3.2.6.1.1 and aseptically poured into each Petri dish. Once dry, a 24 hr bacterial culture in nutrient broth, was streaked on the periphery of the medium.

A hole of 2 mm was made in the lid of the Petri dish to secure a 1000 µl pipette tip containing two *G. mellonella* larvae. Insects were added 1.5 hr before the IJ in order to form a gradient. Approximately 60 IJ suspended in 20 µl of H_2O were added to the centre of the Petri dish and air-dried in the laminar. The nematodes present in each arc were counted after 5, 10, 20, 30 and 40 minutes. Replication was five-fold in the presence and absence of bacterial endophytes and insect hosts. Control treatment one (no bacterial endophytes) received sterile nutrient broth only and control two (no *G. mellonella* host) pipette tip remained empty and was sealed at the top with parafilm. The Mean distance (mm per nematode) travelled in any direction was calculated using the following formula from Grewal *et al.*, (2009):

$$\frac{([10T_1 + 20T_2 + 30T_3]-[10C_1 + 20C_2+30C_3])}{N-1}$$

Where: T represents the treatment quadrant

C represents the control quadrant

N represents the total number of IJ on the plate

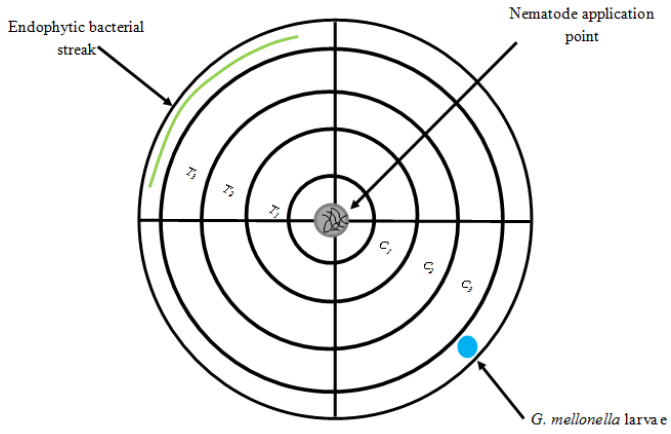

Figure 3.2.2 Experimental design to investigate the effect of endophytic bacteria on nematodes attraction to *G. mellonella* on sucrose glutamic acid agar. 9 cm Petri dishes were graduated with concentric rings 1cm apart. The number of nematodes in each arc were counted after 5, 10, 20, 30 and 40 minutes.

3.2.6.1.1 Preparation of SGA agar (selective for *gfp;kan* labelled bacteria)

To prepare 1L of SGA agar the following components were dissolved in sterile dH_2O and autoclaved for 15 minutes at 15psi and 121^0C.

20g/L^{-1}Sucrose

2 g/L^{-1} Glutamic acid

1 g/L^{-1} K_2HPO_4

15 g/L^{-1} Agar no. 1

After autoclaving, the agar was allowed to cool to approximately 60^0C at which point 5 ml 10 % $MgSO_4$. For a selection of *gfp;kan* labelled endophytes, 1ml of filter sterilised stock Kanamycin solution (50 mg/ml) was added to 1L media to a final concentration of 50 µg/ml. For cultivation wild type strains no antibiotic was added.

3.2.6.1.2 EPN surface sterilisation

To remove surface contaminants from the EPN, a 0.1 % $HgCl_2$ surface sterilisation solution adapted from (Lanau, 1993) was applied. 5 ml of a concentrated EPN suspension was washed twice with sterile dH_2O. The supernatant was removed and the pellet was re-suspended in 15 ml of sterile $^1/_4$ Ringers and washed for 5 minutes at 3800rpm. The supernatant was removed and the pellet was shaken in 15 ml of a 0.1% $HgCl_2$ solution for 10 minutes and subsequently centrifuged at 3800 rpm for 10 minutes. The resulting supernatant was removed and the IJ pellet was washed with sterile dH_2O in triplicate. To test the sterility, 100 µl of each EPN species was added to nutrient agar plates and

incubated at 30ºC for 24 hr and the lack of bacterial growth after 24 hr indicated that surface sterilisation was successful.

3.2.6.1.3 Statistical analysis

A two way repeated measures ANOVA was carried out to determine the effect of endophytic bacteria on EPN attraction to its host on agar. Interactions between the Within-Subjects Factors *host* (direction and distance travelled in the presence or absence) and the Between-Subjects Factors *treatments* (control no bacteria, F113, L321 and L228) were investigated for each nematode species independently. *Post hoc* analysis was carried out using a Bonferroni correction.

3.2.6.2 *S. feltiae* SB 12(1)*, S. feltiae, S. carpocapsae* and *Heterorhabditis bacteriophora* (e-nema) host seeking in sand columns

The effect of the bacterial endophytes F113, S118 and L321 on the ability of EPN to infect and kill *G. mellonella* was also investigated in sand columns. A 24 hr bacterial culture was added to sterile sand and mixed to ensure all sand was moistened (10%w/v). One larva was added to the bottom of each test tube (18 X 150mm) before filling with sand. Approximately 100 IJ contained in 100 µl H_2O was added to each test tube and incubated vertically in the presence and absence of bacterial endophytes for 24 hr. After incubation, the insects were removed from the tubes, washed with sterile dH_2O and incubated for a further 24 hr at 25ºC. *G. mellonella* hosts were scored for mortality and dissected to determine the number of IJ present. Replication was 10 fold per nematode species and bacterial strain, with one insect per replicate.

3.2.6.2.1 Statistical analysis

A one-way between groups analysis of variance was conducted to explore the effect of the bacterial endophytes on insect mortality and the number of IJ establishing in insects at the bottom of sand columns.

3.2.7 Determination of the susceptibility of the black vine weevil (*Otiorhynchus sulcatus*) to entomopathogenic nematodes following bacterial endophyte exposure

Black vine weevil (BVW) larvae were sourced from Greenhill fruit farm, Enniscorthy Co. Wexford. Infected strawberry plants were dug up, the soil was shaken away from the roots and the insects were removed. Larvae were stored in vented plastic pots for transport back to the laboratory. At this point, they were washed in sterile dH_2O and incubated at 10ºC for 48 hr until experimentation began. Similarly sized insects that were moving freely were selected for experimentation. Two separate experiments were carried out to determine any negative or stimulatory effects of bacterial isolates on nematode infectivity. Firstly, a laboratory-based assay utilising Petri dishes of sand and secondly a plant-based experiment using strawberry plants.

3.2.7.1 The infectivity of *S. feltiae* SB 12(1) and *H. bacteriophora* (e-nema) against the black vine weevil (*O. sulcatus*) in a laboratory bioassay

EPN infectivity against the BVW was investigated in 5 cm Petri dishes containing 9 g sterile sand in the presence and absence of the bacterial endophyte strain *P. fluorescens* F113. A 24 hr bacterial culture was homogenised in sterile sand by mixing in a container. Replication was 14 fold, with one insect per in Petri dish. There were two controls; insects in the presence of nematodes and water and insects incubated in the absence of

nematodes and bacteria. The control treatments (No F113 and no nematodes) received sterile dH_2O only. Insect mortality was recorded after three days incubation at 25ºC in the dark.

3.2.7.1.1 Statistical analysis

The number of weevils was limited for experimentation, due to this small sample size and a number of the expected outcome frequencies less than five, the Fisher's exact test was used as it is more accurate than the chi-square test. The Fisher's exact test of independence was conducted to investigate if the proportions of BVW survival in the presence of F113 were significantly different than the control.

3.2.7.2 Susceptibility of the black vine weevil (*O. sulcatus*) to *S. feltiae* SB 12(1) and *H. bacteriophora* (e-nema) in strawberry plants (*Fragaria* x *ananassa*)

The effect of bacterial endophytes on EPN infectivity to the black vine weevil was investigated in strawberry plants (variety: Temptation). Plants were sourced commercially, removed from pots, washed with sterile dH_2O and replanted into sterile soil. Seven larvae were added per pot and there were seven pots (replications) per nematode and bacterial treatment. Pots were kept in bug dorms (60x60x60cm) and incubated in a plant growth room for 7 days at 22°C (± 2°C). Following incubation 10,000 IJ (contained in a 5 ml dH_2O suspension) of the appropriate nematodes, species were added to each pot. Control plants (no nematodes) received sterile dH_2O only. Insects were exposed to nematodes for five days at 22°C (± 2°C). After this, all insects

were removed from the pots, washed with sterile dH_2O and incubated at 25°C for 24 hr. The number of live insects was then counted.

3.2.7.2.1 Statistical analysis

Significant differences in the susceptibility of the BVW to *S. feltiae* SB 12(1) and *H. bacteriophora* (e-nema) in strawberry plants were identified using a *between-subjects* independent t-test between the number of live insects in the bacterial treatment and the control group. The differences between groups were explored for each EPN species (*S. feltiae* 12[1], *H. bacteriophora* and control with no EPN) independently.

3.3 Results

3.3.1 Examination of the effects of endophytic bacteria on nematode ability to cause insect host mortality in a laboratory bioassay

S. feltiae SB 12(1), *S. feltiae*, *H. bacteriophora* and *S. carpocapsae* (e-nema) IJ were exposed to a 10^8CFU/ml bacterial culture in sand, 20 *G. mellonella* hosts were added and scored for mortality after 5 days. Three replicates (each containing 20 insects) were used per trial and three independent trials were carried out to determine reproducibility. Figure 3.3.1 to Figure 3.3.5 display percentage insect mortality in the presence and absence of bacterial isolates. For the purpose of clarity, the insect mortality results are presented separately for the four nematode strains and the control treatment with no nematodes.

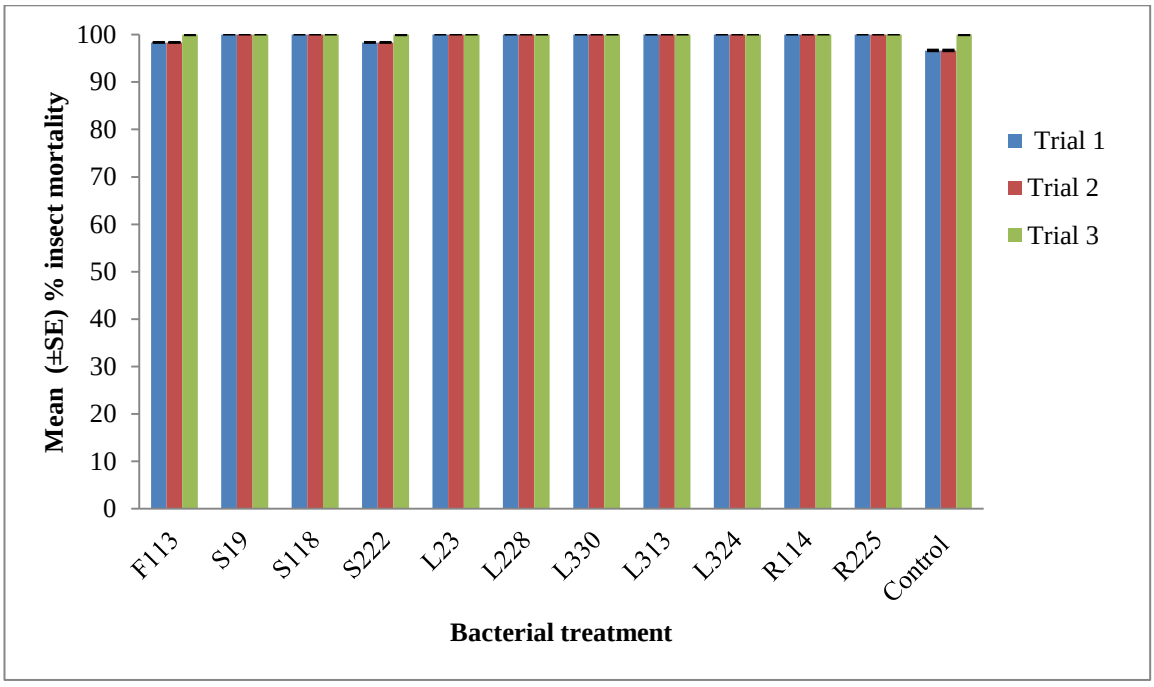

Figure 3.3.1 Percentage (±SE) *G. mellonella* mortality following exposure to *S. feltiae* SB 12(1) and endophytic bacteria in Petri dishes containing sterile sand. n = 3 with 20 insects per replicate. Three independent trials were carried out and results are presented for each trial. Controls consisted of insects exposed to nematodes and sterile dH_2O in the absence of endophytic bacteria

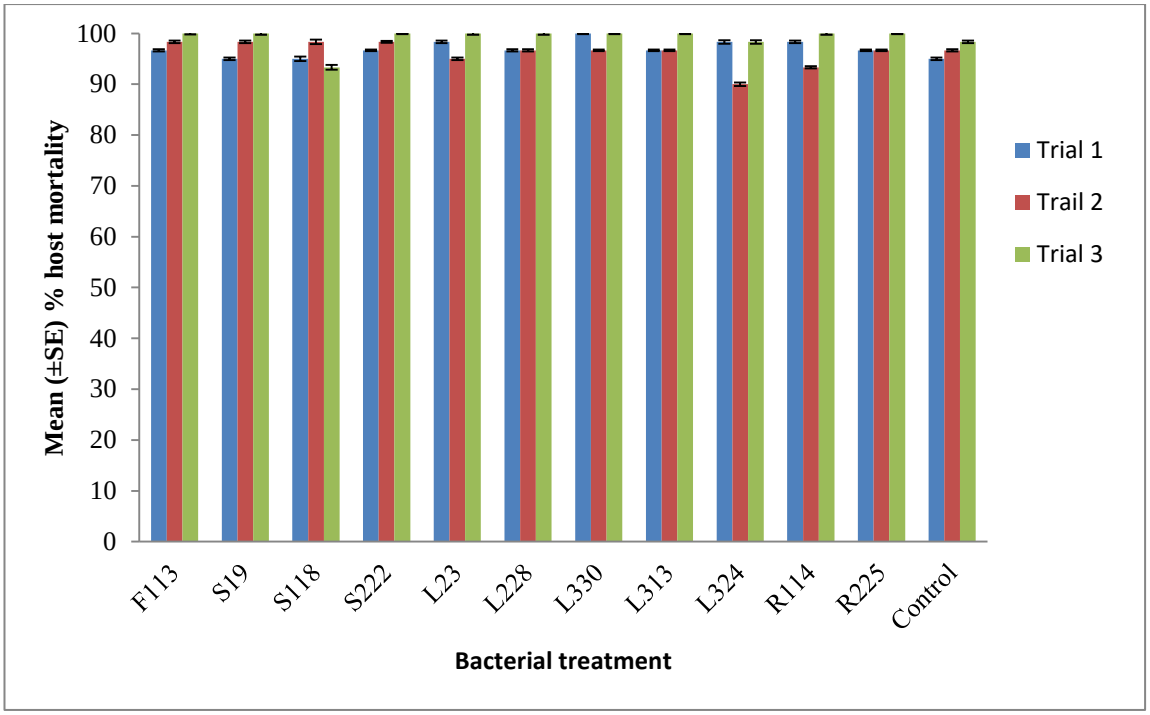

Figure 3.3.2 Percentage (±SE) *G. mellonella* mortality following exposure to *S. feltiae* (e-nema) and endophytic bacteria in Petri dishes containing sterile sand. n = 3 with 20 insects per replicate. Three independent trials were carried out and results are presented for each trial. Controls consisted of insects exposed to nematodes and sterile dH_2O in the absence of endophytic bacteria

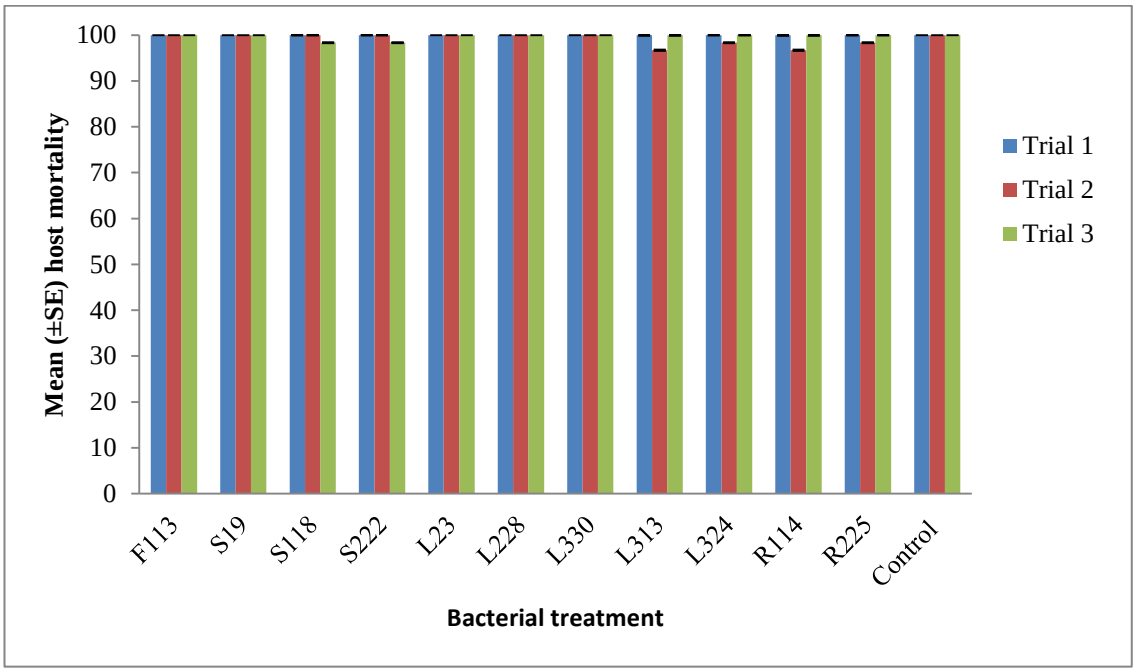

Figure 3.3.3 Percentage (±SE) *G. mellonella* mortality following exposure to *S. carpocapsae* and endophytic bacteria in Petri dishes containing sterile sand. n = 3 with 20 insects per replicate. Three independent trials were carried out and results are presented for each trial. Controls consisted of insects exposed to nematodes and sterile dH_2O in the absence of endophytic bacteria

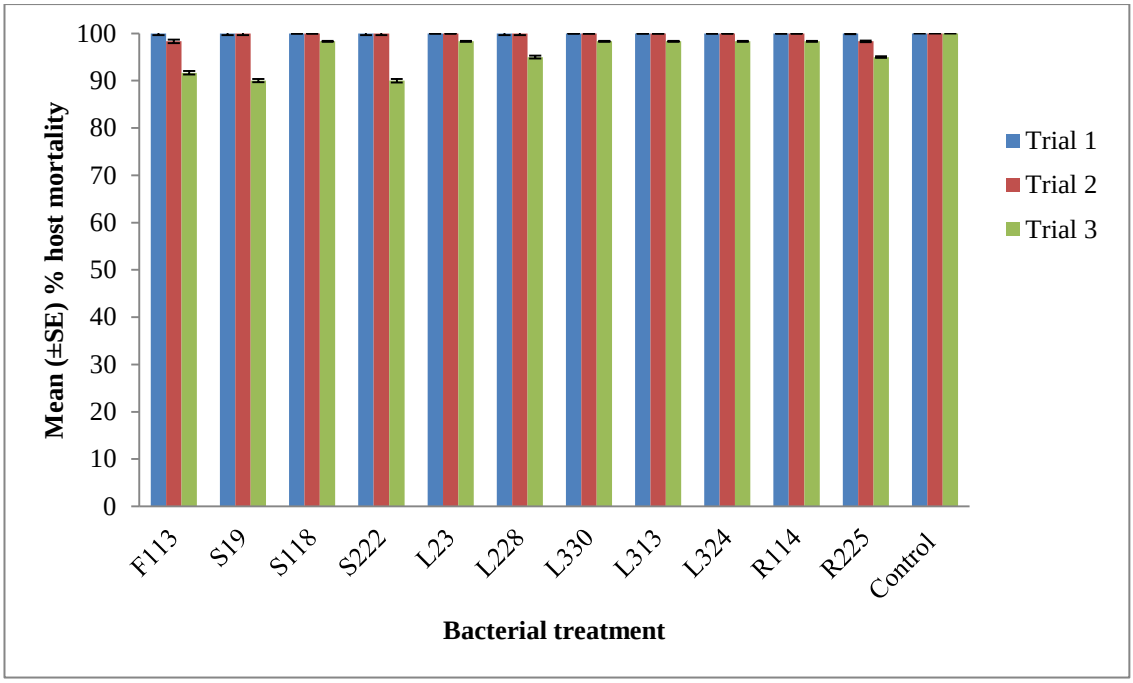

Figure 3.3.4 Percentage (±SE) *G. mellonella* mortality following exposure to *H. bacteriophora* and endophytic bacteria in Petri dishes containing sterile sand. n = 3 with 20 insects per replicate. Three independent trials were carried out and results are presented for each trial. Controls consisted of insects exposed to nematodes and sterile dH_20 in the absence of endophytic bacteria

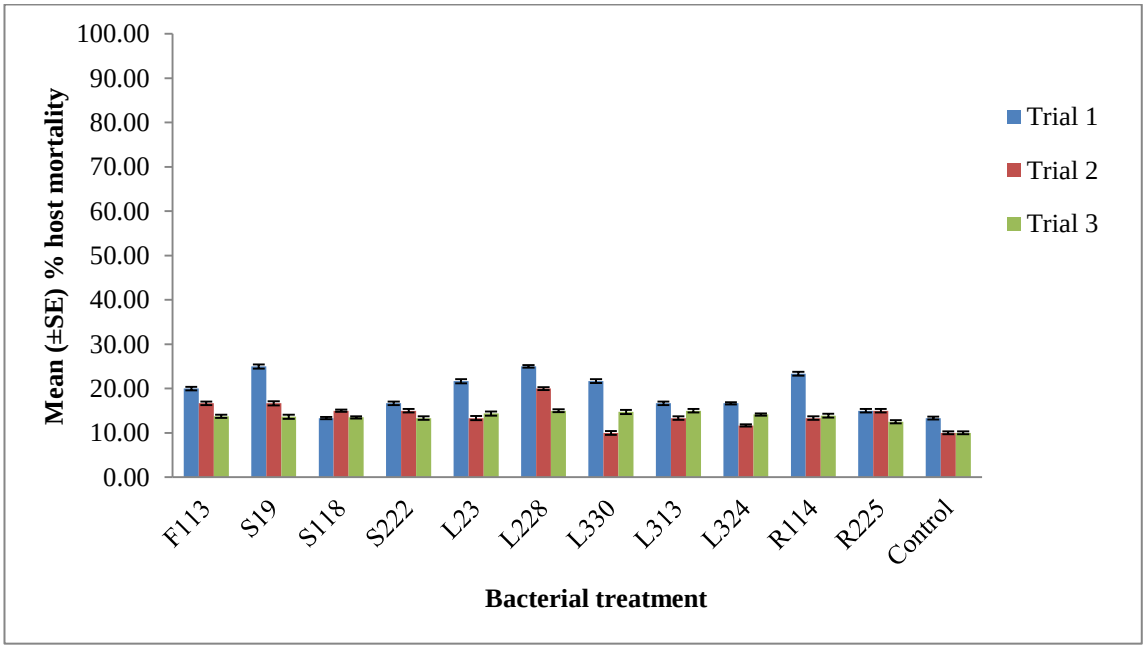

Figure 3.3.5 Percentage (±SE) *G. mellonella* mortality following exposure to endophytic bacteria, in the absence of nematodes. Petri dishes containing sterile sand were the experimental unit. n = 3 with 20 insects per replicate. Three independent trials were carried out and results are presented for each trial. Here, controls consisted of insects exposed to sterile dH_2O in the absence of endophytic bacteria

A Kruskal-Wallis H test was run to determine if there were significant differences ($p<0.05$) in insect mortality between the three experimental trials. There was no significant difference in the mean rank (distributions) between trials for each of the twelve dependent variables. As there was no statistically significant difference between the trials, results for insect mortality were analysed together for each individual EPN species.

A second Kruskal-Wallis H test was run to determine if there were significant differences ($p<0.05$) in the distributions of *G. mellonella* mortality between the bacterial and control treatments for each of the nematode species investigated. There were no significant differences between the bacterial treatments and the control for *S. feltiae* SB 12(1) χ^2 (11) = 14.404, p = .211, for *S. feltiae* e-nema χ^2 (11) = 5.470, p = .906 for *S. carpocapsae* χ^2 (11) = 10.700, p = .469 for H. bacteriophora χ^2 (11) = 12.621, p = .391. Moreover, no bacterial strain caused a significant level of insect mortality in the absence of EPN χ^2 (11) = 19.018, p = .061.

Finally, a Kruskal-Wallis H test was run to determine if there were significant differences in mean rank of insect mortality for the bacterial treatments between the five nematode groups (group 1: *S. feltiae* [SB 12(1)], group 2: *S. feltiae* [e-nema], group 3: *S. carpocapsae*, group 4: *H. bacteriophora* and group 5: control with no EPN). Insect mortality scores were statistically significantly different (p <.05) between groups as shown in Table 3.3.1. *Post hoc* analysis was carried with a Bonferroni correction for multiple comparisons. Across all treatments, the number of dead insects in the control treatment with no EPN is significantly different (p <.05) than the number of dead insects in the four nematode treatments. As there are no significant differences in mortality between the EPN species, we can determine that no individual EPN species examined showed a greater level of insect mortality.

Table 3.3.1 Kruskal- Wallis H test indicating significant differences in insect mortality between at least one of the nematode groups. Group 1: *S. feltiae* SB 12(1) group 2: *S. feltiae* (e-nema) group 3: *S. carpocapsae*, group 4: *H. bacteriophora and* group 5: control with no EPN.

Treatment	N	Test Statistic	Degrees of Freedom	Asymptotic significance (2-sided test)
H_20	45	33.857	4	0.000
F113	45	31.659	4	0.000
S19	45	32.781	4	0.000
S118	45	32.968	4	0.000
S222	45	29.193	4	0.000
L23	45	35.032	4	0.000
L228	45	34.895	4	0.000
L330	45	35.875	4	0.000
L313	45	31.522	4	0.000
L324	45	33.411	4	0.000
R114	45	31.594	4	0.000
R225	45	30.679	4	0.000

*Significance level is 0.05 Combined results for the three experimental trials

3.3.2 Investigation of the effects of endophytic bacteria on EPN infectivity in the Oilseed rape plants

OSR was inoculated with the bacterial strains *P. fluorescens* F113 pcb*rif* and S118 and treated with DAPG (100µg/ml) and sterile dH_2O was added as a control. Plants were grown in a greenhouse for three months until they became established. Two nematode treatments were applied and 15 *G. mellonella* hosts were added to each pot. Plants were further incubated in the presence of nematodes and hosts for three days and the hosts were then scored for mortality. Results are presented below in Figure 3.3.6.

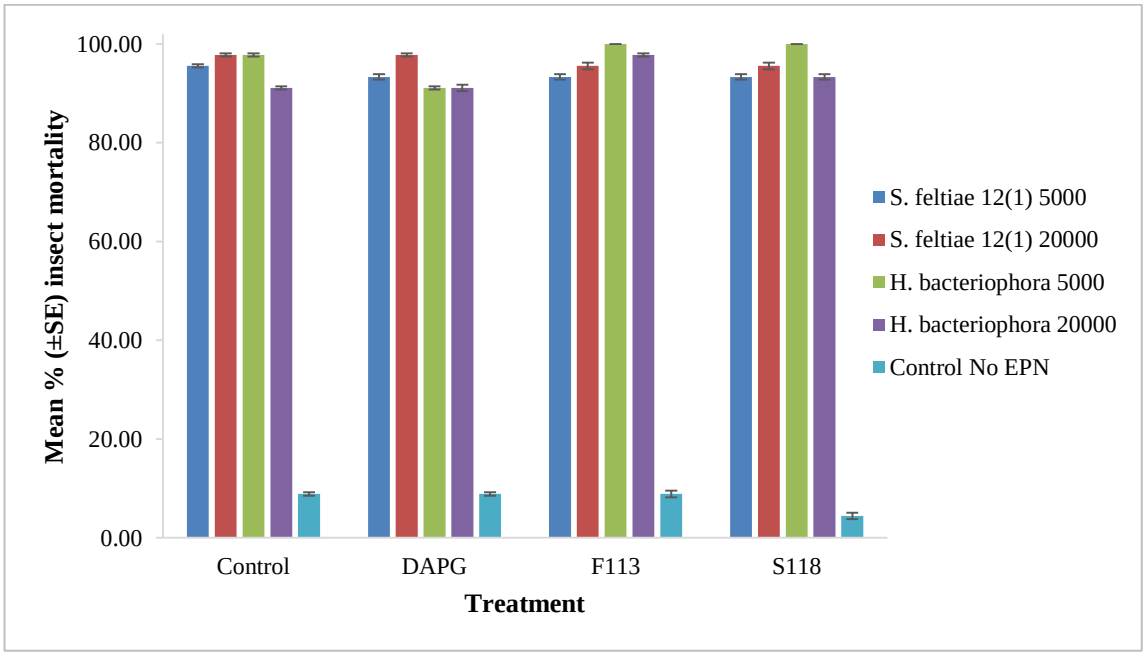

Figure 3.3.6 Mean percentage (±SE) *G. mellonella* mortality in oilseed rape plants inoculated with endophytic bacteria and DAPG independently. Two nematode species and doses (5000 and 20000IJ) were applied. There were two control treatments: 1. Insects exposed to nematodes only and 2. Insects exposed to neither nematodes nor endophytic bacteria or DAPG.

A Kruskal-Wallis H test was conducted to determine if there were differences in percentage insect mortality in OSR plants between bacterial, DAPG and control treatments. Distributions of mortality scores were similar and were not statistically significant for: *S. feltiae* SB 12(1) 5,000 IJ [χ^2 (3) =.349, p = .951], *S. feltiae* SB 12(1)

20,000 IJ [χ^2 (3) =.147, p = .986], *H. bacteriophora* 20,000IJ [χ^2 (3) = 2.750, p = .432] and the control with no nematodes [χ^2 (3) =.1.193, p = .755]. The distributions of percentage mortality scores for the lower *H. bacteriophora* dose of 5,000 IJ were not similar for all groups χ^2 (3) = 8.463, p = .037, and were statistically significantly different between the bacterial and control treatments. *Post hoc* analysis examining pairwise comparisons revealed that were no statistically significant differences in mortality between the Control H_2O (mean rank = 6.67) and F113 (mean rank = 8.50; p = 1.000), Control H_2O and S118 (mean rank = 8.50; p = 1.000), or Control H_2O and DAPG (mean rank= 2.33; p = .461). Adjusted p-values are presented. This indicates that the bacterial or DAPG treatment did not affect the EPN ability to cause insect mortality.

3.3.3 Examination of the effects of bacterial endophytes on EPN virulence

Nematodes, at five increasing doses, were exposed to a bacterial culture for 24 hr prior to adding *G. mellonella* for 48 hr. The experiment was terminated by freezing the insects at -20ºC to halt further nematode development. The insects were dissected and the nematodes present were counted. A linear regression line was drawn by plotting the mean number of nematodes invading per insect over the respective doses (Figure 3.3.7 to Figure 3.3.10). The slope of the line is indicative of nematode virulence, thus a higher slope the more virulent the nematode. Results presented below for all nematode species and strains examined indicate that in the presence or absence of bacterial isolates, the IJ dose applied per larva acts in linear association with the number of nematodes recovered per larva.

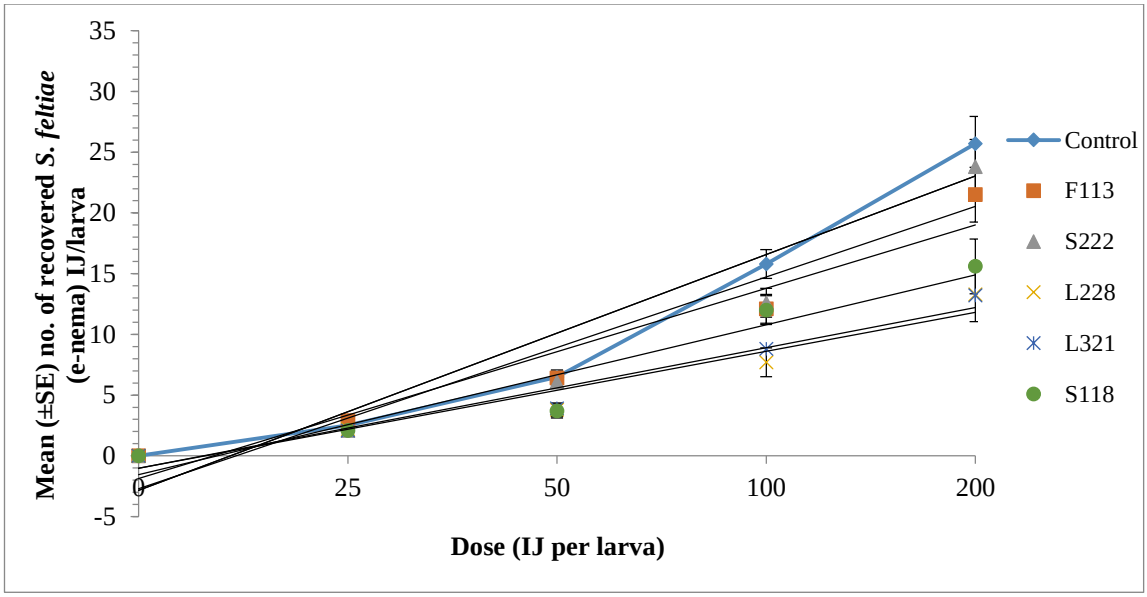

Figure 3.3.7 *S. feltiae* e-nema virulence against *G. mellonella* following exposure to bacterial endophytes. Graph plots mean number (±SE) of nematodes recovered per larva following incubation in the presence of increasing nematode doses (0, 25, 50,100 and 200IJ/larva). Dose-response experiments were carried out in Petri dishes containing sterile sand, with H_2O as a control

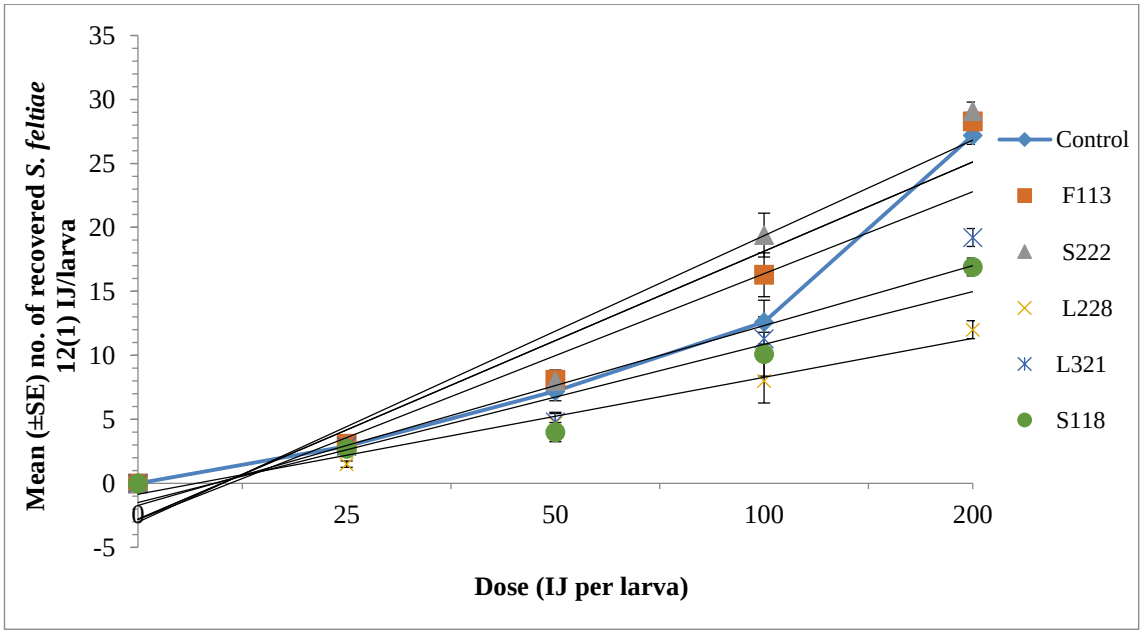

Figure 3.3.8 *S. feltiae* 12 (1) virulence against *G. mellonella* following exposure to bacterial endophytes. Graph plots mean number (±SE) of nematodes recovered per larva following incubation in the presence of increasing nematode doses (0, 25, 50,100 and 200IJ/larva). Dose-response experiments were carried out in Petri dishes containing sterile sand, with H_2O as a control

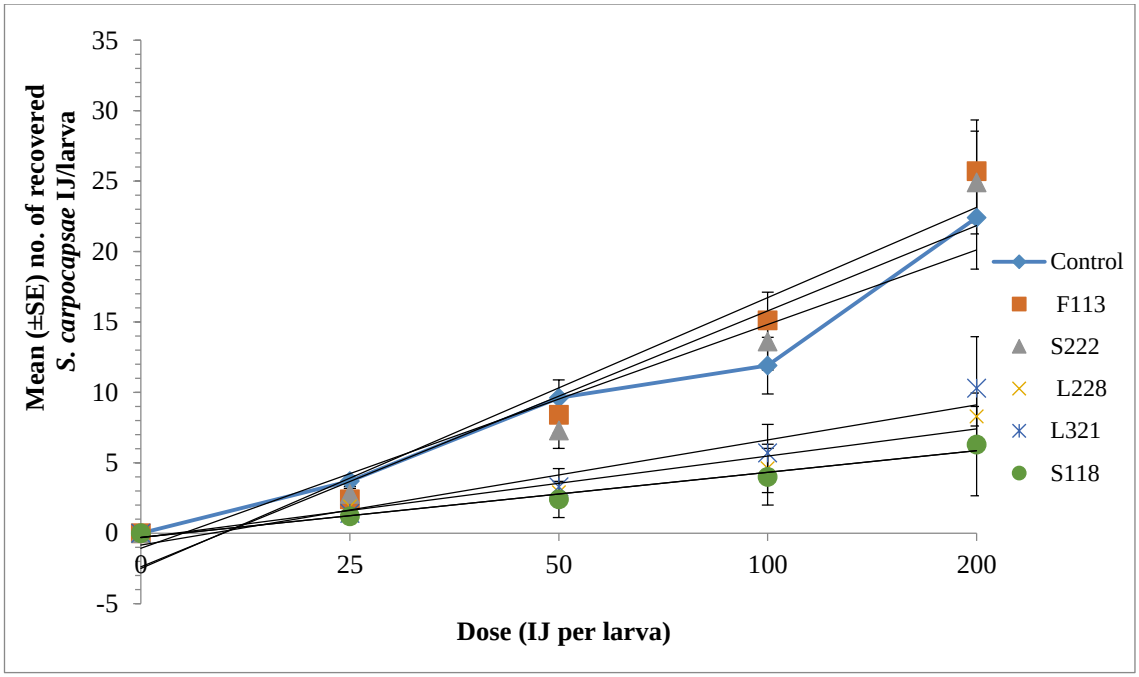

Figure 3.3.9 *S. carpocapsae* virulence against *G. mellonella* following exposure to bacterial endophytes. Graph plots mean number (±SE) of nematodes recovered per larva following incubation in the presence of increasing nematode doses (0, 25, 50,100 and 200IJ/larva). Dose-response experiments were carried out in Petri dishes containing sterile sand, with H_2O as a control

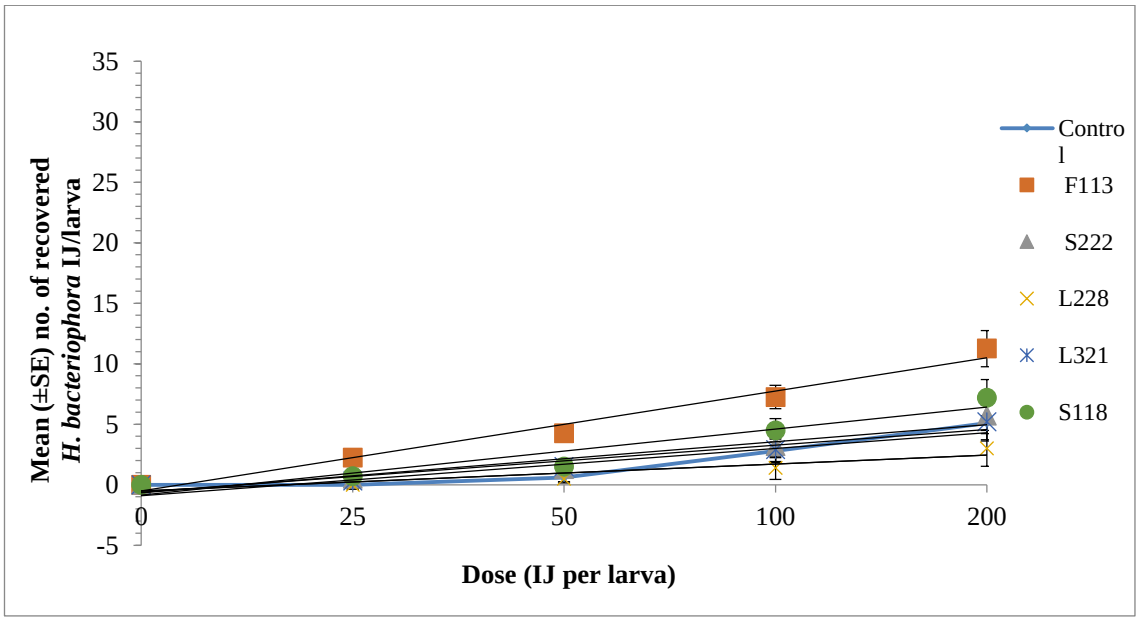

Figure 3.3.10 *H. bacteriophora* virulence against *G. mellonella* following exposure to bacterial endophytes. Graph plots mean number (±SE) of nematodes recovered per larva following incubation in the presence of increasing nematode doses (0, 25, 50,100 and 200IJ/larva). Dose-response experiments were carried out in Petri dishes containing sterile sand, with H_2O as a control

In addition to regression analysis, a Spearman's rank-order correlation was conducted to assess the relationship between the numbers of IJ applied to the mean number of IJ recovered per insect. A value of +1 indicates a strong positive correlation between the two variables and a value of -1 indicates a strong negative correlation. A value close to zero indicates a weaker association between the two variables. Correlation coefficient values presented in Table 3.3.2 indicates that there was a strong positive correlation (p <0.01) between numbers of IJ applied per larva and the number of IJ recovered, for all nematode groups and bacterial treatments. Correlation values calculated for all nematode and bacterial combinations were above 0.830, with the exception of *H. bacteriophora* control treatment (0.588) and L228 treatment (0.474). These values indicate weaker positive correlations between the two variables.

Table 3.3.2 The linear regression equations r^2 and Spearman's ρ values for *S. feltiae* SB 12(1), *S. feltiae* (e-nema), *S. carpocapsae* and *H. bacteriophora* dose-response experiments. The results were expressed by plotting the mean number of nematodes invading per host over the respective nematode doses.

***S. feltiae* 12(1)**	**Treatment**	**Equation**	**r^2**	**Spearman's ρ**
	Control	y=6.41x - 9.25	0.8916	0.847
	F113	y=6.98x - 9.78	0.9386	0.947
	S222	y=7.46x - 10.48	0.9463	0.946
	L228	y=3.05x - 3.91	0.9787	0.867
	L321	y=4.68x - 6.4	0.9283	0.925
	S118	y=4.12x - 5.62	0.9237	0.900
***S. feltiae* e-nema**	**Treatment**	**Equation**	**r^2**	**Spearman's ρ**
	Control	y=6.47x - 9.31	0.9332	0.953
	F113	y=5.22x - 7.08	0.9398	0.908
	S222	y=5.81x - 8.51	0.9148	0.930
	L228	y=3.21x - 4.23	0.9396	0.847
	L321	y=3.31x - 4.33	0.9569	0.918
	S118	y=4.11x - 5.65	0.9264	0.912
S. carpocapsae	**Treatment**	**Equation**	**r^2**	**Spearman's ρ**
	Control	y=0.1069x+1.5025	0.9649	0.888
	F113	y=0.1304x+0.54	0.9833	0.908
	S222	y=0.1252x+0.33	0.9953	0.930
	L228	y=0.0394x+0.585	0.9847	0.876
	L321	y=0.0511x+0.3075	0.9931	0.935
	S118	y=0.0307x+0.4775	0.9717	0.836
H. bacteriophora	**Treatment**	**Equation**	**r^2**	**Spearman's ρ**
	Control	Y = 0.0277x-0.3775	0.9708	0.588
	F113	y=0.0548x+0.8938	0.9701	0.867
	S222	y=0.0294x-0.065	0.9925	0.880
	L228	y=0.018x-0.27	0.9800	0.474
	L321	y=0.0268x-0.03	0.9872	0.877
	S118	y=0.0376x-0.04	0.9767	0.909

To further explore the effect of individual bacterial isolates on nematode virulence at each IJ dose applied a Kruskal-Wallis H test was conducted. Where significant differences ($p<0.05$) were identified, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. Adjusted p-values are presented. The distributions of recovered IJ were not similar between control and bacterial treatments for each nematode strain examined {*S. feltiae* SB 12(1) at IJ dose 200 $\chi 2$ (5) = 24.684, p = .000; *S. feltiae* (e-nema) IJ dose 100 [$\chi 2$ (5) = 12.455, p = .029] and 200 [$\chi 2$ (5) = 22.649, p = .000]; *S. carpocapsae* at IJ dose 50 [$\chi 2$ (5) = 32.619, p = .000], 100 [$\chi 2$ (5) = 31.675, p = .000] and 200 [$\chi 2$ (5) = 44.995, p = .000] and *H. bacteriophora*

at IJ dose 25 [$\chi 2$ (5) = 19.708, p = .001] and 50 [$\chi 2$ (5) = 22.232, p = .000]}. Subsequent *post hoc* analysis and pairwise comparisons revealed statistically significant differences in median scores between control and bacterial treatments for a number of IJ doses applied. Results are presented in Table 3.3.3. The effect of endophytes on nematode virulence varied between nematode and bacterial isolates. No individual isolate significantly and consistently affected all nematode species. Moreover, no nematode species was affected across all doses examined.

Table 3.3.3 Nematode dose response pairwise comparisons were significant differences were detected following *Post hoc* analysis

Nematode	IJ dose applied/larva	Pairwise comparisons		Sig. (p)
S. feltiae 12(1)		**Control**	**L228**	
	200	26.50	11.50	0.013
S. feltiae e-nema		**Control**	**L228**	
	100	15.00	8.00	0.027
	200	27.50	10.50	0.011
		Control	**L321**	
	200	27.50	13.00	0.013
S. carpocapsae		**Control**	**L228**	
	50	8.50	2.50	0.002
		Control	**S118**	
	50	8.50	2.50	0.000
		Control	**L321**	
	50	8.50	3.00	0.007
		Control	**S118**	
	100	9.50	4.00	0.000
		Control	**L228**	
	200	20.50	8.00	0.008
		Control	**S118**	
		20.20	5.50	0.000
H. bacteriophora		**Control**	**F113**	
	25	0.5	1.00	0.009
		Control	**F113**	
	50	0.05	2.00	0.022

3.3.4 Investigation on the effect of endophytic bacteria and DAPG on the fecundity of *S. feltiae* SB 12(1) and *H. bacteriophora* (e-nema) in *G. mellonella*

The effect of bacterial endophytes and DAPG exposure to nematode progeny production was observed in *G. mellonella.* Three larvae were exposed to 100IJ each in the presence and absence of the endophytic bacterial strains F113 and S118 and the synthetic bacterial antibiotic DAPG. Replication was three fold with three insects per replicate. After 72 hr exposure, insect cadavers were removed from the sand and pooled together for each nematode species and bacterial treatment combination. Insects were placed onto White traps (White, 1927) and checked daily for IJ emergence. This assay was carried out a total of three times, with at least two weeks between trials to allow for culture of fresh nematodes. Results are presented below in Figure 3.3.11 to Figure 3.3.13. *S. feltiae* SB 12(1) and *H. bacteriophora*, nematode progeny production is presented separately for each of the three experimental trials.

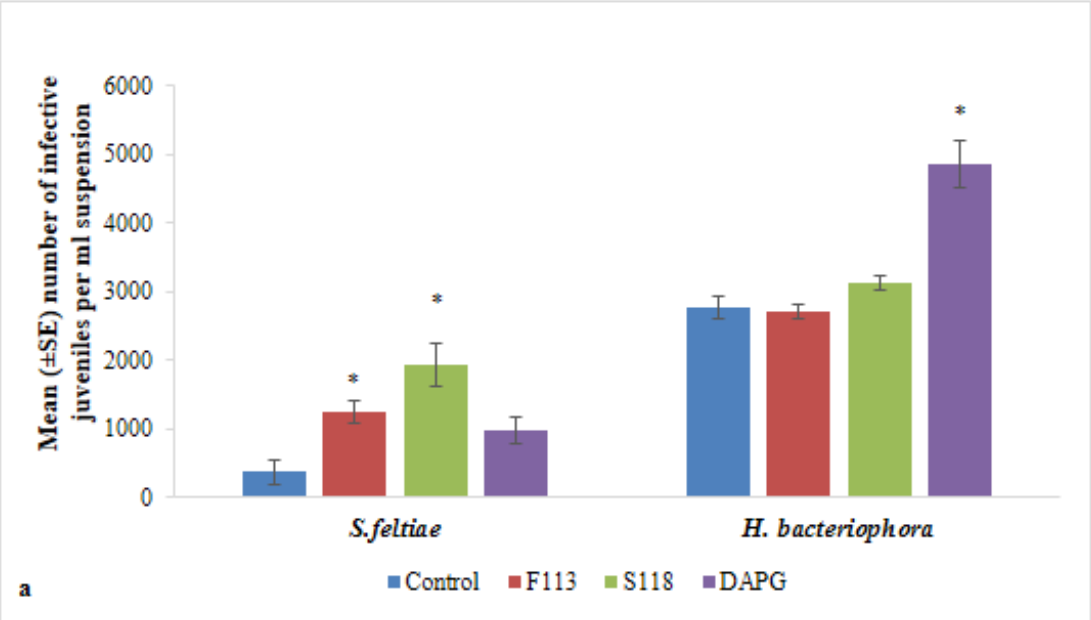

Figure 3.3.11 Nematode progeny production (±SE) in *G. mellonella* following exposure to F113, S118 and the synthetic antibiotic DAPG in trial one. In the absence of bacterial endophytic bacteria and DAPG, sterile dH_2O was used as a control. Asterisk indicates that results are significantly different from the control.

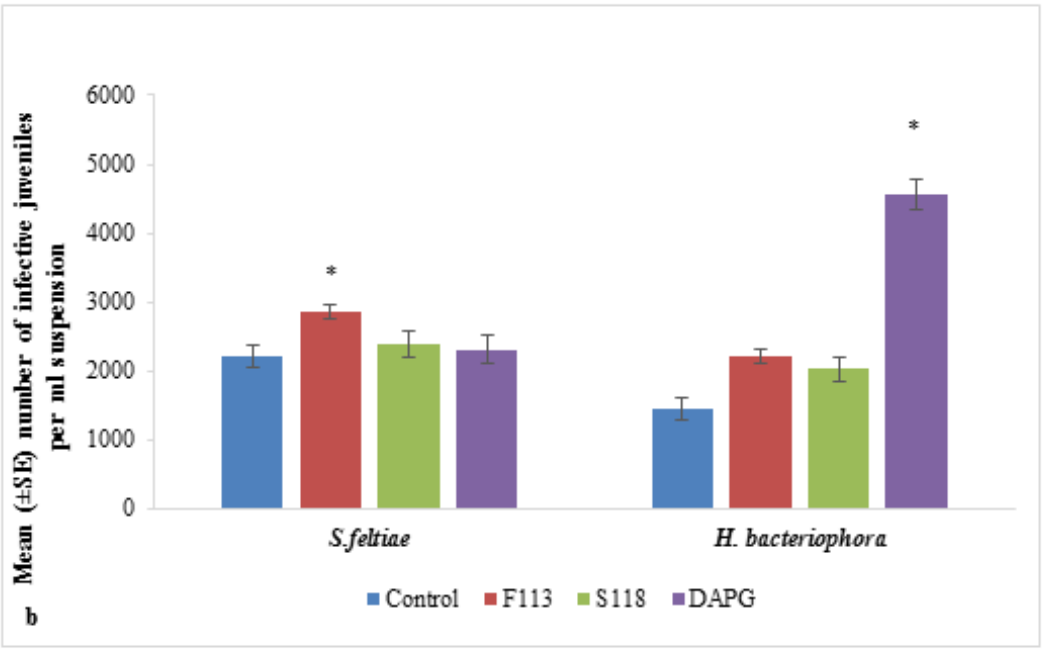

Figure 3.3.12 Nematode progeny production (±SE) in *G. mellonella* following exposure to F113, S118 and the synthetic antibiotic DAPG in trial two. In the absence of bacterial endophytic bacteria and DAPG, sterile dH_2O was used as a control. Asterisk indicates that results are significantly different from the control.

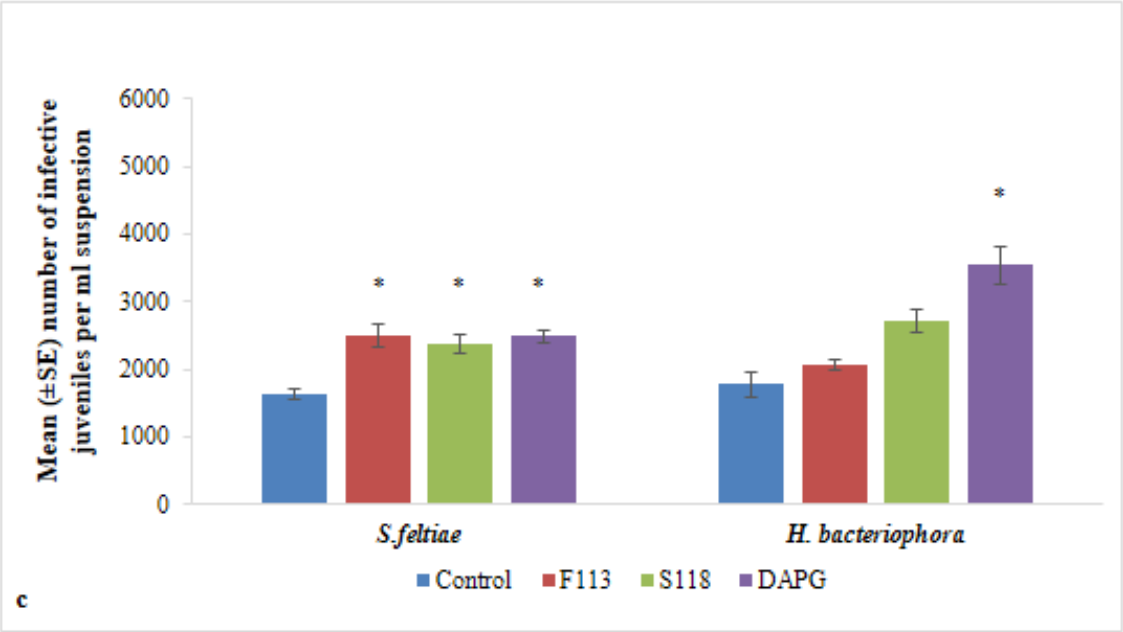

Figure 3.3.13 Nematode progeny production (±SE) in *G. mellonella* following exposure to F113, S118 and the synthetic antibiotic DAPG in trial three. In the absence of bacterial endophytic bacteria and DAPG, sterile dH_2O was used as a control. Asterisk indicates that results are significantly different from the control.

Firstly, a Kruskal-Wallis H test was conducted to determine if the number of emerged IJ between the three independent experimental trials differed significantly. Where significant differences ($p < .05$) were identified, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. The distributions of emerged IJ for each nematode/bacterial combination were not similar for the three trials as shown in Table 3.3.4. There were significant differences ($p < .05$) between progeny production for the three independent trials, for all nematode species and treatments except for *S. feltiae* SB 12(1) and S118. Therefore, results from each trial were assessed independently using a Kruskal-Wallis H test and *post hoc* analysis as outlined above. Where appropriate, adjusted *p*-values are presented.

Table 3.3.4 Kruskal-Wallis H test results comparing nematode progeny production over the three experimental trials. Results indicate that nematode progeny production for EPN examined differed between the three trials for each treatment, except S. feltiae and S118.

	S. feltiae	***H. bacteriophora***
Control	$\chi 2$ (2) = 20.037 , p = .000	$\chi 2$ (2) = 14.243, p = .001
F113	$\chi 2$ (2) = 18.522 , p = .000	$\chi 2$ (2) = 12.389, p = .002
S118	**$\chi 2$ (2) = 5.559 , p = .062**	$\chi 2$ (2) = 12.055, p = .002
DAPG	$\chi 2$ (2) = 17.964, p = .000	$\chi 2$ (2) = 7.285 , p = .026

For the first trial the distribution of emerged IJ was not the same across all categories of treatments *S. feltiae* ($\chi 2$ (3) = 29.984, p = .000) or *H. bacteriophora* ($\chi 2$ (3) = 22.586, p = .000). Subsequent *post hoc* analysis and pairwise comparisons revealed that median scores for *S. feltiae* progeny production for F113 (1210) and S118 (2040) were significantly different than the control with no bacteria or DAPG (370) (p = .005 and p = .000 respectively). Median scores for *H. bacteriophora* progeny production for DAPG (4650) were significantly different than the control with no bacteria or DAPG (2760) (p = .001).

For the second trial the distribution of emerged IJ was not the same across all categories of treatments *S. feltiae* ($\chi 2$ (3) = 10.447, p = 0.015) or *H. bacteriophora* ($\chi 2$ (3) = 24.179, p = 0.000). Subsequent *post hoc* analysis and pairwise comparisons revealed that median scores for *S. feltiae* progeny production for F113 (2970) were significantly different than the control with no bacteria or DAPG (2230) (p = 0.034). Median scores for *H. bacteriophora* progeny production for DAPG (4610) were significantly different than the control with no bacteria or DAPG (1590) (p = 0.001).

For the third trial the distribution of emerged IJ was not the same across all categories of treatments *S. feltiae* ($\chi 2$ (3) = 16.200, p = 0.001) or *H. bacteriophora* ($\chi 2$ (3) = 19.548, p

= 0.000). Subsequent *post hoc* analysis and pairwise comparisons revealed that median scores for *S. feltiae* progeny production for F113 (2560), S118 (2230) and DAPG (2500) were statistically significantly different than the control with no bacteria or DAPG (1640) (p = 0.014. p = 0.007 and p = 0.003 respectively). Median scores for *H. bacteriophora* progeny production for DAPG (3680) were significantly different than the control with no bacteria or DAPG (1790) (p = 0.000).

3.3.4.1 Determining the effect of bacterial endophytes on the attraction of *S. feltiae* SB 12(1), *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* (e-nema) to *G. mellonella*

Two separate bioassays were carried out to investigate the effect of endophytic bacteria on nematode host seeking and the results are presented below.

3.3.4.2 Nematode movement on agar

The effect of bacterial endophytes on the directional movement of EPN towards *G. mellonella* was investigated on SGA agar. Graduated Petri dishes containing SGA agar were inoculated with bacterial endophytes along the periphery as shown in Figure 3.2.2. A 1 ml pipette tip containing two *G. mellonella* larvae was secured in the lid opposite the bacterial streak. 60 IJ were added to the centre of the Petri dish and the nematodes present in each arc were counted after 5, 10, 20, 30 and 40 minutes. Replication was five-fold in the presence and absence of bacterial endophytes and insect hosts. Results are presented in Figure 3.3.14 to Figure 3.3.17 and were analysed using a two way repeated measures ANOVA to determine the effect of endophytic bacteria on EPN attraction to its host.

Interactions between Within-Subjects Factors *host* (distance travelled in the presence or absence) and the Between-Subjects Factors *treatments* (control no bacteria, F113, L321,

L228) were investigated for each nematode species independently. Muchly's test of sphericity was violated ($p<0.05$) thus, an epsilon (ε) correction was carried out to eliminate bias in the analysis. Significance values with a Greenhouse-Geisser correction were interpreted. *Post hoc* analyses were carried out using a Bonferroni correction. Positive values indicate nematode moved towards the host and negative values indicate nematodes moved towards bacterial treatment.

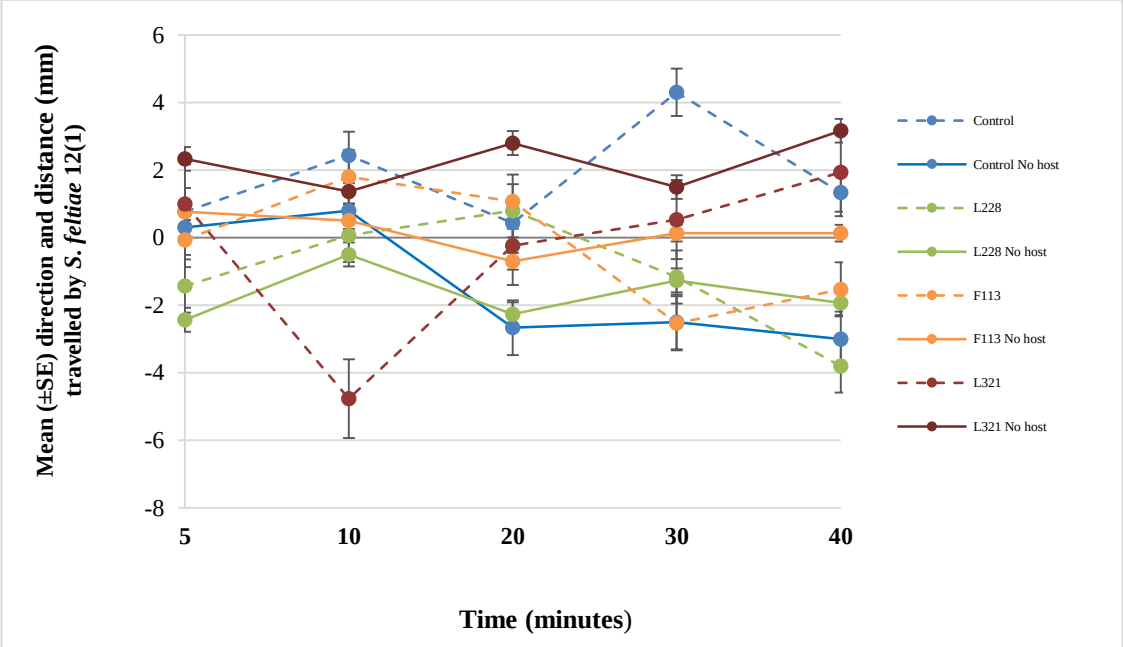

Figure 3.3.14 *S. feltiae* SB 12(1) direction and distance (mm) travelled over 40 minutes. The number of infective juveniles present in each arc on the agar plate were counted at each time point in the presence and absence of *G. mellonella* and endophytic bacteria.

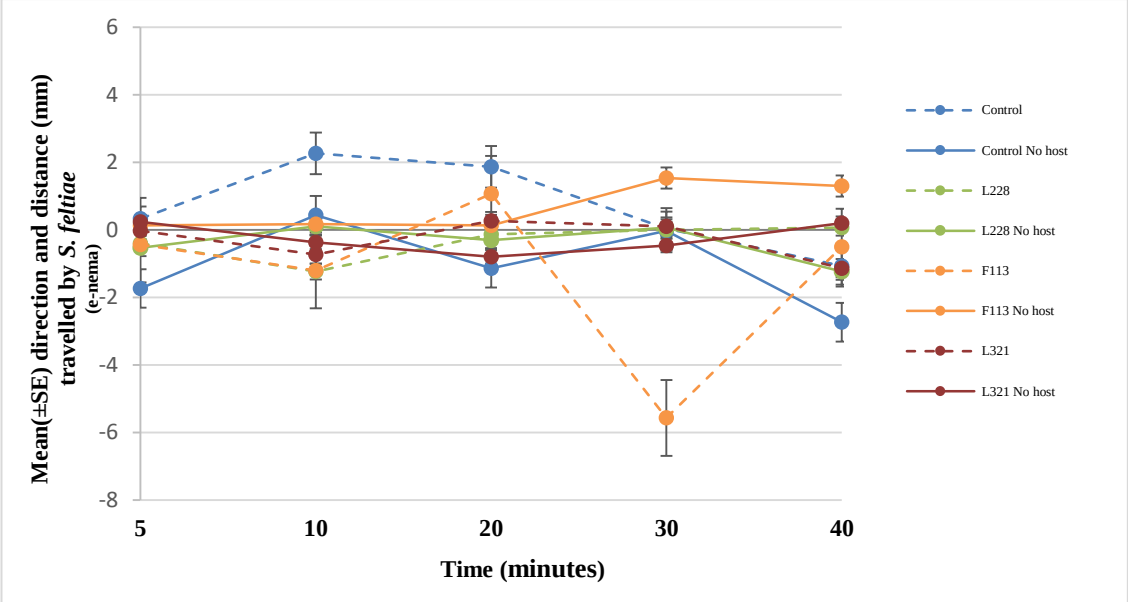

Figure 3.3.15 *S. feltiae* (e-nema) direction and distance (mm) travelled over 40 minutes. The number of infective juveniles present in each arc on the agar plate were counted at each time point in the presence and absence of *G. mellonella* and endophytic bacteria.

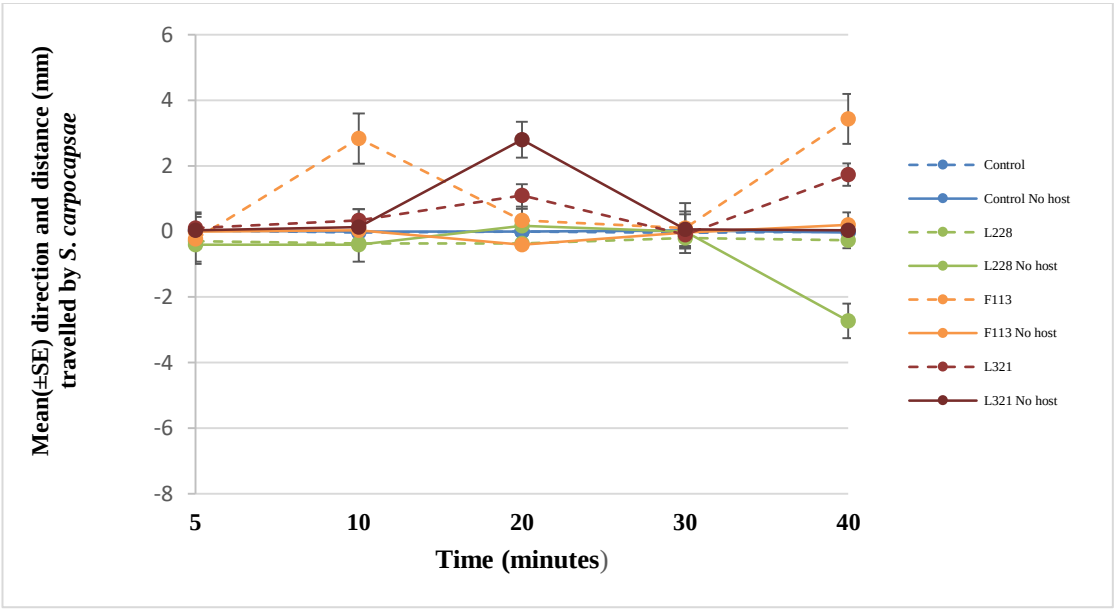

Figure 3.3.16 *S. carpocapsae* direction and distance (mm) travelled over 40 minutes. The number of infective juveniles present in each arc on the agar plate were counted at each time point in the presence and absence of *G. mellonella* and endophytic bacteria.

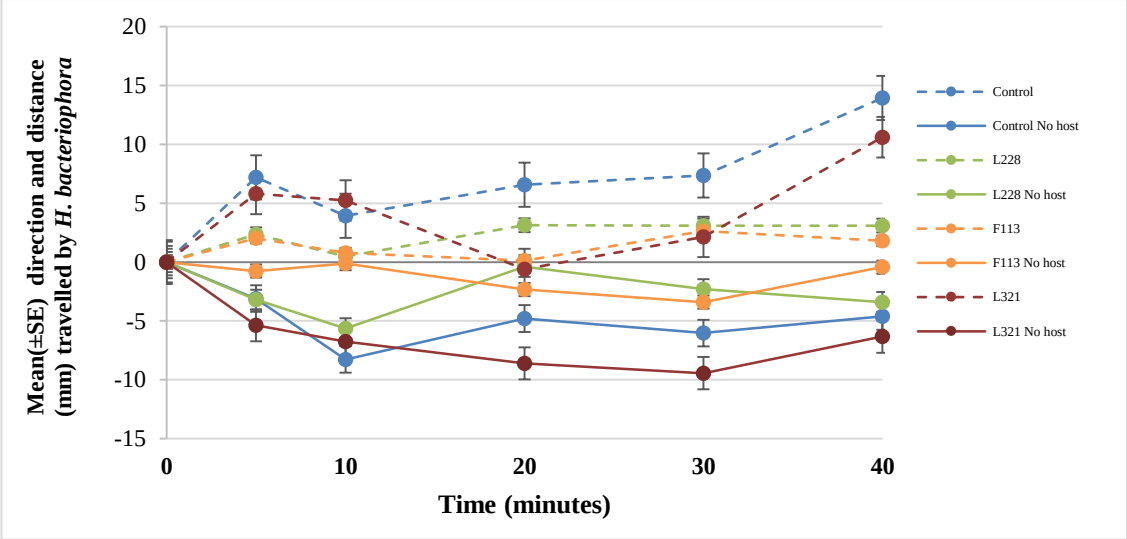

Figure 3.3.17 *H. bacteriophora* direction and distance (mm) travelled over 40 minutes. The number of infective juveniles present in each arc on the agar plate were counted at each time point in the presence and absence of *G. mellonella* and endophytic bacteria

IJ distance travelled on agar was statistically significantly different between the control with no bacteria and bacterial treatments for *S. feltiae* SB 12(1) $F(3, 96) = 16.572$, $p = 0.000$, $\varepsilon = 1.000$, *S. feltiae* (e-nema) $F(3, 96) = 10.225$, $p = 0.000$, $\varepsilon = 1.000$, *S. carpocapsae* $F(3, 92) = 5.811$, $p = 0.001$, $\varepsilon = 1.000$ and H. bacteriophora $F(3, 96) = 7.506$, $p = 0.000$, $\varepsilon = 1.000$. The following effect sizes were calculated using eta (η^2) for the interaction between the host presence and the treatments applied *S. feltiae* 12 (1) $\eta^2 = 0.339$, *S. feltiae* (e-nema) $\eta^2 = 0.2419$ (24.19%), *S. carpocapsae* $\eta^2 = 0.1458$ (14.58%) and *H. bacteriophora* $\eta^2 = 0.1101$ (11.01%). When interpreted as percentages of variance associated within the interaction, *S. feltiae* SB 12 (1) (33.9%) has a large effect size and *S. feltiae* (e-nema) (24.19%), *S. carpocapsae* (14.58%) and *H. bacteriophora* (11.01%) have a medium effect size. The ANOVA strongly confirms that the factors *host* and also the interactions between the *host* and *treatment*s had significant effects on nematode movement. Multiple comparisons in the presence and absence of the host were carried out with a Bonferroni correction, results for such are presented in Table 3.3.5 and Table 3.3.6. An asterisk marks where there are significant differences ($p < 0.05$) as compared to the control with no bacteria.

Table 3.3.5 Nematode direction and distance (mm) travelled on agar plates in the presence of a host. Pairwise comparisons of the Mean distance travelled by infective juveniles between the control with no bacteria and bacterial treatments are presented with a Bonferroni correction. The *p*-value calculated for the control versus the bacterial treatments is shown in the sig. column. *N* is the population size.

Nematode	Treatment	N	Mean	Std. Deviation	Sig.	95 % confidence interval for mean	
						Lower bound	Upper bound
S. feltiae SB 12(1)	Control	25	1.85	2.41	-	0.85	2.8483
	F113	25	- 0.31*	2.38	0.012	-1.29	0.6774
	L228	25	- 1.11*	2.01	0.000	-1.93	-0.2752
	L321	25	- 0.30*	2.73	0.012	-1.43	0.8223
S. feltiae (e-nema)	Control	25	0.68	2.11	-	-0.18	1.5576
	F113	25	- 1.34*	2.67	0.001	-2.44	-0.2441
	L228	25	-0.34	0.67	0.268	-0.61	-0.0623
	L321	25	-0.30	0.82	0.311	-0.64	0.0348
S. carpocapsae	Control	25	-0.00	0.06	-	-0.03	0.0202
	F113	25	1.43*	1.91	0.000	0.64	2.229
	L228	25	-0.30	0.57	1.000	-0.53	-0.0617
	L321	25	0.63	0.93	0.324	0.24	1.0195
H. bacteriophora	Control	25	7.80	9.00	-	4.08	11.5159
	F113	25	1.54*	2.13	0.001	0.66	2.4264
	L228	25	2.43*	3.17	0.007	1.12	3.7421
	L321	25	4.63	5.63	0.300	2.30	6.9573

*The mean difference is significant at the .05 level

Table 3.3.6 Nematode direction and distance (mm) travelled agar plates in the absence of a host. Pairwise comparisons of the Mean distance travelled by infective juveniles between the control with no bacteria and bacterial treatments are presented with a Bonferroni correction. The *p*-value calculated for the control versus the bacterial treatments is shown in the sig. column. *N* is the population size.

Nematode	Treatment	N	Mean	Std. Deviation	Sig.	95% Confidence Interval for Mean	
						Lower Bound	Upper Bound
S. feltiae 12(1)	Control	25	-1.4133	2.40613	-	-2.4065	-.4201
	F113	25	.1387*	.84254	.010	-.2091	.4865
	L228	25	-1.6800	1.50994	1.000	-2.3033	-1.0567
	L321	25	2.2333*	1.64570	.000	1.5540	2.9126
S. feltiae (e-nema)	Control	25	-1.0400	2.43585	-	-2.0455	-.0346
	F113	25	.7053*	1.11445	.001	.2453	1.1654
	L228	25	-.3800	.63560	.775	-.6424	-.1176
	L321	25	-.2400	1.30976	.400	-.7806	.3006
S. carpocapsae	Control	25	.0070	.05989	-	-.0183	.0322
	F113	25	-.0320	.29368	1.000	-.1532	.0892
	L228	25	-.6733	1.76939	.166	-1.4037	.0570
	L321	25	.6133	1.12394	.294	.1494	1.0773
H. bacteriophora	Control	25	-5.3600	7.48040	-	-8.4478	-2.2723
	F113	25	-1.4307*	2.63405	.041	-2.5180	-.3434
	L228	25	-2.9867	3.82364	.589	-4.5650	-1.4083
	L321	25	-7.3000	4.84816	1.000	-9.3012	-5.2988

*The mean difference is significant at the .05 level

3.3.4.3 *S. feltiae* SB 12(1), *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* (e-nema) host seeking in sand columns

The effect of the bacterial endophytes on the ability of EPN to infect and kill *G. mellonella* was also investigated in sand columns. One insect was added to each test tube before filling with sand. Approximately 100 IJ were incubated in the presence and absence of bacterial endophytes and *G. mellonella* for 24 hr. After incubation, the insects were removed from the tubes, washed and incubated for a further 24 hr. Insects were scored for mortality and dissected to determine the number of IJ present. Results for insect mortality and the number of recovered IJ from infected insects are presented in Figure 3.3.18 and Figure 3.3.19, respectively.

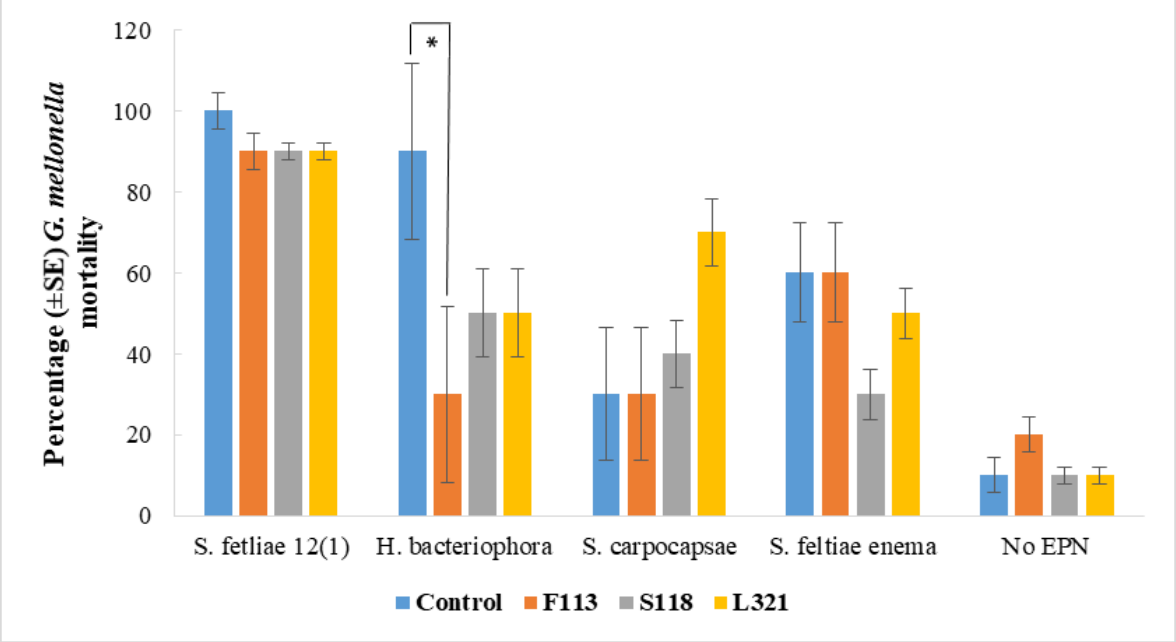

Figure 3.3.18 Percentage (±SE) *G. mellonella* mortality in sand columns. Each test tube received one insect and replication was tenfold per nematode bacterial combination. Asterisk (*) and lines denote significant differences between the control and treatment sample.

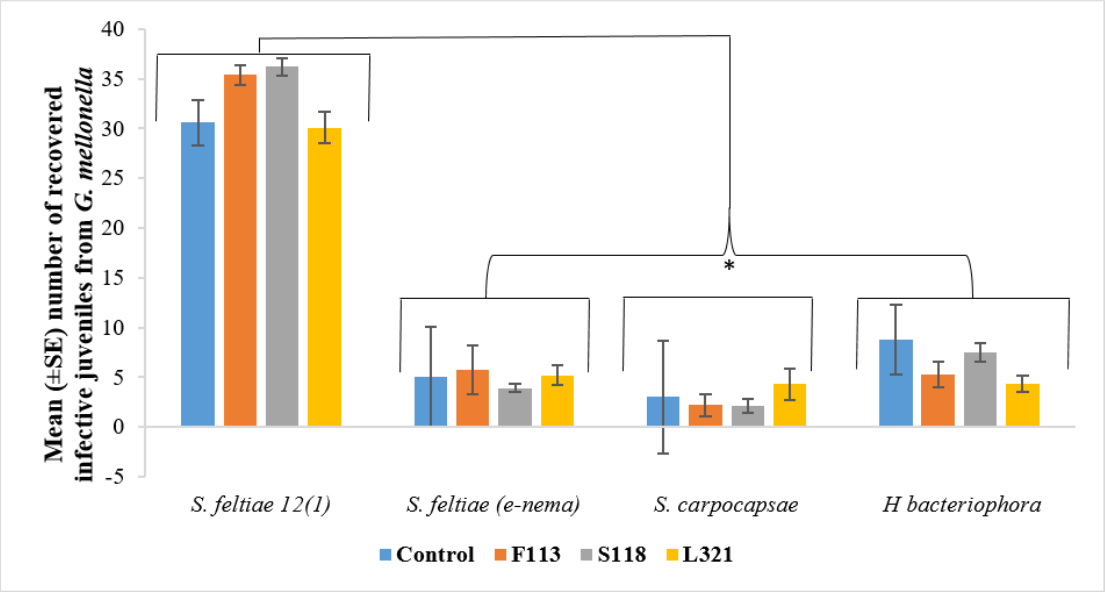

Figure 3.3.19 Mean (±SE) number of recovered infective juveniles from *G. mellonella* in sand columns. Each test tube received one insect and replication was tenfold per nematode bacterial combination. Asterisk indicates significant difference ($p < 0.05$) between nematode treatments.

A one-way between groups analysis of variance was conducted to explore the effect the bacterial endophytes on the number of dead and live insects. There was a statistically significant difference between the control and treatment groups for *H. bacteriophora* as determined by a one-way ANOVA ($F(3,36) = 2.850$, $p = 0.050$). For *H. bacteriophora*, the control treatment with no bacteria had 66.7% more dead insects than the bacterial treatment F113. *Post hoc* analysis using a Dunnett (2-sided) t-test, showed that this was a significant difference ($p = 0.020$). For *S. carpocapsae*, the treatment L321 had 57% more dead insects than the control, however, this difference was not significant ($p= 0.064$) as determined by a one-way ANOVA.

An ANOVA was conducted to explore the effect the bacterial endophytes on the number of IJ establishing in insects at the bottom of sand columns. Each nematode treatment and species was examined independently, but the results remained the same. The number of IJ recovered from insects subjected to the bacterial treatment did not differ significantly ($p >$

0.5) from the control with no endophytic bacteria for *S. feltiae* SB 12(1) F(3,36) = .159, p = .923, *S. feltiae* (e-nema) F(3, 31) = .068, p = 0.977, *S. carpocapsae* F(3, 30) = 2.026., p = 0.131 or *H. bacteriophora* F(3, 36) = 2.748, p = 0.057. Interestingly, the EPN strain *S. feltiae* SB 12(1) was significantly more virulent than the other three nematode strains (F[3, 156] = 146.398, p = 0.000) across all bacterial treatments examined. It can be concluded that no endophytic bacterial strain examined affected the nematodes movement towards its host or virulence, in sand columns.

3.3.5 Determination of the susceptibility of the black vine weevil (*O. sulcatus*) to entomopathogenic nematodes following bacterial endophyte exposure

Two separate experiments were carried out to determine any negative or stimulatory effects of exposure to *P. fluorescens* F113 on nematode infectivity to *O. sulcatus.* Two nematode species were investigated *S. feltiae* SB 12(1) and *H. bacteriophora* (e-nema).

3.3.5.1 The infectivity of *S. feltiae* SB 12(1) and *H. bacteriophora* (e-nema) to the black vine weevil (*O. sulcatus*) in a laboratory bioassay

The effect of the endophytic bacterial isolate *P. fluorescens* F113 on the ability of EPN to cause BVW mortality was investigated in Petri dishes containing sand. There were two controls: insects exposed to nematodes only and insects exposed to neither nematodes nor bacteria. Results are presented in Figure 3.3.20.

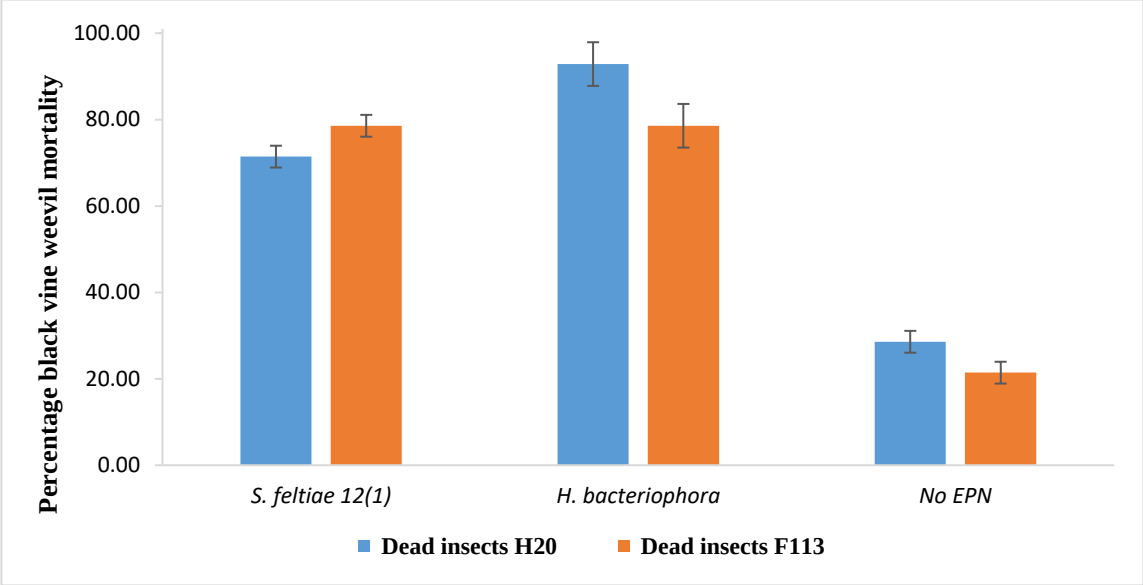

Figure 3.3.20 Percentage (±SE) *O. sulcatus* larvae mortality following exposure to *S. feltiae* SB 12(1) *H. bacteriophora* and the control with no nematodes in the presence and absence of *P. fluorescens* F113. Petri dishes containing sand were the experimental unit (n= 14).

The sample size was small due to a limited number of insects and the number of the expected outcome frequencies were less than five, thus the Fisher's exact test was used as it is more accurate than the chi-square test. The Fisher's exact test of independence was conducted to investigate if the presence of *P. fluorescens* F113 significantly influenced nematode infectivity of BVW when compared to the control with no F113. Two nematode species (*S. feltiae* and *H. bacteriophora*) and a control with no nematodes were tested independently. There was no statistically significant association between the endophytic bacterial treatment and the control (no bacteria) for *S. feltiae* (p = 1.000, OR = 0.682), *H. bacteriophora* (p = 0.596, OR = 3.545) and the control (no nematodes) (p = 1.000, OR = 1.467). As the p-values are not <0.05, the null hypothesis is not rejected, thus the bacterial treatment did not affect *S. feltiae* or *H. bacteriophora* infectivity of *O. sulcatus*. Phi (φ) is a measure of association between variables, with a value of 1 indicating a perfect association and a value of 0 indicating no association or independence of variables. Results indicate that there is very little association between the two variables (variable 1: *P. fluorescens* F113 and variable 2:

control [no bacteria]) for *S. feltiae* (φ = .082 p = 0.663), *H. bacteriophora* (φ = .204 p = 0.208) and control (φ = .082 p = 0.663).

3.3.5.2 Susceptibility of the black vine weevil (*O. sulcatus*) to *S. feltiae* 12(1) and *H. bacteriophora* (e-nema) in strawberry plants

The effect of bacterial endophytes on EPN infectivity to the BVW was investigated in strawberry plants. Seven larvae were added per pot and there were seven pots per nematode and bacterial treatment. Insects were exposed to 10,000 IJ/plant for five days at 22°C (± 2°C). After this, all insects were removed from the pots, washed with sterile dH_2O and incubated at 25°C for 24 hr. The number of live insects was counted and results are presented in Figure 3.3.21.

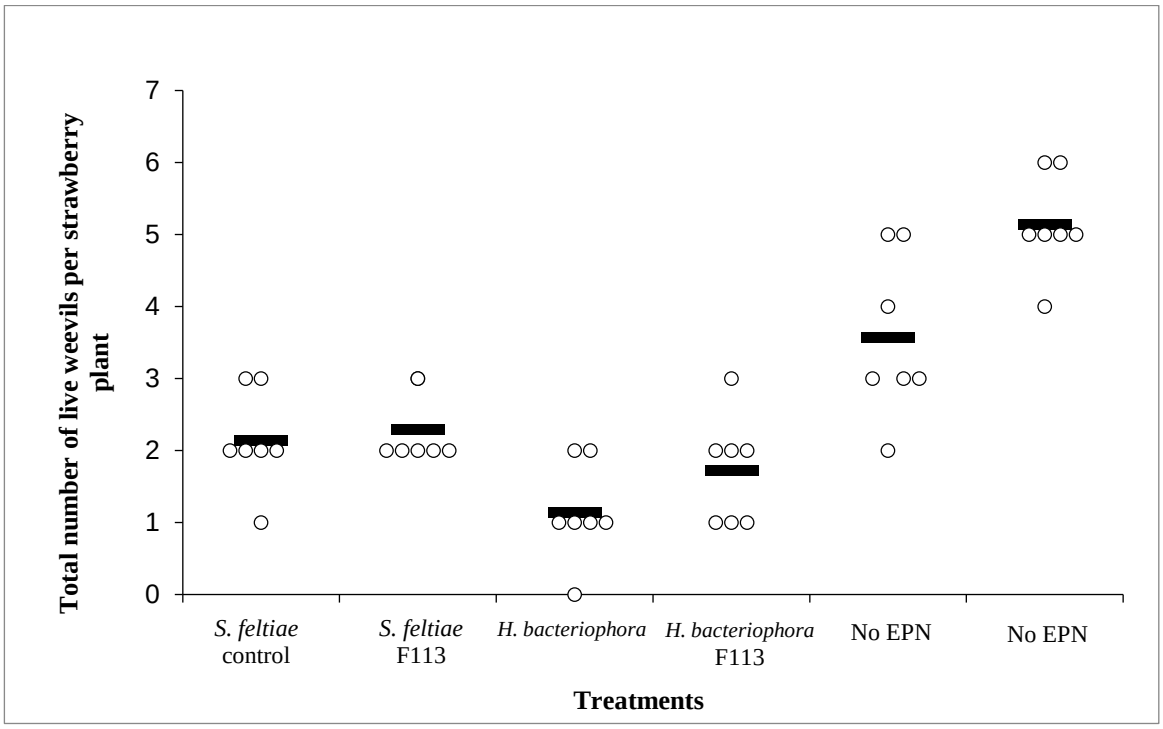

Figure 3.3.21 Scatter plots representing susceptibility of *O. sulcatus* to *S. feltiae* and *H. bacteriophora* in the presences and absence of *P. fluorescens* F113. Pots containing strawberry plants were the experimental unit. Replication was sevenfold with seven larvae per pot. Bold black lines indicate the Mean number of live weevils per pot and each individual replicate is represented by a circle.

A between-subjects (independent) t-test was carried to determine whether the difference between the number of live insects in the bacterial treatment and the control group was statistically significant ($p < .05$). The differences between groups were explored for each treatment (*S. feltiae* 12[1], *H. bacteriophora* and control with no nematodes) independently.

There was homogeneity of variances, as assessed by Levene's test for equality of variances for *S. feltiae* ($p = .667$), *H. bacteriophora* ($p = .583$) and the control with no nematodes ($p = .312$). Outliers in the data were identified by inspection of a boxplot but were included in the analysis. The differences in means were not statistically significant between treatments, for *S. feltiae* $t\ (12) = -.447$, $p = .663$, $g = .22$, *H. bacteriophora* $t\ (12) = -1.477$, $p = .165$, $g = .85$ or the control with no nematodes $t\ (12) = -2.178$, $p = .050$, $g = 1.26$. Hedges's g values are also presented above, this is a correction for the Cohen's d. Effect sizes and corrections were calculated using the formulae shown below in Figure 3.3.22.

$$d_s = \frac{\overline{X}_1 - \overline{X}_2}{\sqrt{\frac{(n_1 - 1)SD_1^2 + (n_2 - 1)SD_2^2}{n_1 + n_2 - 2}}}$$

$$\text{Hedges's } g_s = \text{Cohen's } d_s \times \left(1 - \frac{3}{4(n_1 + n_2) - 9}\right)$$

Figure 3.3.22 Formulae used to calculate Cohen's *d* and Hedges's *g* effect size measurements (Lakens 2013).

Cohen's *d* is used to calculate effect size based on the differences between observations, in the control and treatment groups, divided by the pooled standard deviations. Hedges's *g* incorporates sample size and is calculated to eliminate bias (Lakens 2013). Effect sizes can be interpreted as small ($d = < 0.25$), Medium ($d = < 0.5$) or large ($d = \leq 0.8$) (Cohen 1988).

3.4 Discussion

Modifying and carrying out the laboratory bioassays and plant-based experiments detailed above gives valuable insights on the intrinsic interactions between these two key soil organisms. Determining these effects, be they negative or stimulatory, is a large contribution towards meeting the overall aims of developing a potential biological control product utilising both EPN and endophytic bacteria together and also improving integrated pest management. Understanding how different nematode species and strains interact with naturally occurring soil biota may result in a better approach in insect biocontrol, as well as increased plant growth and health, while also contributing to knowledge on these two environmentally important taxa. Where feasible, different species and strains of EPN, bacterial endophytes and insect species were investigated, aiming to provide vital knowledge on host-parasite interactions.

Two separate laboratory-based bioassay were employed to investigate the effect of endophytic bacterial isolates on the susceptibility of *S. feltiae* SB 12(1), *S. feltiae, S. carpocapsae* and *H. bacteriophora* against *G. mellonella* and *S. feltiae* SB 12(1) and *H. bacteriophora* against *O. sulcatus* larvae independently. In terms of *G. mellonella* susceptibility to EPN, the levels of mortality among the three independent trials were consistent for all bacterial treatments applied, indicating that the bioassay is reproducible and reliable. The presence of bacterial endophytes did not have a significant effect on nematode-induced, insect mortality when compared to control treatments, where insects were not exposed to endophytic bacteria. In addition, the level of insect mortality in the absence of nematodes was similar for the bacterial and control samples. This indicates that no endophytic strain had an effect on insect mortality.

G. mellonella is a commonly used insect in laboratory EPN culturing. Its wide availability commercially, low cost, ability to produce high IJ yields and high susceptibility to most EPN species makes it an ideal laboratory test insect. However, in order to develop an EPN and bacterial endophyte product for insect control a good understanding of the host range and their susceptibility. For this reason, susceptibility tests were carried out on the black vine weevil (*O. sulcatus*). The BVW is a serious economic pest as it has an extensive host plant range, causing serious damage and often plant death in both soft fruits and ornamentals species (van Tol *et al.*, 2001). Both adult and larvae forms feed on the plant, but it is the latter that is most detrimental to plants. Larvae feed on the developing root and stem system resulting in significant plant damage (Moorhouse *et al.*, 1992). EPN control of the insect larvae has been reported previously (Kakouli-Duarte *et al.*, 1997; Lola-Luz & Downes, 2007) as part of integrated pest management strategies. In the laboratory-based mortality trial using *O. sulcatus,* similar results were determined to those for *G. mellonella* mortality. Here, the widely studied soil microbe *P. fluorescens* F113 did not have any significant effect on *S. feltiae* or *H. bacteriophora* induced insect mortality, with little or no association identified between the control and bacterial treatments.

The susceptibility of *G. mellonella* and *O. sulcatus* to EPN in the presence of endophytic bacteria were determined in OSR and strawberry plants, respectively. Although plant-based experiments were carried out in pots containing sterile soil, these experimental arenas still provide a more natural and dynamic environment, than sand based bioassays. The nematode species, bacterial isolates and number of replicates varied between experiments, with *S. feltiae* SB 12(1), *H. bacteriophora* and *P. fluorescens* F113 remaining consistent. Notwithstanding this, the overall result remained the same, the presence of bacterial endophytes did not affect the nematodes' ability to infect and kill *G. mellonella or O. sulcatus*

in OSR or strawberry plants. OSR seeds were inoculated with endophytic bacteria using sodium alginate and subsequently germinated and grown in sterile soil. Whereas juvenile strawberry plants were sourced commercially, removed from pots, vigorously rinsed with sterile dH_2O and replanted in sterile soil. After replanting, strawberry plants were inoculated with liquid bacterial culture. *P. fluorescens* F113 has been shown to successfully colonise the internal tissues of OSR and also persists in the plant rhizosphere for up to 3 weeks (Lally, 2016). However, the colonisation potential of *P. fluorescens* F113 in strawberry plants has not yet been determined. Kunkel & Grewal, (2003) investigated the susceptibility of *Agrotis ipsilon* to *S. carpocapsae* following insect feeding on a host plant colonised by *Neotyphodium lolii*, an endophytic fungus. In this study insects feeding on *Lolium perenne* colonised by *N. lolii* were less susceptible to the EPN. In contrast, (Grewal *et al.*, 1995) showed that the larvae of *Popillia japonica* are more susceptible to *H. bacteriophora* following feeding on plants colonised by the endophytic fungus *Acremonium* sp. Both of these studies examine insect susceptibility to EPN following feeding on endophyte-inoculated plants, whereas the interaction being investigated in this chapter solely examine the effect of the presence of these bacterial soil isolates on nematode infectivity and insect mortality. Together these findings display the importance of testing not only the host range of EPN but also the interaction between plant-associated microorganisms and their effect on herbivore insects.

Nematode dose-response experiments were employed to investigate the effect of endophytic bacterial isolates on nematode virulence. The slope of the regression line was calculated to determine the virulence of each nematode strain in the presence and absence of endophytic bacteria. Nematode dose for both control and bacterial treatments followed linearity as the number of nematodes established in *G. mellonella* hosts were proportional to the respective nematode dose applied, for all nematode species. In addition to regression analysis, the

Spearman's ρ correlation test was used to measure the strength and direction of the association between nematode dose applied and the number of IJ recovered from *G. mellonella* larvae. *S. feltiae* SB 12(1), *S. feltiae* (e-nema) and *S. carpocapsae* showed a strong positive correlation for all bacterial and control treatments examined. *H. bacteriophora* control with no bacteria and bacterial treatment L228 showed a moderately strong correlation.

The effect of endophytes on nematode virulence varied between nematode and bacterial isolates. No individual isolate significantly and consistently affected all nematode species. Moreover, no nematode species was affected across all doses examined. Bacterial isolate L228 had a negative effect on at least one nematode dose of *S. feltiae* SB 12(1), *S. feltiae* (e-nema) and *S. carpocapsae.* The number of established *S. carpocapsae* IJ at dose 50, 100 and 200IJ/host, following exposure to bacterial isolate S118 was less than that recorded in the control; this was the most notable negative effect. *H. bacteriophora* infection was lower overall than those recorded in the other three nematode strains examined. However, no bacterial isolate had a negative effect on virulence across any doses examined. In contrast, *P. fluorescens* F113 was shown to increase the number of IJ establishing inside the host at the lower nematode doses of 25 and 50IJ, with no effect recorded at the higher doses of 100 and 200IJ.

EPN infectivity, ability to cause insect mortality and predictability are all important factors. However, these assays do not contribute knowledge on nematode progeny production and potential persistence in the environment after infection and reproduction. A nematode fecundity assay in sand was employed to determine the effects of endophytic bacteria and the synthetic bacterial antibiotic DAPG on nematode progeny production. The biological control activity of a number of beneficial *P. fluorescens* isolates has been linked to the production of

DAPG (Chiriboga M. *et al.*, 2018; McSpadden Gardener, 2007). DAPG, and in particular DAPG producing pseudomonads, have been shown to increase plant resistance against invading pathogens (van Loon, 2007) , increase crop yields and display biocontrol capabilities against various plant pathogens (Cronin *et al.*, 1997; Fenton *et al.*, 1992; Shaukat & Siddiqui, 2003). The susceptibility of EPN to chemically synthesised synthetic DAPG was investigated previously in Chapter 2 and the effect of DAPG on nematode progeny production was examined in Chapter 3. Experimental results for the three independent trials showed inconsistent results and the effect of bacterial isolates and DAPG differed between trials. For *S. feltiae* SB 12(1), *P. fluorescens* F113 consistently increased nematode fecundity across all three trials. Similarly, *H. bacteriophora* progeny production in the presence of DAPG was higher than that in the control in all trials carried out. Cronin (1997) linked the increase in egg hatch and reduction of juvenile mobility *Globodera rostochiensis*, to the production of DAPG by the DAPG-producing *P. fluorescens* F113 isolate. Contrastingly, Meyer *et al.*, (2009)state that the bacterial-feeding nematodes, *Caenorhabditis elegans, Pristionchus pacificus, and Rhabditis rainai* were not affected by DAPG. It can thus be concluded that production of DAPG by pseudomonads does not negatively affect the non-target beneficial nematodes.

The ability of EPN to disperse throughout the soil and actively seek out their host is a key aspect of insect biological control and any factor that affects this must be identified. Two experiments were employed to investigate the effect of endophytic bacteria on EPN attraction to *G. mellonella.* The first was conducted in sand columns with insect mortality and the number of established IJ recorded. It was found that the presence of endophytic bacteria had no effect on the nematodes attraction to its host in sand columns. Similarly, the number of recovered IJ was the same in the presence and absence of endophytic bacteria. The second

experiment examined nematode attraction to its host in the presence and absence of bacterial endophytes. In the control plates with a host present and no bacterial treatment, *S. feltiae* SB 12(1), *S. feltiae* (e-nema) and *H. bacteriophora* IJ moved toward the host, with *H. bacteriophora* showing a strong directional response to the host. Grewal *et al.*, (1994) also indicate that *H. bacteriophora and S. feltiae* IJ respond directionally to host cues. The presence of endophytic bacteria had a significant effect on nematode movement, in particular, *P. fluorescens* F113. In the presence of *P. fluorescens* F113 and a host, *S. feltiae* SB 12(1), *S. feltiae* (e-nema) and *H. bacteriophora* IJ moved away from the host toward the bacteria. For *S. feltiae* SB 12(1), bacterial isolates L228 and L321 had the same effect and for *H. bacteriophora*, IJ showed movement towards isolate L228 instead of the host. Nematode movement in the control plates with no bacteria and no host varied. *S. feltiae* SB 12(1), *S. feltiae* (e-nema) and *S. carpocapsae* did not significantly move towards the bacteria when compared to the control. *H. bacteriophora* IJ moved directionally toward the test side of the plate, but control and bacterial treatments did not differ significantly. In this experiment, and other experiments carried out where *H. bacteriophora* was examined on agar, IJ were seen to burrow down into the media. *H. bacteriophora* IJ adopts a cruiser technique and it has been suggested that infectivity and mortality assay carried out in sand columns may be more efficient to examine their movement (Grewal *et al.,* 1994). *S. carpocapsae* showed very little directional movement in the presence or absence of a host; this is not unexpected as they use a nictation technique to seek out insects. Grewal *et al.*, (1994) also showed a lack of directional response to host cues by *S. carpocapsae*.

The foraging strategies of EPN can be divided broadly into two groups: those of cruisers and ambushers (Grewal *et al.,* 1994). Nematodes such as *H. bacteriophora*, employ a cruising technique and constantly move through the soil, using chemical (Kaya & Gaugler, 1993) or

heat cues (Byers & Poinar, 1982) to search for an insect host. Ambushers use nictation to find their host. Nictatism is the behaviour whereby a nematode stands on its tail and moves from side to side; *S. carpocapsae* adopts this technique (Gaugler & Campbell, 1993). However, some nematodes such as *S. feltiae* can adopt both strategies and these are denoted as intermediates (Grewal *et al.*, 1994). The foraging technique adopted by EPN determines where they will be located in the soil profile and also what hosts they will infect (Campbell & Gaugler, 1997). Thus, this is a key factor to consider when selecting a nematode species for insect biological control. The foraging technique adopted may also play a role in the degree of contact a particular EPN species will have with bacterial endophytes as it moves through the soil, plant rhizosphere or infects insects that feed on endophyte colonised plants. Examining the effects of bacterial endophytes on EPN from the families Heterorhabditidae and Steinernematidae give a broader insight into potential negative or stimulatory effects of endophytic bacteria to EPN biology and behaviour.

Predictability is one of the biggest challenges in the biocontrol of economically important insects with EPN. In order to establish a more general overview of the interactions between EPN and bacterial endophytes and the effects of the latter on nematode biology and behaviour, different species and strains of both nematodes and endophytic bacteria were examined. This would prove essential for the selection of the best nematode-bacterial combination for insect biological control and PGP. Moreover, as target insect susceptibility and nematode host range can vary considerably, this effective nematode-bacterial combination should be tested specifically against a particular insect pest before field applications in order to increase success rates in integrated pest management.

Chapter 4 Determination of the effects of nematodes on bacterial survival, colonisation of plants and plant growth promoting properties

4.1 Introduction

Inoculation of plants with bacterial endophytes has been shown to promote plant growth and protect plants by deterring insects and plant parasitic nematodes and also reducing fungal, bacterial and viral diseases (Ryan, *et al.*, 2007; Kerry, 2000). In order to use such endophytes as biofertilisers or as biological control agents, they must colonise plant surfaces and also survive and grow along the plant roots in the presence of indigenous microflora (Lugtenberg, *et al*., 2002). The genomes of three *P. fluorescens* strains (L321, L228 and L111) used in this study were recently sequenced and annotated (Lally, 2016; Moreira *et al.*, 2016) and were found to contain genes associated with PGP, plant colonisation and biological control. Insecticidal toxin production by *P. protegens* CHAO and *P. protegens* Pf-5 has been documented (Masson *et al.*, 1995; Olcott *et al*., 2010; Péchy-Tarr *et al*., 2008). These bacteria produce the Fluorescens Insect Toxin (FIT) complex that can induce lethal effects to *Drosophila melanogaster, G. mellonella and Manduca sexta.* The FIT toxin has notable similarities with another insecticidal toxin that is produced by the EPN symbiotic bacteria

Xenorhabdus sp. and *Photorhabdus* sp., called Makes caterpillars floppy (Mcf) toxin Ingestion or injection of this toxin results in insect mortality due to shedding of the insect midgut epithelium and apoptosis (Daborn et al., 2002; Dowling et al., 2004; Ffrench-Constant et al., 2007). These toxins are important in agriculture as they have potential uses in the biological control of insect pests. This Chapter examines the presence of genes associated with these important insect toxins in the genomes of *P. fluorescens* strains F113, L321, L228 and L111.

EPN are also widely used in biological control, both commercially and experimentally. In the environment, endophytes interact with soil fauna, including nematodes, as they are found throughout the soil profile. In addition, results from studies investigating the effects of bacterial-feeding nematodes on plant colonisation are quite promising. For example, *P. fluorescens,* an effective coloniser of plant roots and shoots, has been shown to increase bacterial plant colonisation up to ten-fold when plants are grown in the presence of the bacterial feeding nematodes *Acrobeloides thornei*, *Acrobeloides maximum*, *Cruznema* sp and *Caenorhabditis elegans* (Knox et al., 2004). A detailed literature search has indicated that no other such project, investigating the interactions of these two beneficial types soil organisms, EPN and endophytes, has been undertaken.

Chapters 2 and 3 have focused predominately on the effects of endophytic bacteria on nematode survival and nematode biology and behaviour. The objective of the study presented in Chapter 4 was to investigate the effects of nematodes on endophytic bacteria. These effects were assessed in terms of:

- Effect of nematodes on bacterial growth
- Effect of nematodes on bacterial plant colonisation and PGP

Moreover, endophytic bacterial colonisation of EPN infective juveniles and persistence inside the insect cadaver, post-IJ insect infection was also examined in this chapter. The effect of nematodes on bacterial growth was examined on solid growth media, using surface sterilised nematodes. A large number of isolates, covering three different genera and six species, were screened against the four EPN strains. The EPN examined were *S. feltiae* SB 12(1), *S. feltiae* (e-nema), *S. carpocapsae* and *H. bacteriophora*. The effect of these nematodes on the growth of the following endophytic isolates was examined: *P. fluorescens* F113, S19, S118, S222, L23, L228, L330, L313, L324, R114 and R225; isolate ID can be seen in Table 2.2.2. The number of bacterial strains and nematode species was reduced for the nematode colonisation and plant colonisation trials. For plant trials, the effects of *S. feltiae* SB 12(1) and *H. bacteriophora* (e-nema) and a combination of the two EPN, on the colonisation of oilseed rape (OSR; variety: Tamarin) by *gfp* labelled *P. fluorescens* F113 *gfp* and L321 *gfp,* was investigated in a greenhouse experiment. These bacterial isolates were selected as they both have been shown to successfully colonise OSR (Lally, 2016).

A laboratory-based bioassay was employed to examine the ability of the well-known plant coloniser *P. fluorescens* F113 *gfp* to colonise EPN IJ. EPN from both families of Steinernematidae and Heterorhabditidae were examined. Rt-PCR was used to investigate bacterial colonisation of IJ over time. In addition, the survival of *P. fluorescens* F113 inside the nematode-infected insect cadaver was examined to determine the survival of the endophytic bacteria following direct exposure to the EPN symbiotic bacteria.

4.2 Materials and Methods

4.2.1 Identification of insecticidal genes in the genomes of *P. fluorescens* F113, L321, L228 and L111

A BLASTn sequence alignment was carried out to determine if the genes associated with Fluorescens Insect Toxin (FIT) and Makes caterpillars floppy (Mcf) toxin were present in the genomes of the closely associated *P. fluorescens* strains F113 (Accession No: NC_016830), L321 (Accession No: NZ_CP015637.1), L228 (Accession No: NZ_CP015639), L111 (Accession No: NZ_CP015638) and the EPN symbionts, *P. luminescens* subsp. laumondii TTO1 (Accession No: BX470251), *X. nematophilia* K102 (Accession No: LT966404.1) and *X. bovienii* CS03 (Accession No. NZ_FO818637.1). There is a known association between *fitD* from the FIT complex and the McF toxin, thus an alignment between these was also carried out to act as a control (Ffrench-Constant *et al.*, 2007; Waterfield *et al.*, 2003).

4.2.2 Determination of the effects of *S. feltiae* SB 12(1), *S. feltiae S. carpocapsae* and *H. bacteriophora* (e-nema) on endophytic bacterial growth

A loop full of frozen bacterial stocks (see Section 2.2.2 for aseptic preparation of bacterial stocks) was streaked onto nutrient agar and incubated at 30ºC for 24 hr. Colony morphology was examined to ensure each strain was pure and matched the known morphology (see Appendix 1 for individual strain characterisation). A well isolated bacterial colony was inoculated from nutrient agar into nutrient broth and incubated at 30ºC for 24 hr (100 rpm). The bacterial culture was serially diluted 1:10 until a dilution of 10^{-6} was reached. An endophytic bacterial lawn was prepared by pipetting 100 µl of the 10^{-6} diluted culture onto nutrient agar and spreading throughout using a sterile spreader, with subsequent drying under

laminar flow for 30 minutes. Approximately 100 surface sterilised IJ in 50 µl sterile dH_2O were added to each test agar plate. Control plates were inoculated with endophytic bacteria as described above and received 100 µl of sterile dH_2O only. Replication was six-fold per nematode species and bacterial isolate. Test and control plates were incubated at 30 ºC for 24 hr. To examine the effects of EPN on bacterial growth, bacterial colonies on the test plates with nematodes were counted and compared to the control plates without nematodes.

4.2.2.1 Surface sterilisation of EPN

A 0.1 % $HgCl_2$ (See Appendix 6 for MSDS) surface sterilisation protocol was adapted from Lanau *et al* (1993) and applied to all EPN species to prepare large batches of surface sterilised IJ. 10 ml of a dense EPN suspension was added to a 50 ml centrifuge tube. IJ were washed in 15 ml of sterile Ringer's solution for 5 minutes, using a wrist action shaker. The IJ were pelleted by centrifugation at 3800 rpm for 10 minutes and the resulting supernatant was removed and discarded. The IJ pellet was shaken in 15 ml of a 0.1 % $HgCl_2$ solution for 12 minutes as described above. The $HgCl_2$ was removed and discarded in the designated waste bottle. The surface sterilised IJ pellet were washed once with sterile Ringer's solution and twice with sterile dH_2O, with a final re-suspension in 10 ml sterile dH_2O.

To test the surface sterilisation of the nematode suspension, 100 µl of each EPN suspension was pipetted onto nutrient agar and incubated at 30ºC, and the plates were examined for bacterial growth after 24 hr. Sterilisation was deemed to be successful if no growth was observed after incubation. Surface sterilised EPN suspensions were stored at 4ºC overnight before use for experimentation.

4.2.2.2 Statistical analysis

A Kruskal-Wallis H test using SPSS 23 was conducted to explore the effect of the presence of *S. feltiae* SB 12(1), *S. feltiae S. carpocapsae* and *H. bacteriophora* (e-nema) on endophytic bacterial growth on solid media. This test was run to determine if there were significant differences (p <.05) in the number of bacterial CFU/ml between the control treatment with no EPN and the treatments with EPN IJ. Where significant differences (p <.05) were identified, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons.

4.2.3 An investigation of the effects of *S. feltiae* SB 12(1) and *H. bacteriophora* (e-nema) on bacterial plant colonisation and plant growth promotion in oilseed rape

Two *gfp* labelled PGP bacteria (F113 *gfp* and L321 *gfp*) were used to examine the effect of the EPN *S. feltiae* SB 12(1) and *H. bacteriophora* and a combination of the two nematode species on bacterial plant colonisation and PGP. Topsoil was sieved to remove large debris, autoclaved and air dried for 5 days in a greenhouse. 900 g of soil was added to 1L plastic plant pots and the soil was moistened with 100 ml H_2O. Tamarin, a spring variety of oilseed rape (OSR), seeds were coated with sodium alginate and a *gfp* labelled bacterial strain as described in Section 3.2.3.1. Coated seeds were sown just below the soil surface and allowed to germinate for 3 days. Three coated seeds were planted per pot and two germinated seedlings were removed prior to adding EPN treatments. Individual *S. feltiae* SB 12(1) and *H. bacteriophora* EPN treatments were added to each pot at a density of 10 IJ g^{-1} soil. This was achieved by adding 10 ml of an EPN suspension containing approximately 1000 IJ/ml. For the nematode combination treatment, 5 ml of each individual suspension was added to the pots.

There were two control treatments, control treatment (1) where seeds were coated with endophytes and inoculated with sterile H_2O only (no EPN) and (2) seeds were coated with sterile H_2O and inoculated with H_2O only (no bacterial inoculum and no EPN). Replication was six-fold per treatment with one plant per pot. Growing one plant per pot allowed for the harvest of individual plant root systems.

Plants were grown in a greenhouse maintained at a constant temperature (20ºC, ± 3ºC) for 12 weeks until they reached flowering, with a re-inoculation of endophytic bacteria and EPN after 6 weeks growth.

4.2.3.1 Re-inoculation of EPN and endophytic bacteria

To ensure nematode and bacterial persistence in pots both EPN and bacterial endophytes were re-inoculated onto each plant. EPN were re-inoculated at a reduced density of 5 IJ g^{-1}. This was a 50 % reduction on the initial EPN inoculation. 5 ml of each nematode suspension (containing approximately 1000 IJ/ml) was added per pot by pipetting just below the soil surface. For the EPN combination treatment, 2.5 ml of each EPN species was added per pot.

For bacterial re-inoculation a 24 hr bacterial culture (10^8 CFU/ml) was re-suspended in sterile dH_2O and sprayed onto the plants just above the soil surface, using a sterile aerosol sprayer. Plants were watered every 2 - 3 days by adding 70 - 100 ml of H_2O to the base of the pots.

4.2.3.2 Plant harvest and OSR biomass analysis

OSR plants were harvested after 12 weeks growth. The entire plant was removed, and loose soil was retained in the pots to assess EPN survival. The excess soil was removed from the plant roots and collected in a sterile 50 ml centrifuge tube to assess the survival of the *gfp* labelled bacterial endophytes in the plant rhizosphere. Following harvest, plants were

washed with sterile dH_2O, air dried on paper towels for 30 minutes and weighed to determine the plant fresh weights. A total of 2 g was removed from each plant (1 g from phyllosphere and 1 g from root) to analyse bacterial colonisation. The remaining plant samples were placed individually into heat-resistant aluminium trays and dried at 60°C for 48 hr, and after this, the plant dry weights (g) were recorded.

4.2.3.3 Bacterial colonisation of OSR in the presence and absence of EPN

To determine colonisation (CFU g^{-1}) approximately 1.0 g was removed from the plant root and phyllosphere (stem and leaf). Phyllosphere and root samples were surface sterilised by shaking in 1 % sodium hypochlorite for 10 minutes, the sodium hypochlorite solution was poured off and the samples were rinsed in 95 % ethanol for 30 seconds, before washing in triplicate with sterile dH_2O. After surface sterilisation, the phyllosphere and root samples were finely cut with a sterile scalpel and crushed with a sterile pestle and mortar. To examine bacterial survival in the area surrounding the plant roots, 1 g of the soil collected from the plant rhizosphere was homogenised using a pestle and mortar and any fine roots present were removed.

0.1 g of the homogenised rhizosphere, root and phyllosphere samples were added to 900 µl of Ringer's solution, in 1.5ml microfuge tubes. Samples were vigorously vortexed for one minute and serially diluted 1:10 until a 10^{-7} dilution was reached. 30 µl aliquots from each dilution were plated onto SGA (with 50 µg/µl kanamycin) agar plates in triplicate. Droplets were dried under laminar air flow for 30 minutes and subsequently incubated at 30 °C. Plates were monitored every 24 hr for three days until sufficient growth was observed. The first dilution with colony numbers between 3 and 30 was used to determine the CFU g^{-1} of plant fresh weight.

A number of well-isolated colonies were aseptically removed from growth media to examine for *gfp* expression under UV light, using an epifluorescence microscope. A Nikon eclipse 8i microscope with a fluorescein filter (FITC - excitation wavelength 467-498nm; dichroic cut-off 506nm; emission wavelength 513-556nm) was used to visualise green fluorescent cells. A Nikon DS-5Mc camera with NIS-elements viewer software was used to capture and process the 100 X images.

4.2.3.3.1 Preparation of sucrose glutamic acid agar (SGA) with Kanamycin (selective for *gfp* labelled, kanamycin resistant bacteria)

To prepare 1 L of SGA agar the following components were dissolved in sterile dH_2O and autoclaved for 15 minutes at 15psi and 121^0C.

20 g L^{-1} Sucrose

2 g L^{-1} Glutamic acid

1 g L^{-1} K_2HPO_4

15 g L^{-1} Agar no. 1

After autoclaving, the agar was allowed to cool to approximately 60 ^{0}C and at this point, 5 ml 10 % $MgSO_4$ was added. For selection of *gfp* labelled endophytes, 1 ml L^{-1} of filter sterilised stock Kanamycin solution (50 mg/ml) was added for a final concentration of 50 µg/ml. To prepare a 50mg/ml Kanamycin stock solution, 0.5 g of kanamycin sulphate salt was dissolved in 10 ml sterile dH_2O. The solution was filtered sterilised through a 0.22 µm sterile syringe filter. Aliquots of 1 ml were dispensed into sterile 1.5 ml microfuge tubes and stored at -20°C until required. Agar was aseptically dispensed into 9 cm Petri dishes and dried under laminar air flow.

4.2.3.1 Molecular confirmation of bacterial colonisation of OSR by *gfp* labelled *P. fluorescens* F113 and L321

It is plausible to suggest that bacterial growth on SGA plates with Kanamycin could be an unrelated kanamycin resistant bacterial isolate. Thus, OSR colonisation by *P. fluorescens* F113 *gfp* or L321 *gfp* was confirmed at a molecular level using *gfp* specific primers (Table 4.2.3).

4.2.3.1.1 DNA extraction from plant samples, PCR conditions and gel electrophoresis

Genomic DNA was isolated from the plant phyllosphere, root and rhizosphere using a Power soil DNA extraction kit (MoBio) according to the manufacturer's instructions. Extracted DNA samples were stored at -20°C until required. Standard PCR reactions were performed using the G-storm GS-1 PCR thermocycler. Reactions were performed in 25 µl reaction volumes using GoTaq® Green Master Mix (Promega), primers, sterile ddH_2O and DNA template. For PCR negative controls, the DNA template was replaced with sterile dH_2O. The PCR was carried out under the reaction conditions shown in Table 4.2.1 and Table 4.2.2.

Table 4.2.1 PCR reaction volumes and concentrations used for amplification of the *gfp* gene

Component	Single rxn. (µl)	Concentration
Sterile ddH_2O	8.5	34 %
GoTaq Master Mix 2X	12.5	1X
Primer 10 pmol (Fw & RV)	1 of each	0.4 pmol
DNA sample	2	Template
Total reaction volume	25	-

Table 4.2.2 PCR reaction thermocycling profile for amplification of *gfp* gene

Cycle	Temperature (ºC)	Time (sec)
1	95	300
35	95	30
	60	50
	72	60
1	72	600
-	8	hold

Table 4.2.3 Primer sequences to target *gfp* gene used for standard and qPCR reactions

Primer ID	Fw Sequence 5´- 3´	Amplicon length (bp)	Target
Gfp_P1	gtcagtggagagggtgaagg	135	*gfp* gene

All PCR products from *gfp* gene amplification experiments were visualised via gel electrophoresis with a 2 % agarose gel stained with GelRed™ (Biotium) to a final concentration of 0.05 µg ml^{-1}. Gels were run at 80 V for 70 minutes in 1 X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA at a pH of 8.5) and visualised using Cell Biosciences red gel imagining system. Primers were designed by Lally (2016) using Primer 3 software and examined for sensitivity and specificity for both standard and qPCR.

4.2.3.2 EPN survival in soil following OSR harvesting

Following OSR harvesting, six *G. mellonella* larvae were added to each pot containing soil with bacteria and nematodes and incubated in the greenhouse for three days. The insects were removed from the pots, washed by submerging in sterile dH_2O and incubated for 24 hr at 21°C. After this, the insects were scored for mortality and placed onto White traps to determine if nematode emergence occurred. Nematode emergence was checked daily for 19 days, but the number of emerged IJ was not determined.

4.2.3.2.1 Statistical analysis

OSR biomass data and bacterial colonisation (CFU/ml) data were analysed for statistical significance using the Kruskal-Wallis H test in SPSS 23. Where significant differences (p <.05) were identified, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. A one way ANOVA was used to examine significance of data from the experiment examining EPN survival following plant harvest. Here post hoc analysis was carried out using a *Bonferroni* correction.

4.2.4 Endophytic bacterial colonisation of entomopathogenic nematodes infective juveniles and bacterial persistence post insect infection

Nematodes have been linked with the increased bacterial colonisation of plants. Bacterial colonisation of IJ and subsequent persistence post insect infection may offer a potential explanation for this. Laboratory-based bioassays were employed to examine firstly, the endophytic bacterial colonisation of nematodes and secondly, the survival of the *gfp* labelled bacteria inside the insect cadaver after nematode infection and insect mortality has occurred.

4.2.4.1 *Pseudomonas fluorescens* F113 *gfp* colonisation of *S. feltiae* SB 12(1) and *H. bacteriophora*

The ability of F113 to internally colonise EPN IJ was examined after direct nematode exposure to bacteria. An F113 *gfp* bacterial lawn was prepared on SGA, by adding 100 µl of a 10^8 CFU/ml bacterial culture to the media and dispersing using a sterile spreader. The bacterial lawn was air dried under laminar flow for 30 minutes, and after this 500 IJ in 100 µl sterile dH_2O were added to each plate. Plates were incubated at 25 ºC for 6 hr, 18 hr, 24 hr, 36 hr and 48 hr. Nematodes in the control treatment received sterile dH_2O only. Replication was threefold per nematode species, per incubation time length and in the control treatments.

After each incubation period, EPN were washed off the plates using sterile ringers and retained in a 50 ml centrifuge tube. The IJ were settled at the bottom of the tubes for 2 hr at 4ºC and subsequently washed twice with sterile dH_2O by centrifugation. The IJ were re-suspended in 5 ml sterile dH_2O. A 50 µl aliquot was removed from each tube to examine for *gfp* expression under UV light (Nikon, eclipse 8i). The remaining IJ were surface sterilised using $HgCl_2$ as outlined in Section 4.2.2.1 and visualised as outlined above to determine internal IJ colonisation. The success of surface sterilisation was assessed by inoculation of the nematode suspension onto nutrient agar and incubating at 30°C for 24 hr.

The internal bacterial colonisation of nematodes was verified at a molecular level using *gfp* specific primers. RNA extractions were carried out on surface sterilised nematode samples, followed immediately by cDNA conversion. The remaining template RNA was stored at -70°C until required. Real-time PCR using *gfp* specific primers was carried out on the cDNA to quantify the concentration of bacteria inside the nematodes at each exposure time.

4.2.4.1.1 RNA extractions from surface sterilised IJ and DNase digestion

RNA was extracted from approximately 50 surface sterilised IJ using an E.Z.N.A.® Total RNA Kit (OMEGA bio-tek) according to the manufacturer's instructions. Briefly, IJ were homogenised and disrupted using a syringe and needle in 700 µl TRL lysis buffer. Samples were centrifuged at 10,000 rpm for 5 minutes before transferring supernatant to a clean 1.5 ml tube containing an equal volume of 70 % ethanol. Samples were vortexed before passing through a Hibind® RNA mini column. RNA was washed using RNA Wash Buffer I and Wash Buffer II, with repeated centrifugation at 10,000 rpm for 1 minute, before centrifuging an empty column to remove the remaining buffer. The RNA was eluted in 70 µl of heated (to 70 ˚C) DEPC water.

To prevent DNA contamination in the PCR an RQ1 RNase-Free DNase kit (Promega) was used to digest any potential contaminating DNA. DNase treatment was set up as per reaction volumes and concentrations in Table 4.2.4, and the samples were incubated at 37 ˚C for 30 minutes. Following incubation, 1 µl RQ1 DNase Stop solution was added to terminate the reaction, followed by incubation at 65 ˚C for 10 minutes for DNase inactivation. This solution was used for RT-PCR.

Table 4.2.4 DNase reaction volumes using RQ1 RNase-Free DNase kit

Component	Single rxn. (µl)	Concentration
RNA in DEPC water	5	50 %
RQ1 RNase-Free DNase Reaction Buffer	2	10X
RQ1 RNase-Free DNase	2	20 %
Nuclease-Free water	1	10 %
Total reaction volume	10	-

4.2.4.1.2 Complementary DNA synthesis

A commercially available qScript™ cDNA synthesis kit (Quanta Biosciences™) was used for RNA quantification. The kit components consist of qScript Reaction Mix (a 5X concentrated solution of an optimised buffer, dNTPs, magnesium, oligo [dT] and random primers), 50 X concentration of qScript Reverse Transcriptase and Nuclease-free water. All components were thawed and mixed before placing on ice. Each reaction was carried out in 20 µl reaction volumes in sterile 0.2 ml PCR tubes. The volumes of individual components and RNA are outlined in Table 4.2.5. Tubes were gently vortexed to mix and centrifuged for 10 secs to collect contents at the bottom of the tube. A G-Storm (GS-1) PCR thermocycler was employed to synthesise cDNA using the program detailed in Table 4.2.6 cDNA template was stored at -20 ºC and a $^{1}/_{5}{}^{th}$ or $^{1}/_{10}{}^{th}$ dilution was used for PCR amplification.

Table 4.2.5 cDNA synthesis reaction volumes

Component	Single rxn. (µl)	Concentration
Nuclease-free water	10	50 %
qScript Reaction Mix (5X)	4	1X
qScript Reverse Transcriptase	1	2.5X
RNA Sample	5	Template
Total reaction volume	20	-

Table 4.2.6 cDNA synthesis reaction thermocycling and times

Cycle	Temperature (ºC)	Time (min)
1	22	5
1	42	30
1	85	5
-	4	hold

4.2.4.1.3 Real-time PCR

Real-time PCR (RT-PCR) or quantitative PCR (qPCR) is based on the monitoring of the PCR reaction in real-time. Due to the sensitivity of RT-PCR, all materials (microfuge tubes, PCR tubes, tips, pipettes, 96 well PCR plates) were sterilised by autoclaving, where appropriate, and exposed to UV light for 30 minutes before use. All reactions were prepared under laminar flow on an alcohol sterilised work surface. The Lightcycler Nano (Roche, Germany) was used for optimisation assays and the Lightcycler 480 II was used for 96 well PCR plate assays. Reactions synthesis reagent concentrations and thermocycler profiles are presented below in Table 4.2.7 and Table 4.2.8.

Table 4.2.7 qPCR reaction volumes and concentrations

Component	Single rxn. (µl)	Concentration
SYBR Master Mix	5.5	0.55X
H_2O	11.7	58.5
Primer Fw + Rev	0.4	0.2 pmol μl^{-1}
cDNA	2	template
Total reaction volume	20	

Table 4.2.8 qPCR synthesis reaction thermocycling profile

	Cycle	Temperature (ºC)	Time (sec)	Slope rate
Initial denaturation	1	95	300	
Amplification	45	95	5	
	1	60	5	
		72	10	
Melting	1	95	1	
		65	10	
		95		0.1 ˚C S^{-1}
Cooling	1	30		5 ˚C S^{-1}

Each reaction was carried out in 20 µl reaction volumes using a Roche pre-mixed Master Mix with SYBR green technology, PCR grade H_2O, primers FW 5´- 3´ (Table 4.2.3) and cDNA template. SYBR green is an intercalating dye used in the quantification process. It binds double-stranded DNA during the log exponential amplification, resulting in a fluorescent signal being emitted. This fluorescence is measured at each PCR cycle, allowing

for the display of product accumulation over time. The amount of fluorescence is proportional to the amount of DNA in the reaction. Quantitation cycle (Cq) values recorded during the qPCR reaction correspond to the cycle in which fluorescence was detected. A standard curve of known gene copy numbers (CN) can be constructed and the concentration of the unknown samples can be extrapolated from their respective Cq value.

4.2.4.1.4 Standard Curve for qPCR

Genomic DNA was extracted from a 24 hr 10^8 CFU/ml *Pseudomonas fluorescens* F113 *gfp* bacterial culture using a commercially available genomic DNA extraction kit (Promega), according to the manufacturer's instructions for Gram-negative bacteria. The extracted samples were stored at -20°C until required. The concentration (ng/µl) of the bacterial DNA was quantified using a DeNovix DS-11 spectrophotometer. The genome sequence of the bacterial strain examined here was known thus, it was possible to calculate the genome copy number (CN) for a given bacterial DNA sample of known concentration. The Thermo Fisher Scientific DNA Copy Number and Dilution Calculator was used to calculate the CN μl^{-1} in a given DNA sample of known concentration. From here stock DNA was diluted to obtain standards of 10^8-10^1 CN μl^{-1}.

qPCR was carried out on the sample dilutions as per conditions outlined in Section 4.2.4.1.3. The resulting standard curve was saved as a template on the LightCycler 480 II software and was imported at the analysis stage of each quantification experiment. An internal standard of 10^3 CN was added to each quantification experiment to act as an internal control for standard curve calibration. The gene CN in each of the treated samples was extrapolated from the standard curves and subsequently back calculated to determine the concentration in the original RNA extraction.

4.2.4.2 Endophytic bacterial persistence post insect infection

In Chapter 2 antimicrobial susceptibility testing was carried out to examine the effects of bacterial isolates F113 and L321 against *X. bovienii* and *P. luminescens*. Results displayed in Figure 2.3.10 show this activity. Interactions between colonising *P. fluorescens* F113 and EPN symbiotic bacteria will be examined here *in vitro*, by exposing the EPN *S. feltiae* SB 12(1) and *H. bacteriophora* to F113 *gfp* prior to insect infection. 1000 IJ in 1 ml sterile dH_2O were exposed to 250 µl of a re-suspended 10^8 CFU/ml bacterial culture in 2 ml microfuge tubes for 24 hr at 9ºC. After incubation, the nematodes and bacteria formed a loose pellet and were washed using sterile dH_2O by light centrifugation. IJ were surface sterilised using $HgCl_2$ as outlined in Section 3.2.6.1.2. Nematodes were re-suspended in 1 ml sterile dH_2O and used to infect one *G. mellonella* on a baiting tray. Insect mortality was monitored daily and insects were dissected in Petri dishes (35 x 10 mm) containing 1 ml Ringer's solution, five days following infection.

The insect carcass was removed and disposed of, with the remaining haemolymph homogenised by passing sample through the needle of a syringe (for 1 minute). 100 µl of the haemolymph suspension was serially diluted 1:10 until a dilution of 10^{-9} was reached. 30 µl aliquots of each dilution were pipetted onto SGA (with Kanamycin) to select for the *gfp* labelled isolates. Replication was six-fold per EPN, with one insect per replicate. Control insects received sterile dH_2O only.

4.3 Results

4.3.1 Identification of insecticidal genes in the genomes of *P. fluorescens* F113, L321, L228 and L111

The bacterial genomes of *P. fluorescens* F113, L321, L228 and L111 were examined for the presence of genes associated with the insecticidal toxins FIT and Mcf by carrying out a BLASTn sequence alignment. The FIT complex was identified in *Pseudomonas protegens* CHA0 (Accession No: EU400157.2), it is 28,395bp and consists of 8 ORFs *FitA- FitH*. If an alignment match occurred on the complete sequence, each of the individual ORFs were blasted to identify the exact gene associated match. Results are presented in Table 4.3.1 and for alignments between the FIT toxin and the bacterial endophytes *P. fluorescens* F113, L321, L228 and L111 and the EPN symbionts, *P. luminescens* subsp. laumondii TTO1, *X. nematophilia* K102 and *X. bovienii* CS03). No significant similarities were detected between the FIT complex and the *P. fluorescens* strains F113, L321 and L111 as shown in Table 4.3.1. The only notable exception was a limited association between L228 and the *fitF* gene of the FIT complex.

Table 4.3.1 Sequence alignment results of genes compared with the Fluorescent insect toxin (FIT) complex.

Bacterial genomes	Accession No.	E value	% Ident	Gene
Pseudomonas protegens CHA0 FIT toxin gene	EU400157.2			
P. corrugata F113	NC_016830	-	-	-
P. fluorescens L321	NZ_CP015637.1	-	-	-
P. fluorescens L111	NZ_CP015638.1	-	-	-
P. fluorescens L228	NZ_CP015639.1	2.00E-06	100%	*fitF*
P.luminescens	BX470251.1	0	77%	*fitD*
X.nematophilia	LT966404.1	-	-	-
X. bovienii	NZ_FO818637.1	-	-	-
Photorhabdus temperata McF gene	KJ584647.1	0	78%	*fitD*

Table 4.3.2 Sequence alignments with the Makes caterpillars' floppy (Mcf) gene

Bacterial genomes	Accession No.	E value	% Ident
Photorhabdus temperata *McF* gene	KJ584647.1		
X. bovienii	NZ_FO818637.1	0	83%
P. luminescens	BX470251.1	0	85%
X. nematophilia	LT966404.1	-	-
P. fluorescens F113	NC_016830	-	-
P. fluorescens L321	NZ_CP015637.1	-	-
P. fluorescens L111	NZ_CP015638.1	-	-
P. fluorescens L228	NZ_CP015639.1	-	-

Results presented in Table 4.3.2 display alignments between the McF toxin and the bacterial endophytes *P. fluorescens* F113, L321, L228 and L111 and the EPN symbionts, *P. luminescens* subsp. laumondii TTO1, *X. nematophilia* K102 and *X. bovienii* CS03). Following BLASTn sequence alignment analysis between the Mcf toxin and the bacterial endophytes (F113, L321, L228 and L111) no sequences with noteworthy similarities were detected. No similarities were detected between the Mcf toxin and *X. nematophilia* K102, however as expected significant sequence similarities between *X. bovienii* and *P. luminescens* were detected and they share 83 and 85 % identity, respectively.

4.3.2 Determination of the effects of *S. feltiae* SB 12(1), *S. feltiae, S. carpocapsae* and *H. bacteriophora* (e-nema) on endophytic bacterial growth

Endophytic bacterial growth on solid media was examined in the presence and absence of EPN IJ. 100 surface sterilised IJ were added to an endophytic bacterial lawn on nutrient agar. Control treatment plates were inoculated with an endophytic bacterial lawn and 100 µl of sterile dH2O only. Replication was six-fold per nematode species and bacterial isolate. Bacterial colonies were counted and the average CFU/ml of the bacterial culture was determined after 24 hr incubation at 30 ºC. Results for bacterial growth (CFU/ml) are presented in Figure 4.3.1 to Figure 4.3.11.

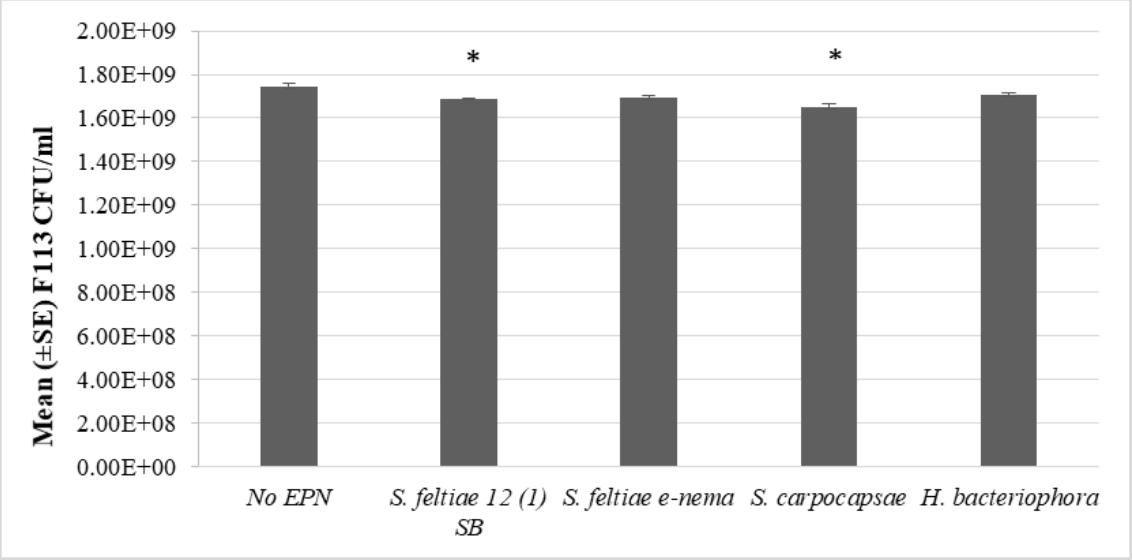

Figure 4.3.1 Mean (±SE) F113 CFU/ ml viable colonies recorded after 24 hr growth at 30 ˚C on nutrient agar following incubation in the presence and absence of surface sterilised EPN infective juveniles. The first grey bar (Control: No EPN) represents the growth of F113 in the absence of EPN, the additional four bars represent F113 growth in the presence of EPN on agar plates. Asterisk (*) indicates that bacterial growth differs significantly from that in the control with no EPN (n=6).

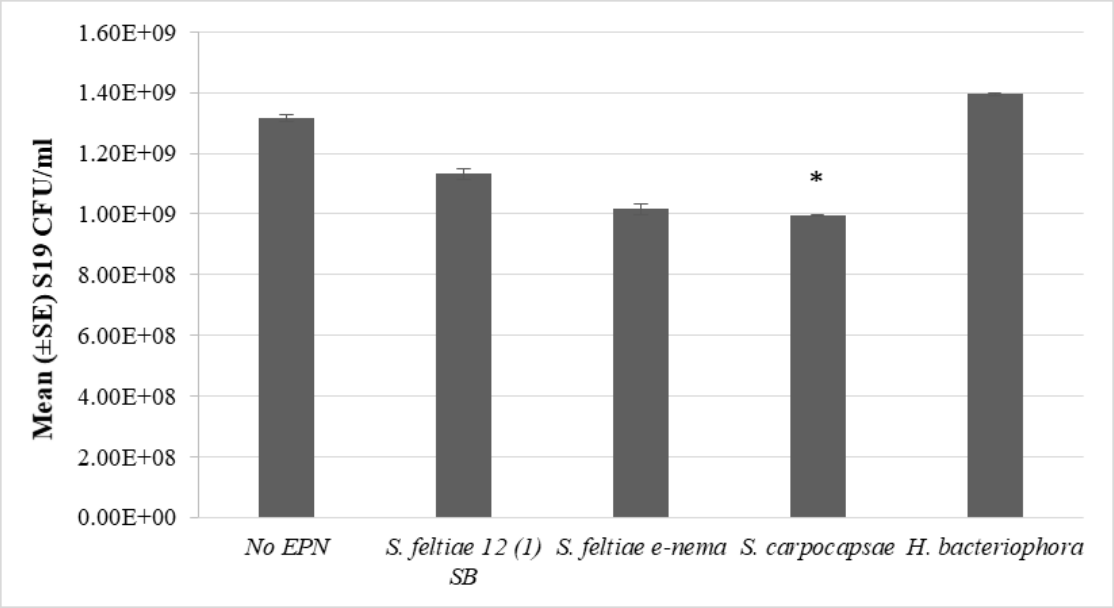

Figure 4.3.2 Mean (±SE) S19 CFU/ ml viable colonies recorded after 24 hr growth at 30 ˚C on nutrient agar following incubation in the presence and absence of surface sterilised EPN infective juveniles. The first grey bar (Control: No EPN) represents the growth of S19 in the absence of EPN, the additional four bars represent S19 growth in the presence of EPN on agar plates. Asterisk (*) indicates that bacterial growth differs significantly from that in the control with no EPN (n=6).

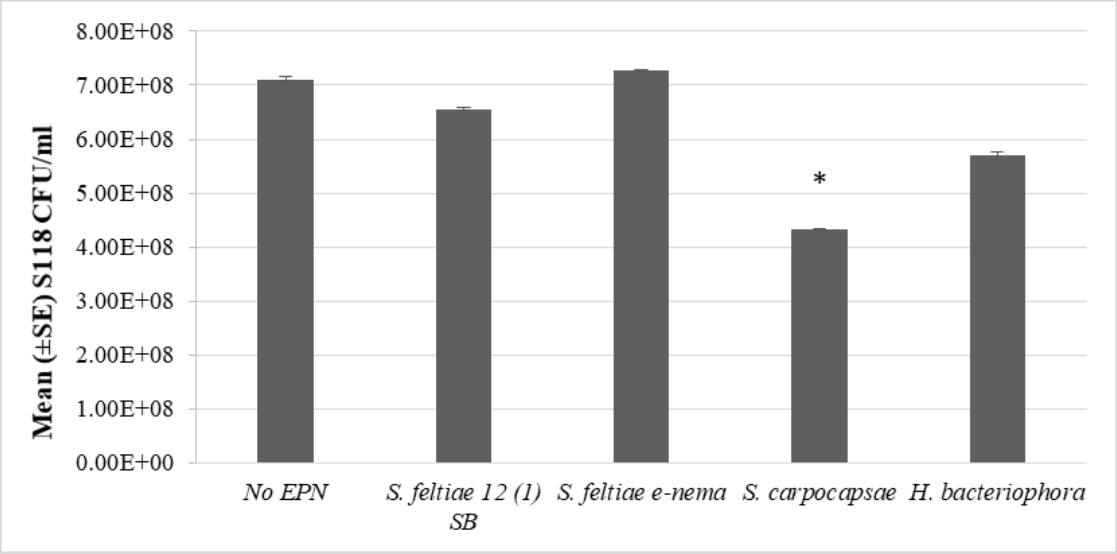

Figure 4.3.3 Mean (±SE) S118 CFU/ ml viable colonies recorded after 24 hr growth at 30 ˚C on nutrient agar following incubation in the presence and absence of surface sterilised EPN infective juveniles. The first grey bar (Control: No EPN) represents the growth of S118 in the absence of EPN, the additional four bars represent S118 growth in the presence of EPN on agar plates. Asterisk (*) indicates that bacterial growth differs significantly from that in the control with no EPN (n=6).

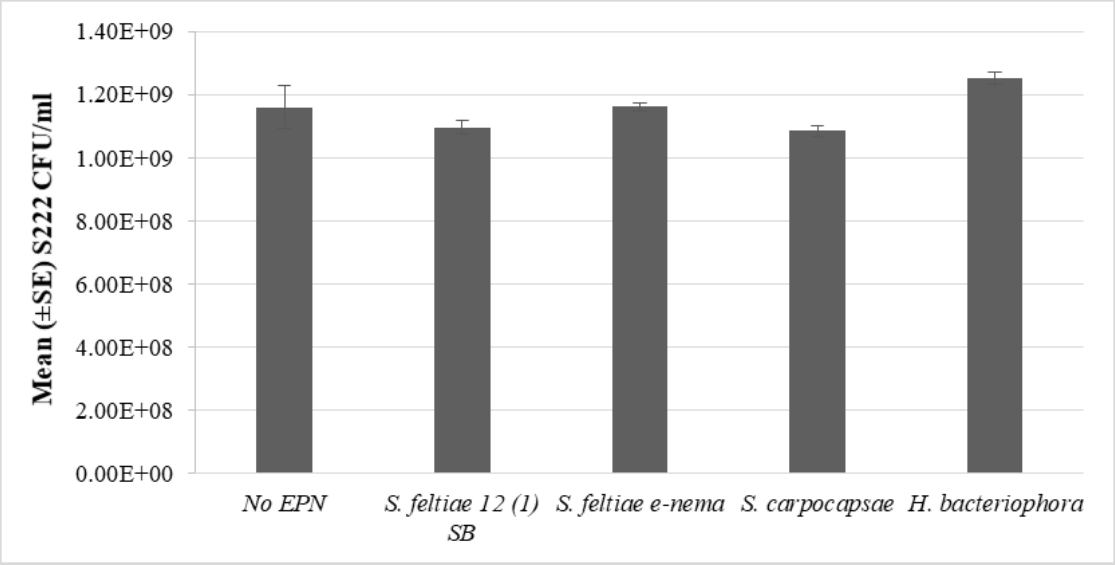

Figure 4.3.4 Mean (±SE) S222 CFU/ ml viable colonies recorded after 24 hr growth at 30 ˚C on nutrient agar following incubation in the presence and absence of surface sterilised EPN infective juveniles. The first grey bar (Control: No EPN) represents the growth of S222 in the absence of EPN, the additional four bars represent S222 growth in the presence of EPN on agar plates (n=6).

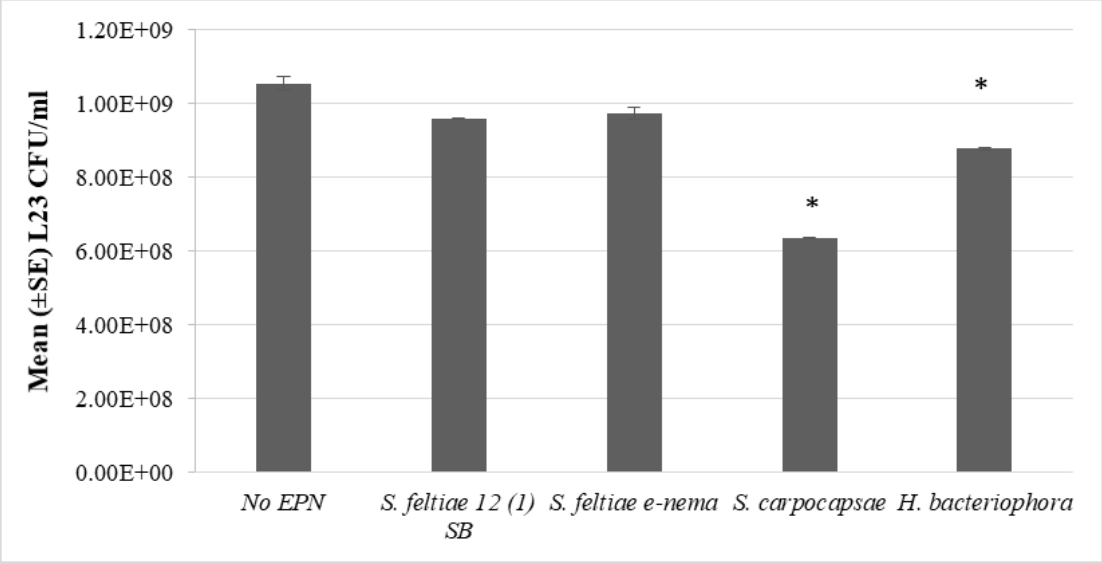

Figure 4.3.5 Mean (±SE) L23 CFU/ ml viable colonies recorded after 24 hr growth at 30 ˚C on nutrient agar following incubation in the presence and absence of surface sterilised EPN infective juveniles. The first grey bar (Control: No EPN) represents the growth of L23 in the absence of EPN, the additional four bars represent L23 growth in the presence of EPN on agar plates. Asterisk (*) indicates that bacterial growth differs significantly from that in the control with no EPN (n=6).

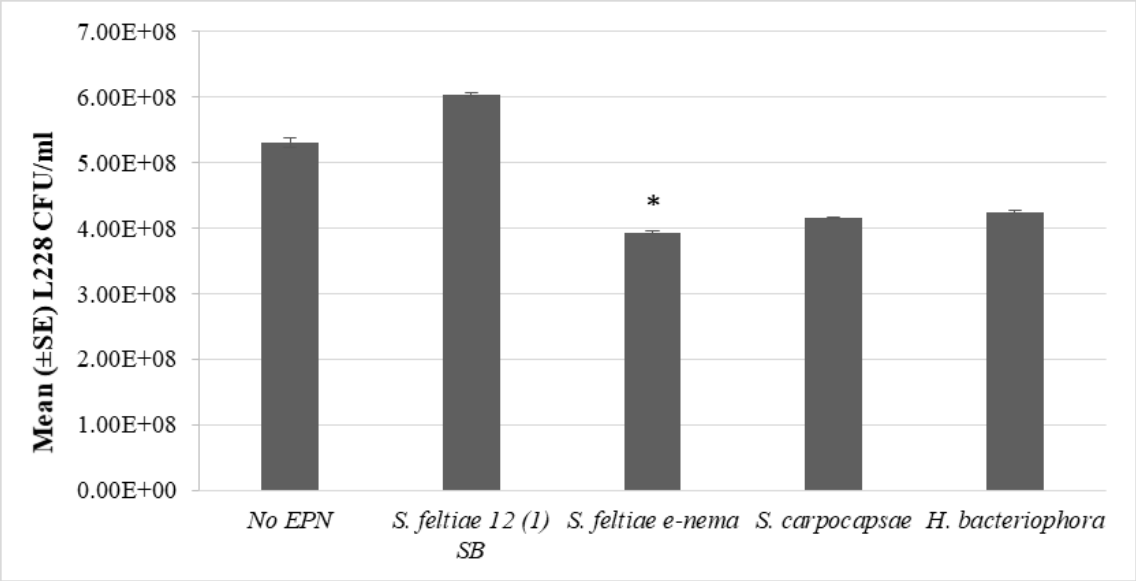

Figure 4.3.6 Mean (±SE) L228 CFU/ ml viable colonies recorded after 24 hr growth at 30 ˚C on nutrient agar following incubation in the presence and absence of surface sterilised EPN infective juveniles. The first grey bar (Control: No EPN) represents the growth of L228 in the absence of EPN, the additional four bars represent L228 growth in the presence of EPN on agar plates. Asterisk (*) indicates that bacterial growth differs significantly from that in the control with no EPN (n=6).

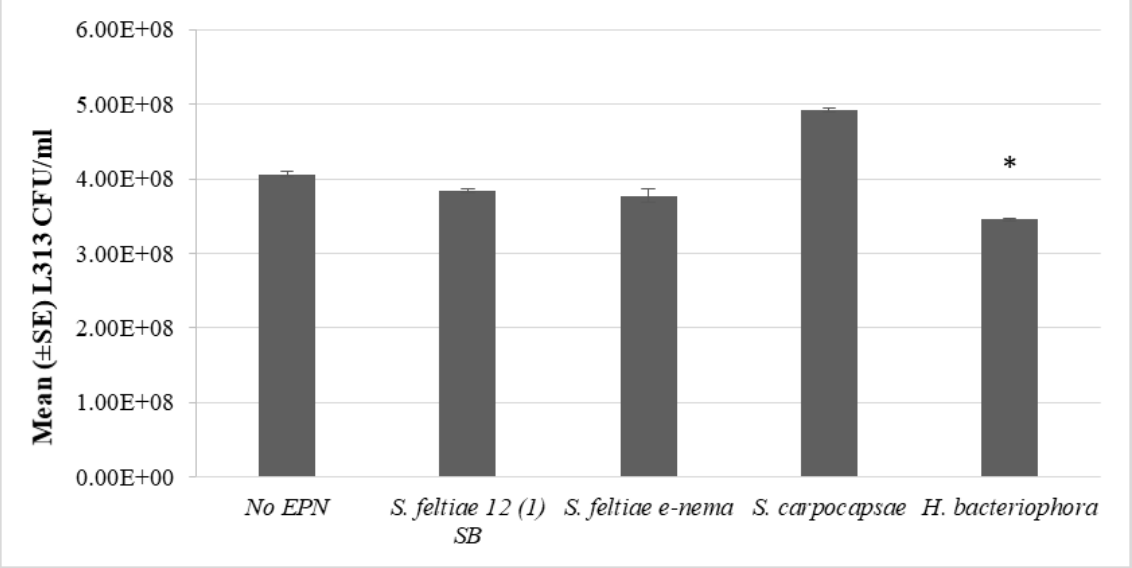

Figure 4.3.7 Mean (±SE) L313 CFU/ ml viable colonies recorded after 24 hr growth at 30 ˚C on nutrient agar following incubation in the presence and absence of surface sterilised EPN infective juveniles. The first grey bar (Control: No EPN) represents the growth of L313 in the absence of EPN, the additional four bars represent L313 growth in the presence of EPN on agar plates. Asterisk (*) indicates that bacterial growth differs significantly from that in the control with no EPN (n=6).

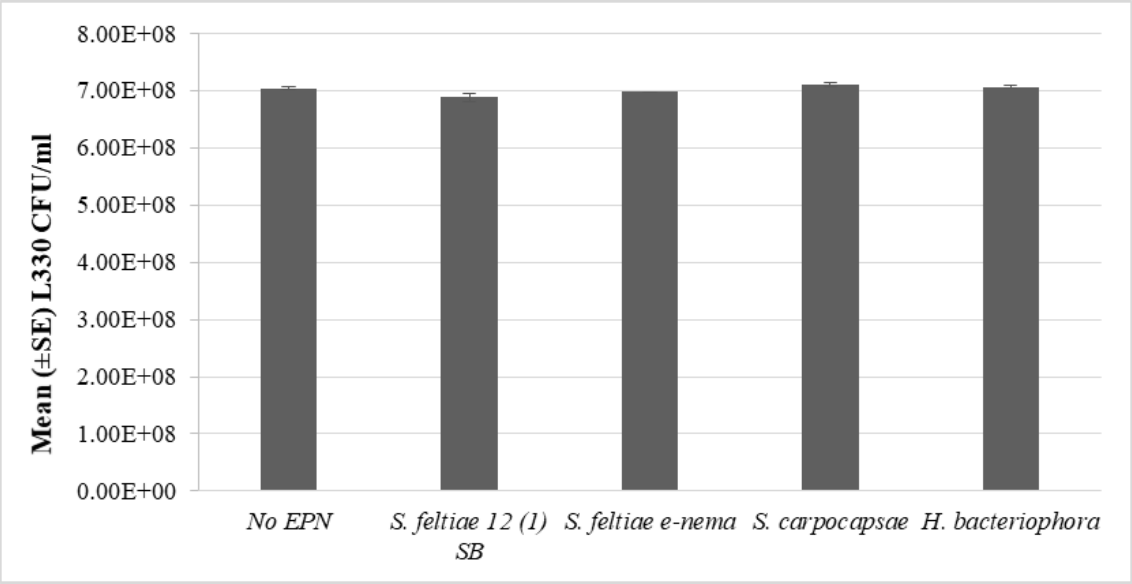

Figure 4.3.8 Mean (±SE) L330 CFU/ ml viable colonies recorded after 24 hr growth at 30 ˚C on nutrient agar following incubation in the presence and absence of surface sterilised EPN infective juveniles. The first grey bar (Control: No EPN) represents the growth of L330 in the absence of EPN, the additional four bars represent L330 growth in the presence of EPN on agar plates (n=6).

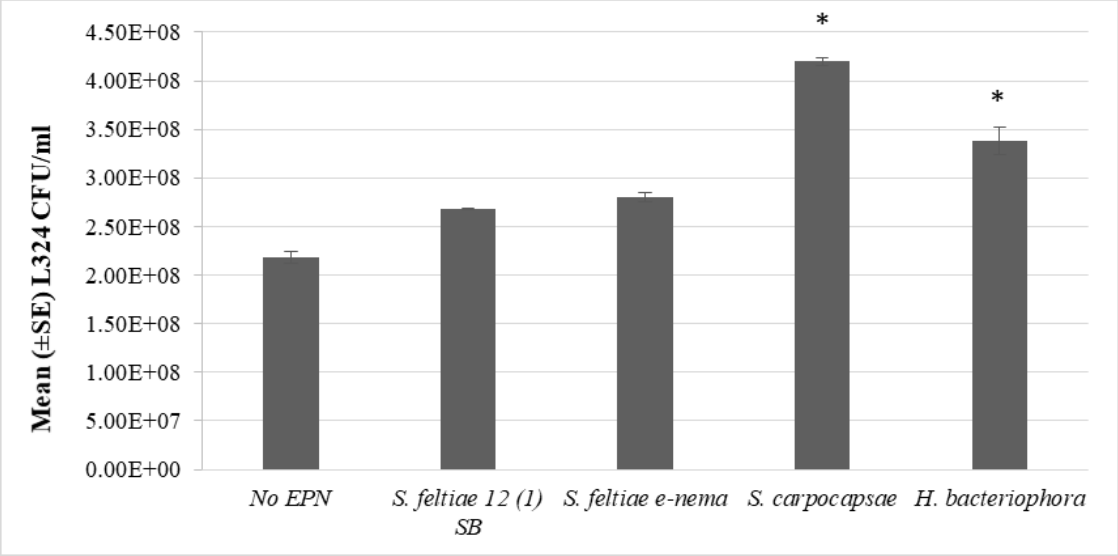

Figure 4.3.9 Mean (±SE) L324 CFU/ ml viable colonies recorded after 24 hr growth at 30 ˚C on nutrient agar following incubation in the presence and absence of surface sterilised EPN infective juveniles. The first grey bar (Control: No EPN) represents the growth of L324 in the absence of EPN, the additional four bars represent L324 growth in the presence of EPN on agar plates. Asterisk (*) indicates that bacterial growth differs significantly from that in the control with no EPN (n=6).

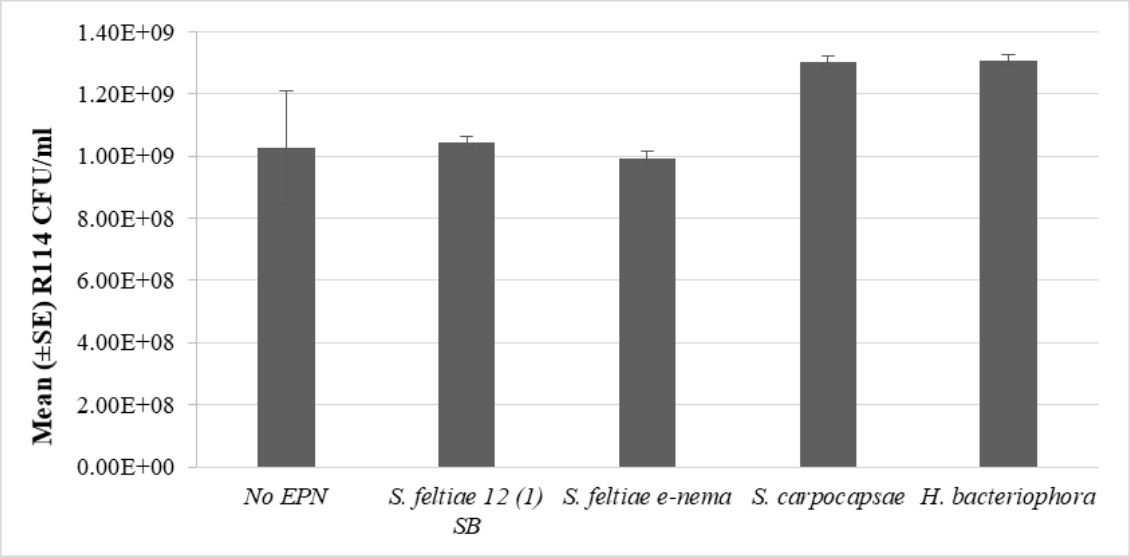

Figure 4.3.10 Mean (±SE) R114 CFU/ ml viable colonies recorded after 24 hr growth at 30 ˚C on nutrient agar following incubation in the presence and absence of surface sterilised EPN infective juveniles. The first grey bar (Control: No EPN) represents the growth of R114 in the absence of EPN, the additional four bars represent R114 growth in the presence of EPN on agar plates (n=6).

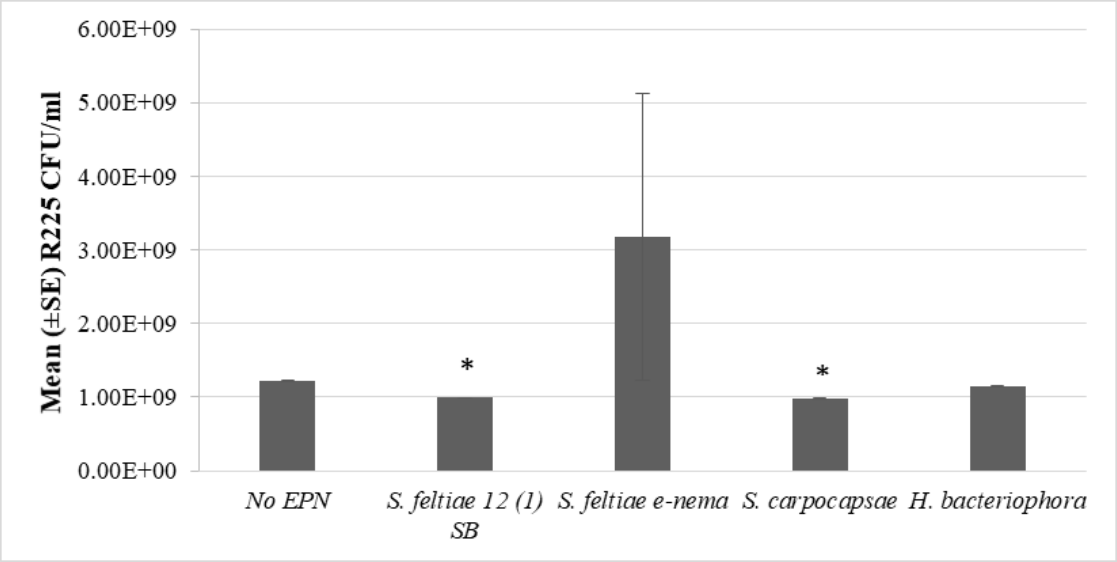

Figure 4.3.11 Mean (±SE) R225 CFU/ ml viable colonies recorded after 24 hr growth at 30 ˚C on nutrient agar following incubation in the presence and absence of surface sterilised EPN infective juveniles. The first grey bar (Control: No EPN) represents the growth of R225 in the absence of EPN, the additional four bars represent R225 growth in the presence of EPN on agar plates. Asterisk (*) indicates that bacterial growth differs significantly from that in the control with no EPN (n=6).

A Kruskal-Wallis H test was conducted to determine if there were significant differences in bacterial growth in the presence and absence of EPN IJ. The distributions of bacterial growth scores in the presence and absence of EPN were statistically significant for **F113** (χ^2 [4] = 17.918, p = .001), **S19** (χ^2 [4] = 27.265, p = .000), **S118** (χ^2 [4] = 27.813, p = .000), **S222** (χ^2 [4] = 16.554, p = .002), **L23** (χ^2 [4] = 26.809, p = .000), **L228** (χ^2 [4] = 27.603 , p = .000), **L330** (χ^2 [4] = 17.721 , p = .001), **L313** (χ^2 [4] = 25.729, p = .001), **L324** (χ^2 [4] = 27.332, p = .000), **R114** (χ^2 [4] = 23.829 , p = .000) and **R225** (χ^2 [4] = 23.030 , p = .000). Post hoc analysis examining pairwise comparisons revealed that were no statistically significant differences in endophytic bacterial growth between the control with no EPN (bacteria only) and the treatment plates with EPN for the bacterial isolates *P. agglomerans* S222, *P. agglomerans* R114 and *Xanthomonas* sp. L330. The percentage difference between the control and treatment plates was < 18 % for all bacterial and nematode combinations, with the exception of the two *S. feltiae* strains. Significant values detected following post hoc analysis are shown below in Table 4.3.3. The growth of *P. amnigenus* R225 was significantly reduced by 18.18 % and 17.52 % in the presence of *S. feltiae* SB 12(1) and *H. bacteriophora*, respectively.

Table 4.3.3 Bacterial and nematode significant interactions. Pairwise comparisons were detected following data analysis using a Kruskal-Wallis H test. Asymptotic (2-sided tests) significant values for individual comparisons between *a* and *b* are displayed.

	Pairwise Comparison				
Bacteria	***a***	***b***	**df**	**Test statistic**	***p* value**
F113	No EPN (control)	*S. carpocapsae*	4	20.42	0.001
		S. feltiae SB 12(1)	4	14.25	0.047
S118	No EPN (control)	*S. carpocapsae*	4	18.17	0.003
L23	No EPN (control)	*S. carpocapsae*	4	24.00	0.000
L228	No EPN (control)	*S. feltiae e-nema*	4	18.00	0.004
L313	No EPN (control)	*H. bacteriophora*	4	17.33	0.006
L324	No EPN (control)	*H. bacteriophora*	4	-18.00	0.004
		S. carpocapsae	4	-24.00	0.000
R225	No EPN (control)	*S. feltiae* SB 12(1)	4	16.75	0.010
		S. carpocapsae	4	16.42	0.012
S19	No EPN (control)	*S. carpocapsae*	4	17.42	0.006

Both nematode and bacterial, species and strain interactions were unpredictable. It was noted during plate examination that nematode sinusoidal trails were present as a result of the IJ moving through bacterial colonies. These were evident as small looping threads, originating from the bacterial colony, that map the path the nematode had travelled. The effects of EPN on bacterial growth were measured by counting viable colonies however, this was an issue that was readily overcome as the bacterial colonies were still easily distinguishable.

There were great variations not only between bacterial species but also within different strains of the same species; this was of particular note for the *Pseudomonas fluorescens* isolates F113, S118, L228, L313 and L324. A reduction in all bacterial growth was recorded for in the presence of at least one EPN examined, with the exception of L324. Here, in the

presence of *H. bacteriophora* and *S. carpocapsae* bacterial growth increased 39 % and 88 %, respectively.

4.3.3 An investigation of the effects of *S. feltiae* SB 12(1) and *H. bacteriophora* (e-nema) on PGP bacterial plant colonisation and plant growth promotion in oilseed rape

Bacterial plant colonisation (by F113 *gfp* and L321 *gfp*) and subsequent PGP in OSR was examined in the presence and absence of the EPN *S. feltiae* SB 12(1) and *H. bacteriophora* (individually) and as a combination of the two nematode species. OSR seeds were coated with one of the *gfp* labelled bacterial strains and sown 2 cm below soil surface. EPN treatments were added to each pot at a density of 10 IJ g^{-1} three days post sowing. There were two control treatments, control treatment (1) seeds were coated with bacteria and inoculated with H_2O only (no EPN) and (2) seeds were coated with sterile H_2O and inoculated with H_2O only (no endophyte inoculum and no EPN). Replication was six-fold per treatment with one plant per pot. Plants were grown in a greenhouse for 12 weeks until they reached flowering, with a re-inoculation of endophytic bacteria and EPN after 6 weeks growth.

4.3.3.1 Plant harvest and OSR biomass analysis

Plant biomass was recorded immediately after harvest to determine the fresh weight. The root/rhizosphere system was rinsed with H_2O and plants were air dried on paper towels for 30 minutes prior to recording fresh weight. Plants were dried at 60°C for 48 hr in aluminium trays to determine the dry weight. Results are presented below in Figure 4.3.12 and Figure 4.3.13.

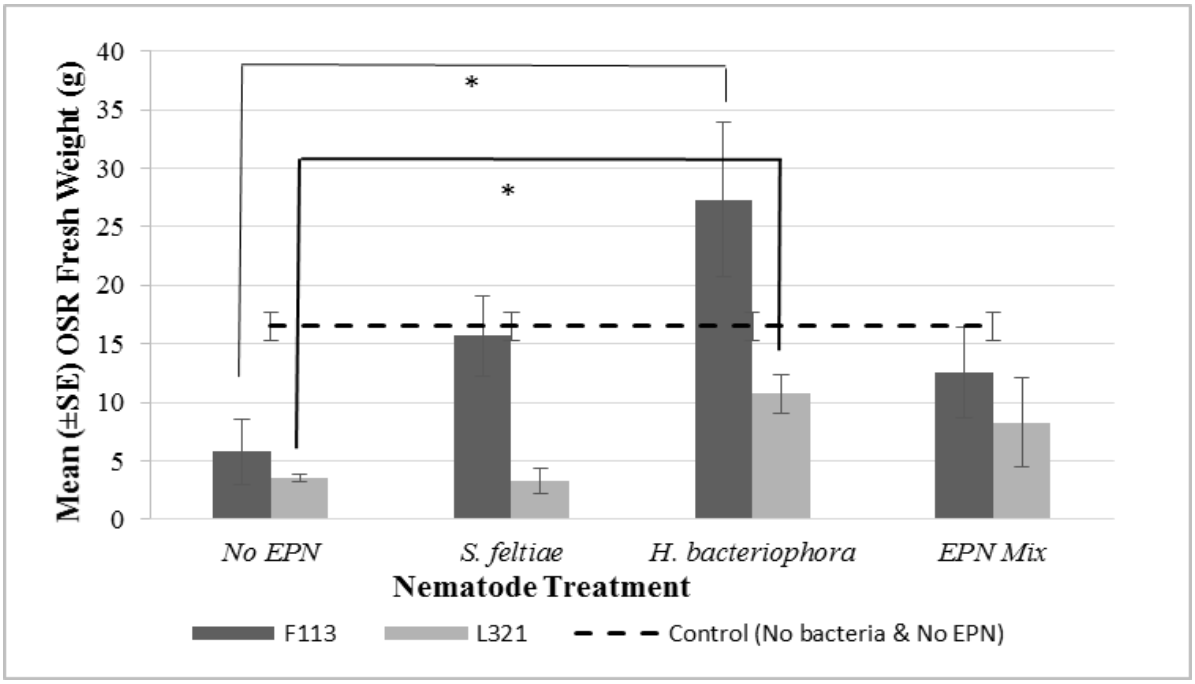

Figure 4.3.12 Mean (±SE) OSR fresh weight following growth in the presence of EPN and bacterial endophytes. The asterisk (*) indicates that samples were significantly different from control with no nematodes. The black dotted line indicates the control with No EPN and no bacteria.

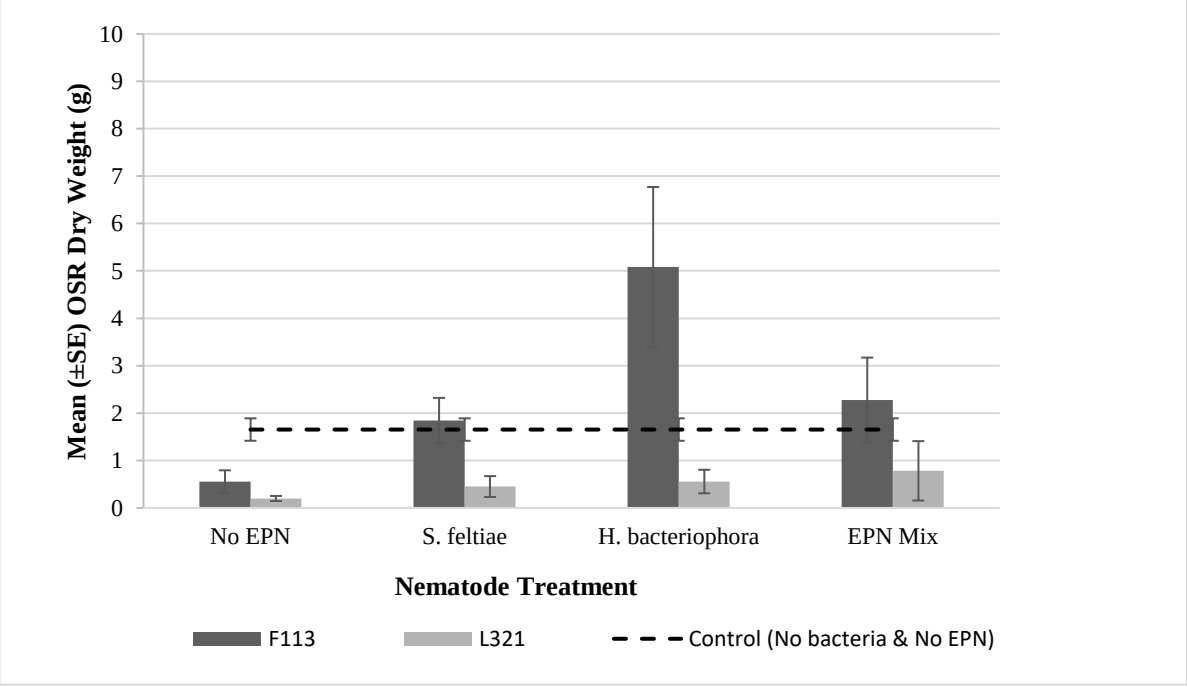

Figure 4.3.13 Mean (±SE) OSR dry weight following growth in the presence of EPN and bacterial endophytes. The black dotted line indicates the control with No EPN and no bacteria.

A Kruskal-Wallis H test was conducted to determine if there were significant differences in plant biomass in the presence and absence of the EPN. Plants were inoculated with two individual bacterial endophytes strains (F113 and L321). The distributions of plant biomass scores in the presence and absence of EPN were statistically significant for fresh weight samples in the presence of *H. bacteriophora* and F113 (χ^2 [3] = -11.417, p = .031) and *H. bacteriophora* and L321 (χ^2 [3] = -11.167, p = .037), when compared to the control with no EPN. No significant differences were recorded for dry weight samples.

4.3.3.2 Bacterial colonisation of OSR in the presence and absence of EPN

Bacterial colonisation of OSR was determined *via* serial dilution of surface sterilised plant extract samples, with subsequent plate counts, on SGA media containing kanamycin. Confirmation of colonisation was determined using *gfp* specific primers and the PCR. Results are presented below in Figure 4.3.14 and Figure 4.3.15.

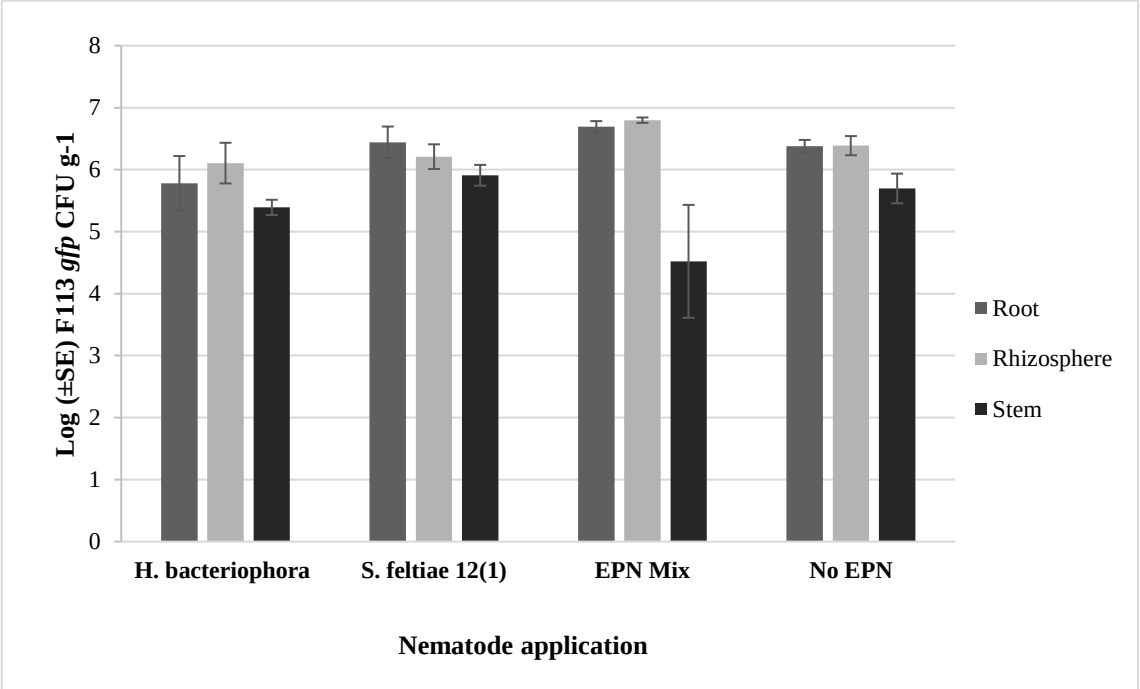

Figure 4.3.14 *P. fluorescens* F113 (±SE) *gfp* colonisation of OSR root, rhizosphere and stem when grown in the presence and absence of EPN.

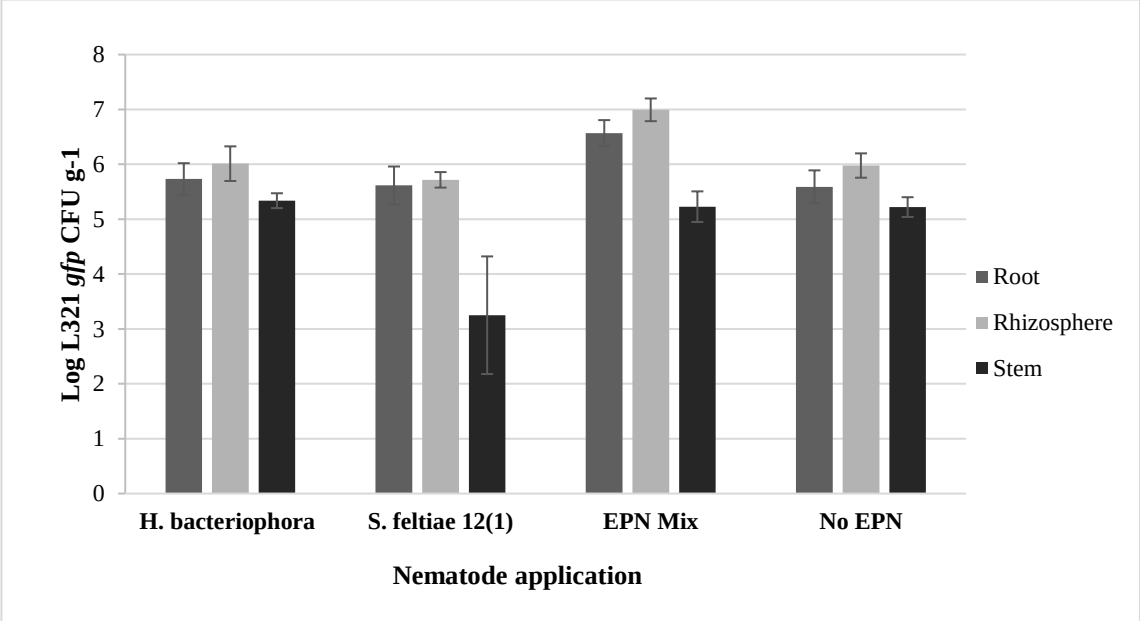

Figure 4.3.15 *P. fluorescens* L321 *gfp* (±SE) colonisation of OSR root, rhizosphere and stem when grown in the presence and absence of EPN.

A Kruskal-Wallis H test was conducted to determine if there were significant differences in bacterial colonisation in OSR root, rhizosphere or stem by F113 and L321 *gfp* in the presence and absence of the EPN. There was no growth on plates from the control treatment pots with no *gfp:kan* labelled bacteria added. The distributions of Log CFU/ml scores in the presence and absence of EPN did not statistically different ($p > 0.05$) for any of the plant samples inoculated with F113, root (χ^2 [3] = 4.713, $p = 0.186$), rhizosphere (χ^2 [3] = 6.465, $p = 0.091$) and stem (χ^2 [3] = 5.744, $p = 0.125$). Similarly, no significant differences were detected in colonisation between presence and absence of EPN in root (χ^2 [3] = 4.764, $p = 0.190$) and stem (χ^2 [3] = 4.329, $p = 0.228$) samples inoculated with L321. However, the distribution of L321 (Log CFU/ml) in the plant rhizosphere was not the same across all EPN samples analysed (χ^2 [3] = 11.022, $p = 0.012$), as shown in Figure 4.3.15. However, post hoc analysis revealed that bacterial concentrations did not differ significantly from the control.

4.3.3.3 Molecular confirmation of bacterial colonisation of OSR by *gfp* labelled *P. fluorescens* F113 and L321

To confirm colonisation of OSR by *gfp* labelled *P. fluorescens* F113 or L321 molecular identification was carried out via the PCR using *gfp* specific primers. Successful amplification of the *gfp* gene, using the reaction conditions and primer set outlined in Section 4.2.3.1.1, was recorded for root, rhizosphere and phyllosphere samples inoculated with F113 and L321 *gfp*. The gel in Figure 4.3.16 displays an example of amplification of a 135bp product from OSR root samples. No amplification was recorded in the control treatment samples with *gfp* labelled bacteria, moreover, there was no amplification in the PCR negative controls.

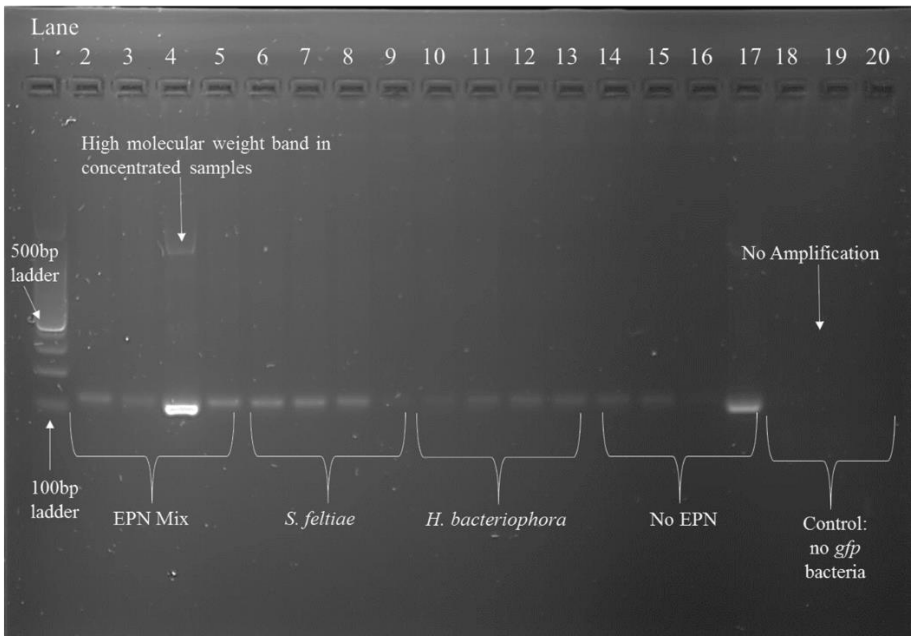

Figure 4.3.16 A 2% agarose gel, run at 70 V for 90 minutes in 1 X TAE, to confirm *gfp* colonisation of OSR roots grown in the presence and absence of EPN IJ. Primers amplified a 135p product and the ladder in lane 1 had a 1000 – 100 bp range. Lanes 2,3,6,7,10,11,14 and 15 are from F113 *gfp* root samples and lanes 4,5,8,9,12,13,16 and 17 are from L321 *gfp* root samples. Lanes 9, 10 and 16 have a band present but show poor amplification. Lane 18-20 are control root, rhizosphere and phyllosphere samples, no amplification was detected.

This can further confirm that colonisation of the *gfp* bacterial isolates occurred in the presence and absence of EPN. In addition, the primer set used in this study showed good specificity for the *gfp* gene, has a small amplicon product and minimal primer dimers thus was deemed appropriate to be utilised for a further qPCR experiment.

4.3.3.4 EPN survival in soil following OSR harvesting

The survival of EPN in pots was determined by adding *G. mellonella* to the remaining soil following plant harvest, recording insect mortality and subsequently visually inspecting for IJ emergence on White traps. The insect mortality results are presented in Figure 4.3.17.

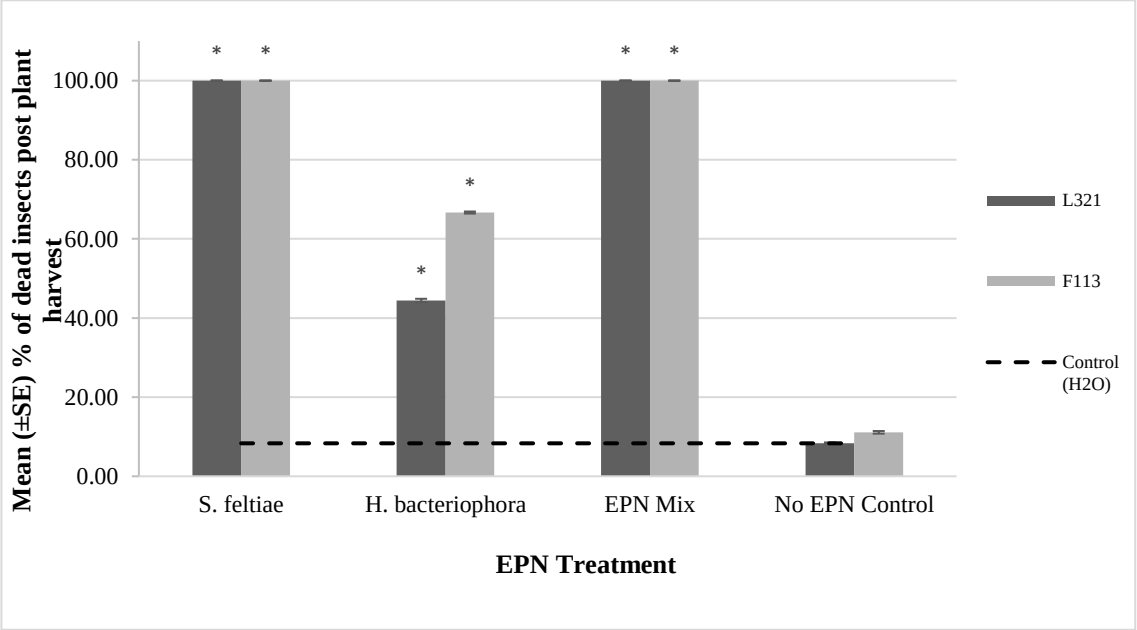

Figure 4.3.17 Mean percentage of *G. mellonella* mortality following insect incubation in soil used for OSR growth. Bars marked with an asterisk (*) were significantly different than the respective bacterial control with no EPN. The black dotted line shows the insect mortality following incubation in Control pots with no Bacterial endophytes and no EPN.

A one-way between groups ANOVA was conducted to explore the effect of bacterial endophytes on the ability of EPN to re-infect insects following bacterial exposure in soil and plant harvesting. The black dotted line in the Figure 4.3.17 shows the percentage of dead insects in the six control pots that had no EPN and no bacterial endophytes. There were no notable differences in the percentage of insect mortality in the control treatment with no bacteria and no EPN and the control treatment with bacterial endophytes and no EPN. This indicates that the bacterial endophytes F113 or L321 did not cause *Galleria* mortality.

Insects incubated in soil inoculated with bacteria (L321 *gfp* or F113 *gfp*) in the absence of EPN were used as the control to determine the effect of bacterial exposure on the ability of EPN to infect *Galleria* larvae following plant growth and harvest. There were statistically significant differences between the control treatment (with bacteria [L321 or F113] and no EPN) and the treatments of both F113 (F (3,20) = 142.50, p = 0.000) and L321 (F (3,20) = 127.927, p = 0.000) with EPN as determined by a one-way ANOVA. A Bonferroni post hoc test revealed that the percentage of insects dead in pots inoculated with EPN were significantly greater ($p < 0.05$) than those in the absence of EPN. Means, standard errors and significance values are reported in Table 4.3.4.

Table 4.3.4 Mean and Significance values for the percentage of infected insects in soil used for oilseed rape growth.

Bacterial Treatment	EPN Treatment	Mean		Sig.
		Statistic	Std. Error	
Control (No bacteria)	**No EPN**	8.33	3.72678	--
F113	**No EPN**	11.11	5.55	0.00
	***S. feltiae* SB 12(1)**	100.00	0.00	0.00
	H. bacteriophora	66.66	4.30	0.00
	EPN mix	100.00	0.000	0.000
L321	**No EPN**	8.33	3.72	0.00
	***S. feltiae* SB 12(1)**	100.00	0.00	0.00
	H. bacteriophora	44.44	7.02	0.00
	EPN mix	100.00	0.00	0.00

After visually assessing mortality, the six insects from each plant pot were placed onto White traps to determine if nematode emergence occurred. IJ emergence was checked daily for 19 days, but the number of emerged IJ was not determined. After 12 days IJ had emerged from larvae exposed to soil inoculated with *S. feltiae* 12(1) SB IJ. The EPN mix larvae all showed emergence after 14 days; these infected insects were dark brown/black in colour. This would indicate that they were infected with higher numbers *S. feltiae* 12(1) SB rather than *H. bacteriophora*. Insects exposed to *H. bacteriophora* emerged after 19 days. In addition, the survival of the resulting emerged IJ was visually examined using a stereoscope, three days after initial emergence. Recovered EPN showed no signs of negative effects from bacterial exposure as IJ were freely moving in the suspension.

4.3.4 Endophytic bacterial colonisation of entomopathogenic nematodes infective juveniles and bacterial persistence post insect infection

Experiments were conducted to examine the bacterial colonisation of nematodes and the persistence of the *gfp* labelled bacteria after nematode infection and insect mortality had occurred.

4.3.4.1 *Pseudomonas fluorescens* F113 gfp colonisation of S. feltiae SB 12(1) and H. bacteriophora

The ability of *P. fluorescens* F113 *gfp* to internally colonise EPN IJ was examined after direct nematode exposure to a bacterial lawn on agar. Colonisation was determined visually by epifluorescent microscopy and at a molecular level by qPCR.

4.3.4.1.1 Examination of internal colonisation via epifluorescent microscopy

H. bacteriophora IJ were difficult to remove from agar plates as they had burrowed down into the media. Therefore, a sterile plastic spreader was used to remove a layer from the surface of the media. The IJ suspension (including some agar and bacterial lawn) was washed off the plates within 45-50 ml sterile dH_2O. The agar was removed by allowing it to settle at the bottom of 50 ml centrifuge tube, and the supernatant (containing IJ and bacteria) was decanted off and passed through a 40 µm stainless steel sieve. The IJ were washed off the sieve with sterile dH_2O and retained in a 50 ml centrifuge tube. IJ were examined for internal colonisation prior to and after surface sterilisation. Results on *H. bacteriophora* and *S. feltia*e 12 (1) SB colonisation by F113 *gfp* are presented in Figure 4.3.18 and Figures 4.3.19.

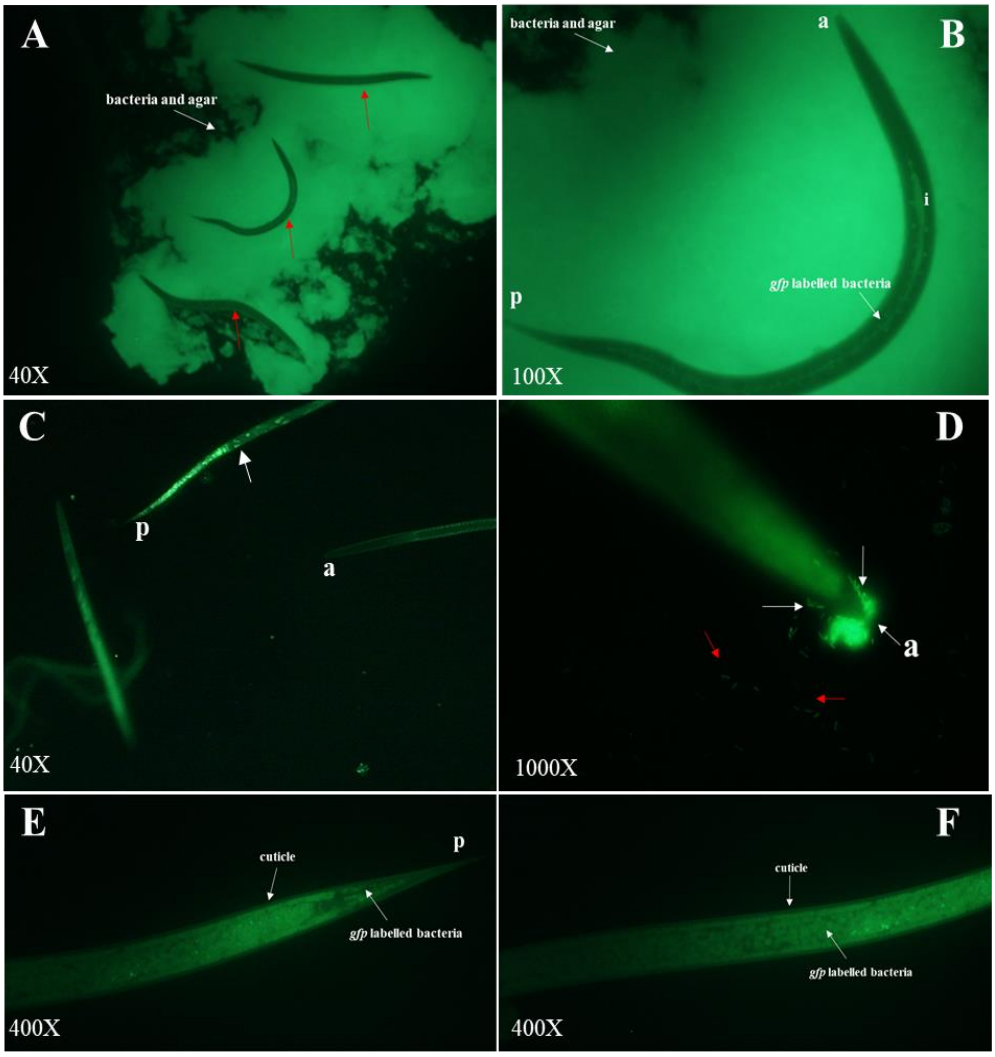

Figure 4.3.18 *H. bacteriophora* IJ following 24 hr exposure to *P. fluorescens* F113 *gfp* on SGA agar. Images were visualised on a Nikon Eclipse microscope with a fluorescent filter. A, B and D were captured prior to nematode surface sterilisation. C, E and F were captured post surface sterilisation with $HgCl_2$ and show internal colonisation. Figure A displays three IJ (red arrows) surrounded by a dense bacterial growth (white arrow). B shows an IJ with visible internal colonisation in the intestine. Figure D red arrows show F113 rods outside IJ and white arrows show dense bacterial population around the nematode anterior. a = anterior, p = posterior and i = intestine.

H. bacteriophora proved very difficult to remove from the agar plates and retained a substantial population of bacteria on its surface as a result; this can be seen in Figure 4.3.18 A and B. However, with additional wash steps it was possible to surface sterilise the IJ. Surface sterilisation was verified by plating an aliquot of the surface sterilised suspension

onto NA and SGA (with kanamycin) and incubating plates for 24 hr. No growth was observed on these plates. Nematodes shown in both Figure 4.3.18 and Figure 4.3.19 were visualised after 24 hr, but colonisation was visible at all time points examined.

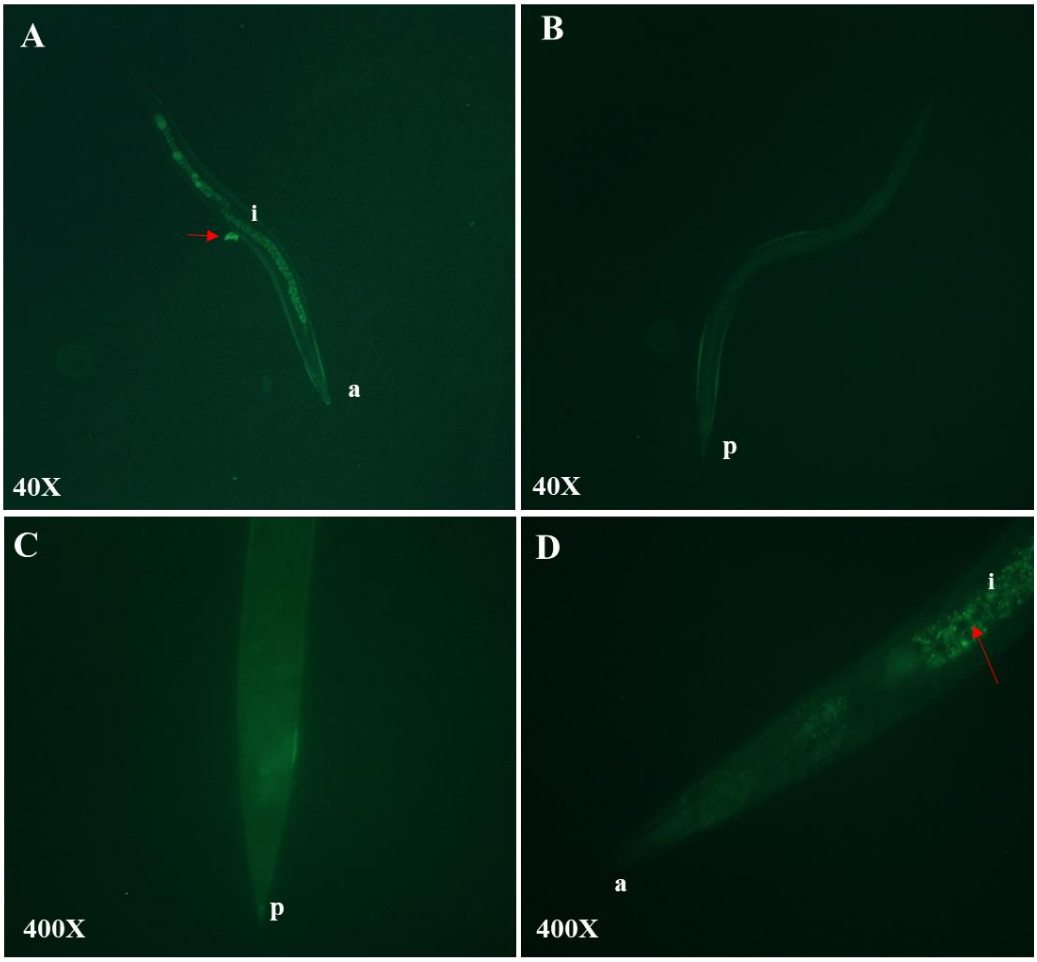

Figure 4.3.19 *S. feltiae* 12(1) SB IJ following 24 hr exposure to *P. fluorescens* F113 *gfp* on SGA agar. Images were visualised on a Nikon Eclipse microscope with a fluorescent filter. Figure A was captured prior to nematode surface sterilisation and B - D were captured following surface sterilisation with $HgCl_2$ and show internal colonisation. The red arrows in Figure A displays a dense bacterial population outside the IJ cuticle. Figure C shows the nematode tail and D head. a = anterior, p = posterior and i = intestine.

Washing nematodes from plates and surface sterilising made maintaining high IJ numbers for subsequent qPCR difficult. For subsequent experiments, IJ were exposed to bacterial cultures in liquid solutions in order to prevent these problems from occurring.

Figure 4.3.18 D shows a dense bacterial population around the IJ head and this was observed numerous times during the experiment. While IJ are a non-feeding stage, nematode movement and behaviour was observed over the 48 hr exposure time and there is insufficient evidence to suggest that nematodes developed into the J3 or J4 stage *in vitro* by feeding on the F113 bacterial lawn.

4.3.4.1.2 qPCR and calculating copy numbers

The bacterial standard curve presented in Figure 4.3.20 was constructed in Microsoft Excel 2013 and displays concentrations determined following qPCR amplification. The standard curves were constructed so that each Cq value recorded by the Roche LightCycler 480 corresponds to the copy number (CN) in the 20 µl qPCR reaction. Here, the CN refers to the *gfp* gene concentration.

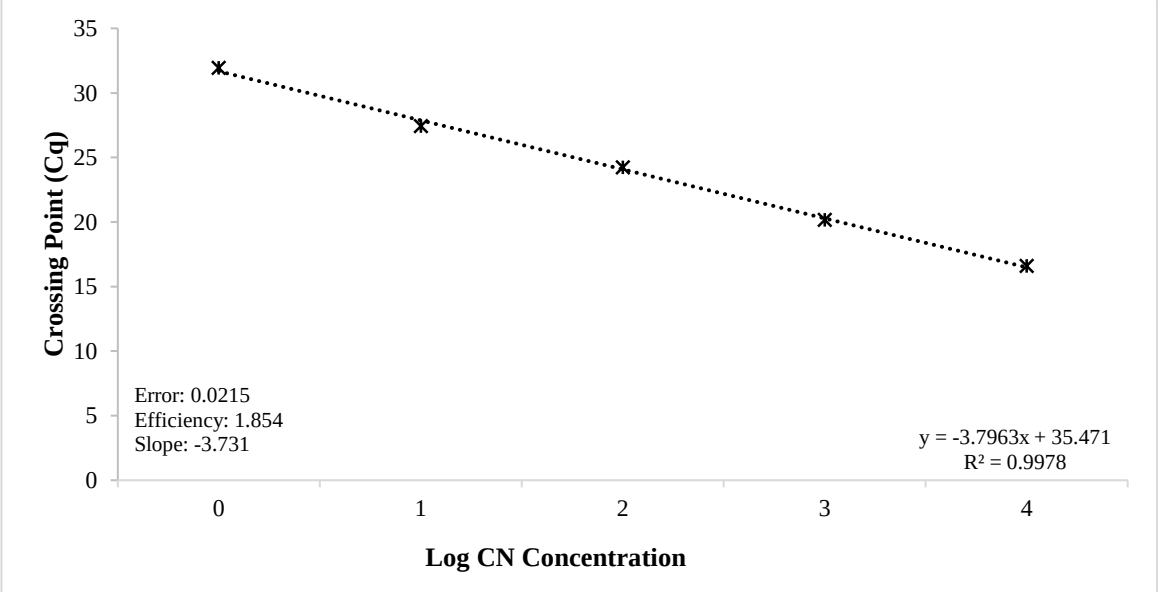

Figure 4.3.20 *P. fluorescens* F113 *gfp* qPCR standard curve. Concentrations are log copy number (CN) μl^{-1}. The standard curve was imported into each experimental reaction on the LightCycler 480 software and the unknown sample concentrations were calculated from these known CN values.

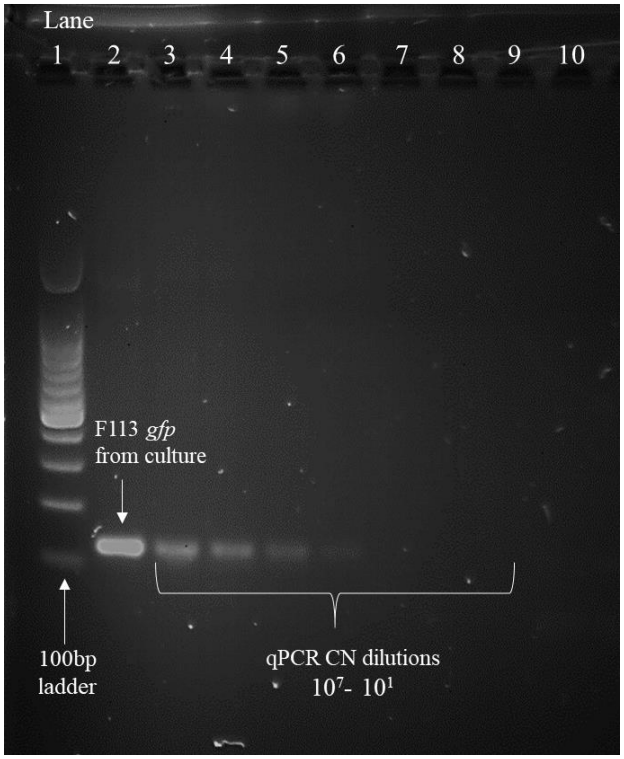

Figure 4.3.21 Agarose gel displaying qPCR standard curve products. Primers amplified a 135p product and the ladder in lane 1 had a 1000 – 100 bp range. Lane 2 displays DNA extracted from an F113 gfp overnight culture as a positive control. Lanes 3-7 contain 15 µl of qPCR product from 10^7 – 10^1 CN dilutions, a very faint band was detected for the 10^4 dilutions, however, no bands were visualised for 10^3, 10^2 or 10 CN dilutions. Reaction conditions are outlined in Section 4.2.4.1.3.

Using an internal standard in each reaction, the standard curve was used to calculate the concentration per 20 µl qPCR reaction for each unknown sample, based on the assumption that there was two copies of the *gfp* gene. These concentration values were then back calculated to account for sample dilutions in the extraction of RNA, DNase digestion, generation of cDNA and qPCR reaction. An example of this calculation is shown below:

CN / 50 IJ suspension = (Extrapolated sample concentration) x (qPCR df) x (Original RNA concentration) x (cDNA df) x (DNase df)

- The extrapolated sample concentration was calculated by the LightCycler 480 software using the previously generated standard curve
- qPCR dilution factor was 10 as 2 µl of the template was used per 20 µl reaction
- Original RNA concentration in ng/µl

- DNase dilution factor was 2 as 5 µl of RNA template was used per 10 µl reaction
- cDNA dilution factor was 4 as 5 µl of DNase treated RNA was used per 20 µl reaction
- Two copies of the *gfp* gene in the transposon thus divide by 2

Calculated example: Following the qPCR cycle the extrapolated sample concentration was calculated from the standard curve as 391 CN μl^{-1} and the original RNA concentration was 31.90 ng/µl. To prevent DNA contamination in the PCR, a DNase reaction was carried out using 5 µl of RNA in a 10 µl reaction volume. 5 µl of this (DNA free) RNA was converted to cDNA and 2 µl of cDNA was used in the 20 µl qPCR reaction.

$$\text{CN / 50 IJ suspension} = \frac{(391) \times (10) \times (31.90) \times (4) \times (2)}{2}$$

$$\text{CN / 50 IJ suspension} = 4.9 \times 10^5$$

During optimisation of the qPCR procedure and generation of the standard curve, CN concentrations of 10^7 and 10^6 were omitted due to late cycle amplification in the more concentrated samples. 10^3-10^1 CN concentrations were not visualised on the gel in Figure 4.3.21 but due to the sensitivity of the instrument, it was possible to amplify down to 10^1 CN during the qPCR cycle as shown in Figure 4.3.22 (c).

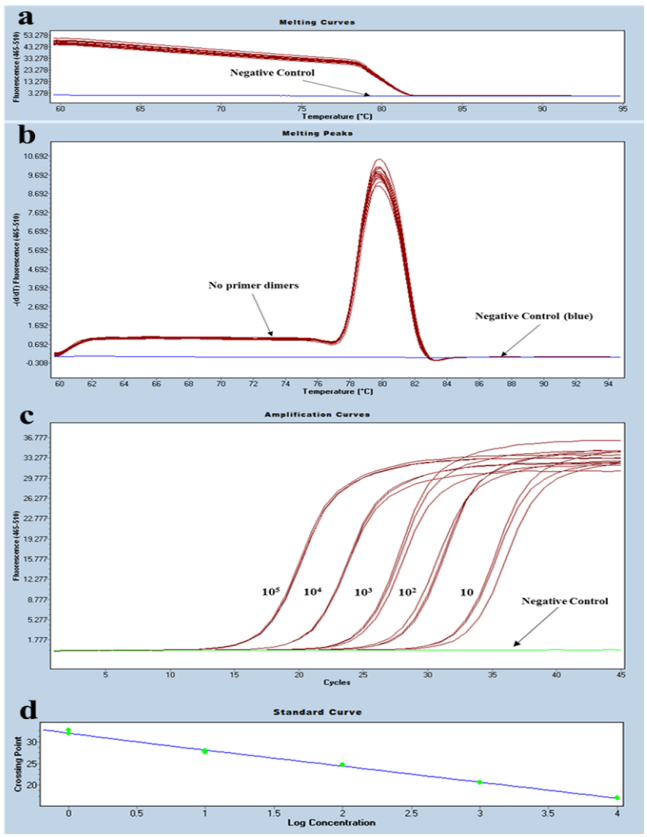

Figure 4.3.22 *P. fluorescens* F113 *gfp* qPCR standard curve quantification generated by the Roche LightCycler ® 480 Software 1.5.1.62, following reaction and thermocycler conditions outlined in Section 4.2.4.1.3. Figures a and b display the melt curve and melt peaks respectively, with no primer dimers and no amplification in the negative control. The Tm values for the melt peaks was between 79.1 and 80.5 ˚C. Figure c shows the amplification curves and concentrations are Log CN μl^{-1}. Figure d displays the standard curve generated from the above Log CN concentration dilutions; here cq is plotted against the Log CN μl^{-1}. This standard curve was used to extrapolate the CN from the cq values recorded for the treatment samples (see Section 4.2.4.1.4 for more detail). An internal standard of 10^3 CN was used in each run to correlate with the standard curve shown in Figure 4.3.22.

Figure 4.3.23 displays an example of the amplification curves and melting peaks for treatment samples *S. feltiae* 12(1), *H. bacteriophora* and the *S. feltiae* 12(1), control treatment with no F113 *gfp* bacteria. For the treatment samples, the amplification occurs later in the qPCR process at approximately cycle 30. From the standard amplification curve shown in Figure 4.3.22 (c) visually this correlates to an approximate CN concentration 10^1. Exact concentrations were calculated from the standard curve and back-calculated to give the concentration per 50 IJ (approx).

There was a slight shift in the Tm of the treatment samples, however, the melt peaks still fall between 79.1 and 80.5 ˚C as shown in Figure 4.3.23 (b and d).

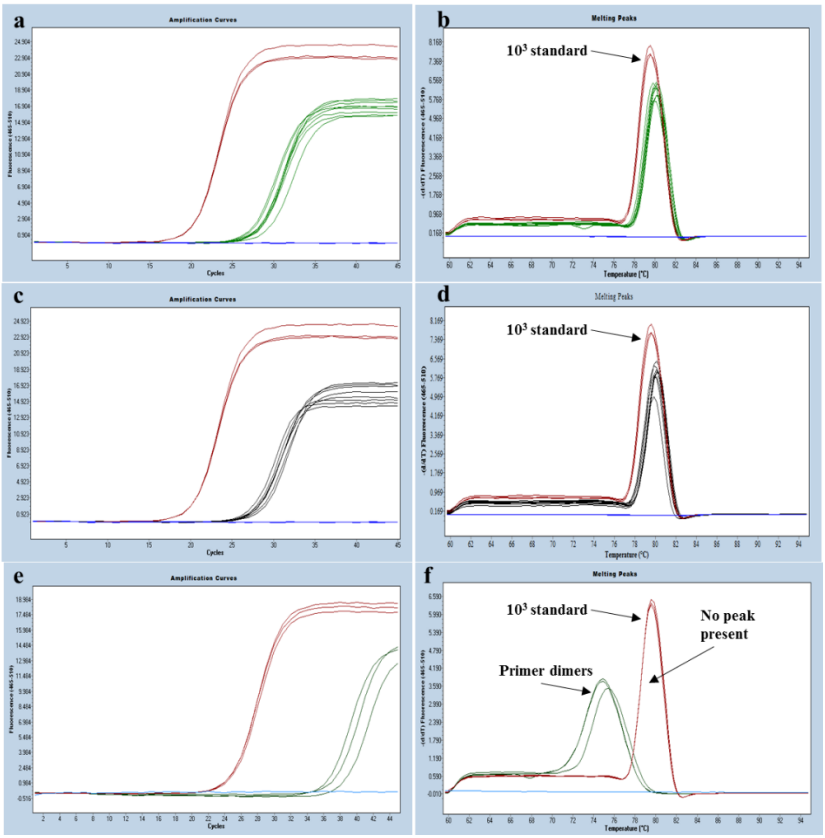

Figure 4.3.23 Displays an example of the amplification curves and melt peaks recorded from cDNA templates for treatment samples *S. feltiae* 12(1), *H. bacteriophora* and the control with no *gfp* bacteria. The red lines represent the 10^3 CN internal standard and blue lines represent the negative control in all figures above. (a and b) displays cDNA from *S feltiae* 12(1) SB following 6, 12 and 24hr exposure F113 *gfp* exposure. (c and d) displays cDNA from *H. bacteriophora* (black lines) following 6, 12 and 24hr F113 *gfp* exposure. (e and f) displays cDNA from Control *S. feltiae* treatment that was not exposed to F113 *gfp.*

Control treatment samples did not result in a peak between 79.1 and 84 ˚C as in the internal standard or F113 treatments. However, other peaks were visible in these samples and an example of such is shown in Figure 4.3.23 (f). Here, peaks were detected between 74 and 76 ˚C, and when the template was run on a gel these bands appeared as primer dimers.

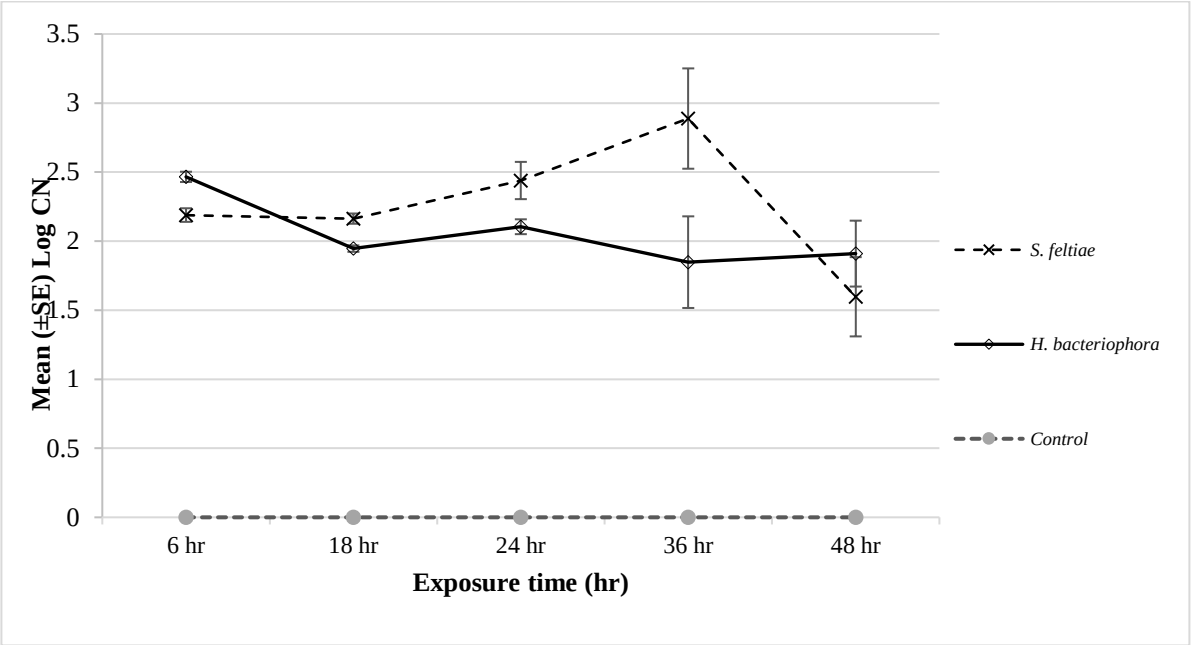

Figure 4.3.24 Displays the Mean (±SE) Log *gfp* gene CN following *S. feltiae* SB 12(1) and *H. bacteriophora* exposure to *P. fluorescens* F113 over various time periods.

A Kruskal-Wallis H test was run to determine if there were significant differences ($p<0.05$) in the distributions of the *gfp* gene CN recovered between *S. feltiae* and *H. bacteriophora* IJ exposed to *gfp* labelled F113 at each exposure time. There were no significant differences in the bacterial concentrations recovered from *S. feltiae* and *H. bacteriophora* after 6 hr (p = 0.111 ±3.672), 18 hr (p = 0.082 ± 3.672), 24 hr (p = 0.789 ± 3.672), 36 hr (p = 0.106 ± 2.105) or 48 hr (p = 0.877 ±3.562). *P. fluorescens* F113 colonisation was examined visually, *via* epifluorescent microscopy, and verified at a molecular level by qPCR thus far. The fate of these colonising microbes following insect infection was examined further in this Chapter.

4.3.4.1.3 Endophytic bacterial persistence post insect infection

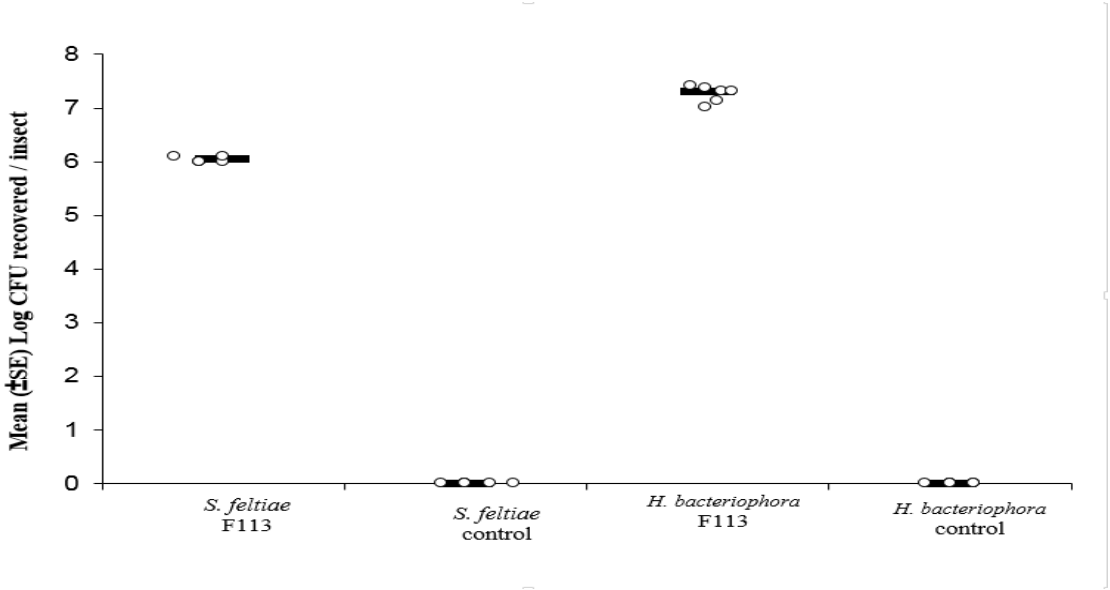

Figure 4.3.25 Displays the mean log (±SE*) P. fluorescens* F113 *gfp* viable colonies recovered per *Galleria mellonella* insect following infection with *S. feltiae* SB 12(1) and *H. bacteriophora* that were exposed to the bacterium.

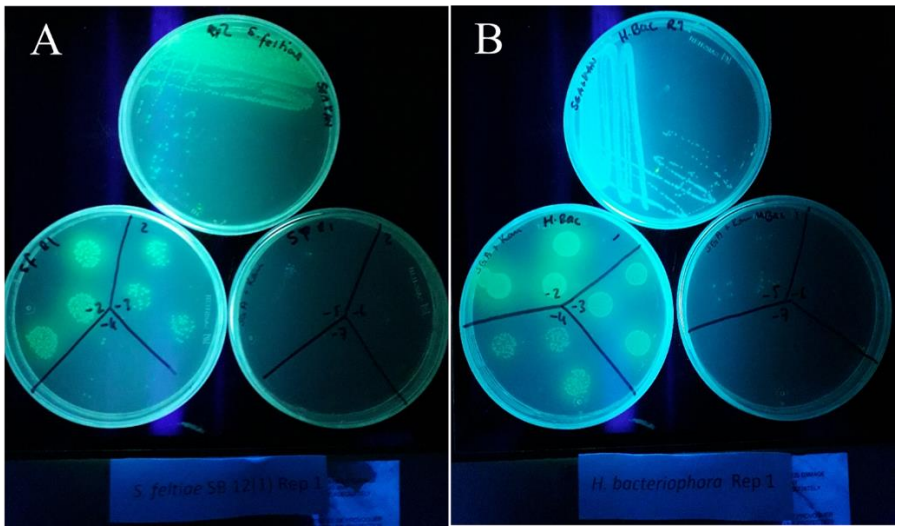

Figure 4.3.26 *P. fluorescens* F113 *gfp* growth on SGA (with Kanamycin). The bacterium was isolated from *G. mellonella* larvae infected with *S. feltiae* and *H. bacteriophora* that were exposed to the *gfp* labelled bacterium prior to infection. Figure **A)** displays fluorescent bacterium isolated from insects infected with *S. feltiae* SB 12(1) and **B)** displays fluorescent bacterium isolated from insects infected with *H. bacteriophora.*

The experimental process of exposing IJ to bacteria in liquid media was adopted as it is a more robust method of recovering high numbers of nematodes in an efficient manner. Following 48 hr incubation, fluorescent colonies were detected on SGA agar, with and without the selective agent kanamycin. This would indicate that following regurgitation of the nematodes symbiotic bacteria during insect infection, F113 can survive. The number of viable colonies was enumerated following serial dilution of the infected insect haemolymph. These results are presented in Figure 4.3.25. The mean F113 *gfp* CFU recovered / insect was 1.08 X 10^6 CFU for *S. feltiae* SB 12(1) and 1.88 X 10^7 CFU for *H. bacteriophora*. Figure 4.3.26 shows fluorescent colonies as pictures under UV light. A selection of colonies was also examined for fluorescence using the epifluorescent microscope. All colonies examined were formed by fluorescent rods corresponding to F113 *gfp*.

4.4 Discussion

Experiments carried out in Chapter 2 and 3 focus predominately on the effects of bacterial isolates on the survival, biology and behaviour of EPN in order to establish the compatibility of these two important taxa, for insect biocontrol and PGP. The objectives set out in Chapter 4 were to examine the presence of insecticidal genes in the genomes of *P. fluorescens* F113, L321, L228 and L111 and also determine whether *P. fluorescens* F113 could persist in insects following infection with EPN IJ. In addition, the effects of EPN on bacterial growth and bacterial colonisation of OSR were also investigated.

Genome sequencing of L321, L228 and L111 indicated the presence of PGP and biocontrol genes (Lally, 2016; Otieno, 2014). In terms of PGP, genes associated with phosphate solubilisation including homologous glucose dehydrogenase (*gcd*), gluconate dehydrogenase (*gad*), pyrrolo-quinoline quinone (PQQ) and two alkaline phosphatases *pho*D (3-phytase *phy*C and acid phosphatase *acp*A) are present in the genomes of L321, L228 and L111. They also produce a siderophore called pyoverdine, which is secreted into the extracellular environment, helping bind available iron making it less available to pathogens and other organisms (Radzki *et al.*, 2013). Genes associated with chemotaxis mechanisms are present in L321 (*aer*, *che*D), L228 (*aer*) and L111 (*aer*, *che*D). The three stains also contain the genes for flagella pilus assembly (*fli*GMND) and motility (*mot*ABD). Motility and chemotaxis are advantageous for soil bacteria as they enable them to detect and move towards the nutrient-rich plant exudates (Bais *et al.*, 2006).

Insecticidal toxin production by a number of bacterial species has been reported and a number of associated genes identified (Bowen *et al.*, 1998; Ffrench-Constant *et al.*, 2007; Loper *et al.*, 2012). Most notable is the FIT complex, produced by some pseudomonads (Péchy-Tarr

et al., 2008; Redondo-Nieto *et al.*, 2013) and the Mcf toxin, produced by EPN symbiotic bacteria (Ffrench-Constant & Dowling, 2014). These toxins can induce lethal effects to *Drosophila melanogaster, G. mellonella* and *Manduca sexta* (Ffrench-Constant *et al.*, 2017; Olcott *et al.*, 2010; Péchy-Tarr *et al.*, 2008). In Chapter 3, the effect of endophytic bacteria on the ability of EPN to cause insect host mortality was examined in both laboratory-based and greenhouse pot experiments. In these experiments, no significant insecticidal activity was recorded in the control treatments with endophytic bacteria only. The full gene complex required for FIT production is not present in the genome of *P. fluorescens* F113, L321, L228 or L111. This is in line with Péchy-Tarr *et al.* (2008) who found no association between the *fit* toxin genes and a number of pseudomonads, including *P. entomophilia*, *P. aeruginosa*, *P. putida* and *P. fluorescens*. The only notable exception was a limited association between L228 and the *fitF* gene of the FIT complex. *fitF* along with *fitG* and *fitH* are associated with regulation of the toxin complex (Péchy-Tarr *et al.*, 2008). *P. luminescens* and Mcf gene locus also had significant alignment similarities with *fitD*, and this is the ORF associated with the insecticidal capability of the FIT as has been previously reported (Péchy-Tarr *et al.*, 2008). Moreover, no Mcf homologous sequences were detected in the three endophytic bacterial strains. This bioinformatic analysis supports the laboratory and greenhouse experiments and would indicate that these isolates have no insecticidal capabilities against *G. mellonella*.

The effect of EPN on bacterial growth was examined in the presence and absence of the two *S. feltiae* stains, *S. carpocapsae* and *H. bacteriophora*. Eleven bacterial isolates were examined covering three different genera (*Pseudomonas, Pantoea* and *Xanthomonas*) and six different species. The soil ecosystem is extremely diverse and examining a wide range of isolates may contribute to increased predictability in integrated pest management and biofertiliser applications. Results presented in Section 4.3.1 show that there was great

variation not only between bacterial species but also within different strains of the same species.

None of the EPN examined affected the growth of isolates *Pantoea agglomerans* S222, *P. agglomerans* R114 or *Xanthomonas* sp. L330. The only significant increase in bacterial growth was recorded for L324 in the presence of *H. bacteriophora* (39.11 %) and *S. carpocapsae* (87.56 %). Intraspecies variation was evident among the *Pseudomonas fluorescens* isolates F113wt, S118, L228, L313 and L324. In the presence of *S. feltiae* SB 12(1), F113 growth was significantly reduced by 3.98 %, but no other *P. fluroescens* strain was significantly affected by this EPN strain. S118 growth was reduced 39.01 % in the presence of *S. carpocapsae*, L228 growth was significantly reduced 24.72 % in the presence of *S. feltiae* e-nema, and L313 growth was reduced 13.25 % in the presence of *H. bacteriophora*. The growth of two additional *Pseudomonas* strains, *P. trivialis* S19 and *P. veronii* L23 were also significantly affected by at least one EPN species. L23 was reduced 15.58 % and 38.94 % in the presence of *H. bacteriophora* and *S. carpocapsae*, respectively. Similarly, S19 growth was reduced 17.53 % in the presence of *S. carpocapsae.*

Examining the interactions between EPN and bacteria under these sterile conditions will give an indication of any immediate negative effects, however, the correlation between these laboratory-based experiments and interactions in the soil are not known. Although bacterial growth was reduced for a number of bacterial and nematode combinations, the overall percentage difference between the control and treatment plates was < 18 % for all bacterial and nematode combinations. Nematodes are known to carry bacteria on their cuticle (Knox *et al.*, 2004; Murfin *et al.*, 2012) and in their intestine (Gaugler & Kaya, 1990), in the case of EPN. The nematode sinusoidal trails visualised on the growth medium demonstrate the

close interactions these microbes have with fauna in the soil ecosystem and indicate the possible mechanism of transporting bacteria throughout the soil profile.

Increased *P. fluorescens* colonisation of wheat has been shown when plants were grown in the presence of free-living bacterial-feeding nematodes, *Acrobeloides* sp, *Cruznema* sp, and *C. elegans* (Knox *et al.*, 2003; Knox *et al.*, 2004). In order to examine if the close interactions between EPN and bacteria could result in increased plant colonisation, OSR was inoculated with *gfp* labelled F113 and L321 and exposed to *S. feltiae* SB 12(1), *H. bacteriophora* and a combination of the two nematodes. The internal bacterial colonisation of the plant rhizosphere, root and phyllosphere were determined following plant harvest. Here, no significant differences were recorded between the control plants with no EPN and the treatment plants with EPN. In addition to bacterial plate counts, colonisation of *gfp* labelled isolates was verified at a molecular level using *gfp* specific primers. Both L321 *gfp* and F113 *gfp* successfully colonised the root, stem and rhizosphere of OSR in the presence and absence of nematodes. However, a number of samples with strong amplifications/ high-intensity band (as in Figure 4.3.16, lane 4) were visualised. This may be due to higher DNA concentrations prior to PCR amplification. Normalising DNA concentrations prior to PCR may remove this problem in the future. The previously discussed laboratory experiment examining bacterial growth in the presence of EPN showed that the growth of a number of *P. fluorescens* strains was reduced in the presence of EPN. This included F113, which when grown in the presence of *S. feltiae* SB 12(1) growth was significantly reduced by 3.98%. Thus, it can be concluded that in a more natural dynamic soil environment EPN shows no negative effect on bacterial survival and plant colonisation. Plant biomass was also assessed to determine if the presence of EPN and bacterial endophytes resulted in increased PGP. When compared to the control with No EPN, the majority of bacterial and nematode

treatment combinations showed notable increases in plant fresh and dry weights. However, the only significant increases were recorded in the fresh weights of plants inoculated with a combination of F113 and *H. bacteriophora* and L321 and *H. bacteriophora*, compared to the control with bacteria and no EPN.

The fate of the EPN in the soil after plant harvest was not known, therefore to examine the survival of the EPN, *G. mellonella* insects were added to the pots after the OSR plants were removed. In Chapter 3 the infectivity of EPN following exposure to bacteria was examined in plants inoculated with bacteria (Section 3.2.3) and subsequently inoculated with EPN IJ; no significant effects on EPN infectivity were recorded. However, in that experiment, IJ were only exposed to bacteria for 7 days before *G. mellonella* insects were added to the pots. In the experiment discussed in Chapter 4, where EPN were incubated in the presence of bacteria for the completed OSR growth cycle, insect mortality was significantly greater in the pots inoculated with EPN than in the control with No EPN.

In addition to assessing insect mortality, nematode emergence was also examined. IJ emerged from larvae exposed to soil inoculated with *S. feltiae* 12(1) SB and the EPN mix, after 12 and 14 days, respectively. The EPN mix larvae were dark brown/black in colour. This would indicate that they were infected with higher numbers *S. feltiae* 12(1) SB rather than *H. bacteriophora*. A *Steinernema* species and *H. bacteriophora* cannot coexist when both species are inoculated directly into the haemocoel of an insect host. However, if inoculated separately, at different time points, they can co-infect with a subsequent increase in insect mortality compared to individual applications (Alatorre-Rosas & Kaya, 1991). Insects exposed to *H. bacteriophora* emerged after 19 days. This is in line with lab culturing experiments where *S. feltiae* 12(1) SB, infected, reproduced and emerged at a faster rate than *H. bacteriophora*. These results indicate that there were no long-term negative effects on

EPN behaviour in the soil, and moreover, the resulting IJ that emerged from insects showed good movement and survival when visually inspected in the IJ suspension.

The bacterial feeding nematode *Cephalobus* sp. was shown to promote *Arabidopsis* root growth when plants were inoculated with the IAA-producing rhizobacteria from *Pseudomonas* and *Burkholderia* sp. Jiangy *et al*. (2012) report that the increase in root growth may be due to the mineral nitrogen and that IAA were increased due to nematode grazing. Similar increases in tomato root growth in the presence of bacterial-feeding nematodes were reported by Xiaofang *et al*. (2006). As discussed above, bacteria adhering to the cuticle of the IJ may be an indication of the relationship between increased plant bacterial colonisation and growth promotion. To add further to knowledge in this area the ability of *gfp* labelled bacteria to internally colonise IJ and subsequently survive once the nematodes infect an insect was examined in this Chapter.

P. fluorescens F113 *gfp* successfully colonised *S. feltiae* 12(1) and *H. bacteriophora* IJ. Subsequent qPCR analysis of F113 *gfp* colonised nematodes showed that there was an initial decrease in bacterial concentration from the first time point of 6 hr to 18 hr for both EPN species. However, the concentration increased again over the next two time points of 24 hr and 36 hr bacterial exposure. Between 36 hr and 48 hr, there was another decrease in bacterial concentration for both EPN strains. Exposing IJ for longer periods of time would give more information as to whether this decline in concentration would continue with increased exposure. Moreover, it is essential to note that the gene copy numbers were calculated based on the assumption that there was not multiple insertion events and the miniTn5 transposon was only inserted once during the construction of the *gfp* labelled strains. Each miniTn5 transposon contains two copies of the *gfp* gene, thus gene copy numbers were calculated accordingly. The number of insertions has not been verified therefore, whole

genome sequencing or restriction digestion of the labelled bacterial strains would be required in order to confirm the total number of *gfp* gene copies in the genomes.

Symbiotic bacteria harboured in the intestine of EPN are regurgitated upon entering an insect, resulting in insect mortality. These bacteria not only act as a nematode food source but also produce compounds to inhibit the growth of competing microbes (Akhurst,1982; Chen *et al*., 1994). The antimicrobial activity of *P. fluorescens* F113 and L321 against the EPN symbionts *X. bovienii* and *P. luminescens* was presented in Chapter 2, where both bacterial isolates (F113 and L321) inhibited the growth of the endosymbionts. Here, *P. fluorescens* F113 *gfp* was successfully isolated from *G. mellonella* insects that were infected with *S. feltiae* 12(1) and *H. bacteriophora* IJ. The evaluation of both sets of results would indicate that there is some level of symbiosis between these organisms.

The overall trend in OSR plant biomass analysis was positive with increases in both dry and fresh plant weights, for plants inoculated with bacterial endophytes and EPN IJ. These results coupled with the results from the bacterial – IJ colonisation experiments and bacterial survival post nematode infection would indicate that a combination of both EPN and bacterial endophytes would be very beneficial for insect control and increased soil and plant health.

Chapter 5 General discussion and future directions

5.1 General Discussion

The work presented here is the first of its kind and was carried out to provide fundamental knowledge on the compatibility of a biopesticide, in the form of entomopathogenic nematodes (EPN), and a biofertiliser in the form of plant growth promoting (PGP) rhizospheric/endophytic bacteria. This study was based on the hypothesis that there is some level of basic fundamental interaction between the EPN and PGP bacteria, as both taxa naturally function in a common habitat in the soil and have widespread applications in biological control and PGP, respectively. This study provides the framework for the development of an environmentally sustainable, reliable, cost-effective multi-function product for biocontrol and PGP. Moreover, examining the effects of bacterial endophytes on EPN contributes to knowledge on the non-target application of endophytes for PGP.

Chapter 2 examined the susceptibility of the EPN (*Steinernema feltiae* SB 12[1], *S. feltiae* e-nema, *S.carpocapsae* and *Heterorhabditis. bacteriophora*) and their symbiotic bacteria (*Xenorhabdus. bovienii* and *Photorhabdus luminescens*) to bacterial endophytes from three different genera (*Pseudomonas, Pantoea* and *Xanthomonas*) using laboratory bioassays. These results were promising and formed the basis for the project going forward. Chapter 3 focused in detail, on the effects of bacterial endophytes on EPN biology and behaviour in

both laboratory bioassays and greenhouse-based pot trials. Chapter 4 dealt with the effects of nematodes on bacterial survival and plant colonisation.

Key Findings

Chapter 2

- *P. veronii* L23 was the most toxic bacterial strain to *S. feltiae* SB 12(1) resulting in a 41 % IJ mortality after 48 hr. However, this was the exception and the mean IJ mortality was approximately 10 % following bacterial exposure.
- *S. carpocapsae* was most susceptible nematode to DAPG, with 18% IJ mortality after 96 hrs exposure to 200 µg/ml DAPG.

Chapter 3

- *S. carpocapsae* virulence was affected by bacterial strains S118, L228 and L321.
- *H. bacteriophora* was the least virulent nematode but was not negatively affected by any bacterial strain and had increased virulence in the presence of F113 compared to the control.
- No bacterial strain negatively affected *S. feltiae* or *H. bacteriophora* progeny production. In addition, DAPG significantly increased *H. bacteriophora* nematode progeny in the three trails. F113 significantly increased *S. feltiae* SB 12(1) progeny production.
- Endophytic bacteria do not affect EPN attraction to or infection of *Galleria mellonella* in sand columns.
- *H. bacteriophora* shows strong directional movement towards *G. mellonella* in the presence of endophytic bacteria. In the absence of a host, *H. bacteriophora* IJ are attracted to endophytic bacteria.

Chapter 4

- Oilseed rape (OSR) fresh weight was significantly increased in the presence of *H. bacteriophora* and bacterial endophytes F113 *gfp* and L321 *gfp*.
- *P. fluorescens* F113 *gfp* successfully colonised *S. feltiae* SB 12(1) and *H. bacteriophora* IJ.
- *P. fluorescens* F113 *gfp* survived inside *G. mellonella* following IJ infection, thus appearing not to be sensitive to antimicrobial compounds produced by EPN endosymbionts.

Chapter 5

- EPN and bacterial endophytes are compatible and show potential as a combined agricultural product, for insect control and increased soil and plant health.

The current applications of chemical fertilisers and insecticides are unsustainable as they are detrimental to the environment. However, they are an essential element in agriculture production as their use not only improves productivity but also reliability. The overuse of common agri-chemicals results in ecological damage in the soil and also in freshwater ecosystems. In addition, the non-target effects of chemical fertiliser applications pose a significant threat to above and below ground ecosystems as well as human health (Camargo & Alonso, 2006). Increased public awareness on the negative effects of chemical input in the environment has driven improvements in governmental policy in relation to their use. This has also created traction in the development and improvement of novel insecticides and fertilisers that are sustainable, reliable, pest-specific, less persistent in the environment and cost-effective (Soberón *et al.*, 2016).

There are a number of processes that can be considered in order to facilitate a reduction in chemical applications. One such approach is the use of genetically engineered crops, for example, the Bt crops resistant to lepidopteran and coleopteran pests (Hellmich *et al.*, 2001; Oestergaard *et al.*, 2006). This has allowed for a reduction in insecticide applications. However, strict EU legislation regarding the use of genetically engineered crops coupled with the growing resistance of insects to the Bt toxins expressed in these crops reduces the suitability of this approach (Tabashnik *et al.*, 2013; Welch *et al.*, 2015). To overcome these restrictions while also reducing chemical inputs, innovative approaches must be examined, such as the introduction of various integrated pest management (IPM) strategies for sustainable and healthy food production. These IPM strategies focus on the implementation of precision agricultural technologies, biocontrol strategies and insect-specific chemical applications as a last resort. Precision agricultural technologies are applied in order to manage farmland in a site-specific manner and design farming practices based on factors such as the soil type, specific crop and varying weather patterns. Precision agriculture also takes account of factors including, profitability, economic efficiency, increased production, and the reduction in environmental harm (Allahyari *et al.*, 2016). The use of species-specific biopesticides and biofertilisers with specific modes-of-action is a key strategy in IPM, and these have the potential to reduce the non-target and long-term environmental effects of agri-chemical applications. The use of plant growth promoting (PGP) bacteria and entomopathogenic nematodes (EPN) individually, fits this criterion. The use of such naturally occurring soil organisms have the potential to eliminate the environmental consequences of continued chemical applications and their promise has been highlighted in a range of publications (Atwa, 2014; Compant *et al.*, 2010; Glick, 2010; Grewal *et al.*, 2005).

Prior to this study, there was very little knowledge about the interactions between EPN and non-pathogenic PGP bacteria. Previous research focused predominately on the effects of entomopathogenic fungi on EPN infectivity and on the control of plant parasitic nematodes (PPN) using rhizosphere and plant-associated bacteria. Shapiro-Ilan *et al.*, (2004) examined the effects of combinations of 1) EPN and entomopathogenic fungi (*Beauveria bassiana* and *Metarhizium anisopliae)* and 2) EPN and insect pathogenic bacterium (*Paecilomyces fumosoroseus* and *Serratia marcescens*) to improve suppression of the Pecan Weevil (*Curculio caryae*) larvae. The results recorded by Shapiro-Ilan *et al*, (2004) were mainly antagonistic with the exception of a combination of *H. indica* and *M. anisopliae*, where additive effects were observed. Previous research examining the effects of various entomopathogenic fungi on EPN focused on insect mortality, with results ranging from synergistic (Ansari *et al.*, 2008) to additive (Shapiro-Ilan *et al.*, 2004) to antagonistic (Barbercheck & Kaya, 1991). The experimental procedures in these experiments were different and variation in results may be due to the specific species interactions between the EPN and entomopathogenic fungi, the application times, application form and insect host examined.

The inhibitory effects of plant-associated bacteria on PPN have been widely reported (Burkett-Cadena *et al.*, 2008; Cronin *et al.*, 1997; Khan *et al.*, 2008), indicating that there is potential for the use of such rhizobacteria for the biocontrol of these agriculturally detrimental pests. Research carried out to date does not provide information on the effects of such bacteria on the non-target beneficial EPN. In addition to assessing the compatibility of EPN and PGP bacteria for use as a dual biocontrol and biofertiliser product, results presented here also provide new knowledge on the non-target effects of bacterial applications for PGP or PPN biocontrol. The majority of previous studies examining the effects of

entomopathogenic fungi on EPN or the biocontrol of PPN using bacteria focus of nematode infectivity and survival, respectively. The study presented here did not solely focus on insect susceptibility to nematodes but provided a comprehensive examination of the overall effects of endophytic bacteria on nematode survival, biology and behaviour.

There was no existing knowledge on the toxic effects of PGP bacteria to EPN thus, it was essential to carry out initial susceptibility assays prior to examining the effects of these bacteria on EPN biology and behaviour. Procedures adopted from Cronin *et al.*, (1997) and Meyer *et al.*, (2009) were applied to determine the toxic effects of bacterial endophytes and chemically produced synthetic 2,4-diacetylphloroglucinol (DAPG) on EPN IJ. Direct IJ exposure in multiwall plates created a very extreme setting and is unlikely to replicate effects occurring in a more dynamic natural soil environment. Nonetheless, carrying out such experiments provides 'worst case scenario' findings and was important to screen out any bacterial strains that were highly toxic to EPN. Initial results were promising as no bacterial strain significantly and consistently affected any of the EPN species and strains examined. A number of bacterial isolates examined throughout this study were also tested for their nematicidal properties against the plant parasitic nematodes *Globodera pallida* and *Meloidogyne javanica* by Egan (personal communication). Results recorded during that study indicate that the bacterial isolates *Pseudomonas fluorescens* F113, *P. fluorescens* S118, *Pantoea agglomerans* S222, *P. fluorescens* L228 and *P. fluorescens* L321 all significantly affected juvenile mortality, with up to 100% mortality recorded, following bacterial exposure.

The only notable negative interaction recorded in this study was between *S. feltiae* SB12 (1) and *P. veronii* L23, with 41 % IJ mortality after 48 hr. However, this was the exception and the mean IJ mortality was approximately 10 % across all nematode taxa examined. This

bacterial strain was included in a subsequent laboratory-based sand bioassay, to determine if this combination of *S. feltiae* SB12 (1) and L23 would result in a reduction in insect mortality in sand. Results from this experiment presented previously in section 3.2.1, indicate that no bacterial strain examined affected *G. mellonella* susceptibility to EPN.

Moreover, in the control treatment with bacteria and no EPN, insect mortality was not significantly different from the control with H_2O only, indicating that none of the bacterial strains examined exhibited insecticidal activity against *G. mellonella.* *Pseudomonas* insecticidal capabilities and their associated toxin genes have been documented previously (Péchy-Tarr *et al.*, 2008; Rangel *et al.*, 2016). The genomes of the bacterial strains L321, L228 and L111 contain numerous PGP genes (Lally, 2016; Otieno, 2014). The full gene complex required for FIT production is not present in the genome of *P. fluorescens* F113, L321, L228 or L111. However, a homologous sequence to *fit*H, associated with toxin regulation, was detected in L228. This bioinformatic analysis findings support those from the laboratory and greenhouse experiments and would indicate that these isolates have no insecticidal capabilities against *G. mellonella*.

The production of antimicrobial compounds, such as DAPG, by bacteria, inhibits the growth of numerous phytopathogens and has been linked to reduced egg hatch, juvenile mortality and mobility in bacterial feeding and plant parasitic nematodes (Cronin *et al.*, 1997; Meyer *et al.*, 2009). Bacterial strains examined here F113 wt and S118 have the *phl*D locus (Menton, 2010) that encodes the genes for production of DAPG (Moynihan *et al.*, 2009). Both strains significantly increased IJ mortality in at least one EPN strain, with a maximum mortality of 31 % for F113 and 23 % for S118 recorded against *S. carpocapsae* and *S. feltiae* (e-nema) respectively. To assess if there was a relationship between DAPG production and IJ mortality, nematodes were exposed to chemically produced synthetic DAPG. *S. feltiae* (e-

nema) IJ mortality was not affected by DAPG at any of the concentrations examined, whereas increased mortality levels were recorded for *S. feltiae* SB 12 (1) and *H. bacteriophora* following exposure to 200 µg/ml DAPG. The maximum IJ mortality recorded for *S. feltiae* SB 12 (1), *S. feltiae* (e-nema) and *H. bacteriophora* was 6 %, 4 % and 6 % respectively. Whereas, *S. carpocapsae* was the most sensitive to DAPG. IJ mortality increased with prolonged incubation time and increasing DAPG concentrations, reaching a maximum of 18 %. EPN infectivity was not affected by DAPG in OSR plants. In addition, *S. feltiae* SB 12(1) fecundity was not affected by DAPG while *H. bacteriophora* progeny production was increased in the presence of DAPG. Meyer *et al*. (2009) state that the bacterial-feeding nematodes, *C. elegans*, *P. pacificus*, and *R. rainai* were not affected by DAPG. Contrastingly, synthetic DAPG and DAPG producing pseudomonads negatively affected plant-parasitic nematodes *Globodera rostochiensis* (Cronin *et al*., 1997), *Globodera pallida* and *M. javanica* (Egan personal communication). Studies carried out by Meyer *et al*. (2009) and Cronin *et al*., (1997) were laboratory-based toxicity tests, however assessing sub-lethal impacts, including fecundity and fertility, as carried out in the current study, can be more sensitive, reliable and practical in eco-toxicological studies (Ford *et al.*, 2003; Peshin & Dhawan, 2009). Taking account of results from susceptibility, fecundity and infectivity experiments in the soil it can thus be concluded that production of DAPG by pseudomonads is highly unlikely to negatively affect the non-target beneficial EPN.

To improve the biocontrol capabilities and predictability of EPN in a dynamic natural soil environment a greater understanding of EPN ecology and interactions with soil fauna is essential, to ensure insect control is consistent and comparable with conventional chemical pesticides. The more virulent a nematode is against an insect the greater the chance of its success is in the environment. There is a linear association between the number of nematodes

applied to and the nematodes recovered from an insect and this is a measure of EPN virulence against a host (Fan & Hominick, 1991). The effect of PGP bacteria on nematode virulence varied but did not affect this linear association. This result was positive as it would indicate that the bacteria would not affect the nematode's ability to infect insects. Moreover, it would indicate that applying these bacteria for PGP or PPN biocontrol is unlikely to have any negative effects to non-target beneficial nematodes. *H. bacteriophora* was the least virulent nematode compared to the other EPN strains and species examined. However, it was also the only nematode to show an increase in the number of IJ establishing inside the host at the lower nematode doses of 25 and 50IJ/larva in the presence of *P. fluorescens* F113, when compared to the control with no bacteria. Bacterial isolate L228 had a negative effect on at least one nematode dose of *S. feltiae* SB 12(1), *S. feltiae* (e-nema) and *S. carpocapsae*. Moreover, L228 significantly affected *S. carpocapsae* IJ establishing when applied at doses of 50 and 100 IJ/larva and L321 resulted in a reduction in IJ establishing when applied at a rate of 50 IJ/larva. In terms of virulence alone, the least compatible and thus least suitable combination for a dual PGP-biocontrol product was the combination of *S. carpocapsae* and *P. fluorescens* S118. S118 exposure resulted in a significant reduction in the number of recovered IJ at three of the four IJ doses examined.

In order to use EPN and bacterial endophytes for biocontrol and PGP, the nematodes' ability to kill insects must not be inhibited. In both laboratory-based and greenhouse pot experiments, endophytic bacterial exposure did not affect the susceptibility of *G. mellonella* to *S. feltiae* SB 12(1), *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* or *O. sulcatus* susceptibility to *S. feltiae* SB 12(1) and *H. bacteriophora*. The microbial interactions in the soil are very complex and more so when plants and insects are introduced. This is the first study of its kind examining the effects of PGP bacteria on EPN, thus comparisons must be

drawn from existing knowledge on the interactions between various endophytic fungi and EPN. There are contrasting results in terms of the interactions between insects feeding on plants colonised by endophytic fungi and the resulting EPN virulence. Kunkel & Grewal, (2003) reported that, following feeding on perennial ryegrass colonised by *Neotyphodium lolii,* the black cutworm (*Agrotis ipsilon*) was less susceptible to *S. carpocapsae*. Although insect susceptibility to *S. carpocapsae* was not affected by PGP bacteria in this study, it was the most susceptible nematode to DAPG and virulence was reduced by the DAPG-producing pseudomonad S118 and the non-DAPG-producing L228 and L321. In contrast, Grewal *et al.*, (1995) showed that the larvae of the Japanese beetle (*P. japonica*) are more susceptible to *H. bacteriophora* following feeding on plants colonised by the endophytic fungus *Acremonium* sp. In the current study, *H. bacteriophora* was the only EPN strain/species that showed increased virulence in the presence of F113 and also consistently increased progeny production in the presence of DAPG. These findings demonstrate the importance of testing the host range of EPN but also the interactions between plant-associated microorganisms and their effect on herbivore insects.

The foraging technique adopted by EPN is a key factor to consider when selecting a nematode species for insect biological control as it determines where the nematodes will be located in the soil profile, what hosts they will infect and the degree of contact an EPN species will have with the plant rhizosphere and the insects that feed on endophyte colonised plants (Campbell & Gaugler, 1997). EPN foraging strategies can be predicted through analysis of responses to host volatile cues and dispersal behaviour on agar. In sand columns, nematode movement was not inhibited by bacteria as insect mortality and the number of IJ establishing in the insects did not differ from the controls. *H. bacteriophora* is most suited of the nematodes examined for attraction experiments on agar as it is a cruise forager and shows

strong direction movement (Grewal *et al.*, 2009). In this study *H. bacteriophora* IJ moved towards the host, indicating that the bacteria did not interfere with host emitted cues. Moreover, in the absence of a host, *H. bacteriophora* IJ consistently moved towards the endophytic bacteria examined, but this was not the case with *S. feltiae* SB 12(1) and *S. feltiae* (e-nema) or *S. carpocapsae*. *S. feltiae* SB 12(1) and *S. feltiae* (e-nema) exhibited limited movement and attractiveness towards the host, which were not affected by the presence of bacteria. As expected *S. carpocapsae* showed no directional movement as it is an ambush forager, indicating that these bacteria do not affect the nematodes' natural behaviour.

Insect-induced plant chemicals, such as (E)-β-caryophyllene, are emitted from roots damaged by feeding insects and have been shown to attract EPN (Rasmann *et al.*, 2005; van Tol *et al.*, 2001). Increased production of this has been linked to colonisation of plants by *Pseudomonas* sp. but the mechanism by which these bacteria influence (E)-β-caryophyllene production is unknown (Chiriboga M. *et al.*, 2018). (E)-β-caryophyllene production by the bacterial strains examined in this study has not been determined. The complexity of the belowground interactions and the subsequent cascading effects on the soil ecosystem are evident here. Examining the interactions between plants, their associated bacteria, herbivores and entomopathogens will provide new insights into these multitrophic interactions and potentially increase efficiency in biocontrol and PGP.

EPN IJ are a non-feeding developmental stage and little is known about the internal colonisation of bacteria other than their endosymbionts. Although not demonstrated in this study, nematodes have been shown to increase bacterial colonisation of plants (Knox *et al.*, 2003, , 2004). To examine the close interactions between EPN and PGP bacteria and the potential link between nematodes and increased bacterial colonisation of plants, the ability of *P. fluorescens* F113 *gfp* to colonise IJ and persist post-IJ insect infection was determined.

P. fluorescens F113 *gfp* successfully colonised *S. feltiae* SB 12(1) and *H. bacteriophora* IJ. EPN symbiotic bacteria are essential for EPN survival and any factor affecting these symbionts will directly affect the EPN ability to cause insect mortality. Both *P. fluorescens* F113 and L321 inhibited the growth of *P. luminescens* and *X. bovienii* endosymbionts on agar, but despite the close interactions have no negative effect on the nematodes ability to infect insects. Furthermore, *P. fluorescens* F113 *gfp* persisted in insects that were infected with *S. feltiae* SB 12(1) and *H. bacteriophora* IJ previously exposed to the bacterium. This indicates that not only is this rhizospheric bacterium not susceptible to antimicrobial metabolites produced by the EPN symbionts, but it may also indicate a mode of increased plant colonisation. The evaluation of these results would indicate that there is a close interaction between EPN and other soil microbes in the environment and there is some level of interaction between them.

Both of these bacterial isolates have shown biocontrol capabilities against the plant pathogens *Fusarium* sp. and *Pythium* sp. (Menton, 2010; Otieno, 2014) and also nematicidal capabilities against the plant parasitic nematodes *G. pallida* and *M. javanica* (Egan, personal communication). Suppression of PPN using EPN has also been reported, with results varying from limited inhibition (Kunkel *et al.*, 2006) to significant repulsion (Molina *et al.*, 2007). A combination of EPN and bacterial endophytes offers threefold biocontrol potential. Firstly, for the infection of herbivorous insects, secondly for the inhibition of plant pathogens and finally for the suppression of PPN. Such an approach has the additional benefits of promoting soil ecosystem health, due to a reduction of chemical input and increasing plant health and crop yield due to the addition of endophytic bacteria with PGP traits.

The selection of bacterial strains with PGP properties was an integral aspect of this project. Plants benefit from a symbiotic relationship with their microbes and this may result from the

microorganism's ability to acquire nutrients from the soil, to protect the plant from pathogenic organisms and pests, or to relieve the effects of environmental stresses on the plant host. Genes associated with phosphate solubilisation, production of siderophore, chemotaxis and motility are present in the genomes of L321, L228 and L111. Moreover, nematodes have been linked to increased plant bacterial colonisation and PGP (Mao *et al.*, 2006; Xu *et al.*, 2015).

In this study *H. bacteriophora* significantly increased OSR fresh weight when grown in the presence of F113 *gfp* or L321 *gfp.* This result becomes more interesting when combined with the results from the previous experiment, examining nematode movement on agar. Here, *H. bacteriophora* was consistently attracted to bacteria in the absence of an insect host. Although EPN did not significantly increase F113 *gfp* or L321 *gfp* colonisation of OSR total bacterial colonisation was not examined. This may be a better indication of the ability of EPN or *H. bacteriophora* in particular to increase plant bacterial colonisation. The overall trend in OSR plant biomass analysis was positive with increases in both dry and fresh plant weights, for plants inoculated with bacterial endophytes and EPN IJ. The previously discussed laboratory experiment examining bacterial growth in the presence of EPN showed that the growth of a number of *P. fluorescens* strains was reduced in the presence of EPN. This included F113, which when grown in the presence of *S. feltiae* SB 12(1) growth was significantly reduced by 3.98 %. Thus, it can be concluded that in a more complex soil environment EPN shows no negative effect on bacterial survival and plant colonisation.

5.2 Conclusion

This study examined the basic fundamental interactions between two beneficial soil taxa, EPN and PGP bacteria, to contribute to sustainable agriculture by facilitating a move away from the use of traditional agri-chemicals to more environmentally safe alternatives. Despite the widespread agricultural benefits and applications of PGP bacteria and EPN, little was known about the interactions between the two. This work was the first of its kind and as a result, drew comparisons from previous research on the control of PPN using plant-associated bacteria and the interactions between entomopathogenic fungi and EPN. However, both PPN and entomopathogenic fungi are eukaryotes that carry out very different functions within the soil ecosystem than EPN and rhizospheric bacteria, respectively.

EPN virulence against insects varies greatly thus, when selecting EPN for insect control it is essential to match the correct EPN species to each insect pest. Likewise, results from bacterial and DAPG susceptibility and nematode virulence assays indicate that it is also necessary to have a suitable combination of EPN and PGP bacteria. Laboratory-based assays indicated that *S. carpocapsae* was most affected by DAPG after prolonged exposure and also bacterial strains S118, L228 and L321 significantly affected *S. carpocapsae* virulence. No such negative interactions were recorded in experiments examining the effects of DAPG or PGP bacteria on the ability of EPN to cause insect mortality in a greenhouse pot experiment.

This study provides the framework for the development of a product combining EPN and PGP bacteria, however, there are still factors that require examination before a product reaches application stage. This is in no small part due to the complex nature of the belowground interactions between plants, their associated microbiome, herbivores and entomopathogens. This is demonstrated in the variation in results between laboratory-based

bioassays and greenhouse pot experiments, similar variations would inevitably arise when moving to large-scale greenhouse or field trials.

By carrying out this comprehensive examination it can be concluded that PGP bacteria do not significantly affect EPN survival, biology and behaviour. Moreover, in a more dynamic natural soil environment, EPN do not affect bacterial plant colonisation. Thus, it can be concluded that both taxa are compatible and a product consisting of both EPN and PGP bacteria has great potential for insect control and increased plant growth, while also contributing to improved soil health and sustainable agricultural practices.

5.3 Future Directions

The results presented above have demonstrated the compatibility of EPN and bacterial endophytes for use as a combined biocontrol and biofertiliser product. This work leads to the possibility of the following investigations that could be pursued:

- Genome sequencing of L321, L228 and L111 has contributed significant knowledge on PGP genes. Greenhouse experiments showed that bacterial strains L321 and L228 exhibited PGP in *B. napus*. Moreover, L321 was observed to contribute to significant plant length in a field trial (Lally, 2016; Moreira *et al.*, 2016). Thus, it is logical to focus future research on the bacterial strains L321, L228 and L111. With future aims of developing a biocontrol-biofertiliser product, continued research should focus on the EPN species *S. feltiae* SB 12(1) and *H. bacteriophora* to examine nematodes' from two different families and most importantly, as both nematodes are compatible with the bacterial endophytes examined.
- Now that compatibility and potential bacterial-EPN combinations have been identified, the optimum product formulation and application method must be

examined. Hiltpold *et al.*, (2012) reported that encapsulated nematodes survived the encapsulation and were able to break through the shell and subsequently infect hosts. Moreover, Bashan *et al.*, (2002) used encapsulation to inoculate seeds with plant growth-promoting bacteria. Thus, the co-encapsulation of EPN and bacterial in an alginate capsule provides a novel starting point to examine application methods.

- Interactions between *H. bacteriophora* and the plant associated bacteria were overwhelmingly positive and very interesting. Of particular importance for investigation is the attraction of *H. bacteriophora* to endophytic bacteria in the absence of a host and the potential stimulation of plant growth, increased progeny production in the presence of DAPG and increased virulence in the presence of the rhizospheric bacteria *P. fluorescens* F113. Further investigation may lead to new knowledge on the link between the presence of nematodes and increased plant colonisation. This, in turn, would benefit the development of a robust biocontrol-PGP product.
- Sciarid flies (Diptera: Sciaridae) are a predominant agricultural pest and the larval stage feed on mushroom mycelium, destroying both developing mushrooms and mature mushrooms (Jess *et al.*, 2007). The adult/flying stage of these insects can act as vectors of agriculturally detrimental pathogens (Scarlett *et al.*, 2014). Sciarid flies are attracted to and can develop on *Fusarium* sp. (Frouz & Nováková, 2001). This is a pathogenic fungus that causes significant plant damage (Li *et al.*, 2018). EPN have been shown to act as biocontrol agents for sciarid flies (Grewal, 2007; Lacey & Georgis, 2012) and a number of bacterial endophytes examined throughout this project have been shown to inhibit the growth of *Fusarium* sp. A large-scale greenhouse trial examining the suppression of both insects and fungi would

demonstrate one of the potential application of such a combined product for biocontrol of two agriculturally important pests.

- There is great potential deriving from this work to increase plant growth and control of economically important insects. However, before applying EPN and endophytes the bacterial colonisation of plant tissues, commonly attacked by insects susceptible to nematodes, must be determined. Bacterial endophytes did not affect EPN infectivity of *O. sulcatus* in strawberry plants, however bacterial colonisation or PGP was not examined. A large-scale trial with strawberry plants, examining insect mortality, root damage from larval feeding and plant biomass, following inoculation with bacterial endophytes and EPN, would be a logical step forward.

Chapter 6 References

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Appendix 1

Bacterial endophyte morphological characterisation. Growth characteristics were assessed following 24 hr incubation on nutrient agar at 30 ˚C.

Endophyte label	Strain ID	Colour	Surface	Elevation	Margin	Mucoidal
F113	*Pseudomonas fluorescens*	cream*	shiny	convex	entire	yes
S19	*Pseudomonas trivialis*	cream	shiny	convex	entire	no
S118	*Pseudomonas fluorescens*	pale yellow	shiny	convex	entire	yes
S222	*Pantoea agglomerans*	Pale orange**	dull	convex	entire	no
L23	*Pseudomonas veronii*	cream	dull	raised	entire	yes
L124	*Pseudomonas fluorescens*	cream	dull	convex	entire	yes
L228	*Pseudomonas fluorescens*	cream	dull	flat	undulate	yes
L313	*Pseudomonas fluorescens*	pale yellow**	shiny	convex	entire	yes
L321	*Pseudomonas fluorescens*	cream	shiny	convex	entire	yes
L324	*Pseudomonas fluorescens*	cream*	shiny	flat	entire	yes
L330	*Xanthomonas sp*	pale orange**	dull	convex	entire	yes
R114	*Pantoea agglomerans*	cream	shiny	convex	entire	yes
R225	*Pantoea amnigenus*	cream	shiny	flat	entire	yes

* cream colonies turn to dark cream/pale brown with prolonged incubation

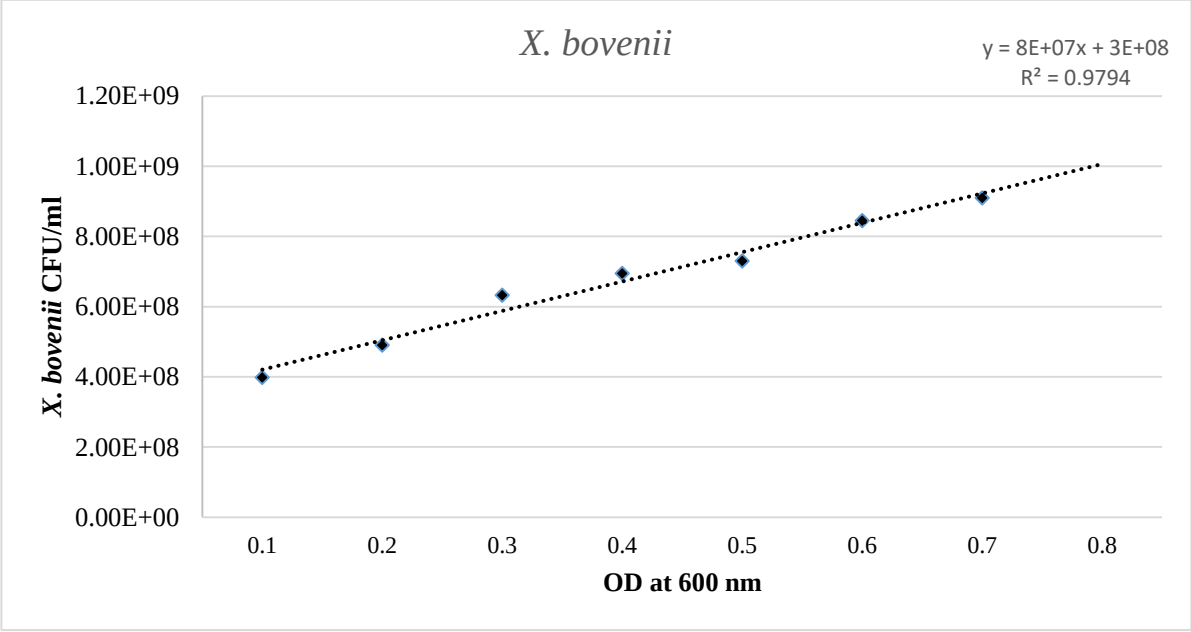
** Orange and yellow pigments are faint and develop with prolonged incubation

Appendix 2

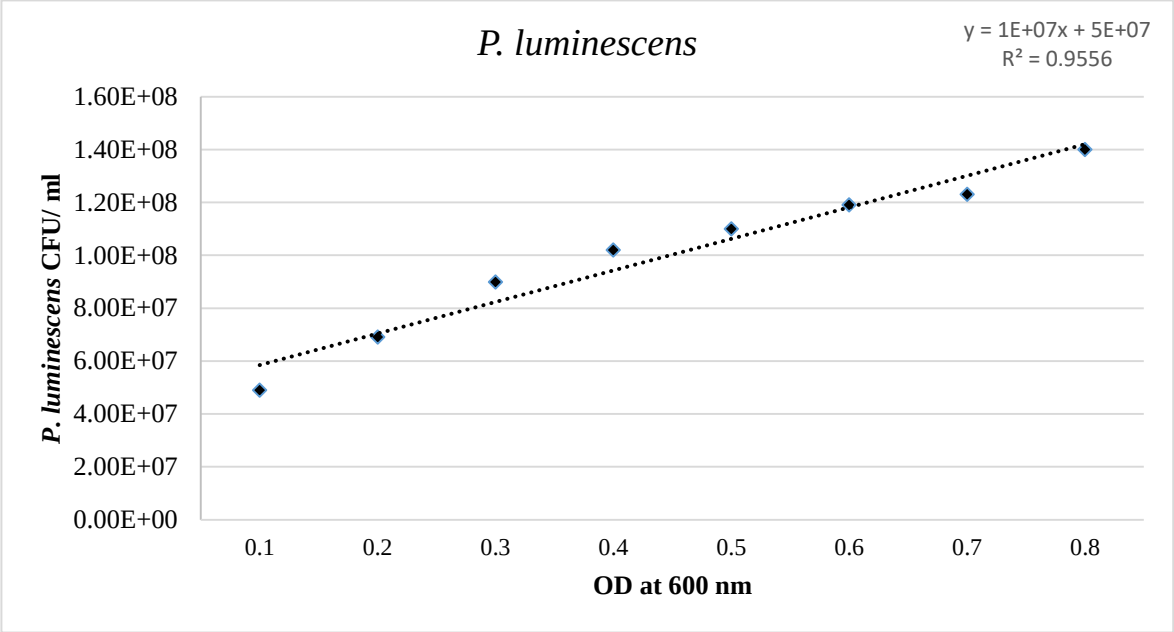
Endophytic bacterial and EPN symbiotic bacterial standard curves

The standard curves below were constructed to determine the bacterial concentration (in colony forming units per volume unit [CFU/ml]) causing nematode mortality after 24 and 48hrs. Cultures were incubated for 24hrs at 30^0C after which the CFU/ml was determined. The CFU/ml was plotted against the bacterial OD to generate standard curves. Standard curves were constructed using Microsoft excel for the endophytic bacterial strains, *Pseudomonas fluorescens* F113 PCBrif, S19, S118, S222, L23, L228, L330, L313, L321, L324, R114 and R225. Standard curves for the entomopathogenic nematode symbiotic bacteria were prepared using the same process.

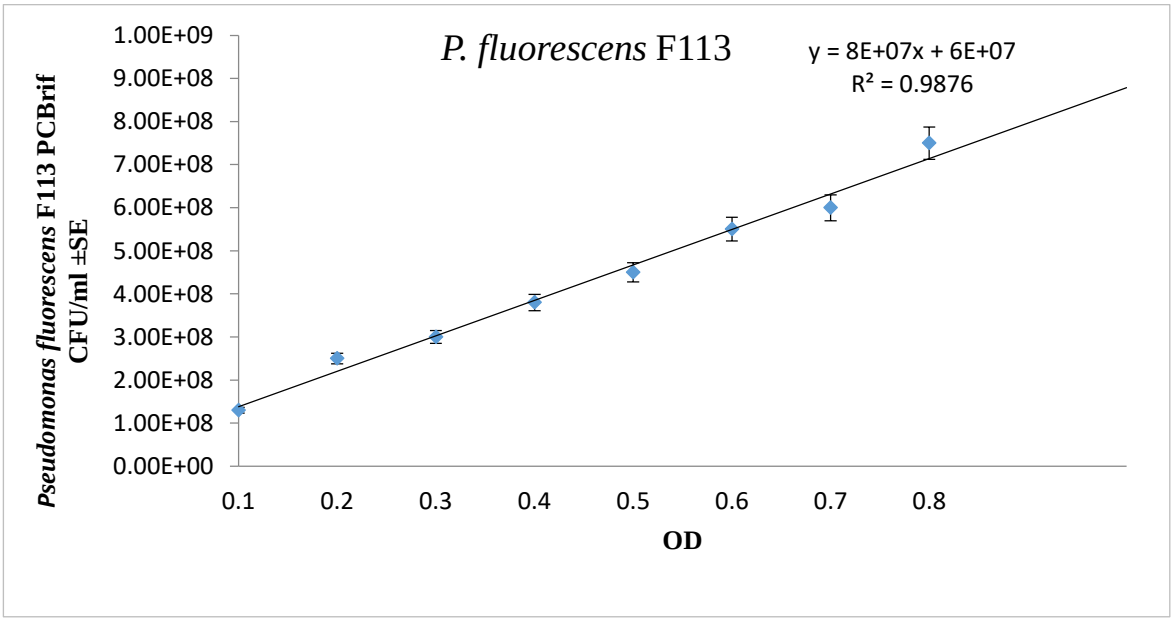
A)



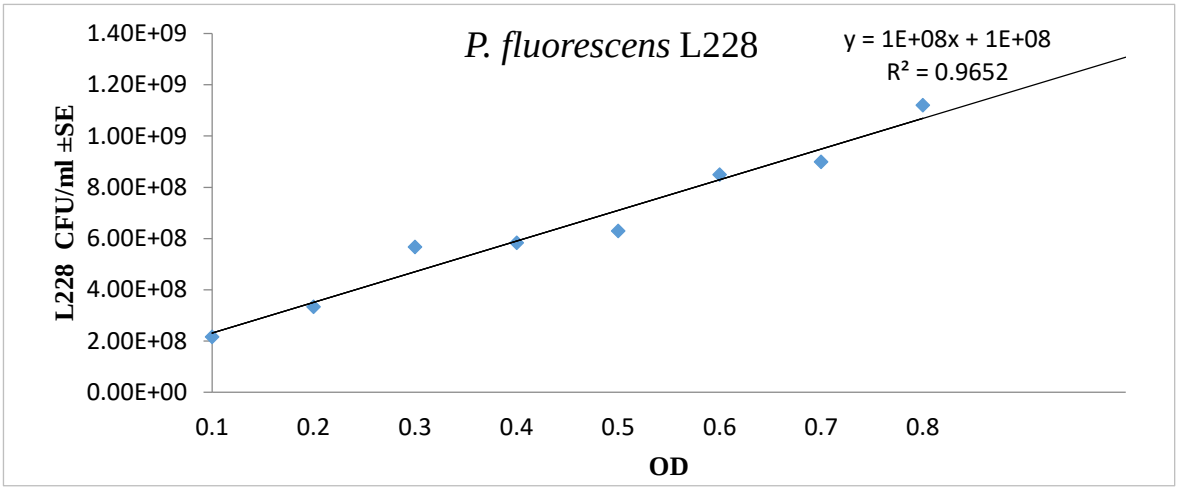
B)


P. luminescens
y = 1E+07x + 5E+07
R² = 0.9556
1.60E+08
1.40E+08
1.20E+08
1.00E+08
8.00E+07
6.00E+07
4.00E+07
2.00E+07
0.00E+00
P. luminescens CFU/ ml
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
OD at 600 nm

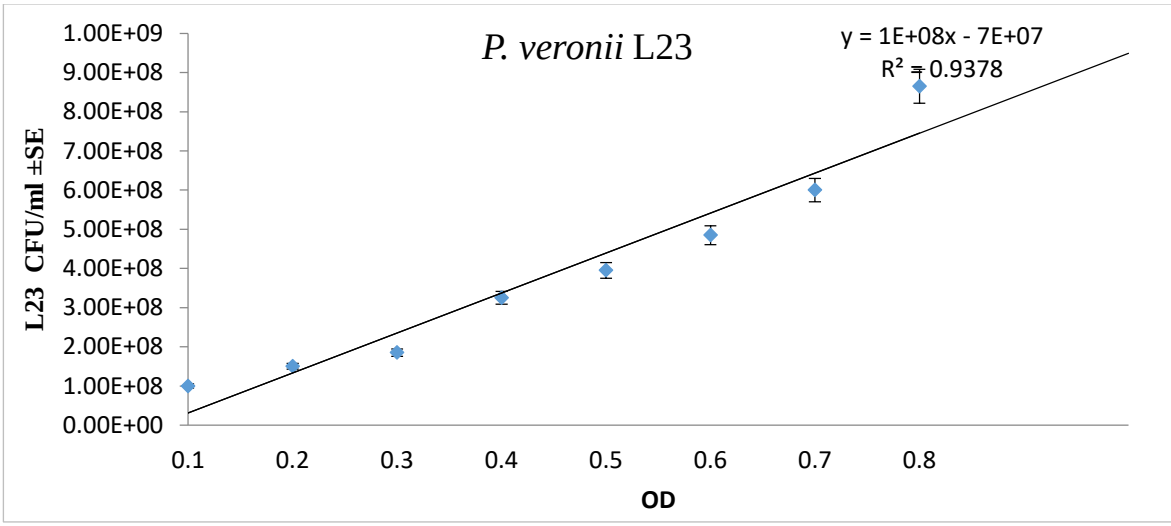
C)


P. fluorescens F113
y = 8E+07x + 6E+07
R² = 0.9876
1.00E+09
9.00E+08
8.00E+08
7.00E+08
6.00E+08
5.00E+08
4.00E+08
3.00E+08
2.00E+08
1.00E+08
0.00E+00
Pseudomonas fluorescens F113 PCBrif CFU/ml ±SE
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
OD

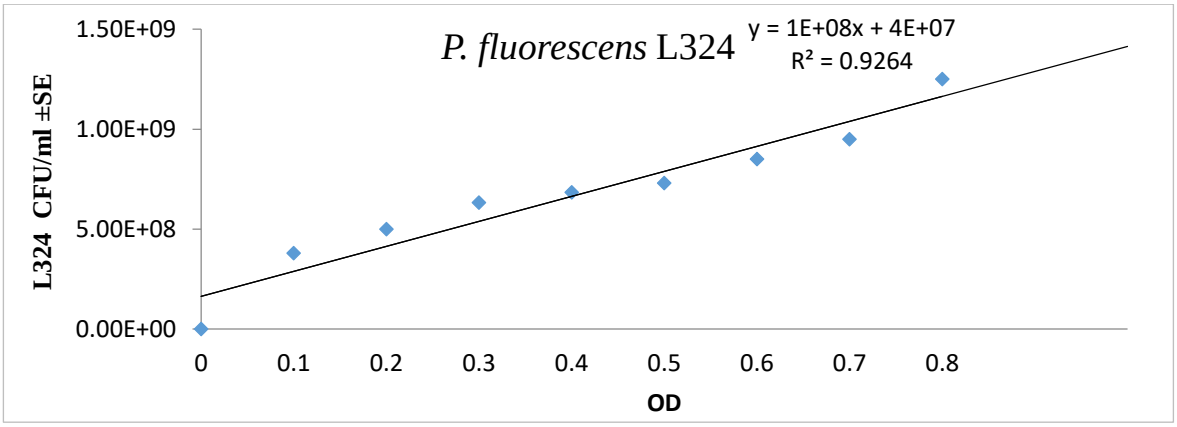
D)


P. fluorescens L228
y = 1E+08x + 1E+08
R² = 0.9652
L228 CFU/ml ±SE
1.40E+09
1.20E+09
1.00E+09
8.00E+08
6.00E+08
4.00E+08
2.00E+08
0.00E+00
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
OD

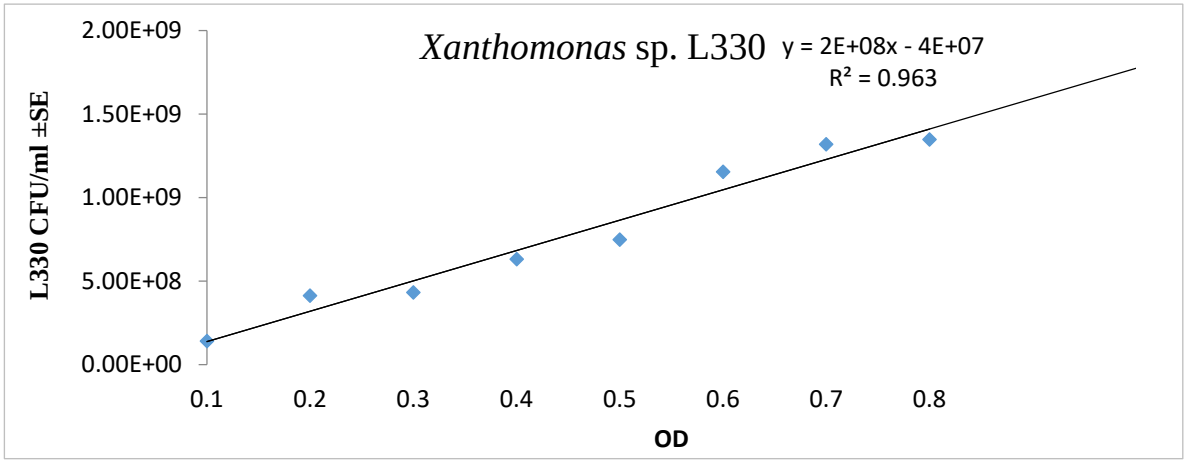
E)


P. veronii L23
y = 1E+08x - 7E+07
R² = 0.9378
L23 CFU/ml ±SE
1.00E+09
9.00E+08
8.00E+08
7.00E+08
6.00E+08
5.00E+08
4.00E+08
3.00E+08
2.00E+08
1.00E+08
0.00E+00
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
OD

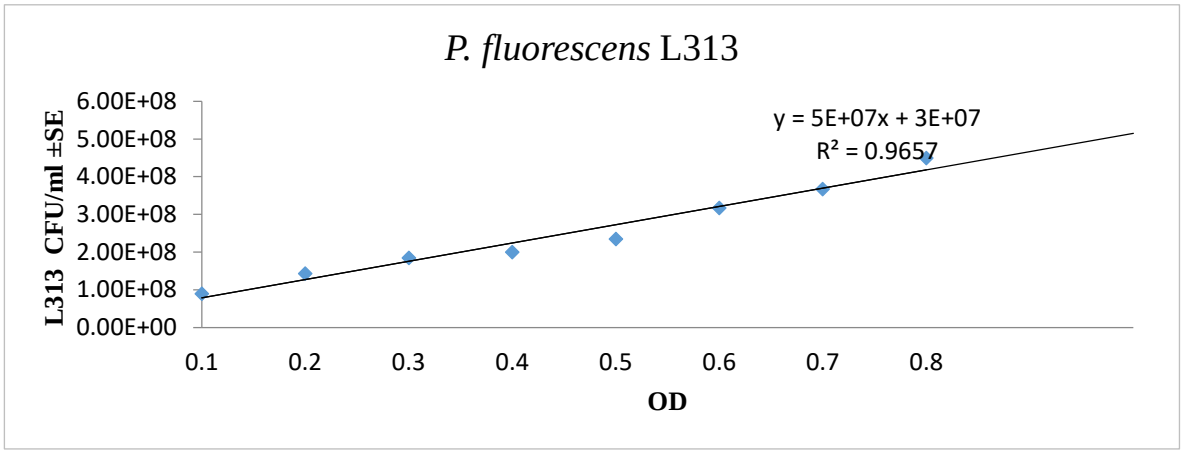
F)


P. fluorescens L324
y = 1E+08x + 4E+07
R² = 0.9264
L324 CFU/ml ±SE
1.50E+09
1.00E+09
5.00E+08
0.00E+00
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
OD

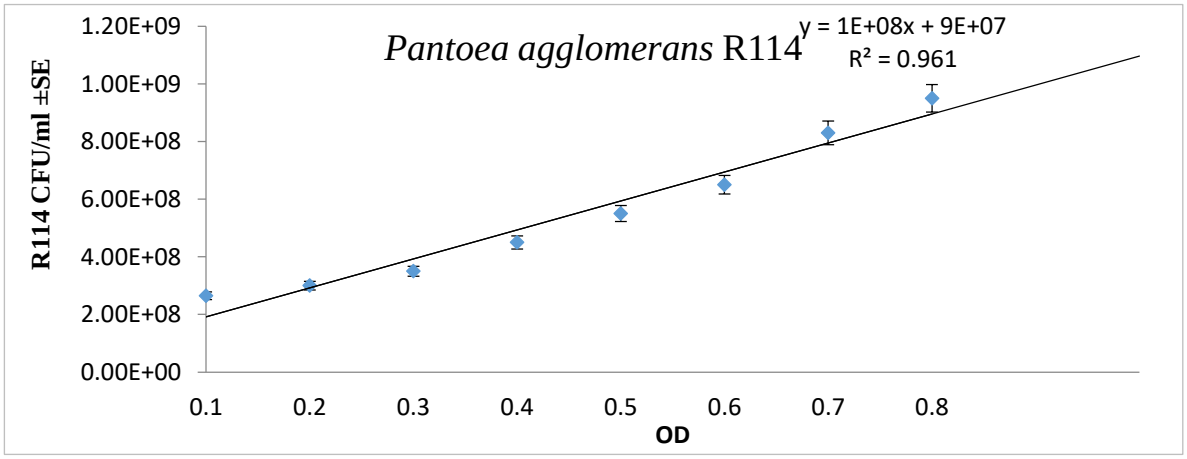
G)


Xanthomonas sp. L330
y = 2E+08x - 4E+07
R² = 0.963
L330 CFU/ml ±SE
2.00E+09
1.50E+09
1.00E+09
5.00E+08
0.00E+00
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
OD

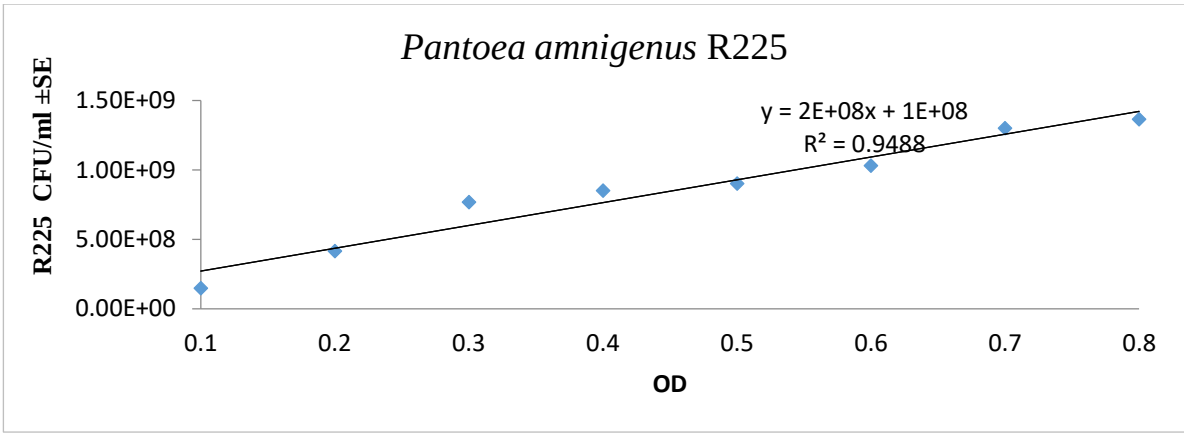
H)


P. fluorescens L313
y = 5E+07x + 3E+07
R² = 0.9657
L313 CFU/ml ±SE
6.00E+08
5.00E+08
4.00E+08
3.00E+08
2.00E+08
1.00E+08
0.00E+00
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
OD

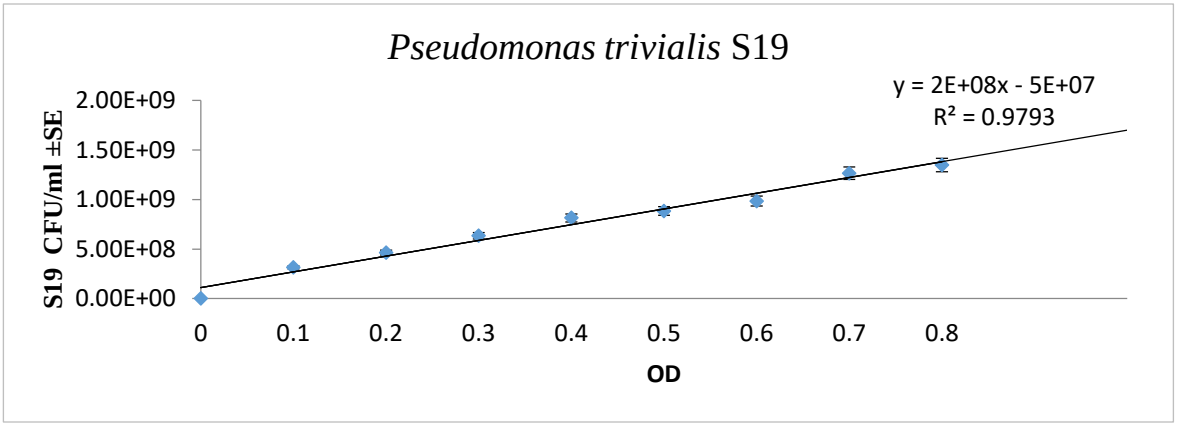
I)


Pantoea agglomerans R114
y = 1E+08x + 9E+07
R² = 0.961
R114 CFU/ml ±SE
1.20E+09
1.00E+09
8.00E+08
6.00E+08
4.00E+08
2.00E+08
0.00E+00
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
OD

J)



K)



L)

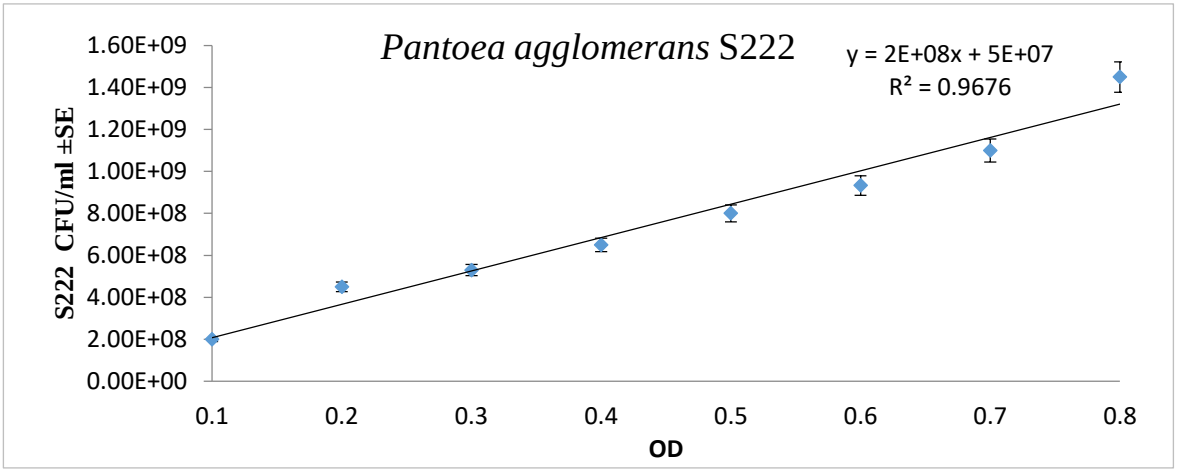


Figure 5.3.1 **(A-L)** Bacterial dilutions were carried out using sterile ringers and measured at 600nm on a spectrophotometer to construct a standard curve for the bacterial strain. Viable colonies were derermined via plate counts on nutrient agar for 24hrs, replication was 3fold per bacterial strains per dilution.

Appendix 3

Bacterial growth curves for *X. bovenii* and *P. luminescens*

A 10 ml of *X. bovienii* and *P. luminescens* grown in Luria-Bertani broth was pelleted by centrifugation and re-suspended in Ringer's solution. 20 µl aliquots of these bacterial suspensions were inoculated into 180 µl of sterile Luria-Bertani broth in a 96 well multiwell plates, the OD at 600nm was recorded every 60 minutes for 24 hr. The following growth curves were constructed in Microsoft Excel 2013 and display the bacterial growth over time. .

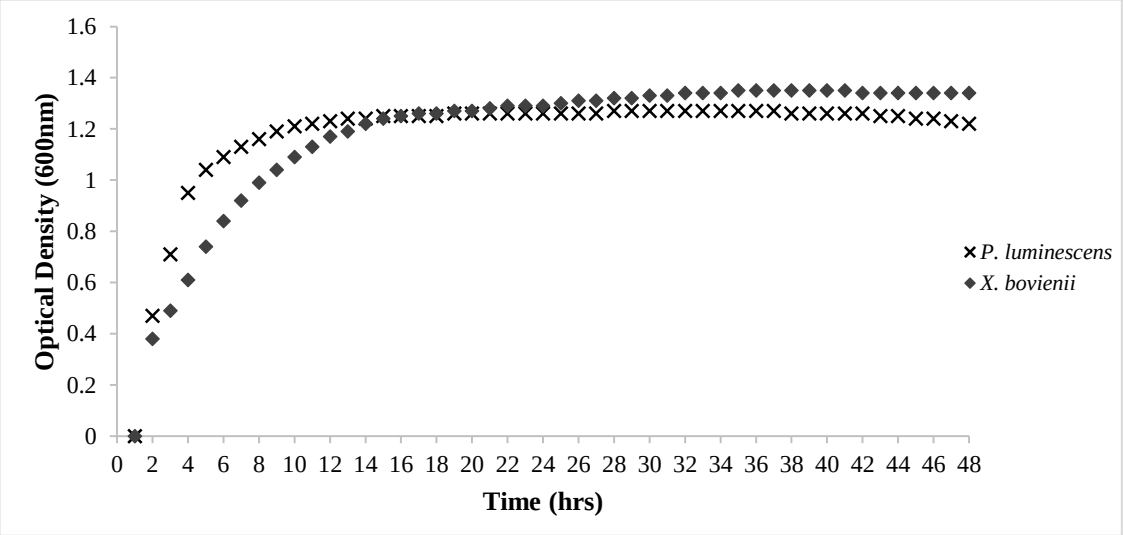


Figure 5.3.2 *X. bovienii* and *P. luminescens* bacterial growth curve

Bacterial growth curves and total viable colonies were determined for each strain to ensure that after 24 hr incubation bacteria would reach stationary phase and have sufficient and reliable numbers for future experimentation. This is essential to ensure repeatability and reliability of experiments. In addition, the production of idiolites (secondary metabolites), these include antimicrobial agents, occurs during stationary phase. This is of particular importance when examining the susceptibility of *X. bovienii* and *P. luminescens* to endophytic bacteria.

Appendix 4

Sequence of the *gfp* insert from Mini Tn5 transposon vector pFAJ1819. The primers from the experimentation were designed from this sequence (Otieno, 2014).

ttat ttgtagagct catccatgcc atgtgtaatc ccagcagcag ttacaaactc aagaaggacc atgtggtcac gcttttcgtt gggatctttc gaaagggcag attgtgtcga caggtaatgg ttgtctggta aaaggacagg gccatcgcca attggagtat tttgttgata atggtctgct agttgaacgg atccatcttc aatgttgtgg cgaattttga agttagcttt gattccattc ttttgtttgt ctgccgtgat gtatacattg tgtgagttat agttgtactc gagtttgtgt ccgagaatgt ttccatcttc tttaaaatca ataccttta actcgatacg attaacaagg gtatcacctt caaacttgac ttcagcacgc gtcttgtagt tcccgtcatc tttgaaagat atagtgcgtt cctgtacata accttcgggc atggcactct tgaaaaagtc atgccgtttc atatgatccg gataacggga aaagcattga acaccataag agaaagtagt gacaagtgtt ggccatggaa caggtagttt tccagtagtg caaataaatt taagggtaag ctttccgtat gtagcatcac cttcaccctc tccactgaca gaaaatttgt gcccattaac atcaccatct aattcaacaa gaattgggac aactccagtg aaaagttctt ctcctttgct agccat

Appendix 5

$HgCl_2$ Material Safety Data Sheet

1. Product and Company Information

Product Name: Mercury(II) chloride, 99.5+%, a.c.s.

Reagent Product Number: 215465

Company: Sigma-Aldrich Ireland Ltd. Airton Road, Tallaght, Dublin, 24 Ireland

Technical Phone No.: 353-1404-1900

2. Composition/Information on Ingredients

Formula HgCl2 Molecular Weight 271.5 AMU

Synonyms Abavit B * Bichloride of mercury * Bichlorure de mercure (French) * Calochlor * Chlorid rtutnaty (Czech) * Chlorure mercurique (French) * Cloruro di mercurio (Italian) * Corrosive mercury chloride * Corrosive sublimate * Dichloromercury * Fungchex * Mercuric bichloride * Mercury bichloride * Mercury(2+) chloride * Mercury dichloride * Mercury perchloride * NCI-C60173 * Perchloride of mercury * Quecksilber chlorid (German) * Sulem * Sulema (Russian) * Sublimat (Czech) * Sublimate * TL 898

3. Hazards Identification

Special indication of hazards to humans and the environment

Very toxic if swallowed. Causes burns. Toxic: danger of serious damage to health by prolonged exposure in contact with skin and if swallowed. Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.

4. First Aid Measures

After inhalation

If inhaled, remove to fresh air. If not breathing give artificial respiration. If breathing is difficult, give oxygen.

After skin contact

In case of skin contact, flush with copious amounts of water for at least 15 minutes. Remove contaminated clothing and shoes. Call a physician.

After eye contact

In case of contact with eyes, flush with copious amounts of water for at least 15 minutes. Assure adequate flushing by separating the eyelids with fingers. Call a physician.

After ingestion

If swallowed, wash out mouth with water provided person is conscious. Call a physician immediately.

5. Fire Fighting Measures

Extinguishing media

Suitable: Water spray. Carbon dioxide, dry chemical powder, or appropriate foam.

Special risks

Specific Hazard(s): Emits toxic fumes under fire conditions.

Special protective equipment for firefighters

Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes.

6. Accidental Release Measures

Personal precaution procedures to be followed in case of leak or spill

Evacuate area.

Procedure(s) of personal precaution(s)

Wear self-contained breathing apparatus, rubber boots, and heavy rubber gloves.

Methods for cleaning up

Sweep up, place in a bag and hold for waste disposal. Avoid raising dust. Ventilate area and wash spill site after material pickup is complete.

7. Handling and Storage

Handling

Directions for Safe Handling: Do not breathe dust. Do not get in eyes, on skin, on clothing. Avoid prolonged or repeated exposure.

Storage

Conditions of Storage: Keep tightly closed. Store in a cool dry place.

Special requirements: Light sensitive. Moisture sensitive.

8. Exposure Controls / Personal Protection

Engineering controls

Safety shower and eye bath. Use only in a chemical fume hood.

General hygiene measures

Wash contaminated clothing before reuse. Wash thoroughly after handling.

Exposure limits - UK

Source:OEL Type: TWA Value: 0.025MG(HG)/MG

Personal protective equipment

Respiratory Protection: Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU). Where risk assessment shows air-purifying respirators are appropriate use a full-face particle respirator type N100 (US) or type P3 (EN 143) respirator cartridges as a backup to engineering controls. If the respirator is the sole means of protection, use a full-face supplied air respirator. Hand Protection: Compatible chemical-resistant gloves. Eye Protection: Chemical safety goggles.

9. Physical and Chemical Properties

Appearance Physical State: Solid

Property	Value	At Temperature or Pressure
pH	N/A	
BP/BP Range	302 °C	760 mmHg
MP/MP Range	277 °C	

Flash Point	N/A	
Flammability	N/A	
Autoignition Temp	N/A	
Oxidizing Properties	N/A	
Explosive Properties	N/A	
Explosion Limits	N/A	
Vapor Pressure	1.3 mmHg	236 °C
SG/Density	5.44 g/cm3	
Partition Coefficient	N/A	
Viscosity	N/A	
Vapor Density	N/A	
Saturated Vapor Conc.	N/A	
Evaporation Rate	N/A	
Bulk Density	N/A	
Decomposition Temp.	N/A	
Solvent Content	N/A	
Water Content	N/A	
Surface Tension	N/A	
Conductivity	N/A	
Miscellaneous Data	N/A	
Solubility	N/A	

10. Stability and Reactivity

Stability

Stable: Stable.

Conditions to Avoid: Moisture. Light.

Materials to Avoid: Strong oxidizing agents, Strong bases.

Hazardous decomposition products

Hazardous Decomposition Products: Mercury/mercury oxides.

Hazardous polymerization

Hazardous Polymerization: Will not occur

11. Toxicological Information

Acute toxicity

LDLO
Oral
Man
143 mg/kg
Remarks: Kidney, Ureter, Bladder:Changes in tubules (including acute renal failure, acute tubular necrosis). Blood: Changes in leukocyte (WBC) count. Behavioral:Excitement.
LDLO Oral Man 86 mg/kg
Remarks: Gastrointestinal:Ulceration or bleeding from stomach. Gastrointestinal:Necrotic changes. Vascular:Change in plasma or blood volume.
LDLO Oral Human 29 mg/kg
Remarks: Gastrointestinal:Ulceration or bleeding from large intestine. Gastrointestinal:Nausea or vomiting. Gastrointestinal:Ulceration or bleeding from duodenum.
LD50 Oral Rat 1 mg/kg
LD50 Skin Rat 41 mg/kg
LD50 Intraperitoneal Rat 3210 UG/KG
Remarks: Kidney, Ureter, Bladder: Changes in tubules (including acute renal failure, acute tubular necrosis).

Signs and symptoms of exposure

Material is extremely destructive to tissue of the mucous membranes and upper respiratory tract, eyes, and skin. Inhalation may result in spasm, inflammation and edema of the larynxand bronchi, chemical pneumonitis, and pulmonary edema. Symptoms of exposure may include burning sensation, coughing,wheezing, laryngitis, shortness of breath, headache, nausea, andvomiting. Prolonged exposure can cause: Stomach pains, vomiting, diarrhea.

Route of exposure

Skin Contact: Causes burns.

Skin Absorption: May be fatal if absorbed through skin.

Eye Contact: Causes burns.

Inhalation: Material is extremely destructive to the tissue of the mucous membranes and upper respiratory tract. May be harmful if inhaled.

Ingestion: May be fatal if swallowed.

12. Ecological Information

No data available.

13. Disposal Considerations

Substance disposal

Contact a licensed professional waste disposal service to dispose of this material. Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an after burner and scrubber. Observe all federal, state, and local environmental regulations.

14. Transport Information

RID/ADR
UN#: 1624
Class: 6.1
PG: II
Proper Shipping Name: Mercuric chloride

15. Regulatory Information

Classification and labelling according to EU directives

ANNEX I INDEX NUMBER: 080-010-00-X
INDICATION OF DANGER: T+-N
Very toxic. Dangerous for the environment.
R-PHRASES: 28-34-48/24/25-50/53
Very toxic if swallowed. Causes burns. Toxic: danger of serious damage to health by prolonged exposure in contact with skin and if swallowed. Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.
S-PHRASES: 36/37/39-45-60-61
Wear suitable protective clothing, gloves, and eye/face protection. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible). This material and its container must be disposed of as hazardous waste. Avoid release to the environment. Refer to special instructions/safety data sheets.