The interaction between root knot nematodes (*Meloidogyne* spp.) and soft rot *Enterobacteriaceae* (*Pectobacterium* spp.) and their host *Solanum tuberosum*

by

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DECLARATION

I declare that the dissertation, which I hereby submit for the degree Magister of Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution and the work from other sources has been duly acknowledged.

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Aobakwe Oratile Mongae

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SUMMARY

The interaction between root knot nematodes and soft rot *Enterobacteriaceae* and their effect on *Solanum tuberosum*

by

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ABSTRACT

Meloiodgyne incognita, one of the most aggressive plant parasitic nematodes species on potato in South Africa, belongs to a group of plant parasitic nematodes commonly known as root knot nematodes (RKN). This group of nematodes is widely distributed throughout the world. *Meloidogyne* spp. cause major economic losses to important crops such as potato and therefore decrease their market value in many countries across the world. The second stage juveniles are the only mobile and infective phase of the root knot nematode. As they infect host roots, they create wounds that can be used by other plant pathogens to penetrate the host in large numbers. The most effective management strategy for root knot nematodes is the use of nematicides such as Temik and Methyl bromide. However, these have been banned due to adverse on the environment. Therefore, *Meloidogyne* spp. will inevitably become a big problem in the potato industry of many countries due to the lack of effective alternatives to banned chemicals.

Pectobacterium carotovorum subsp. *brasiliensis* (*Pcb*) is one of the most important soft rot-causing agents in South Africa. This pathogen belongs to a group of pathogens commonly known as soft rot *Enterobacteriaceae* (SRE). Bacteria belonging to this group of pathogens are known to cause soft rot and blackleg diseases on potato and other crops. *Pcb* is known as an opportunistic pathogen that can only penetrate host root tissue through natural openings or wounds that result from a variety of agents. Post penetration, the bacteria will increase in number and cause soft rot and blackleg. As rotting plant tissue disintegrates the bacteria escapes into the soil where it serves as inoculum and can infect healthy hosts.

Many interactions have been documented between *Meloidogyne* spp. and other plant pathogens but to our knowledge there are no interactions that have been reported between *Meloidogyne* spp. and *Pectobacterium* spp. Considering the life cycles of RKN and SRE, we hypothesised that there could be an interaction between the two pathogen groups. Since both RKN and SRE are potato pathogens, they share the same space in the rhizosphere. This likely can lead to synergies and complex formation between the two pathogens. Likely, the wounds created by RKN J2s as they penetrate plant tissue can potentially be used by opportunistic *Pcb* to infect various hosts. It is from these identified overlaps that the first part of this study focused on investigating the potential interaction between *M. incognita* and *Pcb*. The first objective was to determine whether *Pcb* can attach onto *M. incognita* J2s and, if this was the case, to determine whether the J2s can disseminate the bacteria as they move around in the environment. The second objective was to determine whether there is a synergistic interaction between RKN and SRE and the combined effect of the two pathogens on their host *Solanum tuberosum* cv Mondial. The results obtained in the first part of the study strongly suggested that *Pcb* can attach onto *M. incognita* J2s and can be disseminated as the J2s move around in the environment. Thus, this indicated that there is a synergistic interaction between *M. incognita* and *Pcb* as there was increased disease severity and incidence in plants inoculated with both pathogens compared to those inoculated with individual pathogens. Significantly higher *Pcb* concentrations were found in plants inoculated with both pathogens. There was no breakage of tolerance to *Pcb*-caused blackleg on an otherwise resistant cultivar, BP1.

The second aim of this study was to determine whether the induction of natural resistance using environmentally friendly resistance inducing chemicals can potentially be used as an alternative to chemical control. To this end, the effect of three inducers at different concentrations, amongst DL-β-aminobutyric acid, Acibenzolar-s-methyl and Messenger on potato plants infected with RKN was compared. The most effective resistance inducer amongst the three was 20mM BABA as it was able to reduce the number of J2s that penetrated host tissue, the number of females in the roots and the rate of egg production. Furthermore, the galling index observed in potato roots was significantly lower when plants were treated with 20mM BABA. Additionally, the reduced rate of RKN infection in plants primed with 20mM led to a decrease in the rate of *Pcb* infection.

Interaction between soft rot *Enterobacteriaceae* and root knot nematodes

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ABSTRACT

The study of plant parasitic nematodes such as *Meloidogyne spp.* and their interactions with phytopathogenic bacteria remains underexplored. One of the challenges towards establishing such interactions is the dependence on symptom development as a measure of interaction. In this study, mCherry was employed as a reporter protein to investigate the interaction between the soft rot *Enterobacteriaceae* (SRE) *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pcb*) and root knot nematode (*M. incognita*). *Pectobacterium carotovorum* subsp. *brasiliensis* was transformed with pMP7604 generating *Pcb_mCherry* strain. This strain was shown to attach to the surface coat of *M. incognita* J2 at the optimum temperature of 28°C. This suggests that RKN juveniles may play a role in disseminating *Pcb* in soils that are heavily infested with *Pcb*. The presence of RKN juveniles was shown to play a role in introducing *Pcb_mCherry* into potato tubers potentially acting as a source of latent tuber infections.

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CHAPTER THREE: INDUCTION OF RESISTANCE IN *SOLANUM TUBEROSUM* AGAINST ROOT KNOT NEMATODE (RKN) INFECTION

LIST OF ABBREVIATIONS

$	dollars
%	percentage
°C	degrees Celsius
ANOVA	analysis of variance
BABA	DL-β-aminobutyric acid
bp	base pair
CaCl2·2H2O	calcium chloride
CFU	colony forming units
CLSM	confocal laser scanning microscopy
cm	centimetre
cv	cultivar
CVP	crystal violet pectate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside-5'-triphosphate
g	gram
HRBC	Human Red Blood Cells
i.e.	that is
IGS	intergenic spacer region
ISR	Induced Systemic Resistance
J2	second stage juvenile
KH_2PO_4	Potassium di-hydrogen phosphate
LB	Luria-Bertani
M	Molar
mg	microgram
mg/L	micrograms per liter
MgSO4	magnesium sulphate
min	minute
ml	millilitre
mM	millimolar

mm	millimetre
NaCl	sodium chloride
$NaNO_3$	sodium nitrate
NaOH	sodium hydroxide
$Na_2HPO_4 \cdot 2H_2O$	Disodium phosphate
ng/µl	nanograms per microliter
NH_4Cl	ammonium chloride
nm	nanometer
Pcb	*Pectobacterium carotovorum* subsp. *brasiliensis*
*Pcb*_mCherry	*Pectobacterium carotovorum* subsp. *brasiliensis* tagged with mCherry fluorescent protein
Pcc	*Pectobacterium carotovorum* subsp. *carotovorum*
PCR	polymerase chain reaction s second
$rev.min^{-1}$	centrifugal force
RKN	Root Knot Nematodes
SAR	Systemic Acquired Resistance
sec	seconds
SEM	scanning electron microscopy
spp.	species
SRE	soft rot *Enterobacteriaceae*
subsp.	subspecies
Tet	tetracycline
U	unit
USA	United States of America
v/v	volume per volume
w/v	weight per volume
µl	microlitres

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CHAPTER ONE

ROOT KNOT NEMATODES AND THEIR RHOZOSPHERE INTERACTIONS

CHAPTER ONE

Root Knot Nematodes and their rhizosphere interactions

1.1 Background on Root Knot Nematodes (RKN)

Root knot nematodes (RKN), also known as *Meloidogyne* spp., are sedentary obligate endoparasites with complex and specialised relationships with their hosts (Caillaud et al., 2008). They are the most successful plant parasitic nematodes in terms of distribution and host range. They have the ability to infect and reproduce on hundreds of unrelated plant species (Sasser, 1977; Sasser et al., 1983; Abad and Williamson, 2010). This genus is currently known to have more than 90 species and these include some of the most economically important plant parasitic nematodes (Bird and Koltai, 2000; Brito et al., 2008). The most widely distributed RKN species include *Meloidogyne incognita, M. javanica, M. arenaria, M. chitwoodi, M. hapla,* and *M. fallax*. Amongst these *Meloidogyne* spp., *M. incognita, M. javanica and M. arenaria* are found mainly in tropical regions whilst *M. chitwoodi, M. fallax and M. hapla* are found in areas with lower temperatures (Santo et al., 1980; Trudgill and Block, 2001; Adam et al., 2007). Although many *Meloidogyne* species are known to be important pests worldwide, the above-mentioned species are regarded as the most important in globally in potato production (Hunt and Handoo, 2009).

Infections by *Meloidogyne* spp. have been reported globally. Some of these areas include Florida (Chitwood, 1949) and Saudi Arabia (Al-Hazmi et al., 1993). Recently, RKN have been reported in banana, potato, peanut, soybean and sugarcane growing regions in South Africa (De Waele and Davide, 1998; Fourie et al., 2001; Cadet and Spaull, 2003; De Waele and Elsen, 2007). A recent study in South Africa was conducted by Onkendi and Moleleki (2013) on the distribution of root knot nematodes in potato farms. From that study, *M. incognita*, *M. arenaria*, *M. javanica*, *M. hapla*, *M. chitwoodi* and another highly virulent species in South Africa, *M. enterolobii*, were identified. Information about the different species of parasitic nematodes, especially root knot nematodes, present in different regions around South Africa, and other parts of the world, is important as effective pest management programmes can be put in place to control them (Onkendi and Moleleki, 2012; 2013)

1.2 The life cycle of RKN

Generally, the life cycles of plant parasitic nematodes occur over an average period of three weeks to several months, depending on environmental conditions such as temperature, moisture, availability of a suitable host and the parasitic nematode species involved. *Meloidogyne* spp. can reproduce both sexually and asexually, a phenomenon referred to as mitotic parthenogenesis (Castagnone-Sereno, 2006). In RKN, second stage juveniles hatch from the eggs and will start to search for roots of a compatible host. Once inside the root of a compatible host, they migrate towards the root tip. They have been shown to move to the root apex first before they migrate backwards to invade the vascular cylinder (Dropkin, 1969; Mitkowski and Abawi, 2003; Williamson and Gleason, 2003) (Figure 1.1). In the vascular cylinder, each female J2 will then induce several parenchyma cells in the zone of protoxylem development to become large, multinucleate cells. Subsequent to that, female J2s become sedentary and this period is characterized by feeding from the multinucleate cells that they have induced (Mitkowski and Abawi, 2003; Caillaud et al., 2008). Since males do not play a role in the reproduction process of most RKN spp., they migrate out of the roots whilst females start the process of egg production. As females produce eggs they increase in size and become pear-shaped. Their posterior ends then break the root epidermis of the galls in which they reside, this allows them to lay eggs on the root surface in a gelatinous matrix that serves as a protective barrier to physical damage and microbial attack (Mitkowski and Abawi, 2003; Caillaud et al., 2008; Abad and Willamson, 2010). In the eggs, embryogenesis will occur, followed by the first molt that will produce first stage juveniles (J1s). These will mature into second stage juveniles (J2s) inside the eggs which will hatch and infect compatible host plants once in the soil (Mai and Abawi, 1987). The second stage juveniles of RKN represent the only mobile and infective stage in the life cycle of *Meloidogyne* spp. (Mitkowski and Abawi, 2003).

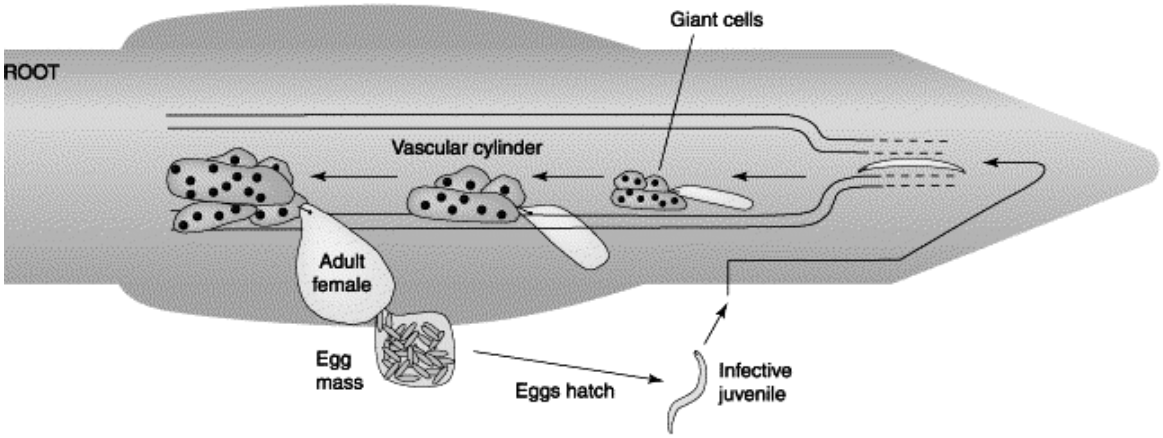

Figure 1.1 Summary of the life cycle of *Meloidogyne* spp. Second stage juveniles (J2s) hatch from eggs in a gelatinous matrix on the surfaces of root knots and start the process of searching for compatible host roots in the soil. The J2s then penetrate the roots, migrate towards the root apex where they gain access into the vascular cylinder containing parenchyma cells. Each female J2 then modifies few parenchyma cells into nutrient sinks from where they will feed during the course of the cycle (Williamsom and Gleason, 2003).

1.3 Hosts and symptoms of RKN

Although root knot nematodes are generally known to cause symptoms on plant roots in the form of galls, infection can also result in foliar deformations of their hosts. *Meloidogyne* spp. are very difficult to identify as causal agents of crop damage without assessment of roots as the symptoms observed on the foliar regions of infected hosts are nonspecific and can result from infections by other parasitic nematodes and/or other biotic and abiotic factors (Jardine, 1990; Tylka, 1994). A classic example is that of chlorosis which can be a symptom of RKN infestation or nutrient deficiency (Tylka, 1994). Another example is poor growth stands of crops which can be caused by poor soil fertility, water stress symptoms or RKN damage. Other above-ground symptoms caused by RKN infection include patchy and stunted growth (Mitkowski and Abawi, 2003; Cerkauskas, 2004), wilting even in adequate water supply (Mitkowski and Abawi, 2003), low yields (Davis, 2007) and the collapse and premature death of the host crop (Mitkowski and Abawi, 2003; Cerkauskas, 2004). This shows that for effective RKN management, foliar symptom development cannot be used to identify the causal agent of a particular deficiency. Perhaps the lack of clear links in symptoms observed to RKN is one of the reasons plant parasitic nematode research remains neglected.

Below-ground symptoms include swellings or galls on roots or tubers and a stubby root system. When attacked by RKN, root crops such as carrots usually undergo severe galling and forking (Widmer et al., 1999). Crops infected by RKN become vulnerable to several environmental stresses and therefore become more susceptible to other pathogens (Mitkowski et al., 2002). Crops such as onions produce significantly smaller bulbs since mineral absorption from the soil is impaired as a result of RKN infestation (Widmer et al., 1999). Indirect damage caused by RKN includes the formation of blisters on the surfaces of vegetables such as potato tubers and brown spots on the inside where females are usually found. These deformities caused by RKN on vegetables such as potato and carrots lower their market value and most importantly play a role in the spread of RKN to new areas (Volvas et al., 2005).

1.4 Importance of RKN

Meloidogyne spp. belong to a group of economically important plant parasitic nematodes called sedentary endoparasites. Sedentary endoparasites include cyst nematodes (*Globodera* and *Heterodera*) which belong to the family heteroderidae (Williamson and Gleason, 2003). Some of the migratory plant parasitic nematodes are also known to cause damage and these include those belonging to the genera *Pratylenchus* and *Radopholus* (Bird and Koltai, 2000). Sasser and Freckman (1987) estimated the average yield loss caused by plant parasitic nematodes to collectively be 14.6% with some crops approaching losses of about 20%. The monetary value of the losses caused by these plant parasitic nematodes was estimated to exceed $100 billion annually (Sasser and Freckman, 1987; Koenning et al., 1999). In 1994, it was estimated that annually RKN caused looses of 8% to major food and fiber crops. In the USA, when calculated, in monetary terms, RKN damage was estimated to be about $8 billion. When these estimations were done for the rest of the world the value came to about $78 billion annually (Baker et al., 1994; Koenning et al., 1999).

The global distribution of *Meloidogyne* spp. combined with their wide host range and their ability to form synergies and complexes with fungi, bacteria and viruses makes them even more important pests economically (Wallace, 1963; Sasser, 1980; Grundler, 1996; Dervant and Sogut, 2011). Besides their wide host range, important *Meloidogyne* spp. are difficult to manage due to their short generation time and high reproductive rates (Trudgill and Block, 2001).

1.5 Interactions between RKN and plant pathogens

The first report of an interaction between *Meloidogyne* spp. and a soil-borne parasitic microorganism was in 1892 and this interaction was between RKN and *Fusarium oxysporum* on cotton. Since then, more interactions have been discovered and reported (Powell, 1971; Powell et al., 1971; Mai and Abawi, 1987; Back et al., 2000; Riedel, 1988; 2002). The attention received by the subject of interactions between parasitic nematodes and soil-borne plant pathogens was not only as a result of their economic importance but also due to the insights of the interactions between the pathogens and their hosts (Riedel, 1988).

It has long been known in plant pathology that for the development of disease there has to be an interrelation amongst the host, pathogen and the environment but in the case of soil-borne pathogens, opportunistic microbes occupying the same niche establish secondary interactions in disease development (Back et al., 2002; Agrios, 2005). A lot of work has been done on the interactions between plant parasitic nematodes, particularly *Meloidogyne* spp., and fungi (Riedel, 1988). Other endoparasitic nematodes such as *Globodera* spp., *Heterodera* spp., *Rotylenchulus* spp. and *Pratylenchus* spp. and ectoparasites such as *Xiphinema* spp. and *Longidorus* spp. have also been associated with soil-borne fungal pathogens (Riedel, 1988). *Meloidogyne* spp. have been shown to interact with different plant pathogens such as fungi (Golden and van Gurdy, 1975; Negron and Acosta, 1989), bacteria (de Moura et al., 1975; Walker et al., 1998) and viruses (Alam et al., 1990; Udo et al., 2003). Reports of interactions between fungi and RKN are more frequent than those of viruses or bacteria. This is due to the fact that RKN and fungi are represented by many species in the soil and since their inoculum levels are usually found in high proportions there is a higher chance for interaction between them. On the other hand, nematode-nematode interactions are rarely reported and this is solely due to lack of studies (Riedel, 1988).

Even though interactions between plant parasitic nematodes and phytopathogenic bacteria have been neglected, they are important and warrant investigation (Siddiqui et al., 2012).

In 1989, Negron and Acosta demonstrated the interaction between RKN and fungi using coffee plants with *M. incognita* and *F. oxysporum* f.sp coffee. They observed that the necrosis on the roots and chlorosis on foliage were more severe only when plants were inoculated with *M. incognita* several weeks prior to *F. oxysporum* f.sp coffee inoculations. When the fungus was inoculated two or four weeks post inoculation with *M. incognita* it was found in the xylem vessels and giant cells. Some of the giant cells had slightly depleted contents compared to others. Plants which were simultaneously inoculated with *M. incognita* and the fungus had fewer galls colonized by the fungus and there was no fungus observed in the xylem vessels.

More evidence of interactions between RKN and plant pathogens was provided by de Moura et al. (1975), using *M. incognita* and *Corynebacterium michiganese* (now known as *Clavibacter michiganensis*), a bacterial pathogen that causes canker on tomatoes. In their experiments, they demonstrated the variations of interaction between RKN and the pathogenic bacteria using different inoculation time points and different hosts. They observed that plants, both susceptible and resistant to the bacteria, had more severe canker in nematode-bacteria combination inoculations compared to those inoculated with *C. michiganensis* alone. It took fewer days for resistant plants inoculated with both *M. incognita* and *C. michiganensis* to develop symptoms and more days in those plants which were inoculated with the bacteria alone. There were no significant differences in canker severity when both the susceptible and resistant varieties were inoculated with bacteria ten days prior inoculation with RKN compared to simultaneous inoculation with the two pathogens. This implied that in this particular interaction the severity of disease from the synergistic interaction between the RKN and bacteria is not related to when both pathogens got into contact with the host. A different trend was observed when using *Lycopersicon hirsutum* whereby plants that were inoculated immediately after transplanting had more sever canker compared to those inoculated ten days later with the bacteria post RKN inoculation. This might have been due to the fact that these plants grow slowly and therefore require several days to establish. The differences in disease severity in the mentioned interactions are an indication that interactions between parasitic nematodes and secondary pathogens with

their hosts are unique and to understand them, they must be studied individually and not generalised.

When Griffin and Hunt (1972) performed experiments on alfafa where they were determining the effect of simultaneous inoculation of *Corynebacterium insidiosum* and 30 days post inoculation with *M. hapla*, they did not observe any significant differences in disease severity. In contrast to Griffin and Hunt's observations, Johnson and Powell (1969) reported significant differences when they inoculated one group of tobacco plants with *Ralstonia solanacearum* four weeks post inoculation with RKN and another group with both pathogens simultaneously. In these experiments, significantly higher disease severity was observed on plants inoculated with *R. solanacearum* several weeks post nematode inoculation compared to plants that were simultaneously inoculated with both pathogens. These results indicated that some nematode-bacterial combinations are dependent on the time of inoculation by both pathogens whilst in some combinations time of inoculation is not essential.

In 1998, Walker and co-workers discovered the interaction between *M. incognita* and *Thielaviopsis basicola*, a pathogen responsible for the black root-rot of cotton. Neither of these pathogens has the ability to cause any severe damage to cotton and plants rarely die when solely infected by either these pathogens. However, co-infection has been found to cause the death of seedlings, root necrosis, suppressed early seedling growth and reduction in the percentage of cotton bolls. An increase in disease severity was also observed in a combination-inoculation between *M. incognita* and tomato mosaic virus on tomato plants as a result of their synergistic relationship (Alam et al., 1990).

In cases where other microorganisms can only cause disease on plants which have been attacked by plant parasitic nematodes, the latter were referred to as "secondary invaders". However this term can also be used to refer to organisms which invade dead tissue resulting from a prior infection. Powell (1971) suggested that the term "secondary pathogen" be adopted in place of "secondary invaders". This is because the term explains that these microorganisms infect their hosts prior contact with other pathogens. The use of the term "secondary invaders" also highlights the ability of these organisms to play a role in causing disease, albeit secondary to primary invaders.

Root knot nematodes have also been demonstrated to have the ability to break the resistance of their hosts to infection by secondary pathogens. Johnson and Powell (1969) provided evidence to this concept using RKN *M. incognita* and the soil-borne pathogen *Ralstonia solanacearum*. In their experiment, in which they used susceptible, moderately resistant and highly resistant varieties of flue-cured tobacco inoculated with both *M. incognita* and *R. solanacearum*, they found that both susceptible and moderately resistant varieties equally wilted even though the symptoms were more severe in susceptible plants. On all their sampling days, they also observed that there were no significant differences when assessing the highly resistant and the moderately resistant plants for disease severity indices. There was also more disease development when the bacteria was inoculated three or four weeks post inoculation with RKN compared to when they were inoculated simultaneously. The same trend was observed when mechanically wounding plants before bacterial inoculation. Regardless of the variety, the wilt incidence was higher in those plants which were inoculated with RKN three weeks prior to bacterial inoculation. A synergistic relationship was also observed between *M. incognita* and *R. solanacearum* on tomato where disease caused by the phytobacteria was significantly higher in the presence of RKN (Napiere and Quinio, 1980). These results indicated that RKN have the ability to break resistance/tolerance of their hosts to a range of pathogens which would, in the absence of RKN, not have been able to cause disease on the same level of severity.

Based on the examples provided, it is clear that the role of secondary pathogens in disease complexes with RKN deserves intensive study. The correct identification of the causal agents of any disease is therefore not as simple and straight forward due to synergies and complexes that form between pathogens in the soil. This emphasises the importance of studying pathogens not as individuals but as complexes since this better emulates the natural environment. The literature clearly highlights the fact that as the role of these secondary pathogens is investigated, further more efficient control measures can be developed (Powell et al., 1971).

Although RKN, unlike free-living nematodes, do not ingest bacteria, they are able to carry the bacteria on their cuticles and thus serve as disseminators. Hallmann et al. (1998) proved this using a phytobacteria *Enterobacter asburae* and *M. incognita*. In their experiments, after the two organisms were co-incubated, dissemination of *E. asburae* by J2s of *M. incognita* was demonstrated by trails of bacteria on nutrient medium away from the intial point onto which the

bacteria-nematode suspension was originally pipetted. In 2008, Maghodia and colleagues conducted similar experiments that supported the attachment of microorganisms on the surfaces of RKN using *Meloidogyne javanica* and *Escherichia coli.* They found that *E. coli* can attach to the RKN under specific bacteria-nematode ratios and temperature conditions.

Many interactions have been reported between RKN and other important plant pathogens but the potential of an interaction between *Meloidogyne* spp. and soft rot enterobactericeae (SRE) has not been studied.

1.6 Soft Rot *Enterobacteriaceae* (SRE)

In potatoes and other tuber crops, soft rot and blackleg are known to be mainly caused by SRE genera *Pectobacterium* and *Dickeya* spp. Blackleg caused by *Pectobacterium* spp. is characterised by slimy, wet, black rot lesion on the stem and this has been shown to originate from planting latently infected mother tubers (Perombelon and Kelman, 1980; Toth et al., 2003). Alternatively, similar symptoms can develop from inoculum that is deposited by insects as they feed between healthy and infected plants (Agrios, 2005). *Pectobacterium* spp. belong to the family *Enterobacteriaceae* and have been shown to produce enzymes which degrade the cell walls of their hosts in order to gain access to nutrients, multiply and colonise host tissue (Barras et al., 1994; Hauben et al., 1998). *Pectobacterium* spp. that have been identified as major soft rot causing agents are *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) and *Pectobacterium atrosepticum* (*Pa*), both formerly classified under soft rot causing Erwinias (Dye, 1969). Recently in South Africa, two species of importance for the potato industry have been reported on. Firstly *Pectobacterium carotovorum* subsp. *brasiliense* (*Pcb*), a Brazilian isolate shown to be more virulent than some of the commonly known *Pectobacterium* spp. on potatoes has been found. (van der Merwe et al., 2009). Secondly, Moleleki et. al. (2012) reported on the presence of *Pectobacterium wasabiae* strain in South Africa.

Soft rot-causing phytobacteria can infect mother tubers of potato latently and only start causing rot when the conditions are favourable. These type of bacteria can also originate from the soil as a result of tuber infections from the previous planting seasons (Serfontein et al., 1991; Perombelon, 1992; Rowe et al., 1995; Agrios, 2005; Tsror et al., 2009). Another source of

inoculum is rotting tubers in the soil (Agrios, 2005). Insects that feed between diseased and healthy plants have also been shown to act as a source of inoculum.Water has also been reported as a source of inoculum for *Pectobacterium* spp. (Harrison et al., 1987; Laurila et al., 2008). The bacteria are able to move up into the stem from mother or progeny tuber infections or from the stem down to the tubers underground (Czajkowski et al., 2010). As soon as the conditions are favourable, the bacteria start to build up in numbers and once they have reached their population threshold, they start to produce effectors and pectolytic enzymes that will result in rotting of the mother and progeny tubers (Perombelon, 2002). Rotting mother tuber or progeny tubers as well as roots release bacteria into the soil leading to increased bacterial load in the soil (Rowe et al., 1995 Roberts et al., 2007). Inoculum that has spread up into the stem above ground can be transmitted by feeding insects to healthy hosts (Perombelon 1992; Rowe et al., 1995).

1.7 Factors affecting RKN interactions with other plant pathogens

Interactions between plant parasitic nematodes and other plant pathogens are affected by several factors. These include carbohydrate moieties on nematode surface coats, openings formed by J2s as they penetrate host roots, physiological modifications (galled areas), nutritional status of the host, resistance breakdown in the host to other pathogens by RKN, the release of root exudates, nematode species involved and environmental conditions.

Root-knot nematodes, like free-living and other plant parasitic nematodes, have a layer that covers the outer surface of their bodies. This layer is commonly known as the surface coat and it protects the inside delicate parts of the nematode bodies from the external environment. In addition, the surface coat serves as an exoskeleton for the nematodes (Spiegel and McClure, 1995). The surface coat is composed of carbohydrate moieties onto which several molecules and microorganisms can bind (Spiegel et al., 1983; Zuckerman and Jansson, 1984; Wright, 1987; Bird and Zuckerman, 1989; Hallman et al., 1998; Maghodia et al., 2008). To demonstrate that these carbohydrate moieties play a role in attachment of molecules onto the surface of nematodes in general, Spiegel et. al. (1995) used Human Red Blood Cells (HRBCs). The experiments were conducted by exposing the plant parasitic nematodes to various sugars and then subsequently exposing them to a HRBC suspension. When comparing HRBCs that were bound to plant

parasitic nematodes that were exposed to sugars and those that were not, a significantly higher number of HRBCs was able to attach onto the surfaces of those that were not exposed to sugars prior HRBC co-incubation. This was confirmation that parasitic nematode surfaces have carbohydrate moieties that play a role in the attachment of molecules onto nematode surfaces. These carbohydrate moieties on the surface coat of plant parasitic and free-living nematodes have also been suggested as sites for microbial attachment. Furthermore, fungi that predate nematodes produce conidia and fungal traps which attach to carbohydrate binding sites on nematode surfaces (Spiegel and McClure, 1995). Additional evidence of carbohydrate moieties, and maybe other components of the surface coat, playing a role in attachment of microorganisms onto nematode surfaces was provided by Bird and Zukerman (1989) and McClure and Spiegel (1991) when they demonstrated that without the surface coat, attachment does not happen.

The surface coat of nematodes also serves as a platform onto which microorganisms can secrete exctracellular material such as exopolyssacharides that enable them to attach onto nematode surfaces. The use of exopolyssacharides and therefore the formation of biofilms on nematode surfaces, as a tool for bacterial attachment was demonstrated by Darby et. al. (2002). In their experiments they used *Yersinia pestis*, a plaque-forming pathogen in mammals, and *C. elegans*, a commonly known free living nematode. *Yersinia pestis* were shown to attach onto the surface of the nematode using an exopylossacharide layer without the cells being in direct contact with the surface of the nematode. The bacteria embedded themselves in the exopolyssacharide layer that was attached onto the nematode surface, forming a biofilm.

The openings on host roots created by infective stages of plant parasitic nematodes have also been demonstrated to play a pivotal role in interactions between plant parasitic nematodes and secondary pathogens. Plant parasitic nematodes, depending on their different life cycles and life stages, are able to form openings on the roots of their hosts during the infection process (Dropkin, 1969; Inagaki and Powel, 1989; Mitkowski and Abawi, 2003; William and Gleason, 2003). *Pratylenchulus* (migratory ectoparasites) and *Meloidogyne* spp. (sedentary endoparasites) cause far more destruction to the host roots than other known plant parasitic nematodes. The J2s penetrate the roots behind the growing root tips and either migrate intracellularly (*Globodera* and *Heterodera*) or intercellularly (*Meloidogyne*) to the vascular cylinder where feeding commences (Doncaster and Seymour, 1973; Back et al., 2002). These wounds created by infective stages of

respective parasitic nematode species have been suggested to play a pivotal role in many interactions (Inagaki and Powell, 1969).

Inagaki and Powell (1969) provided evidence that supported the hypothesis that openings created by plant parasitic nematodes' infective stages play a significant role in the secondary infection process of fungi. In their experiments, one group of plants was subjected to artificial wounding and exposed to the fungus, another exposed to the pathogenic fungus without wounding and the third group was inoculated with the fungus several days post inoculation with the RKN. The fourth group was inoculated with both RKN and the fungus simultaneously. Both the artificially inoculated plants and those inoculated simultaneously with both pathogens had higher disease severity compared to those inoculated with the fungus alone. Plants inoculated with the fungus one week post inoculation with RKN showed elevated disease development compared to those that were inoculated with the fungus two or three weeks post inoculation with RKN. It was concluded that minute openings, created by the RKN J2s as they penetrated the roots during the infection process, acted as entry points for the fungus. To explain the differences in their observations, they reasoned that after two or three weeks post inoculation, the nematode-created minute openings on the roots were probably closed hence not as many as the ones created at one week post nematode inoculation. This, consequently, resulted in higher fungal inoculum levels that elevated the severity of disease in plants inoculated with the fungus a week post inoculation with RKN compared to plants inoculated with the fungus alone.

The importance of wounds in the increased infection rates of secondary pathogens was also demonstrated by Storey and Evans (1987) using *Verticillium dahliae* and *Globodera pallida* on potatoes. They found that disease caused by *V. dahliae* depends on the timing of invasion by the cyst nematode. The fungus was found to use the channels created by the nematode when both pathogens were simultaneously inoculated. They also found that the disease symptoms caused by the fungus were less severe when the fungus was inoculated eight days post inoculations with the nematode, compared to inoculations with the fungus alone. This further highlights the importance of wounding as an essential factor in interactions between parasitic nematodes and plant pathogens.

The feeding sites induced by the sedentary endoparasitic nematodes, such as galls caused by RKN, are areas of high metabolic activity which favour microbial colonization and have been

shown to also play a role in interactions between plant parasitic nematodes and secondary pathogens (McLean and Lawrence, 1993). In 1970, Mayol and Bergeson demonstrated this phenomenon when tomato plants were inoculated with *M. incognita*. After several weeks post inoculation, necrosis was observed on the roots of inoculated plants, and no such symptoms were observed on negative control tomatoes. The fungi isolated from the galls included *Trichorderma* spp., *Fusarium* spp. and *Rhizoctonia solani*. This shows that without the physiological modifications induced by RKN on the host roots, the fungi in the soil was not able to cause disease on its own.

In 1975, Golden and Van Gurdy did a comprehensive study on the role of physiological modifications of the plant by RKN. In their study they used *M. incognita, Rhizoctonia solani* and fumigants (ethylene dibromide and methyl bromide, in separate plots). In plots which were not treated with the fumigants, *R. solani* was isolated from the *M. incognita*-induced galls a week after their formation and two weeks later black sclerotia were found embedded in the galls. The sclerotia were not seen on non-galled root parts and after four weeks a large amount of root decay was observed on roots inoculated with *M. incognita* and *R. solani*. The fungus appeared to be using galled tissue to infect plants.

The effect of physiological modifications on interactions between plant parasitic nematodes and secondary pathogens can be systemic, such that factors or substances induced by parasitic nematodes which might be beneficial to secondary pathogens are translocated within the plant whereby secondary pathogens can severely infect host tissue that is distal from the tissue infected by parasitic nematodes. In the absence of parasitic nematodes, secondary pathogens would have been able to successfully or severely infect the host. To prove this hypothesis, Bowman and Bloom (1966) used the "split root" technique whereby the root system of the plants of interest were split into two equal portions and planted in two separate pots. One portion of the roots was inoculated with infective stages of the parasitic nematode whilst the other was inoculated with the fungus. They found out that the development of disease was dependent on the presence of both the plant parasitic nematode species and the fungus. Even though the "split root" technique did not identify systemic physiological modifications or any other mode of interaction, it did indicate that there might be an interaction between the parasitic nematodes involved and the fungus. Amongst the other possible reasons, the nutritional status of the plants might have

improved in favour of fungal growth or nematode infestation might have caused a loss of resistance or stress to the host. Another reason might be that nematode infestation disabled the host's ability to produce compounds which would have been toxic for secondary pathogens.

The nutritional status of the host is also an important factor as it influences host colonization. Physiological modifications on host roots also alter the nutritional status of the host. Melendez and Powel in 1967 demonstrated that *Fusarium* spp. selectively colonized galled root tissue compared to the healthy root parts. These findings were confirmed by Emmanouili and Wood (1981) whereby they identified the nutritional status of the host as an important element in interactions between parasitic nematodes and secondary pathogens. They concluded that the injection of amino acids and sugars into the host cells by parasitic nematodes could be a way in which susceptibility to *Verticillium* is increased in tomato by *Pratylenchus* spp. Parasitic nematodes have also been shown to influence the growth factors which are suggested to influence the hosts' susceptibility to pathogens. This was demonstrated by increased levels of Indole Acetic Acid (IAA) in tomato plants after inoculation with *M. incognita* and *Fusarium oxysporum f.sp. lycopersici*. Growth factors play a role in keeping host cells in a rapidly dividing juvenile state which might make it easier for pathogens to colonize them (Riedel, 1988).

Root exudates that leak out into the soil as infective stages of plant parasitic nematodes penetrate into the roots also play a significant role in interactions between plant parasitic nematodes and other pathogens by attracting secondary pathogens and other plant parasitic nematodes to primary infection sites. For example, when plant parasitic nematodes penetrate the host roots, root exudates leak out and attract bacteria and other soil-borne pathogens to the damaged areas (Zentmyer, 1961). If some of the attracted microbes are pathogenic, the oozing wounds serve as entry points. Root exudates can also serve as a source of nutrition (Rovira, 1956) and thus provide energy for host penetration by secondary pathogens (Schroth and Hendrix, 1962).

In 1977 Van Gurdy and co-workers used a hydroponic system to demonstrate the attraction of *R. solani* to *M. incognita*-infested tomato plants. The treatments in their experiments included plants inoculated with *M. incognita* J2s alone, plants inoculated with *M. incognita* J2s and the fungus, plants inoculated with the fungus alone and untreated plants. All the treatments were confirmed free of root-necrosis five weeks prior to inoculation. When root lechates were taken from the roots previously infected with *M. incognita* and applied to plants inoculated with *R.*

solani alone, necrosis developed but when, in the same experiment, lechates from untreated plants were used no necrosis developed. Furthermore, when the trial was repeated in soil without a hydroponic system, plants only developed root-rot when exposed to both organisms. These results implied that *M. incognita*-infested plants produced special exudates attractive to most pathogens including *R. solani*. The exudates were found to contain high levels of ^{14}C metabolites. Interestingly and contiguous with this, it is known that at the time of sclerotial development, the major constituents of the ^{14}C-labeled metabolites include amino acids and proteins which are essential in the virulence of *R. solani* (Weinhold et al., 1972).

Root knot nematodes facilitate infection by secondary pathogens by breaking down plant resistance. Even though there are reports of loss of resistance during attacks by plant parasitic nematodes and secondary pathogens, the significance of these complex infections is seldomly reported (Marley and Hillocks, 1994; Back et al., 2002). There are few reports on how this loss or reduction of resistance occurs and a classic example was that provided by Marley and Hillocks in 1993 and 1994 when working on *Cajanus cajun* (pigeonpea) and *Fusarium udum*. These authors noted that rapid accumulation of a phytoalexin cajanol in *C. cajun* was responsible for resistance *F. udum*. Interestingly, when plants were inoculated with *M. incognita* or *M. javanica* simultaneously with the fungus, the cajanol content was 62% lower and resistance was lost. The disease incidence and severity were significantly higher than in plants inoculated with *F. udum* alone. This was an indication that RKN changed the plants' physiology making it more vulnerable to fusarium wilt. The authors reasoned that infection by RKN could have resulted in reduction of the overall metabolic rate of the plant or specific changes in the host that had adverse effects on the synthesis of defense chemicals such as isoflavanoids (Marley and Hillocks 1994).

There are various synergistic interactions between plant parasitic nematodes and secondary pathogens which result in disease development only under specific conditions. Some interactions are dependent on the nematode species and the genotype of the secondary pathogen (in case of fungi) (Riedel, 1985; Botseas and Rowe, 1994). Riedel and colleagues (1985) observed that the severity of potato early dying disease caused by *Verticulium dahliae* was significantly increased by populations of *Pratylenchus penetrans* but not *P. arenutus* or *P. scribneri*. These findings were recently confirmed by Hafez and co-workers in 1999 when they proved that *V. dahliae*

synergistically interacts with *P. neglectus* from Canada (Ontario) and not populations from Parma (Idaho). Restriction enzyme analysis of the ITS1 region on the rDNA gene of *Pratylenchus* spp. from Idaho and Ontario revealed unique fragments for each population which imply variations within the species.

Environmental conditions also affect disease complexes between plant parasitic nematodes and secondary pathogens. These include temperature, soil type and pH. In 1994, France and Abawi saw visible wilt symptoms on a bean genotype which was shown to have dual resistance against *F. oxysporum* f.sp. *phaseoli* and *M. incognita* when inoculated together at 27°C. They confirmed that high temperatures caused a break in resistance to *M. incognita* and in turn broke the resistance against the fungus. Uma Maheswari et. al. (1997) observed that soil type also affects interactions between plant parasitic nematodes and other soil-borne pathogens. They used *M. javanica* and *F. oxysporum* f.sp. *ciceri* on chickpeas. They found that disease development was higher in clay soil and loamy soil for *F. oxysporum* f.sp. *cicery* and *M. javanica*, respectively. This implies that it will be difficult for these pathogens to form an interaction in either soil type since one of them will be in an environment in which conditions are not optimal for survival.

1.8 Management of RKN

Root knot nematodes can be managed using various methods which include sanitation, resistance breeding, summer fallowing, crop rotation, heat, biological control and chemicals (nematicides and resistance-inducing chemicals). Sanitation includes use of seed that is free of RKN and clean equipment that is thoroughly washed and sterilised between farms to avoid the spread of plant parasitic nematodes. It also includes removal of infected plants from the farm/nursery. Removed plants from nurseries must be burned, and if fed to livestock like potatoes, they should be boiled first to avoid RKN spread (Agrios, 2005). High standard hygienic principles are critical in maintaining nurseries free of RKN infestation. Even though these guidelines are set and available for everyone to use, they are not adhered to and that is the reason behind the presence of RKN inoculum in nursery material. Breeding for resistance against plant parasitic nematodes has also been used as a control strategy. So far it has been one of the most successful control strategies

for RKN. It has been used in tomato (Williamson, 1998), pepper and cucumber (Walters et al., 1997; Thies and Fery, 1998).

Root knot nematodes are very sensitive to dry and hot conditions, and therefore, turning the soil over a period of time before planting exposes the plant parasitic nematodes to direct sunlight and high temperatures. This has been shown to be fatal for RKN and other plant parasitic nematodes. This method of control is called summer fallowing and thus conducted during hot summer days (Agrios, 2005; Olsen, 2011). However summer fallowing is only effective in bare areas with long periods of hot conditions. Another form of parasitic nematode control is crop rotation. Its success is dependent on thorough knowledge of the species, race and the host range of the RKN population in the soil. The basic principle of crop rotation is to ensure that the rotation schedule includes crops that will not allow the nematode population numbers to reach a virulence threshold. Through crop rotation the nematode numbers in the soil can be decreased significantly. This includes planting a RKN non-host/resistant crop for several seasons, since without the host plant, RKN will not survive and therefore inoculum levels will decrease significantly. This puts less pressure on the host plants that are next in line in the rotation schedule (Noling, 2009). Heat and biological control have also been shown to be effective is parasitic nematode control but not always practical (Stirling, 1984; Elmore et al., 1997; de Villiers, 2002).

Chemical control in South Africa includes the use of nematicides which belong to four groups of pesticides. These include the halogenated hydrocarbons, methyl isothiocyanates, organophosphates and methyl carbamates. Halogenated hydrocarbons and methyl isothiocyanates are fumigants that can kill plant parasitic nematodes and their eggs. The other two groups are non-volatile in their mode of action and can kill plant parasitic nematodes by contact or ingestion. The common and effective chemicals include Temik and Methyl Bromide. Even though these chemicals are effective against plant parasitic nematodes, there has been problems in terms of pressure from environmental lobbyists and other groups to phase them out of the market due to the potential damage they can cause in the environment. These chemicals have also been shown to have adverse effects on humans and animals (Smith et al., 1977; Giannakou et al., 2002; Dervan and Sogut, 2011).

Due to environmental issues such as ground water contamination, mammalian and avian toxicity and residues in food, tighter restrictions have been put into place to control the use of nematicides such as Temik and Methyl Bromide in many countries, including South Africa (Grundler, 1996; Caillaud et al., 2008; Dervan and Sogut, 2010; Dervan and Sogut, 2011). Temik, one of the banned chemicals, has been proven to have adverse health effects such as neurotoxicity and also adversely affects the liver and kidneys in humans. Symptoms to longterm exposure to Temik are diarrhea, blurred vision, dyspnoea and pulmonary oedema (Rother and Jacobs, unknown year). In 2002, Temik was banned in the European Union due to the residues found on crops such as potato. In South Africa Temik could only be used only up to December 2011 (Macleod, 2011).

Another chemical which is very effective against plant parasitic nematodes, methyl bromide, has been banned in European Union (Council of Trade Unions, 2010). Its ban makes it very difficult, if not impossible, to establish crops in some parts of Europe due to RKN infestation (Giannakou et al., 2002). The chemicals used to control RKN have also been proven to increase the cost of crop production (Mitkowski et al., 2002). This makes the studies on the interactions between RKN and other soil-borne pathogens with their hosts even more relevant as safe and sustainable cropping systems will need to be developed using information generated from such studies as more and more of these effective chemicals are taken off the shelves (De Waele and Elsen, 2007; Abad and Williamson, 2010). With the production and use of these effective chemicals being terminated, host resistance as an alternative measure remains the most practical and environmentally friendly control strategy to manage RKN. In crops such as cucumber and peppers, where a resistant cultivar is available, host resistance has proven to be a very valuable commodity (Walters et al., 1997; Thies and Fery, 1998).

Plant resistance can be enhanced by modifying plants to constitutively express genes which are required in resistance against parasitic or pathogenic organisms or priming them so that they can more rapidly respond to attack by these organisms. Constitutive gene expression has a large fitness cost and is accompanied by low yields. Due to allocation of resources towards manufacturing of defence products and the possible toxicity of such products when accumulated, the resulting yield and economical losses can be devastating (Heil, 2002). Hence priming, which

will be discussed in the next section, appears to offer more advantages and as a result is becoming more and more popular.

1.9 Priming

In all living organisms, "priming" is a very important phenomenon as it helps many organisms survive infections by pathogenic microorganisms. Several researchers have given various forms of definitions of what priming is. In simple terms, it can be defined as the preparation of an organism's defense system to rapidly respond to infections by pathogenic/parasitic organisms. It has been scientifically defined as "the augmented capacity to mobilize cellular defense responses" (Conrath et al., 2002). This phenomenon has further been defined as the development of an enhanced form of resistance by more rapid activation of defense responses, the second time a host encounters an attack by a pathogen, even in distal parts from the initial points of attack (Conrath et al., 2006). Plants which have been primed are said to be in a "prime state" and this is defined as a physiological condition in which plants are able to better or more rapidly mount defense responses, or both, to biotic or abiotic stresses (Beckers and Conrath, 2007). The concept of priming has been known to exist for many years but only until recently has more work been done to try and understand it and use the knowledge to enhance plant resistance (Conrath et al., 2006). Priming can result in Systemic Acquired Resistance (SAR) or Induced Systemic Resistance (ISR).

Systemic Acquired Resistance is a broad-spectrum form of resistance that is usually triggered in a host post exposure to a low dosage of pathogens or avirulent pathogens. It has has also been shown to be triggered by application of chemicals that induce resistance in plants. It is associated with an increase in signaling molecules such as salicylic acid and pathogenesis related (PR) proteins (Sticher et al., 1997; Durrant and Gong, 2004; Agrios, 2005). On the other hand, Induced Systemic Resistance can be induced by non-pathogens or, like SAR, the application of various chemicals (Van Loon et al., 1998; Conrath et al., 2001; Jakab et al., 2001; Pieterse et al., 2001). The major distinguishing factor between SAR and ISR is that the latter is independent of salicylic acid and pathogenesis-related proteins but rather on ethylene and jasmonic acid as signaling chemicals. Even though this is the case, exogenous application of salicylic acid has

been shown to elicit ISR (Van Peer et al., 1991; Kessmann et al., 1994; Gorlach et al., 1996; Duijff et al., 1998; Van Loon et al., 1998; Mauch, et al., 2001).

When compared to other methods/strategies of resistance induction in plants, priming stands out as the more practical strategy to use (Heil, 2002). There are several researchers who have conducted experiments which highlighted the benefits of priming and costs of priming in comparison with other forms of induced resistance (Agrawal et al., 1999; Cipollini, 2002; Korves and Bergelson, 2004). Korves and Bergelson (2004) highlighted the cost of constitutively or overexpressing resistance genes in plants against pathogen attack. In their experiments, using susceptible Arabidopsis plants that contained the RPS2 gene, they observed that plants that were overexpressing this R gene produced less seed compared to susceptible lines when exposed to *Pseudomonas syringae*. This was attributed to the fact that plants that constitutively/overexpress the R gene do so even in the absence of the pathogen and therefore spend more energy expressing this foreign gene instead of promptly responding to pathogen infection. In priming, plants can more efficiently express pathogenesis-related genes in the presence of a pathogen. Therefore in the absence of a pathogen primed plants do not waste energy synthesising pathogenesis-related protein that are not needed but rather focus that energy and resources on seed/fruit production (Siegrist et al., 2000; Conrath et al., 2002; Beckers and Conrath, 2007; Heil and Kost, 2006).

Although it is generally accepted as better than overexpressing R genes, priming sometimes has costs associated with it. Heil et. al. (2000) demonstrated that wheat plants primed with Bion when growing in nitrogen-deficient soils developed fewer shoots and ears and therefore produced less seed compared to their non-primed counterparts in the absence of the pathogen. In another study, plants primed with methyl-jasmonate by soil drench against herbivous insects produced more seed than their unprimed counterparts under pathogen attack. Another example of priming used against herbivores was provided by Heil and Kost (2006). It is known that plants under attack by herbivores produce extrafloral nectar that attracts carnivorous arthropods to feed on attacking herbivores. In experiments conducted by Heil and Kost (2006) it was shown that plants primed with volatile organic compounds produced increased levels of secreted extrafloral nectar that attracted significantly more carnivorous arthropods, therefore increasing predation

pressure on the attacking herbivores. This provided protection for primed plants whilst unprimed plants were vulnerable to more continuous attack from the herbivores.

The use of chemicals to induce resistance in plants under attack has also been demonstrated against viruses, fungi, bacteria and plant parasitic nematodes (Hwang et al., 1997; Siegrist et al., 2000; Scarponi et al., 2001; Chinnasri et al., 2006). The efficiency of priming was demonstrated on tobacco against tobacco mosaic virus using β-aminobutyric acid (BABA), γ-aminobutyric acid (GABA), and α-aminobutyric acid (AABA). Significantly higher resistance in tobacco against the virus was observed in plants treated with BABA whilst other isomers were not active against the pathogen. BABA activity was associated with the synthesis of superoxide and hydrogen peroxide. Pathogenesis-related protein synthesis and salicylic acid levels also significantly increased in BABA-treated plants. Those prompt responses to viral attack enabled BABA-treated plant to resist disease development (Siegrist et al., 2000).

Priming has also been shown to be effective against fungal pathogens. In experiments conducted by Hwang et. al. (1997) using *Phytophthora capsici* and pepper plants, it was demonstrated that BABA-treated plants were significantly less diseased compared to unprimed plants. This was solely dependent on the dramatic increase of salicylic acid and PR proteins such as chitinases and β-1.3-glucanases. Similar results were observed by Cohen et. al. (1994) using BABA on tomatoes against *Phytophthora infestans*. In those experiments, a significant increase in PR protein accumulation was observed in tomato foliage. BABA provided 97% protection against the fungus compared to other resistance inducers whose protection levels were negligible. Bion has also been shown to be a potential priming agent against fungi in several host plants. Benhamou and Belanger (1998) demonstrated the ability of Bion to prime for resistance against *Fusarium oxysporum* f.sp. *radias-lycopersici* (FORL) in tomato. In Bion-treated plants, fungal growth was restricted to the upper plants layers and this was attributed to the development of callose-enriched wall appositions at fungal penetration sites. Harpin has also been shown to be a potential priming agent. This was demonstrated against *Peronospora parasitica* attacking Arabidopsis plants (Dong et al., 1999).

Against bacterial pathogens, the efficiency of priming was demonstrated against *Pseudomonas syringae* pv. tomato, the causal agent of bacterial speck disease. Plants that were treated with Bion prior bacterial inoculations had significantly less degrees of infection. Bion-treated plant had smaller lesion diameters and bacterial growth *in planta* was also significantly lower (Scarponi et al., 2001) compared to those in non-primed plants. Harpin has also been shown to be effective in eliciting resistance against bacterial pathogens. This was demonstrated by Dong et. al. (1999) using Arabidopsis against *Pseudomonas syringae*. There was reduced bacterial growth on leaves of treated plants. Similar results were obtained by Herman et. al. (2008) using *Pseudomonas syringae* on tomato. In their experiments they observed that Bion-treated tomato plants had significantly reduced levels of bacterial speck incidence caused *Pseudomonas syringae* pv. tomato. This was linked with the fact that in Bion-treated plants increased salicylic levels were detected.

Plant parasitic nematodes belong to the group of pathogens against which priming agents have been tested. In experiments conducted by Chinnasri et. al. (2006) uing *Rotylenchulus reniformis* on pineapple, it was found that acibenzolar-s-methyl (active ingredient of Bion) decreased egg production of *R. reniformis* by 58%. In the same experiments, BABA decreased egg production of *M. javanica* by 60%. In work done by Oka and Cohen (2001), it was demonstrated that BABA is also effective against *Heterodera avanae* and *Heterodera latipons* on cereal. BABA was shown to decrease the number of cysts produced by *H. avanae* and *H. latipons* by 90% and 79%, respectively. On tomato plants, BABA was shown to reduce the rate of *M. javanica* J2s development into adults (Oka and et al., 1999). Another example of how priming can be used as a method to control plant parasitic nematodes was provided by Owen et. al. (2002) using Bion on grapevine against *Meloidogyne* spp. In those experiments, Bion-treated plants had significantly less eggs on their root surfaces compared to untreated plants. On pineapple, it was observed that Bion decreased egg production of root knot nematodes by 40% (Chinnasri and Sipes, 2005).

1.10 Aims of the study

The aims and questions addressed in this study were:

1. To determine whether there is an interaction between RKN and SRE.
    a. Can SRE attach to RKN?
    b. Can RKN disseminate SRE in the rhizosphere?
    c. Do RKN and SRE form synergies/complexes in the rhizosphere?
    d. Do RKN have the ability to break the tolerance of potato BP1 plants to SRE stem infections?
2. To determine the effect of different priming agents on potatoes against RKN.
    a. Which resistance inducer, amongst DL-aminobutyric acid, Acibenzolar-s-methyl and Messenger, can reduce RKN infection in potato?
    b. Can the most effective resistance inducer be used against simultaneous RKN and SRE infections?

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CHAPTER TWO

THE ASSOCIATION BETWEEN ROOT KNOT NEMATODES (RKN) AND SOFT ROT *ENTEROBACTERIACEAE* (SRE) AND ITS EFFECT ON THE HOST PLANT, *SOLANUM TUBEROSUM*

CHAPTER TWO

The association between root knot nematodes (RKN) and soft-rot *Enterobacteriaceae* (SRE) and its effect on the host plant *Solanum tuberosum*

2.1 Introduction

Meloidogyne spp. is a genus of plant parasitic nematodes on which intense research has been conducted due to their importance in the agricultural sector worldwide (Bowman and Bloom, 1966; Powell, 1971; Bergeson, 1972; Bird and Zuckerman, 1989; Bellafiore et al., 2008). Nematodes belonging to this genus cause severe plant disease epidemics that may result in the total loss of crops and therefore great loss of income (Sasser and Freckman, 1987). Root knot nematodes (RKN), as they are commonly referred to, have also been shown to have the ability to form disease complexes with other soil-borne phytopathogens (Barker et al., 1994; Handoo, 1998). A disease complex is formed through an interaction between two organisms whereby the magnitude of their damage is greater than that of their individual effects combined (1+1>2). A combination of two organisms resulting in less damage than the sum of the disease severities of the two organisms combined is regarded as antagonistic (1+1<2). Where two organisms combine and the magnitude of the disease caused is the same as the combined form of the two organisms separately then the association is neutral (1+1=2) (Back et al., 2002).

Meloidogyne spp. have been shown to interact with different plant pathogens such as fungi (Golden and van Gurdy, 1975; Negron and Acosta, 1989), bacteria (de Moura et al., 1975; Walker et al., 1998) and viruses (Alam et al., 1990; Udo et al., 2003). In 1971, Powell regarded pathogens that infect their hosts more severely after being in contact with RKN as “secondary pathogens”. In most instances, where interactions between RKN and secondary pathogens have been reported, it was found that the severity of disease caused by secondary pathogens in disease complexes was significantly higher compared to those pathogens on their own (de Moura et al., 1975; Golden and van Gurdy, 1975; Negron and Acosta, 1989; Alam et al., 1990; Walker et al., 1998; Udo et al., 2003).

There are several factors known to promote the interaction between plant parasitic nematodes and plant pathogenic bacteria. These include the surface coat and the carbohydrate binding sites on the surface of the nematodes, wounds or openings on host root tissue created by infective stages of the parasitic nematodes, parasitic nematode-induced physiological modifications of host tissue, timing of invasion of the parasitic nematode and secondary pathogen involved, nutritional status of the host, root exudates and environmental conditions such as soil type and pH. Interactions between RKN and other soil-borne pathogens have been well studied. However, there is currently no information available regarding the potential interaction between RKN and *Pectobacterium* spp. *Pectobacterium carotovorum* subsp *brasiliense* (*Pcb*) is one of the most virulent *Pectobacterium* spp. in the South African potato industry. The aim of this study was therefore to investigate the interaction between *Meloidogyne spp*. and *Pectobacterium* spp. using *Meloidogyne incognita* as one of the most aggressive *Meloidogyne* spp. on potatoes and *Pectobacterium carotovorum* subsp. *brasiliense* (*Pcb*) as an important and recently isolated soft-rot causing agent in South Africa.

2.2 Materials and Methods

2.2.1 *Meloidogyne incognita* inoculum

Pure cultures of freshly hatched *M. incognita* J2s were supplied by Prof D. Fourie from the University of North West (Potchestroom, South Africa) and Ms Nancy Ntidi (ARC Potchestroom, South Africa). These cultures were maintained and isolated from tomato cv Rodade in the form of egg masses. The egg masses were harvested from the surfaces of the root knots using tweezers to hold the knots in place and a sterile blade to detach the egg masses from the knots. To facilitate hatching, the egg masses were suspended in sterile tap water and were then transferred onto a 20µm sieve (Universal Test Sieves, South Africa). The sieve was placed in a plastic container with enough water to cover the egg masses on the sieve, closed and incubated in a dark cupboard at room temperature for 48-72 hours. Before being used in attachment and dissemination experiments, the freshly hatched J2s were surface sterilised using 1.0% [v/v] of sodium hypochlorite and were rinsed using sterilised distilled tap water on a 20µm sieve.

2.2.2 Bacterial culture conditions

Pectobacterium carotovorum subsp. *brasiliense* (*Pcb*) that was used in this study was tagged with an mCherry fluorescent protein. This strain was obtained from Gugulethu Khubheka from the Host-Pathogen Interaction Research Group at the University of Pretoria, South Africa. Bacterial cultures were grown in a shaking incubator (200 rev.min^{-1}) for 24 hours in Lubria-Bertani (LB) broth (0.8% [w/v] tryptone, 0.4% [w/v] NaCl, 0.4% [w/v] yeast extract; 1% [w/v], pH 7.4). The bacteria were streaked and incubated for 24 hours at 28°C on nutrient agar. The culture media was supplemented with 12.5µg.ml^{-1} of tetracycline hydrochloride (Duchefa Biochemie, Nertherlands). The pectolytic ability of *Pcb*_mCherry strain was assessed by transferring single colonies from the nutrient agar onto crystal violet medium (CVP) (90.1% [w/v] tryptone, 0.5% [w/v] tri-sodium citrate, 0.2% [w/v] $NaNO_3$, 0.4% [w/v] bacteriological agar, 0.18% polypectate (Coen Bezuiddenhout Seed Testing Center), 0.25% [w/v] of 5M NaOH, 1.02% [w/v] of 10% [w/v] $CaCl_2$•$2H_2O$, 0.2% [w/v] of 0.0075% [w/v] aqueous crystal violet; pH 7). For long term storage the bacterial strain was maintained at -70°C as a frozen stock culture in 30% glycerol. All the chemicals, unless otherwise stated, were obtained from Merck, South Africa.

2.2.3 Bacterial attachment to *M. incognita* J2s

To determine whether *Pcb*_mCherry can attach onto *M. incognita* J2s and to quantify this attachment, about 3000 J2s were co-incubated with overnight cultures of *Pcb*_mCherry (1×10^8cfu/ml) in 10ml minimal media (6.4% [w/v] $Na_2HPO_4\cdot7H_2O$, 15% [w/v] KH_2PO_4, 0.25% [w/v] NaCl, 0.5% [w/v] NH_4Cl). *Pcb*_mCherry overnight cultures (1×10^8cfu/ml) were centrifuged for 5min at 10000 rev.min^{-1} and the supernatant was decanted. To wash off the remaining broth from the *Pcb*_mCherry cells they were suspended in 20ml sterile distilled water, centrifuged at 1000 rev.min^{-1} for 3min and the supernatant was decanted. This wash step was repeated twice. Co-incubations were conducted at 4, 28 and 37°C for 24, 48 and 72 hours to determine the optimal temperature and incubation period at which most attachment occurs. Co-incubations were done in triplicates for quantification and attachment studies, separately. At each incubation time point, *M. incognita* J2s were transferred onto a 20µm sieve and washed gently

using sterilised distilled water. The two minute wash step was repeated three times. The washed J2s were suspended in 10ml of sterile distilled water and centrifuged at 1000 rev.min^{-1} for 3min to remove loosely attached bacterial cells. The J2s were then suspended in 1ml of sterile distilled water and transferred into clean 1.5ml Eppendorf tubes. Subsequently the *M. incognita* J2s were ground using Eppendorf microspestles and a serial dilution was prepared. From each dilution factor of ground J2s, 100µl aliquots were plated in triplicate onto nutrient agar supplemented with tetracycline. Bacterial colonies on nutrient agar from each co-incubation time point and temperature were counted. The negative control included ground *M. incognita* J2s alone, without *Pcb*_mCherry. Another set of *M. incognita* J2s from those that were co-incubated with *Pcb*_mCherry was prepared for scanning electron microscopy (SEM). In this study all the quantitative assays were analysed using analysis of variance (ANOVA) at the 95% level and Student-T Test for mean comparison. The statistical analysis was performed using JMP v.5 software (SAS Institute Inc., NC, USA).

2.2.3.1 Scanning Electron Microscopy (SEM)

After washing, *M. incognita* J2s were suspended in sterile distilled water and then filtered through a 0.22µm polycarbonate filter (Millipore Cat. No.GTTP01300) on a 13mm plastic filter holder (Gelman Sciences Inc., Ann Arbor, Milwaukee, USA), connected to a 2ml disposable syringe. Nematode samples were fixed for 30min in 2.5% [v/v] gluteraldehyde in 0.075M phosphate buffer (pH 7.4) and washed three times in 0.075M phosphate buffer (pH 7.4) each for 15min. Samples were then post-fixed in 1% [v/v] osmium tetra-oxide for 1 hour and washed again three times using 0.075M phosphate buffer (pH 7.4). The samples were dehydrated by sequential treatment for 15min each, in increasing concentration of ethanol 50%, 70%, 90% and 100% [v/v]. The treatment with 100% ethanol was repeated three times. The samples were placed in the Bio-Rad E3000 critical point dryer (Bio-Rad, Watford, England) and the filters containing the samples were sputter coated with gold using an Emitech K550X sputter coater (Emitech, Ashford, England), before viewing on a JEOL JSM-5800LV scanning electron microscope (JEOL Tokyo, Japan).

2.2.3.2 Confocal Laser Scanning Microscopy (CLSM)

Following co-incubation of *M. incognita* J2s and *Pcb*_mCherry, the J2s were washed with sterile distilled water and subjected to CLSM (Carl Zeiss, Jena, Germany) using the following conditions: excitation 543nm emission filter BP 560-615nm to view *Pcb*_mCherry and excitation 488nm, emission filter BP 505-560nm to view the surfaces of *M. incognita* J2s.

2.2.4 Bacterial dissemination by RKN

Co-incubations of 100 *M. incognita* J2s and 10ml *Pcb*_mCherry (1×10^{8}cfu/ml) were conducted as described in section 3.2.3 using optimal temperature of 28°C and duration of incubation of 72 hours. The following controls were included: *Pcb*_mCherry alone and *M. incognita* J2s alone. These were suspended in minimal medium. At 72 hours post co-incubation, *M. incognita* J2s were washed as previously described without centrifugation since that would affect the mobility of J2s, and suspended in 5ml of sterile distilled water. Aliquots of washed J2s (200µl) were pipetted onto nutrient agar that was supplemented with tetracycline. The same wash steps were followed for the *M. incognita* control. The *Pcb*_mCherry and water controls were not washed. The plates were dried inside a laminar flow hood, sealed with parafilm and incubated at 28°C for 48 hours upright. Selective CVP media, PCR with *Pcb*-specific primers and confocal microscopy were used to confirm the identity of the disseminated mCherry tagged *Pcb*.

2.2.4.1 Confirmation of identity of disseminated bacteria

The identity of disseminated *Pcb*_mCherry was confirmed by PCR, CLSM and selective CVP media. For PCR, genomic DNA extraction was performed using the boiling method. In this method, a loop-full of bacteria from a single test colony was suspended in 200µl sterile distilled water in 2ml Eppendorf tubes and boiled at 100°C for 10min. The solution was then centrifuged at 14000 rev.min^{-1} for 30sec and the supernatant was transferred into a clean 2ml microcentrifuge tube. The DNA was cleaned and concentrated using a Zymo Research DNA Clean and Concentrator™-25 Kit (Orange, CA, USA) and the procedures were performed in accordance with the manufacturer's instructions. Species-specific primers for *Pcb* based on

partial 16S-23S rDNA intergenic spacer region (IGS), L1r (5'-CAG GGC ATC CAC CGT-3') and BR1f (5'-GCG TGC CGG GTT TAT GAC CT-3') (Duarte et al., 2004), were used. The primers were synthesised by Inqaba Biotech (Pretoria, South Africa). The PCR was done as described by Duarte et. al. (2004). Each PCR reaction contained DreamTaq buffer with 20mM $MgCl_2$, 2.5mM deoxyribonucleotide triphosphates (dNTPs), 0.4µM of each primer, 0.5U DreamTaq polymerase, 10ng/µl template DNA in a final volume of 50µl. The PCR mixtures were placed in a Biometra T1 thermocycler® (Germany). The thermal profile that was used was as described by Duarte et. al. (2004). Unless otherwise stated all the reagents were obtained from Fermentas. PCR products were resolved by agarose gel electrophoresis in 1% [w/v] agarose (Lonza, USA) in 1 × TAE buffer. PCR products were then loaded into the agarose gels with GelRed™ (Biotium, Hayward, Carlifornia – USA) that was used for visualization of the DNA on a Unitec UV transilluminator (Cambridge, UK). A standard DNA molecular weight marker was included. DNA obtained from *Pectobacterium carotovoroum* subsp. *carotovorum* and water were used as a negative controls.

To determine the pectolytic ability and therefore confirm the identity of the disseminated bacteria, the colonies that were subjected to PCR were spotted on CVP media and the plates were incubated for 48 hours at 28°C. The colonies were also viewed under the CLSM under the conditions described in section 2.2.3.2.

2.2.5 Virulence assays

In order to evaluate the combined effect of *M. incognita* and *Pcb* on incidence and severity of disease on potato plants, freshly hatched *M. incognita* J2s and *Pcb*_mCherry overnight cultures (1×10^8cfu/ml) suspended in 10ml sterile distilled water, were soil drench-inoculated on five week old *Solanum tuberosum* cv Mondial. In each trial, the 25 plants used comprised of five groups of plants, five plants per treatment. These were plants that were drench-inoculated with 6000 *M. incognita* J2s and 50ml *Pcb*_mCherry (1×10^8cfu/ml), 200 *M. incognita* J2s and 50ml *Pcb*_mCherry (1×10^8cfu/ml), 50ml *Pcb*_mCherry (1×10^8cfu/ml) alone, 6000 *M. incognita* J2s alone and the water control. Plants that were used were grown in 250cm^3 pots, containing

200cm^3 of sterilised soil. Three independent replicates of greenhouse trials were conducted for this experiment.

To test the ability of *M. incognita* to break the tolerance of potato plants against soft-rot pathogens, five week old *Solanum. tuberosum* cv Mondial plants (susceptible) and cv BP1 (tolerant) were grown in 250cm^3 containing 200cm^3 of sterilised soil. Plants were drench-inoculated with 6000 *M. incognita* J2s while controls were drenched with water. Four weeks, post nematode inoculations, plants were stem-inoculated with 10µl of overnight cultures of *Pcb*_mCherry (1×10^8cfu/ml). Controls included plants that were initially drenched with water and then stem-inoculated with $MgSO_4$. In each of three independent replicate trials, each treatment had five plants. Post stem-inoculation with *Pcb*_mCherry plants were maintained for a period of four weeks and monitored daily for disease development.

The incidence of disease was measured by calculating the average number of stems that were wilted compared to the total number of stems per plant and the severity of disease was calculated in the form of a wilt index percentage. Both the incidence and severity of disease were measured per treatment in each trial and the data from the independent trials was combined to calculate the averages of both incidence and severity of disease for each treatment. Severity of disease was measured by combining the scale provided by He et. al. (1983): no symptoms (class 0); 1 leaf wilted (class 1); two or three leaves wilted (class 2); four or more leaves wilted (class 3); plant dead (class 4); and the percentage wilt index formula provided by Powell and co-workers (1971):

Wilt Index (%) = [(Number of plants in class 1 × 1) + (Number of plants in class 2 × 2) + (Number of plants in class 3 × 3) + (Number of plants in class 4 × 4)] / (Number of plants in the treatment × 4)

To evaluate the root galling in different treatments, plants were first uprooted and the roots were washed to get rid of excess soil. The rate of galling on the roots was evaluated visually. Root galling was grouped into five classes as described by Taylor and Sasser (1978). No galls (No galling); 1 = one or two galls (Slight galling); 2 = three to ten galls (Mild galling); 3 = eleven to thirty galls (Moderate galling); 4 = thirty one to hundred galls (Heavy galling); 5 = more than hundred galls (Extreme galling).

2.2.5.1 *Pcb*_mCherry in roots and mother tuber tissue

The presence of *Pcb*_mCherry inside the roots was determined by randomly selecting ten roots per plant that were dissected longitudinally and divided into cross-sections that could fit in pitted slides (±5mm). Each root was checked systematically for the presence of *Pcb*_mCherry using the CLSM. The mother tuber was also analysed to check for *Pcb*_mCherry penetration with CLSM. To determine the presence of *Pcb*_mCherry in the mother tubers of plants inoculated with *M. inocgnita* J2s in combination with *Pcb*_mCherry, and those inoculated with *Pcb*_mCherry alone, small pieces of the mother tuber tissue were cut and chopped to fit in pitted slides in a film of sterile distilled water. The pitted slides containing mother tuber tissue were viewed under the CLSM using the following conditions: excitation 543nm emission filter BP 560-615nm.

A combination of roots and mother tuber tissue was harvested from plants inoculated with *Pcb*_mCherry alone, *M. incognita* alone, a combination of *M. incognita* J2s and *Pcb*_mCherry and lastly from water-drenched plants. The sampled roots and mother tuber tissue were surface sterilised with 10% [v/v] sodium hypochlorite and rinsed with sterile distilled water. The amount of bacteria from 12g total tissue representative of the samples roots and mother tuber was then determined. The roots and mother tuber tissue per plant were processed in combination by blending them in sterile distilled water and a serial dilution was prepared. From the different dilution factors per root and mother tuber combination sample, 100µl aliquots were plated on nutrient agar plates, supplemented with tetracycline. The nutrient agar plates were incubated for 24-48 hours at 28°C. Colonies were confirmed as *Pcb*_mCherry by selecting random colonies from different plates and viewing them under the CLSM.

2.3 Results

2.3.1 The association of *Pcb* with *M. incognita*

Under the stereo microscope (Olympus, SZX10; Japan), at all the time points, *M. incognita* J2s that had been incubated at 28°C appeared to be as mobile as they were just after hatching whilst there was a noticeable decrease in the mobility of J2s incubated at 4 and 37°C. From the plate counts, significantly more *Pcb*_mCherry cells were observed attached onto *M. incognita* J2s at

28°C compared to at 4 and 37°C (Figure 2.1 and 2.2). There were no significant differences in attachments of *Pcb* to J2s at 24, 48 and 72 hours post co-incubation at each given temperature. This was an indication that the association of *Pcb*_mCherry cells with *M. incognita* J2s is temperature dependent but none of the tested incubation periods appeared to have any significant effect. The optimum temperature observed here for attachment is also the optimum and favourable conditions for growth of both organisms.

On the scanning electron micrographs, relatively more bacterial cells appeared to have attached onto the J2s at 28°C, relatively fewer cells attached at 37°C, whilst bacterial cells hardly attached to J2s at 4°C (Figure 2.2 A-C). Scanning electron microscopy results thus corroborated earlier observations using viable cell counts that 28°C is optimum for attachment. It is interesting to note that *Pcb*_mCherry cells appeared clustered together as microcolonies associated with a lot of extracellular materials at 37°C (Figure 2.2 C). This was hardly observed in the association between *Pcb*_mCherry and *M. incognita* J2s at 4 and 28°C, where the bacteria were seen as individual cells after 72 hours.

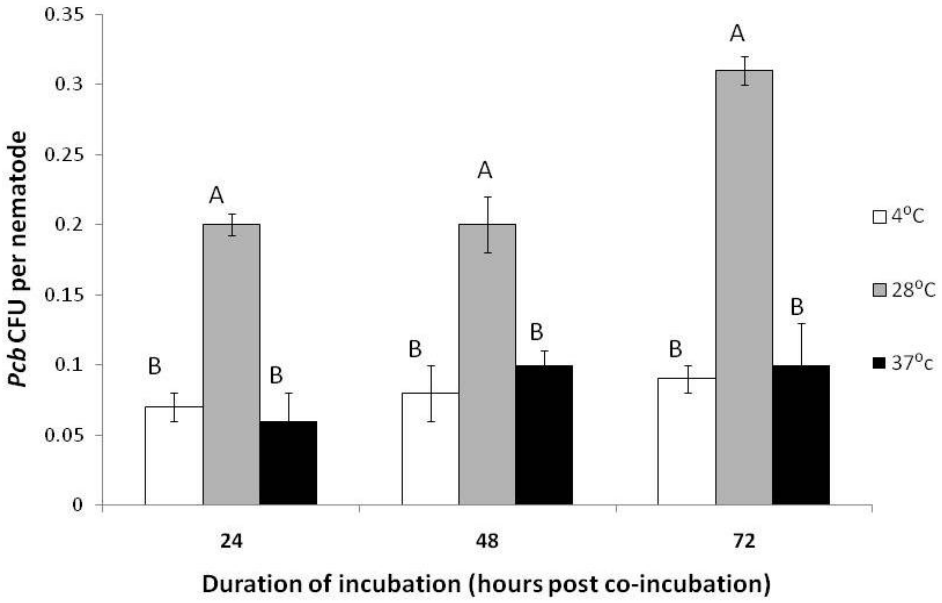

Figure 2.1 *Pcb*_mCherry cells attached onto *M. incognita* J2s post co-incubations at different temperatures and time points. *M. incognita* J2s were co-incubated with *Pcb*_mCherry overnight cultures at 4, 28 and 37°C for 24, 48 and 72 hours. At each time point *M. incognita* J2s were washed to remove unattached *Pcb*_mCherry cells, ground, serially diluted and plated on tetracycline-supplemented media to select for *Pcb*_mCherry cells. The plates were incubated for 24-48 hours at 28°C and colonies were counted. Different letters indicate significant differences. Means followed by common letters are not significantly different according to the Student–t test (0.05% confidence interval).

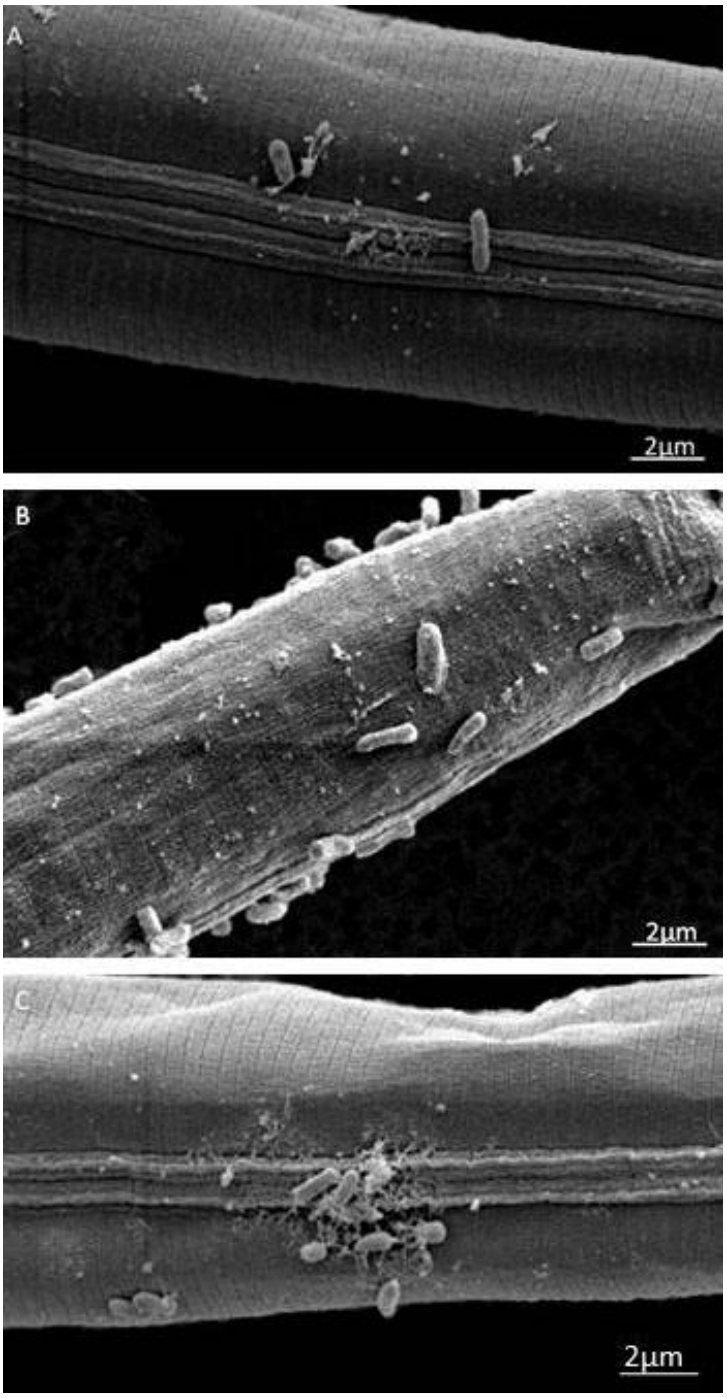

Figure 2.2 Scanning electron microscopy micrographs showing attachment of *Pcb*_mCherry cells on the surface of *M. incognita* J2s. *M. incognita* J2s were co-incubated with overnight cultures of *Pcb*_mCherry in minimal media for 72 hours at (**A**) 4, (**B**) 28 and (**C**) 37°C. After co-incubation for 72 hours, at different temperatures, the *M. incognita* J2s were washed to remove unattached *Pcb*_mCherry cells and prepared for analysis with scanning electron microscopy.

The optimum conditions established in the previous section, 28°C and 72 hours, were used to determine whether the bacteria observed attached to *M. incognita* J2s (Figure 2.2) was *Pcb*_mCherry. In this regard, confocal scanning electron microscopy was used. *Pcb*_mCherry cells were readily identified as red colonies at wavelength of 560-615 nm on the green auto-fluorescencing surface of *M. incognita* J2s at 505-560nm (Figure 2.3). The use of the 543nm emission filter only allowed visualisation of *Pcb*_mCherry cells (Figure 2.3 B) and 488nm emission filter only allowed the visualisation of the green auto-fluorescing surface of the *M. incognita* J2s (Figure 2.3 A). The images captured using the two emission filters were superimposed onto each other to obtain images that showed *Pcb*_mCherry cells can attach onto the surface of *M. incognita* J2s (Figure 2.3 C).

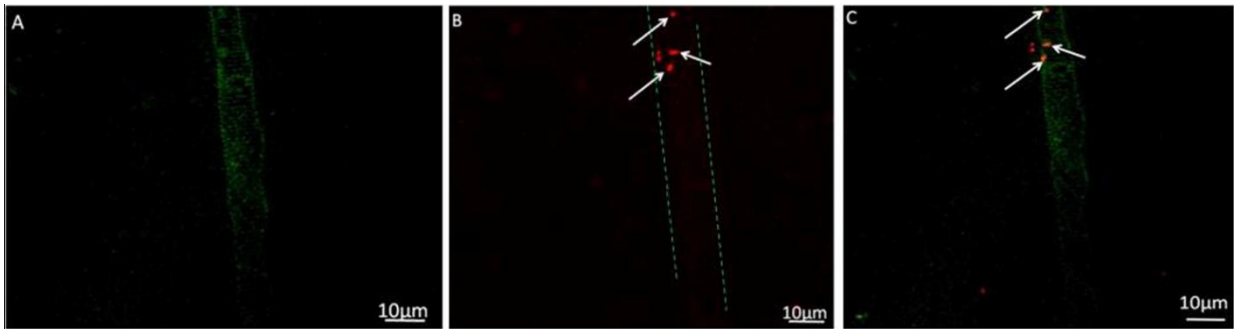

Figure 2.3 Visualisation of *Pcb*_mCherry cells on *M. incognita* J2s under the confocal laser scanning microscope after 72 hours of incubation at 28°C. After the 72 hour incubation period *M. incognita* J2s were washed to remove unattached *Pcb*_mCherry cells and were viewed using the confocal laser scanning microscope. (**A**) *M. incognita* J2s viewed at wavelength 505-560nm with the 488nm emission filter. (**B**) *Pcb*_mCherry viewed at 543 emission filter wavelength 560-615nm. The nematode surface is represented by a faint red shade outlined with green dotted lines in the background. (**C**) Superimposition of Figure 2.2 A and B showing the attachment of *Pcb*_mCherry cells on the surface of *M. incognita* J2s. The red-fluorescing *Pcb*_mCherry cells represented by the red spots on the surface of *M. incognita* J2s are highlighted with white arrows.

2.3.2 *Pcb* is disseminated by *M. incognita* J2s *in vitro*

The nutrient agar plates onto which the nematode-bacteria suspensions were aliquoted had trails of bacterial cells on the tracks of *M. incognita* J2s as they moved from the initial inoculum spot to the edges of the plate. Bacterial cells grew as far as 5cm from the initial inoculation spot (Figure 2.4 A). As expected, the *Pcb*_mCherry alone control did not have any colonies radiating from the initial inoculum spot. The bacteria grew and formed one mass of bacteria at the centre of nutrient agar plates (Figure 2.4 B). There was also no bacterial growth, or any form of growth, on the *M. incognita* J2s alone (Figure 2.4 C) and water control plates (not shown).

To confirm the identity of the radiating bacteria (Figure 2.4 A), PCR using *Pcb*-specific primers, CVP selective media and confocal microscopy were used. A single discrete expected amplicon size of about 322 bp was obtained from all the colonies, similar to the *Pcb* wild type strain (*Pcb*_1962) (Figure 2.5 A). In contrast, no amplification products were observed in the negative controls in which template DNA was omitted or where *Pectobacterium carotovorum* subsp. *carotovorum* was included.

The identity of the selected bacterial colonies that were disseminated *in vitro* by *M. incognita* J2s was also confirmed to be *Pcb*_mCherry cells by red mCherry fluorescence under 543nm emission filter BP 560-615nm (Figure 2.5 B). Furthermore the confirmation of *Pcb*_mCherry was established by spotting the randomly selected colonies on CVP selective media. After 48 hours of incubation of CVP the plates all the colonies formed pits, typical of pectolytic bacteria (Figure 2.5 C).

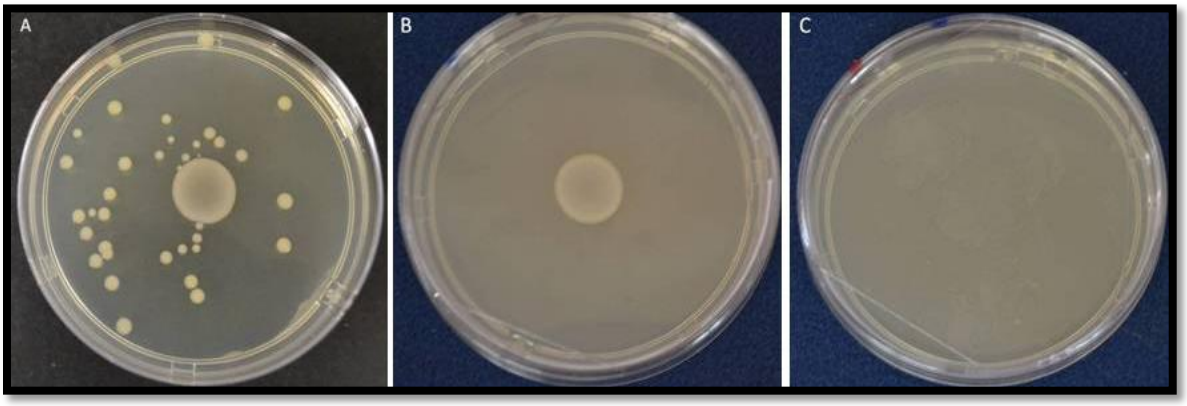

Figure 2.4 Dissemination of *Pcb*_mCherry cells by *M. incognita* J2s *in vitro*. *M. incognita* J2s were co-incubated with *Pcb*_mCherry cultures in minimal media at 28°C for 72 hours. After 72 hours the J2s were washed and aliquoted onto nutrient agar supplemented with tetracycline to select for *Pcb*_mCherry growth. Movement of *M. incognita* J2s from initial inoculum spots to the periphery of the plates resulted in *Pcb*_mCherry dissemination. (**A**) *Pcb*_mCherry cells disseminated by *Meloidogyne incognita* J2s. (**B**) *Pcb*-mCherry alone control. (**C**) *M. incognita* J2s alone control.

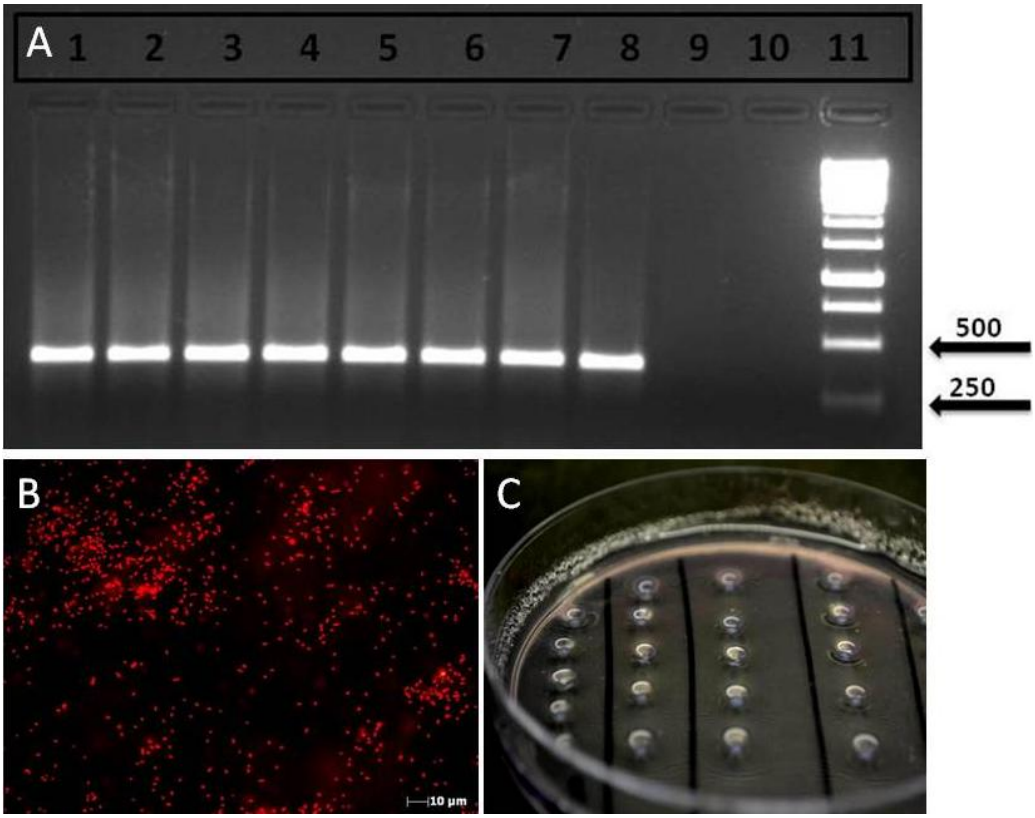

Figure 2.5 Identification of randomly selected bacteria disseminated by *M. incognita* J2s on tetracycline-supplemented nutrient agar. (**A**) Agarose gel electrophoretic analysis of the amplification products, using 16S-23S intergenic spacer (IGS) based Pcb specific primer pairs BR1f and L1r (Duarte et al., 2004): Lanes 1-7 represent the randomly selected colonies. Lane 8 represents the positive control in which *Pcb*_1962 wild type was used. Lanes 9 and 10 represent negative controls in which *Pectobacterium carotovorum* subsp *carotovorum* and water were used, respectively. Lane 11 represents the 1Kb ladder. (**B**) Confocal laser scanning electron microscope micrograph showing red fluorescing bacterial cells. (**C**) The pectolytic ability of the disseminated *Pcb*_mCherry cells reflected as pits on CVP media, 48 hours post incubation.

2.3.3 Synergistic relation between *M. incognita* and *Pcb* and its effect on potato

2.3.3.1 Disease incidence and severity

Associations between *Meloidogyne* spp. and secondary pathogens have been shown to increase the incidence and severity of disease caused by secondary pathogens (de Moura et al., 1975; Golden and van Gurdy, 1975; Negron and Acosta, 1989; Walker et al., 1998).

After four weeks post inoculation, in all the three independent replicate trials conducted, the plants co-inoculated with *M. incognita* and *Pcb*_mCherry showed a significantly higher incidence of disease compared to those inoculated with *Pcb*_mCherry alone, *M. incognita* alone and the water controls (Figure 2.6). In all the experiments in which plants were co-inoculated with *M. incognita* and *Pcb*_mCherry, there was on average at least 50% (two out of four stems per plant) disease incidence recorded at four weeks post inoculation. Plants inoculated with *Pcb*_mCherry alone had, on average at the most, 25% (one out of four stems per plant) disease incidence. The severity of disease was also significantly higher in co-inoculated plants compared to plants inoculated with individual pathogens. The highest severity of disease in plants inoculated with *Pcb*_mCherry alone was less that 40% whilst the least percentage of wilt index in plants inoculated with both pathogens was more than 65% (Figure 2.7). Figure 2.8 A-D shows representative plants of the treatments and controls. Both treatments, *Pcb*_mCherry alone and a combination of *Pcb*_mCherry and *M. incognita* J2s, showed some level of wilting (Figure 2.8 B and D) whilst the *M. incognita* alone inoculated plants and the water control showed no signs of wilt (Figure 2.8 A and C).

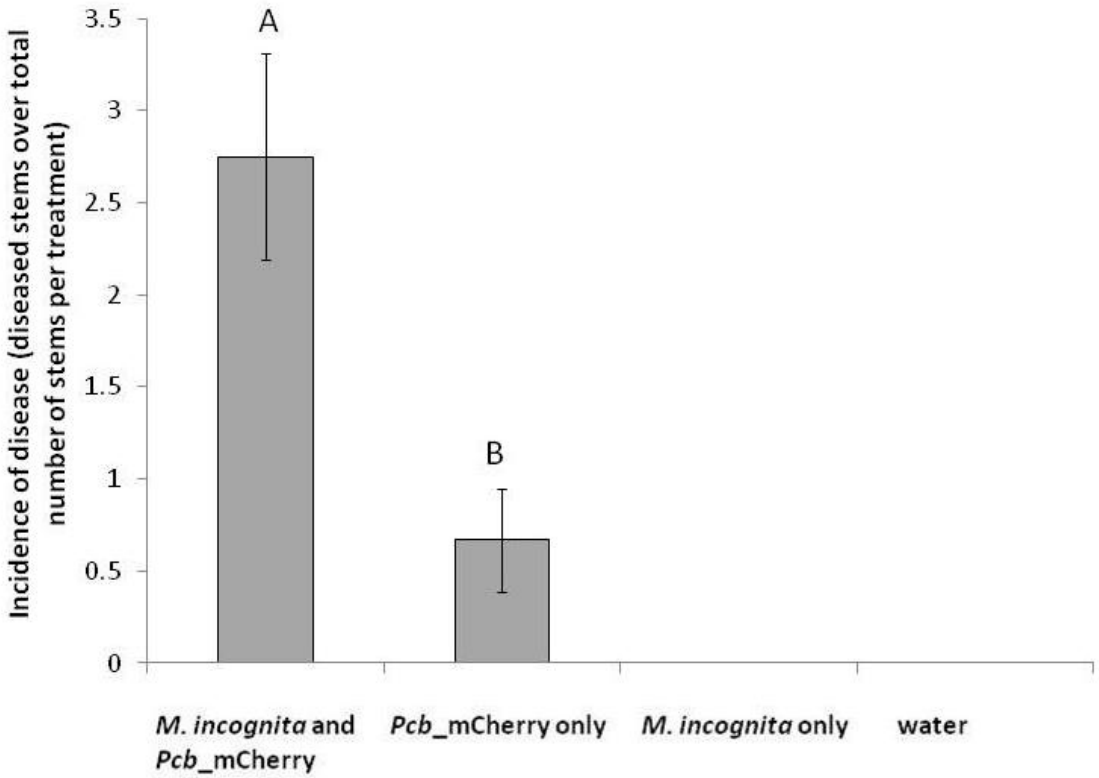

Figure 2.6 The disease incidence in plants treated simultaneously with *M. incognita* J2s and *Pcb*_mCherry, *Pcb*_mCherry alone, *M. incognita* J2s alone and water as a negative control. Five weeks old potato plants were inoculated by soil drench. Plants were rated for disease development after four weeks using the formula provided by He et. al. (1983). Each bar represents the mean disease incidence of three independent trials, each containing five plants per treatment. The incidence of disease following inoculation with both *M. incognita* J2s and *Pcb*_mCherry was significantly different from that of *M. incognita* J2s and *Pcb*_mCherry alone. Different letters indicate significant differences. Means followed by common letters are not significantly different according to the Student–t test (0.05% confidence interval).

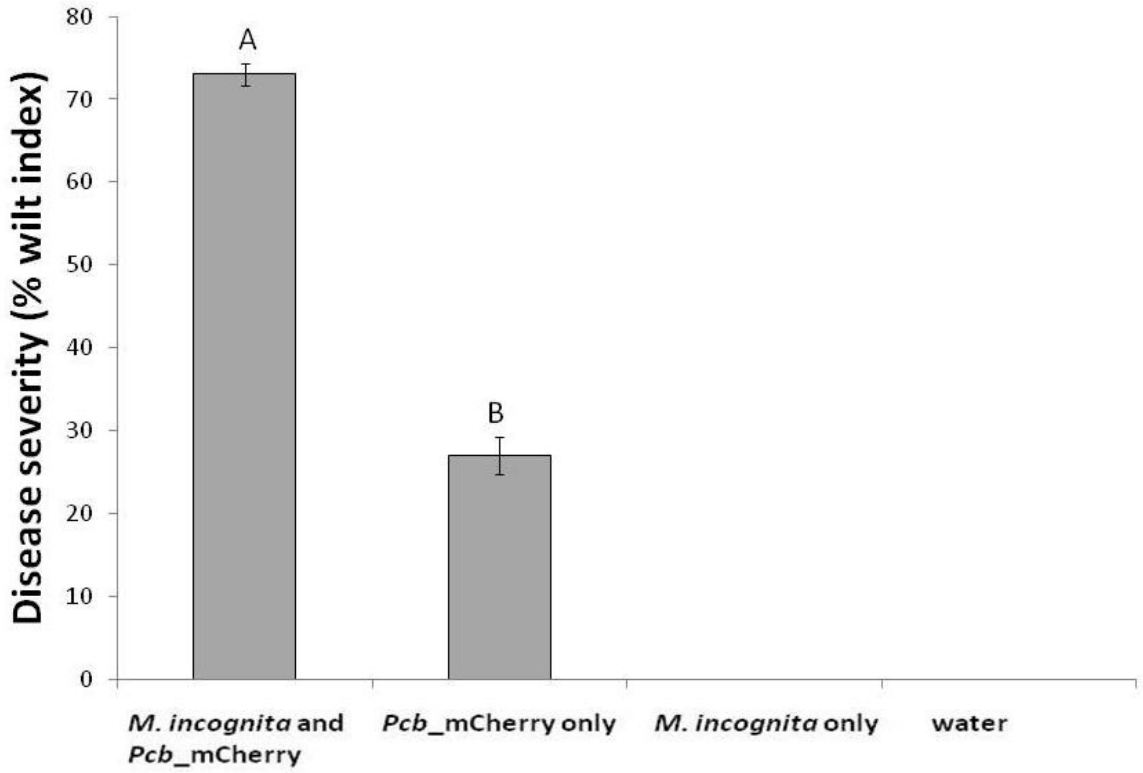

Figure 2.7 The severity of disease in plants treated with *M. incognita* J2s and *Pcb*_mCherry simultaneously, *M. incognita* alone and those treated with *Pcb*_mCherry alone. Five weeks old potato plants were inoculated by soil drench. In these assays, plants inoculated with sterile water were included as negative controls. Plants were rated for disease severity four weeks post inoculation using the wilt index percentage formula provided by Powel (1971). Each bar represents the mean wilt index percentage which represents the average disease severity of three independent trials, each containing five plants per treatment. The severity of disease following inoculation with both *M. incognita* J2s and *Pcb*_mCherry was significantly different from that of *Pcb*_mCherry alone and *M. incognita*. Means followed by common letters are not significantly different according to the Student–t test (0.05% confidence interval).

Figure 2.8 Foliar disease symptoms on potato plants inoculated with (**A**) water alone, (**B**) *M. incognita* J2s and *Pcb*_mCherry simultaneously, (**C**) *M. incognita* J2s alone and (**D**) *Pcb*_mCherry alone. Plants were assessed for symptom development, disease severity and incidence at four weeks post inoculation.

To evaluate whether *Pcb*_mCherry had an effect on the damage caused by *M. incognita* on potato roots, root galling on plants inoculated with both *M. incognita* and *Pcb*_mCherry and those inoculated with *M. incognita* alone was evaluated. As expected, only plants inoculated with a combination of *M. incognita* J2s and bacteria or *M. incognita* J2s alone showed galling symptoms (Figure 2.9). There was no difference in root galling in roots of plants inoculated with both *M. incognita* and *Pcb*_mCherry and those inoculated with *M. incognita* alone (Figure 2.9). Moreover, plants in these two treatments had root weights that were significantly higher than those of plants treated with either *Pcb*_mCherry alone or water (Figure 2.10).

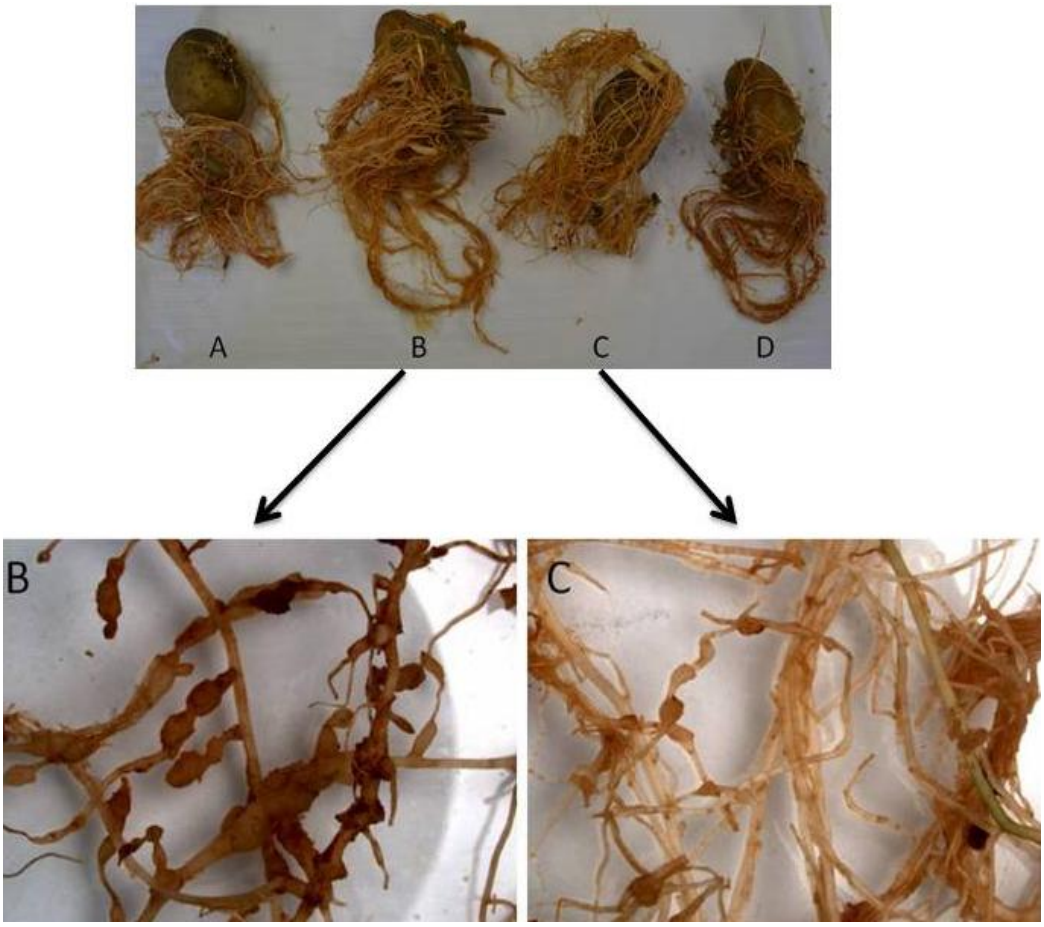

Figure 2.9 Root galling on potato plants. (**A**) Roots of uninoculated plants. (**B**) Roots of plants inoculated with *M. incognita* J2s and *Pcb*_mCherry simultaneously. (**C**) Roots of plants inoculated with *M. incognita* J2s alone. (**D**) Roots of plants inoculated with *Pcb*_mCherry alone.

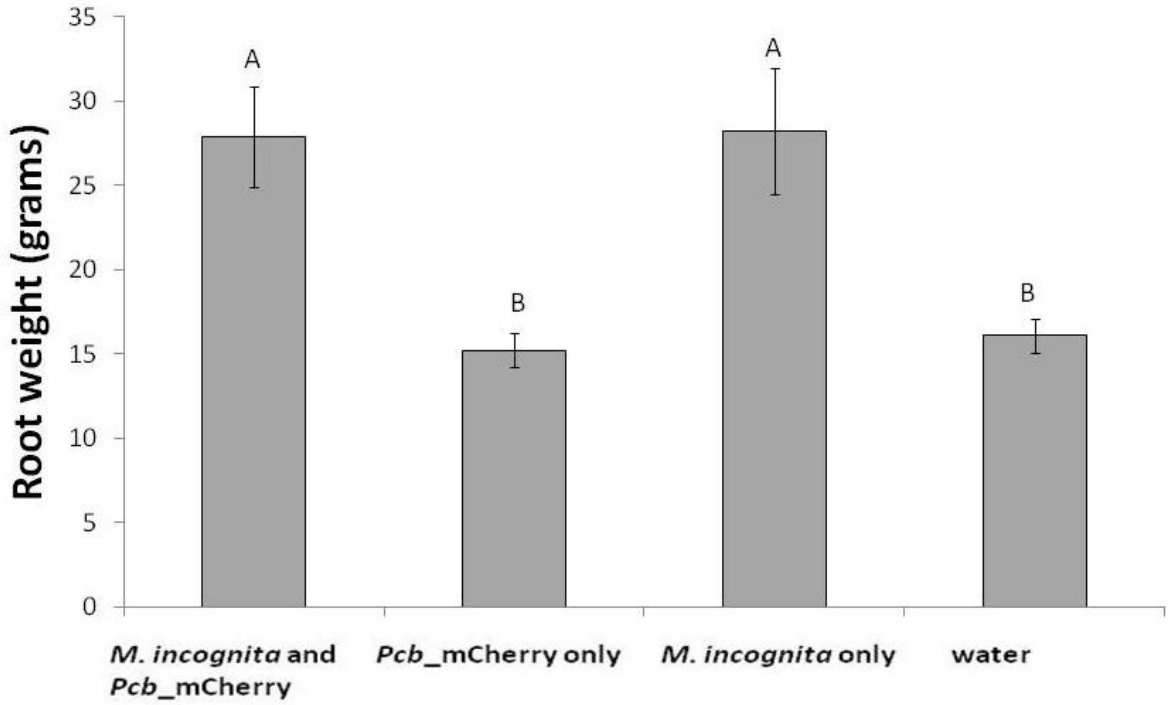

Figure 2.10 The mean root weights of plants that were simultaneously inoculated with *Pcb*_mCherry and *M. incognita* J2s, *M. incognita* alone, *Pcb*_mCherry alone and the water control. The roots were weighed and the mean root weights were calculated per treatment in each trial. There were significant differences in the average root weights between plants inoculated with J2s and those without J2s. Different letters indicate significant differences. Means followed by common letters are not significantly different according to the Student–t test (0.05% confidence interval).

Meloidogyne spp. have been shown to have the ability to break the tolerance of many hosts, allowing invasion by secondary pathogens. Potato cv BP1 has previously been shown to be tolerant to *Pectobacterium* spp. stem infections and therefore does not develop blackleg when stem inoculated with *Pcb* under greenhouse conditions (Gugulethu Kubheka, unpublished). All *S. tuberosum* cv BP1 plants, drench inoculated with *M. incognita* J2s and stem inoculated with bacteria and those inoculated with bacteria only, did not show blackleg symptoms nor any foliar symptoms in all the independent trials conducted (Figure 2.11 A and B). Regardless of whether drench inoculated with J2s or water, cv Mondial plants that were stem inoculated with *Pcb*_mCherry showed blackleg development (Figure 2.11 C and D). In both cultivars, plants that were stem-inoculated with $MgSO_4$ and those drench-inoculated with J2s alone without stem inoculations did not show any blackleg development. Upon uprooting, plants inoculated with either *M. incognita* J2s alone or with both *M. incognita* J2s and *Pcb*_mCherry showed typical galling symptoms. Plants of both cultivars drenched with water and those stem-inoculated with *Pcb*_mCherry alone or with $MgSO_4$ (without *M. incognita* soil drench), did not have galls on their roots as expected.

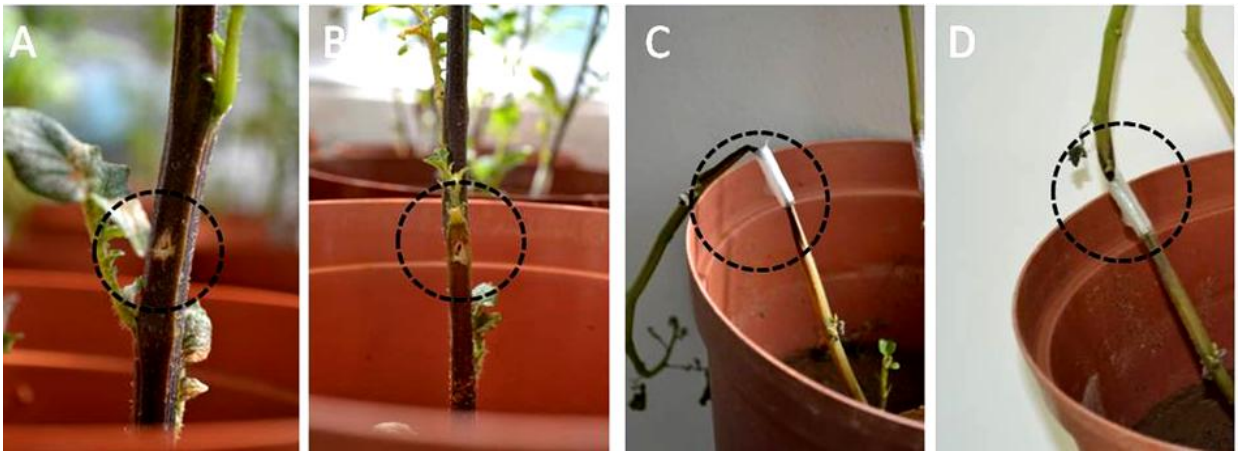

Figure 2.11 Determination of the ability of *Meloidogyne incognita* J2s to break the tolerance of cv BP1 plants to *Pcb* under the tested conditions. Blackleg development assessment on potato BP1 stems inoculated with *Pcb*_mCherry four weeks post inoculation with *M. incognita* J2s. No blackleg development was observed on *Pcb* stem-inoculated potato cv BP1 (**A**) drench inoculation with *M. incognita* J2s and (**B**) those that were not previously inoculated with *M. incognita* J2s. Blackleg development was observed on *Pcb* stem-inoculated potato cv Mondial (**C**) drench inoculated with *M. incognita* J2s and (**D**) those that were not previously inoculated with *M. incognita* J2s.

2.3.3.2 The effect of RKN inoculum load on *Pcb* infection of potato roots

There were significantly higher *Pcb*_mCherry concentrations found in plant root masses inoculated with *M. incognita* J2s and *Pcb*_mCherry in comparison to those inoculated with *Pcb*_mCherry alone (Figure 2.12 and 2.13). Higher *Pcb*_mCherry concentrations were found in plants inoculated with 6000 J2s compared to those inoculated with 200 J2s. These results therefore indicated that *M. incognita* facilitates host plant invasion by this phytobacteria.

The red-fluorescing *Pcb*_mCherry cells were observed inside the mother tuber tissue of plants inoculated with both *Pcb*_mCherry and *M. incognita* and those inoculated with bacteria alone using the CLSM (Figure 2.13 D, E and F). A higher concentration of *Pcb*_mCherry cells in the mother tuber tissue was found in plants co-inoculated with the *M. incognita* J2s and bacteria (Figure 2.12). Interestingly, no *Pcb*_mCherry were visualised inside the roots using the CLSM.

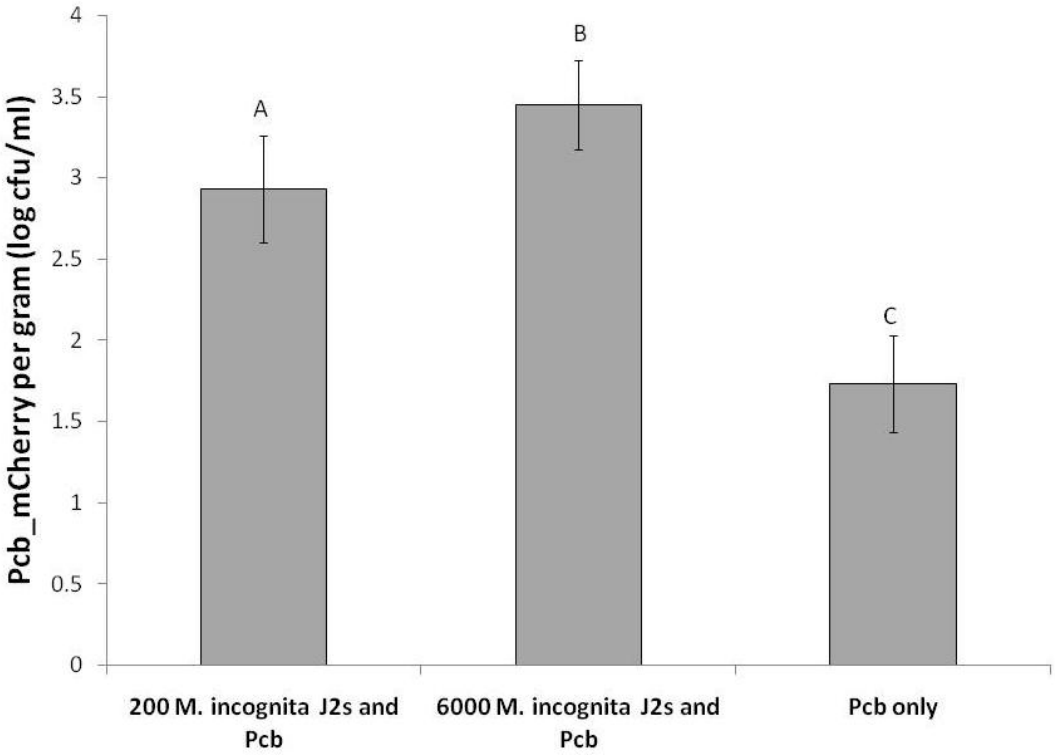

Figure 2.12 *M. incognita*-facilitated potato root mass invasion by *Pcb*_mCherry. The concentration of recovered *Pcb*_mCherry from a combination of roots and mother tuber tissue inoculated with 6000 *M. incognita* and *Pcb*_mCherry, 200 *M. incognita* and *Pcb*_mCherry and control plants that were inoculated with *Pcb*_mCherry alone, four weeks post inoculation. Each bar represents the mean bacterial concentration of the combination of independent trials, each containing five plants per treatment. The concentration of recovered viable *Pcb*_mCherry following inoculation with both 6000 and 200 *M. incognita* J2s in combination with *Pcb*_mCherry was significantly higher than that of the *Pcb*_mCherry alone. Different letters indicate significant differences. Means followed by common letters are not significantly different according to the Student–t test (0.05% confidence interval).

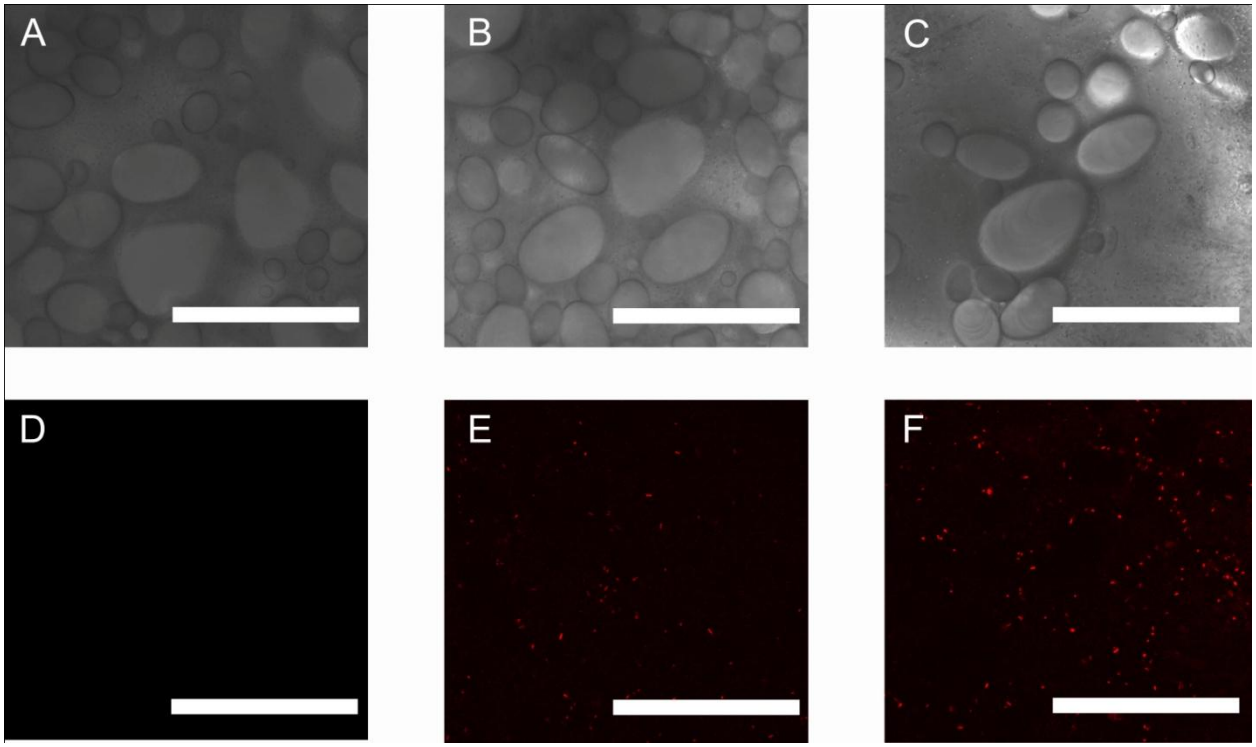

Figure 2.13 The presence of *Pcb*_mCherry in the intracellular spaces of plant cells in the mother tubers confirmed using CLSM using the 543nm emission filter BP, wavelength 560-615nm. The mother tubers from uninoculated plants, plants inoculated with both pathogens and those inoculated with *Pcb*_mCherry alone were surface sterilised and viewed using the CLSM with UV and white light. (A) and D) represent the uninoculated mother tuber tissue. (B) and (E) represent *Pcb*_mCherry cells that were found in mother tubers inoculated with *Pcb*_mCherry alone. (C) and (F) represent the *Pcb*_mCherry cells inside mother tubers inoculated with *M. incognita* and *Pcb*_mCherry. Image A, B and C were viewed under white light whilst image D, E and F were viewed under red UV light. The scale bar in each image represents 20µm.

2.4 Discussion

Several studies have been conducted whereby it was demonstrated that there is an interaction between bacteria and both plant parasitic and free living nematodes. In these studies, bacteria were shown to attach onto *M. incognita* J2s and be disseminated by juveniles as they move in the environment (Hallmann et al., 1998; Darby et al., 2002; Anderson et al., 2003; Caldwell et al., 2003a and b). Caldwell et. al. (2003a and b) demonstrated that free living nematodes such as *Caenorhabditis elegans* have the ability to ingest bacteria that is pathogenic to humans and excrete it in its viable form at a location away from where the bacteria was originally ingested. This way, these nematodes protect the bacteria residing in their guts from chemicals that might subsequently kill them in the environment such as sanitizers. More evidence of this kind of nematode-bacteria interactions was provided by Anderson et. al. (2003) when they demonstrated that *C. elegans* can ingest bacteria such as *E. coli*, *Salmonella typhimurium*, *Listeria welshimeri* and *Bacillus cereus*. These migrate from the initial location of ingestion and excrete the bacteria in its viable form at another location. Plant parasitic nematodes such as *Meloidogyne* spp. and *Anguina* spp. are not bacterivorous but have the potential to carry bacteria on their surface coats from one location to another (Spiegel and McClure, 1991; Hallmann et al., 1998; Maghodia et. al. 2008). McClure and Spiegel (1991) demonstrated that *Clavibacter* spp. can attach onto the *Anguina tritici* and *A. funesta.* Furthermore, Hallmann et. al. (1998) showed that *Enterobacter asburae* can attach onto *M. incognita* J2s and also be disseminated by the J2s *in vitro*. Maghodia et. al. (2008) demonstrated that *E.coli* can attach onto the surface of *M. javanica*. The currently available literature strongly suggests that plant parasitic nematodes, just like free living nematode spp., have the potential to disseminate pathogenic bacteria.

To determine whether *Pectobacterium* spp. can attach onto the surface of RKN, *M. incognita* J2s were co-incubated with *Pcb*_mCherry at different temperatures. Using plate counts on nutrient agar to quantify the attachment and scanning electron microscopy micrographs for visualisation, it was found that *Pectobacterium* spp. can attach onto RKN. Several factors on which attachment of bacteria onto nematodes, specifically RKN, is dependent on have been identified in literature and amongst them are mobility of the nematodes, lability of the surface coat, presence of certain elements of the surface coat and exopolysaccharides (Bird et al., 1989; Bird and Zuckerman,

1989; Page et al., 1992; Spiegel et al., 1995; Spiegel et. al. 1997; O'Toole and Kolter, 1998; Darby et al., 2002; Wairuri et al., 2012).

Temperature ranges have been shown to play a pivotal role in the attachment of molecules or microorganisms to nematode surfaces. Temperatures ranging from 4°C and below and from 37°C and above were noted by Kleynhans (1999) as hostile for RKN development and survival, a scenario that was observed in this study. The reduced mobility at 4 and 37°C seems to have played a role in less *Pcb*_mCherry cells attaching onto the surface of *M. incognita* J2s.

There are other factors that might have contributed to less *Pcb*_mCherry cells attaching onto the surface of *M. incognita* J2s at 4 and 37°C.The lability of the surface coat and the possibility of certain essential constituents of the surface coat being absent due to the age of the J2s might have influenced the number of *Pcb*_mCherry that adhered onto *M. incognita* J2s (Page et al., 1992; Spiegel et al., 1997). At temperatures that are not optimal for survival of RKN, such as those as low 4°C and as high as 37°C, the surface coat of *M. incognita* J2s which harbors carbohydrate moieties that play a role in the attachment of lectins and bacteria might not develop optimally, making it difficult for *Pcb*_mCherry to attach onto *M. incognita* J2s. This phenomenon of the temperature-dependent development of the surface coat was demonstrated by Spiegel et. al. (1997). Several constituents of the surface coat, when absent or not fully developed, can also result in decreased attachment of bacteria or lectins onto RKN (Bird et al., 1989; Bird and Zuckerman, 1989). This might have been the case in this study whereby the essential elements for attachment on young freshly hatched *Meloidogyne* spp. J2s' surfaces possibly developed at a much slower rate at temperatures that were not optimal for RKN survival, resulting in significantly lower attachment rates. Another observation that was made was that of an increase in the rate of attachment with an increase in temperature (4 to 28°C) and a decrease past a certain point (28 to 37°C). The directly proportional increase of *Pcb*_mCherry cells onto *M. incognita* J2s was contrary to what was found by Maghodia and colleagues (2008) when they found that a decrease in temperature yielded more attachment of *Escherichia coli* onto *M. javanica* J2s. In thier experiments, maximal adhesion occurred at 4°C. This is another example that illustrates the diversity of the interactions between RKN and various pathogens and therefore, to be able control these interactions and control disease, they must be individually studied in detail and not generalised.

Exopolysaccharide formation is one of the common mechanisms with which bacteria are able to firmly attach onto surfaces (Wairuri et al., 2012). O'Toole and Kolter (1998) demonstrated that substances secreted by bacteria aid in bacterial attachment onto surfaces. Darby et. al. (2002) also illustrated that bacteria can use extracellular material to attach onto the nematode surfaces without the cells being in direct contact with the nematode surfaces. In this study, a similar observation was made. At all the temperatures investigated, *Pcb*_mCherry cells on *M. incognita* J2s had a white substance protruding from them that appeared to be exopolysaccharides. At 37°C, after 72 hours, exopolysaccharide production was in its advanced stages. The observation made of *Pcb*_mCherry cells found as clumps embedded in exopolysaccharide layers (Figure 3.2 C) further supports the hypothesis that exopolysaccharides play an important role in the attachment of *Pectobacterium* spp. onto RKN J2s

Root knot nematodes are giants in the rhizosphere and can move longer distances in a short space of time relative to bacteria (De Waele and Elsen, 2007). Here we demonstrated that *M. incognita* J2s, as they moved around on nutrient agar, disseminated *Pcb*_mCherry cells in a similar manner to what was observed by Hallmann et al. (1998). The fact that *M. incognita* can carry *Pcb*_mCherry cells as they move from one point to the next *in vitro* suggests that *Pectobacterium* inoculums from one area in the rhizosphere to the next or from diseased to healthy plants can be spread faster. This also implies that *Pectobacterium* inoculum can be carried and spread much faster compared to when the bacteria is moving on its own in a water film in the soil. The faster movement of *Pectobacterium* spp. in the soil can result in the rapid spread of soft-rot and blackleg epidemics in potato fields as these pathogens will be able to infest the rhizospheres of many hosts with the help from *Meloidogyne* spp. Furthermore, since the time it takes for dissemination will be shortened, this can lead to acceleration of such epidemics (Cadet et al., 1989). On all the control plates, no bacterial colonies radiating from the initial inoculum spot were observed. This was an indication that *Pectobacterium* spp. cells, as can be expected, are unable to move on their own *in vitro* on solid medium, and that surface sterilised RKN J2s that were used were free from all contamination that could have affected the result in this aspect of the study. Thus, these observations, combined with the fact that *Pcb*_mCherry colony-trails were observed only where the bacteria was co-incubated with *Meloidogyne* J2s, suggest that RKN have the potential to disseminate *Pectobacterium* inoculum even in the field.

Both *Pectobacterium* spp. and *Meloidogyne* spp. can cause severe epidemics in potato fields. *Meloidogyne* spp. J2s create wounds when they penetrate the host roots and at some point in their life cycles both *Meloidogyne* spp.and *Pectobacterium* spp. need to be inside their hosts' roots and mother tuber where, if they co-infect the same root tissue, will share minute spaces. Nabhan et. al. (2012) stated that *Pectobacterium* spp., unlike other soft rot causing pathogens such as *Dickeya*, depend on wounds and openings to infect their hosts. We thus hypothesised that there can be synergistic interactions between the RKN and *Pectobacterium* spp. that can lead to increased disease incidences due to the two pathogens. To investigate this, five week old potato cv Mondial plants were simultaneously co-inoculated with freshly hatched *M. incognita* J2s and overnight cultures of *Pcb*_mCherry by soil drench. Other treatments included plants that were inoculated with *M. incognita* J2s alone, *Pcb*_mCherry alone and the water control. Post inoculation plants were maintained for a period of four weeks with regular monitoring for disease development. The effect of the co-inoculation using *M. incognita* J2s compared to other treatments was measured using disease incidence and severity. In all the three independent replicate trials conducted, plants that were co-inoculated with *Pcb*_mCherry and *M. incognita* J2s were significantly more wilted than plants in other treatments. Plants inoculated with *M. incognita* alone and the water control did not show foliar symptoms.

The observations made in this study suggest that the co-infection of potato cv Mondial plants with RKN and *Pectobacterium* spp. can result in more severe disease compared to when *Pectobacterium* spp. infect the host alone. Several *Pectobacterium* spp. have been reported to be opportunistic pathogens and therefore infect their hosts through natural openings or those created by other agents on the roots (Nabhan et al., 2012). Based on this and the results obtained in this study, wounds created by the *M. incognita* J2s as they penetrated potato cv Mondial plant roots seem to have significantly contributed to higher disease incidence and severity in co-inoculated plants. The results strongly suggest that as *M. incognita* J2s penetrated the host roots and *Pcb*_mCherry used those openings as entry points to gain access to root tissue. This was supported by the higher levels of *Pcb*_mCherry found in roots masses of plants inoculated with 6000 J2s and *Pcb*_mCherry compared to those inoculated with 200 J2s and *Pcb*_mCherry from the dilution series results. The importance of wounds created by various agents as entry points for the entry of *Pectobacterium* spp. is highlighted by Czajkowksi et. al. (2011) in a review and Perombelon and van der Wolf (2002). The latter authors highlighted the importance of wounds

using those that form during handling and how they serve as entry points for *Pectobacterium* inoculum from nearby rotting tubers. It is also possible that natural wounds such as lenticels played a role as entry points for *Pcb*_mCherry into the mothers since *Pcb*_mCherry cells were found in mother tissue of plants inoculated with the bacteria alone. Kastelein et. al. (1999) conducted experiments in which the protection of lenticels significantly reduced infection by *Pectobacterium atrosepticum*, thus highlighting the importance of lenticels in the introduction of *Pectobacterium* spp. into host roots. Through confocal microscopy it was also demonstrated that more *Pcb*_mCherry cells were able to enter the mother tuber tissue in higher numbers in co-inoculated plants compared to when *Pcb*_mCherry was inoculated alone. Similar results were obtained by Inagaki and Powell (1969) and Storey and Evans (1987), the latter using *Globodera pallida* and *Verticilium dahliae* where higher disease severity was observed in plants inoculated with plant parasitic nematodes and the secondary pathogens compared to when the secondary pathogens were inoculated alone. No *Pcb*_mCherry was observed in the roots. This was more likely to be as a result of the upward movement of the bacteria into the mother tuber or into the stem. This upward movement of *Pcb* in potato plants was demonstrated by Kubheka (unpublished) from the Host-Pathogen Interaction Research Group at the University of Pretoria. The results obtained in this study further suggest that wounds created by RKN J2s on the host roots shorten the period it takes for *Pectobacterium* spp. to infect the same host and this therefore results, as observed, in the earlier development of disease in the host plant.

Physiological alterations have been shown to play a role in synergistic interactions between RKN and other soil-borne pathogens whereby secondary pathogens were found concentrated around galled areas. Alternatively, secondary pathogens could be attracted to galled roots since they could obtain nutrients or use galled tissue as entry points into the healthy parts of the roots (Mayol and Bergeson, 1970; Golden and van Gurdy, 1975; McLean and Lawrence, 1993; Hallmann et al., 1998). Similar observations were not made in this study as no *Pcb*_mCherry cells could be visualised inside the galled root parts. This could have been due to the upward movement of water in the xylem. As the water moves from the roots into the stem and other part of the plant it can carry the bacteria with and therefore translocate it into the stem tissue. Thus, not being visualised in the roots four weeks post inoculation under the confocal microscope. On the contrary, more bacteria could be visualised in potato tuber tissue. This can be correlated with

the sink/storage nature of potato tuber tissue. Hence, bacteria can only accumulate in storage tissue without being translocated.

There are interactions between RKN and secondary pathogens that involve host resistance breakdown allowing secondary pathogens to infect hosts which were previously known to be resistant towards those particular secondary pathogens. Even though such interactions do occur they are seldomly reported (Marley and Hillocks, 1993; Back et al., 2002). Marley and Hillocks (1993; 1994) demonstrated that RKN can break the resistance of hosts against secondary pathogens using *Cajanus cajun* that was known to be resistant to *Fusarium udum.* Plants inoculated with *F. udum* post inoculation with *M. incognita* were susceptible to *F. udum.* Potato cv BP1 has previously shown high levels of tolerance against *Pcb* in glasshouse trials whereby no blackleg developed subsequent to stem inoculations (Kubheka, unpublished). To investigate whether RKN can break the tolerance of potato cv BP1 plants, five week old plants were drench-inoculated with freshly hatched *M. incognita* J2s and four weeks later they were stem-inoculated with overnight cultures of *Pcb*_mCherry. Other treatments included plants that were stem-inoculated with *Pcb*_mCherry four weeks post water-drench and those that were drench-inoculated with *M. incognita* J2s without stem inoculations with *Pcb*_mCherry. The controls included uninoculated plants and those stem-inoculated with $MgSO_4$. Potato cv Mondial plants were also included in the experiment as positive controls in each treatment. Three independent replicate trials were conducted. Unlike in experiments conducted by Marley and Hillocks, no resistance-breakage was observed in cv BP1 plants whilst the cv Mondial plants that were inoculated with RKN and *Pcb*_mCherry and those inoculated with *Pcb*_mCherry alone developed blackleg. There is a wide range of possible reasons why the tolerance in cv BP1 against *Pcb*_mCherry was not broken and these are discussed in the following paragraph.

Resistance breakage has sometimes been linked to a decrease in the levels of various chemicals that play a role in defense (Marley and Hillocks, 1993). This might have been the case in this study but it is possible that the chemicals involved in defense, such as isoflavanoids, might not have been decreased below the levels where they become inactive against the pathogen, thus cv BP1 plants maintaining tolerance against *Pcb*_mCherry. The use of different pathogen populations has also been shown to affect synergistic interactions between RKN and secondary pathogens. This was demonstrated by Riedel et. al. (1985) when they conducted experiments

from which they observed a synergistic interaction between *Verticillium* spp. and certain *Pratylenchus* spp. but not others. In those experiments the combination between *Verticillium dahliae* and *P. penetrans* resulted in more severe disease but not with *P. arenutus* or *P. scribneri*. Environmental conditions have also been shown to contribute to resistance breakage by RKN. In their experiments France and Abawi (1994) demonstrated that growing chick peas which had a dual resistance to *M. incognita* and *F. oxysporum* f.sp. *ciceri* at 27°C broke the their resistance to *M. incognita* which resulted in resistance breakage to *F. oxysporum* f.sp. *ciceri*. With different environmental conditions, inoculation of cv BP1 potato plants with *Pcb*_mCherry post inoculation with *M. incognita* might produce different results. Based on these examples we cannot conclude that RKN cannot break the tolerance of cv BP1 against *Pcb*_mCherry as inoculations with another RKN spp. prior inoculation with *Pcb*_mCherry or change in environmental conditions can result in a different outcome.

2.5 Conclusion

In the natural environments, plants are never exposed to one pathogen and in the soil environment we can be sure that plants are in a constant battle against a range of pathogens. These pathogens are most likely to affect the way in which each one of them interacts with the host since they share the same habitat. This study, through *in vitro* experiments and glasshouse trials, has provided results that suggest that there is a possible synergistic interaction between *Meloidogyne* spp. and *Pectobacterium* spp., the two pathogens with significant importance in the potato industry worldwide. This is limited proof as no field trials were conducted. Disease complexes involving plant parasitic nematodes and other plant pathogens are a major economic hazard since they result in diseases that are difficult to manage. To be able to control these pathogens they need to be understood in terms of their pathogenesis mechanisms and these include their interactions with other pathogens in disease complexes. It is clear that *Meloidogyne* spp. do not only possibly interact with *Pectobacterium* spp. in disease complexes by enhancing the disease caused by soft rot-causing enterobacteriaceae but could also contribute to their dissemination. The observation of increased disease incidence and severity caused by *Pectobacterium* spp. and their dissemination by RKN suggests that there is a synergistic relationship between the two pathogens. This observation has potential to contribute to the

development of efficient control strategies in future since more research can be focused on identifying the key phases within this interaction that can be interrupted to manage the damage caused by the resulting disease complex. These experiments were performed under glasshouse and therefore optimum conditions. However, the real impact of this interaction on potato yields warrants further investigation using large scale field trials.

2.6 Acknowledgements

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CHAPTER THREE

INDUCTION OF RESISTANCE IN *SOLANUM TUBEROSUM* AGAINST ROOT KNOT NEMATODE (RKN) INFECTION

CHAPTER THREE

Induction of Resistance in *Solanum tuberosum* against Root-knot nematode Infection

3.1 Introduction

Root knot nematodes (RKN) are important pests in the agricultural sector worldwide as they can cause disease epidemics that can result in the total loss of crops (Widmer et al., 1999; Mitkowski and Abawi, 2003; Cerkauskas, 2004; Davis, 2007). Their importance is also linked with the fact that they can interact with other soil-borne pathogens to cause severe disease outbreaks (de Moura et al., 1975; Golden and van Gurdy, 1975; Sasser and Freckman, 1987; Alam et al., 1990). After RKN infect their hosts, they are known to reduce host roots to galls and this affects normal host growth through reduced uptake of water and minerals. In addition to affecting water and mineral absorption from the soil, galls give the edible parts of the roots (i.e potato tubers and carrots) a rough texture and deformed appearance, resulting in a decrease in their market value. Galling of roots also result in stunting and therefore lower yields (Widmer et al., 1999; Mitkowski and Abawi, 2003; Davis, 2007).

Meloidogyne spp. are managed using various strategies and these include biological control, crop rotation, incorporation of resistance genes in hosts, nematicides, sanitation and physical methods such as soil solarisation. These methods for RKN management are not always practical (Elmore et al., 1997; Walters et al., 1997; Thies and Fery, 1998; Williamson, 1998; de Villiers, 2002; Noling, 2009; Olsen, 2011). The use of chemicals has, for years, been the control method on which farmers solely relied on for parasitic nematode management. However, the withdrawal of many of these chemicals has left growers in need of alternative control strategies. One such alternative control strategy is the use of resistant cultivars. Although natural resistance has previously been demonstrated against RKN, it is generally very rare. This is mainly due to the polyphagous nature of RKN. Hence, artificially induced resistance appears to be a viable alternative.

There are two methods with which resistance can be induced in plants and these include direct induction and priming. Direct induction refers to constitutively enhancing resistance in plants irrespective of pathogen presence (Conrath et al., 2006). Priming, on the other hand, is preparing plants to more efficiently react to pathogen infection (Conrath et al., 2006). Priming using chemical analogues of salicylic acid for SAR and jasmonic acid for ISR has been demonstrated for various crops. Hence, the aim of this chapter was therefore to determine the efficacy of chemical resistance inducers, such as BABA, BTH and Messenger, against RKN on potato. The efficacy of each of the inducers was assessed based on: galling index, egg production, the number of RKN J2s that penetrated the roots and the proportion of those J2s that developed into females. The second objective was to determine whether the most effective resistance inducer against RKN infection on potato has an effect on RKN-assisted SRE infection of potato roots.

3.2 Materials and Methods

3.2.1 *Meloidogyne incognita* inoculum

Meloidogyne incognita inoculum was maintained in the glasshouse on roots of tomato cv Rodade. Prior to use, egg masses were harvested from roots on the surface of the knots. The egg masses were removed using a sharp blade and tweezers and suspended in sterile tap water. They were then transferred onto a 25µm sieve (Universal Test Sieves, South Africa) that was placed in a plastic container with enough water to cover the eggs on the sieve. The container was closed and incubated in a dark cupboard at room temperature for 48-72 hours for second stage juveniles (J2s) to hatch.

3.2.2 Effect of chemicals on RKN

To ensure that the selected resistance induction chemicals do not have any direct toxic effects on RKN J2s, BABA (5mM and 20mM), Bion (50mg/L and 100mg/L) and Messenger (0.4g/L) were prepared and 100 freshly hatched *M. incognita* J2s were suspended in 50ml of each of the resistance inducers at various concentrations. *M. incognita* J2s that were suspended in sterile tap water served as controls. These were incubated at 25°C for five days. The effect of the resistance

inducer chemicals on J2s was determined by assessing the percentage of J2s that were immobile (dead) in the water control compared to those that were immobile in respective chemical concentrations. Each treatment was done in triplicates and the experiment was conducted once.

3.2.3 Effect of priming against RKN infection

Five week old potato cv Mondial plants in pots were primed by soil-drench with 100ml of 5mM or 20mM BABA, 50mg/L or 100mg/L Bion three days prior inoculation. Messenger was applied onto seed tubers by immersing them in 0.4g/L Messenger solution for 3min and then leaving them to dry in the glasshouse at 26˚C for 24 hours. The seed tubers were planted and Messenger was continuously applied at 14 day intervals post planting by spraying plant leaves with the solution. Inoculation of plants treated with Messenger was done at the same time with plants treated with BABA and Bion. Each primed plant was inoculated with 500 freshly hatched *M. incognita* J2s and the trials were conducted for a period of eight weeks post inoculation. Roots were harvested at four weeks and eight weeks post inoculation. At each harvest time point, the efficacy of the various resistance inducers at each concentration against RKN infection was measured using the following parameters: galling index, egg numbers, RKN population numbers, number of J2s and the percent of females in RKN populations from different treatments and untreated controls.

3.2.3.1 Galling index

Galling index was measured using a scale provided by Taylor and Sasser (1978): 0 = no galls (No galling); 1 = one or two galls (Slight galling); 2 = three to ten galls (Mild galling); 3 = eleven to thirty galls (Moderate galling); 4 = thirty one to hundred galls (Heavy galling); 5 = more than hundred galls (Extreme galling). To obtain the average galling indices, galling indices of all plants in each treatment per trial and per harvest time point were added and divided by the number of plants in each treatment. At each harvest time point, in all the three independent trials conducted, nine plants were harvested.

3.2.3.2 Egg numbers extracted from roots

Egg production was measured by calculating the average number of eggs extracted in each treatment at each harvest time point compared to control plants. Eggs were extracted from 20g of roots. Roots were suspended in 300ml of 3.0% [v/v] sodium hypochlorite and were shaken vigorously for 3min in 500ml glass bottles. The suspension was then decanted onto a 20µm sieve and the residual sodium hypochlorite on the surface of the eggs was washed off using sterilised tap water. The eggs were subsequently suspended in 50ml of sterile tap water and counted using a McMaster counting slide under the stereo microscope (Olympus, SZX10; Japan).

3.2.3.3 *M. incognita* J2s and females extracted from roots

Populations of *M. incognita* consisting of J2s and females were extracted from 20g roots by cutting them into small pieces and blending them in 3% [v/v] sodium hypochlorite for 1min. The blended root suspension was then decanted onto a stack of sieves, 1000µm, 150µm, 45µm, 38µm and 20µm, in that descending order, and washed thoroughly with sterile tap water. The washed down root suspension from the 20µm was suspended in 50ml sterile tap water in sterile falcon tubes. A teaspoon of Kaolin was added to each falcon tube containing the blended root material and, subsequent to that, centrifugation was performed at 3500rpm for 7min. The supernatant was discarded and the sedimented root material containing RKN J2s and females was suspended in 40ml sugar solution (450g of sugar in 1L) and subjected to centrifugation at 3500rpm for 3min. The supernatant containing RKN was decanted onto a 20µm sieve. Residual sugar solution on nematode surfaces was washed off using sterile tap water. RKN extracted from roots of each plant in each treatment were then suspended in 10ml sterile tap water and then counted on a McMaster plate using a stereo microscope. From each RKN population extracted from each plant, the total number of RKN was counted and the nematodes were divided into J2s and females based on size and shape. The percentage of females in each population was thereafter calculated.

3.2.4 Effect of priming against RKN and SRE

After determining the most effective priming agent on potato plants amongst BABA, Bion and Messenger, it was important to determine the effect of reduced nematode infectivity on the rate of RKN-assisted SRE infection. For this purpose, five week old *S. tuberosum* cv. Mondial plants were primed with 100ml 20mM BABA three days prior inoculation. Plants were simultaneously inoculated with freshly hatched 500 *M. incognita* J2s and 50ml overnight cultures of *Pcb*_mCherry (1×10^{8}cfu/ml). The following controls were included: 1. Plants that were primed (20mM BABA) and inoculated with *Pcb*_mCherry or *M. incognita* J2s alone; 2. Plants that were not primed but inoculated with with *Pcb*_mCherry and *M. incognita* simultaneously; 3. Untreated plants. Post inoculation, plants were maintained for a period of four weeks and the effect of priming on RKN or *Pcb* was assessed. *Pcb*_mCherry concentration inside the roots was determined by grinding 20g of surface sterilised mother tuber tissue followed by preparation of serial dilutions. Serial dilutions were plated on nutrient agar supplemented with 12.5µg.ml^{-1} of tetracycline. Plates were incubated for 48 hours at 28˚C, colonies were counted and the average concentrations of *Pcb*_mCherry in the roots on each treatment were calculated. Three independent trials were conducted and in each trial ten plants were used.

3.3 Results

3.3.1 Effect of chemicals on RKN

For a chemical to be classified as a resistance inducer, it is important that it should not have any direct biocidal effects on the pathogens. Different concentrations of chemicals that were used to prime potato cv Mondial plants were prepared and 100 freshly hatched *M. incognita* J2s were suspended in each concentration. There were no significant differences in the percentages of J2s that were mobile amongst the different treatments. There were also no significant differences between the different treatments and the water control (Figure 3.1). This therefore suggests that none of the chemicals have any direct biocidal effects on RKN J2s.

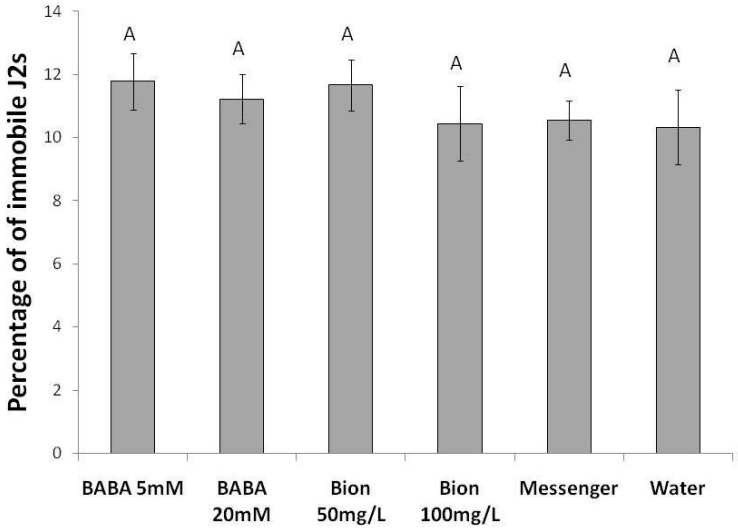

Figure 3.1 The effect of chemical inducers on *M. incognita* J2s. Freshly hatched *M. incognita* J2s were suspended in BABA, Bion and Messenger at various concentrations for five days at 25°C to determine whether these resistance inducing chemicals have a direct biocidal effect on the J2s. Sterile distilled water was used as a control. There were no significant differences in the percentage of immobilised J2s in the different treatments and the control. Different letters indicate significant differences. Means followed by common letters are not significantly different according to the Student–t test (0.05% confidence interval).

3.3.2 Evaluation of root galling

Galling index is one of the parameters that are commonly used to evaluate the virulence of RKN on various hosts or to determine the efficacy of resistance inducers against RKN infection (Taylor and Sasser, 1978). The average galling index for untreated plants in all the three independent trials conducted and at both four and eight weeks post inoculation was significantly higher than that of treated plants, averaging between 3.5 and 4. This was an indication that all the tested decreased galling index significantly, even though it was at varying degrees.

At four weeks post inoculation there were no significant differences in the average galling indices of plants treated with 5mM BABA, 20mM BABA, 100mg/L Bion and Messenger, all averaging between 2.25 and 2.75. At eight weeks post inoculation there were no significant differences in the average galling indices amongst plants treated with 5mM BABA, 50mg/L Bion and 100mg/L Bion. Of the three treatments, 20mM BABA-treated plants had significantly lower average galling index compared to 5mM BABA, 50mg/L Bion, 100mg/L Bion and Messenger which were not significantly different from each other at four weeks post inoculation. At eight weeks post inoculation a similar trend was observed whereby 20mM BABA-treated plants had the lowest significant average galling index whilst Messenger-treated plants, unlike at four weeks post inoculation, had the highest average galling index. At the same harvest time point, there were no significant differences amongst 50mg/L Bion, 100mg/L Bion and Messenger-treated plants in terms of average galling indices. As can be expected, at both four and eight weeks post inoculation, untreated plants had significantly higher galling indices compared to all treated plants. At the second harvest time point, there were no significant differences between the average galling index of plants treated with Messenger and untreated plants. It is possible that the use of BABA reduces cell development into galls, therefore limiting reducing the development of J2s into feeding females. This was demonstrated by Oka et al., (1999) where BABA was shown to reduce the rate of development of *M. javanica* J2s into adults. This, therefore, resulted in a significant reduction in the average galling index of treated plants. Alternatively, 20mM BABA induced resistance in potato plants to a level where the infection rate of *M. incognita* J2s was significantly reduced. This, in turn, could have resulted in lower female numbers in the roots of 20mM BABA-treated plants and therefore significantly lower galling indices.

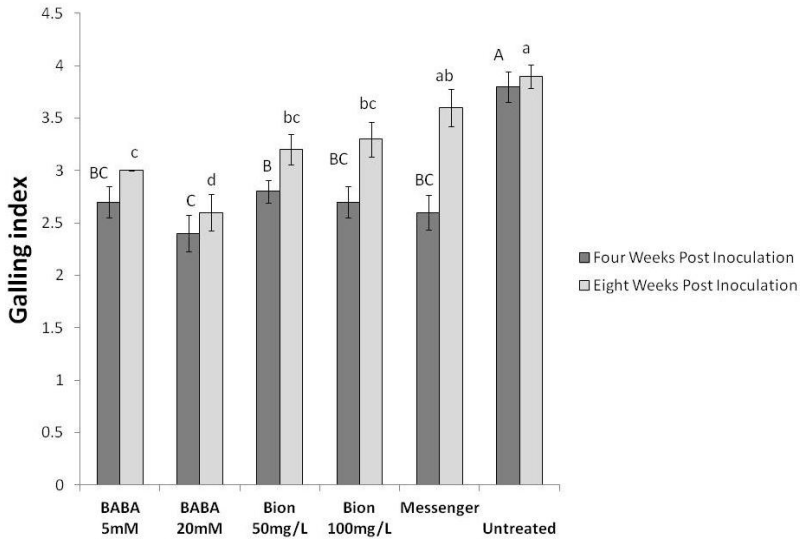

Figure 3.2 The galling index of potato cv Mondial plants treated with various concentrations of resistance inducing chemicals at various harvest time points post inoculation. Galling index of each plant in all the treatments was evaluated using the formula provided by Taylor and Sasser (1978) and the average galling indices from the three independent trials conducted was calculated per treatment at each harvest time point. Different letters indicate significant differences. Means followed by common letters are not significantly different according to the Student–t test (0.05% confidence interval). Capital letters represent significant differences at four weeks post inoculation and small letter represent significant differences at eight weeks post inoculation.

3.3.3 Egg production

The number of eggs, from the egg masses on the surface of roots, was used as a second criteria to evaluate the efficacy of the different chemicals against RKN infection. At both four and eight weeks post inoculation, control plant roots had significantly larger egg numbers indicating higher RKN infestation and multiplication or fertility. Contrary to control plants, all the other treatments showed a level of reduced egg production. The most significant reduction was observed for plants treated with 20mM BABA. (Figure 3.3). At four weeks post inoculation there were no significant differences in the number of eggs extracted from 5mM BABA- and 50mg/L Bion-treated plants. At the same harvest time point, no significant differences in egg production were observed in plants treated with 50mg/L Bion, 100mg/L Bion and Messenger (Figure 3.3).

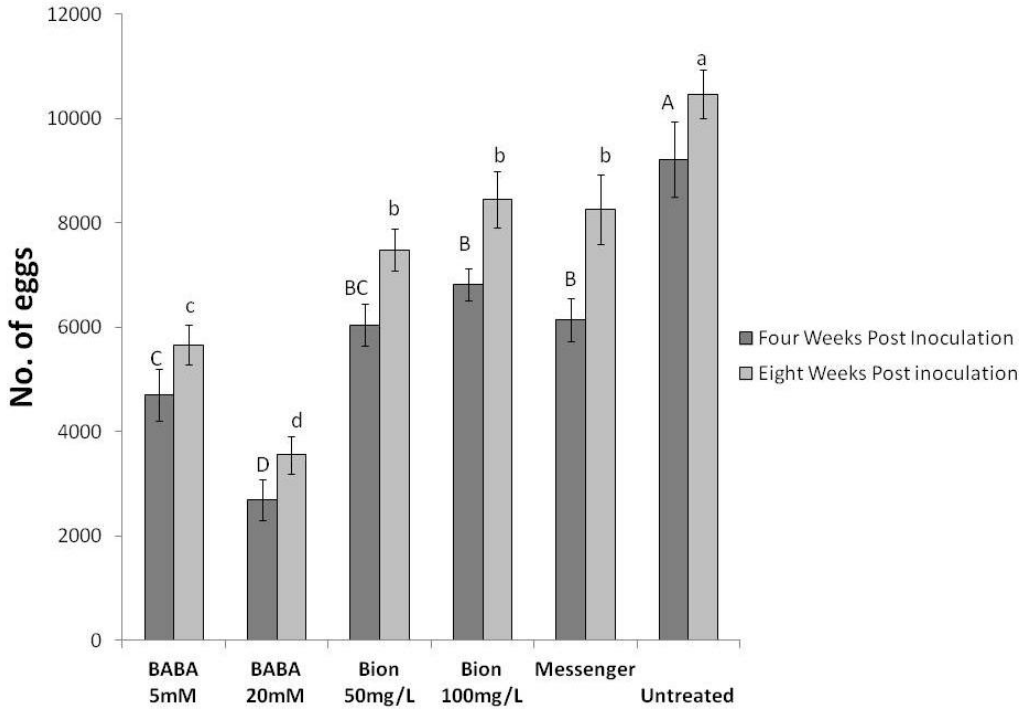

Figure 3.3 The average number of eggs produced per treatment at different harvest time points post inoculation. The average number of eggs per treatment from the three independent trials was calculated. Plants treated with 5mM BABA and 20mM BABA had a significantly lower number of eggs compared to plants treated with other chemicals. Different letters indicate significant differences. Means followed by common letters are not significantly different according to the Student–t test (0.05% confidence interval). Capital letters represent significant differences at four weeks post inoculation and small letter represent significant differences at eight weeks post inoculation.

3.3.4 RKN numbers

3.3.4.1 Second stage juveniles

The total numbers of RKN extracted from roots in different treatments were counted and second stage juveniles and females from 20g of roots per plant in each treatment were also counted separately in each population. There were no significant differences observed at four weeks post inoculation when comparing the total number of RKN extracted from roots treated with 5mM BABA, 20mM BABA and Messenger. RKN extracted from roots treated with 50mg/L Bion and 100mg/L Bion were also not significantly different from each other at the same harvest time point. At eight weeks post inoculation, there were no significant differences in the RKN numbers extracted from roots treated with 5mM BABA, 100mg/L Bion and Messenger. However, at both harvest time points, compared to all other treatments and the untreated control, 20mM BABA significantly reduced RKN populations in the roots of potato plants (Figure 3.4).

The average number of juveniles in each treatment at each harvest time point was calculated and compared to that of untreated plants. As can be expected, in all the three independent trials and at both harvest time points, significantly more J2s were extracted from roots of untreated plants compared to all treatments. At four weeks post inoculation, there were no significant differences in the number of J2s extracted from plants treated with 5mM BABA, 50mg/L Bion, 100mg/L Bion and Messenger. At eight weeks post inoculation, there were no significant differences in J2 numbers extracted from plants treated with 50mg/L Bion, 100mg/L Bion and Messenger. However, at both time points, plants treated with 20mM BABA had the least significant number of J2s in their roots whilst untreated plants had the highest number of J2s in their roots (Figure 3.5). Thus, it can be concluded that at both time points 20mM BABA is the most significant treatment leading to a significant reduction in the number of J2s counted.

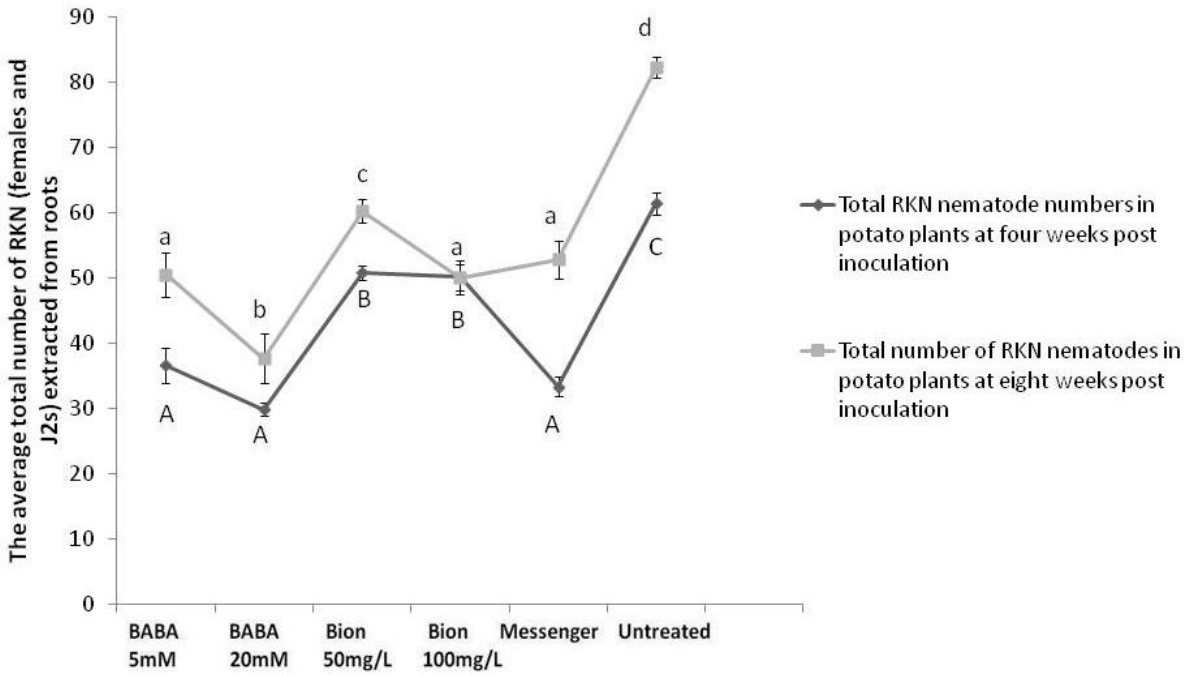

Figure 3.4 The total numbers of RKN (J2s and females) extracted from roots treated with various resistance inducer chemicals at four and eight weeks post inoculation. Different letters indicate significant differences. Means followed by common letters are not significantly different according to the Student–t test (0.05% confidence interval). Capital letters represent significant differences at four weeks post inoculation and small letter represent significant differences at eight weeks post inoculation.

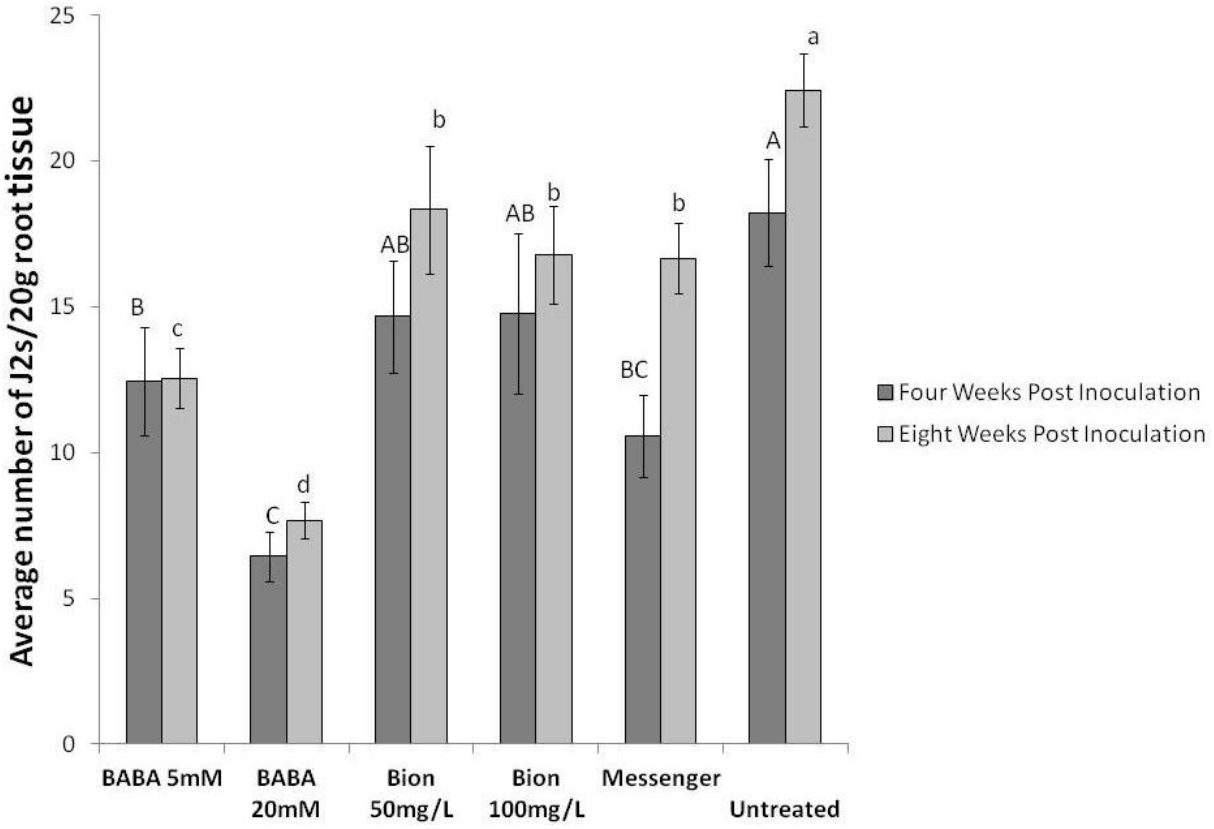

Figure 3.5 The average number of J2s that were extracted from 20g potato roots at four and eight weeks post inoculation in plants treated with different concentrations of resistance inducers and untreated plants. Different letters indicate significant differences. Means followed by common letters are not significantly different according to the Student–t test (0.05% confidence interval). Capital letters represent significant differences at four weeks post inoculation and small letter represent significant differences at eight weeks post inoculation.

3.3.4.2 Percentage of females

To determine the effect of each resistance inducer chemical on the development of J2s into adult female stage, the average percentage of females in 20g of root tissue was calculated. There were significant differences in the percentages of J2s that developed into females post infection amongst the different treatments and the untreated control plants. At four weeks post inoculation, 5mM BABA reduced the average percentage of females the most. Comparing the different treatments, this low female percentage in 5mM BABA-treated plants was not significantly different from the female percentages in RKN populations in all the other treatments including the untreated control except 20mM BABA. There were also no significant differences noted amongst 20mM BABA, 50mg/L and 100mg/L Bion, Messenger and the untreated control in the first harvest time point. At eight weeks post inoculation there were no significant differences in the percentage of females in RKN populations extracted from plants treated with 5mM BABA, 50mg/L and 100mg/L Bion and untreated controls. At both harvest time points, 20mM BABA-treated plants had the highest percentage of females in RKN populations extracted from them. This result implies most of the J2s that penetrated potato roots developed into females. Furthermore, these results indicate that none of the used resistance inducers can reduce the rate of development of RKN from J2s to adult females (Figure 3.6).

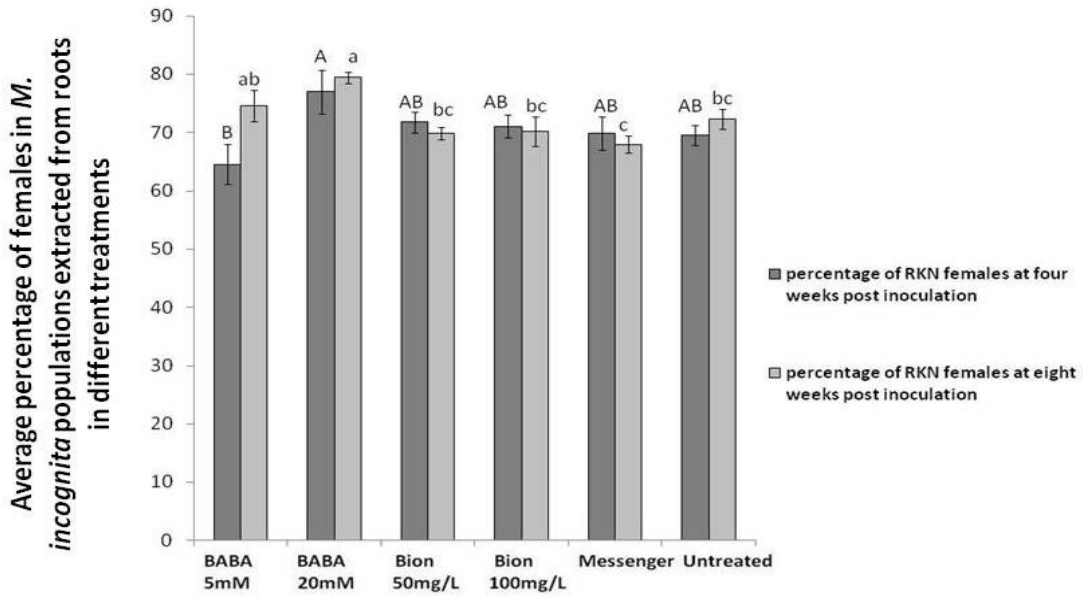

Figure 3.6 The percentages of females from the nematodes that were extracted from 20g of root mass. Different letters indicate significant differences. Means followed by common letters are not significantly different according to the Student–t test (0.05% confidence interval). Capital letters represent significant differences at four weeks post inoculation and small letter represent significant differences at eight weeks post inoculation.

3.3.5 Effect of priming on the synergistic interaction between RKN and SRE

The effect of priming agents on RKN infection and subsequently on *Pcb* infection rates was determined by comparing the amount of *Pcb*_mCherry in 20g roots tissue obtained from primed and control (without priming) plants. There was a significant difference between the amount of *Pcb*_mCherry found in roots of primed and untreated plants inoculated with *M. incognita* J2s and *Pcb*_mCherry. Untreated plants that were inoculated with both pathogens had significantly higher amounts of *Pcb*_mCherry compared to their primed counterparts. No significant differences in the amounts of *Pcb*_mCherry in roots of primed and untreated plants inoculated with *Pcb*_mCherry alone were found. The amounts of *Pcb*_mCherry found in primed and untreated plants inoculated with *Pcb*_mCherry alone were not significantly different from that of primed plants that were inoculated with both *M. incognita* J2s and *Pcb*_mCherry. This is an indication that BABA, as a resistance inducer chemical, has the potential to be used against the RKN-SRE disease complex as it can reduce the amount of SRE that enter potato roots in the presence of RKN. Based on the results obtained, BABA also has the potential to render the presence of RKN insignificant in this disease complex since the amount of SRE that enter primed roots in the presence of RKN is not significantly different from that in primed or untreated plants inoculated with *Pcb*_mCherry alone.

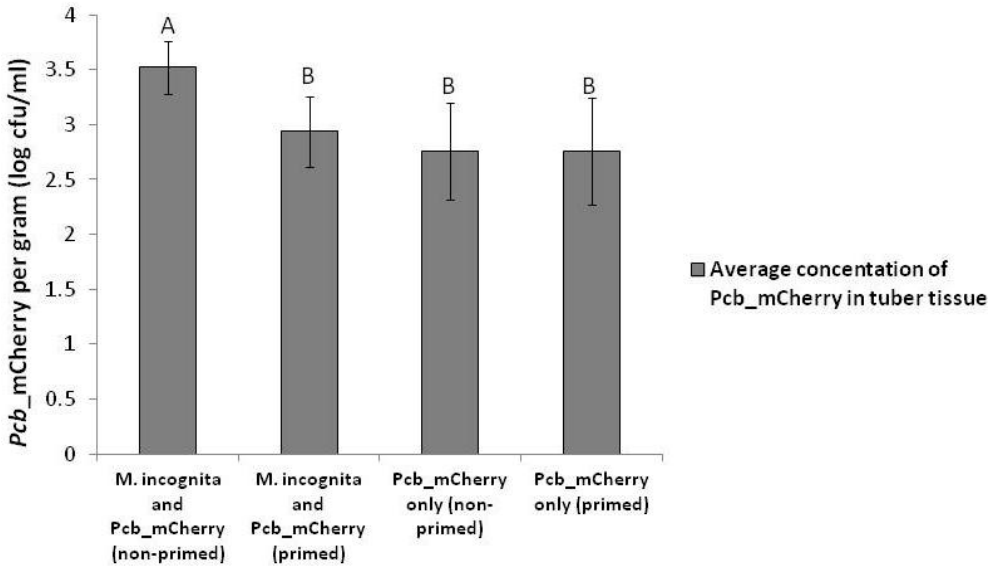

Figure 3.7 The concentration of *Pcb*_mCherry isolated from roots of plants primed with 20mM BABA and untreated plants inoculated simultaneously with *M. incognita* J2s and *Pcb*_mCherry and *Pcb*_mCherry alone. Non-primed plants inoculated with both pathogens had the highest concentration of *Pcb*_mCherry in comparison to all the other treatments. Different letters indicate significant differences. Means followed by common letters are not significantly different according to the Student–t test (0.05% confidence interval).

3.4 Discussion

Systemic acquired resistance (SAR) is a type of resistance that is induced by avirulent pathogens. It spreads throughout the host and gives plants the ability to become more tolerant or resistant to pathogen attack prior to contact with the pathogen. This usually occurs when a host plant encounters a pathogen that subsequently potentiates it to react more efficiently in the next encounter with another or the same pathogen (Ryals et al., 1994, 1996; Agrios, 2005). The recognition of the initial pathogen by the host plant results in the production of key signaling molecules which rapidly induce the expression of pathogenesis-related genes. These signaling molecules, such as salicylic acid, will be distributed endogenously throughout the plant to distal areas from the initial inoculation site (Ryals et, al., 1994, 1996; Stitcher et al., 1997). Alternatively SAR can be induced by the application of resistance inducing chemicals which are analogues of salicylic or jasmonic acid. The process where resistance inducing chemicals are applied onto plants to potentiate them against pathogen attack is known as priming. Resistance induction chemicals used in this study included DL-β-aminobutyric acid (BABA), Acibenzolar-s-methyl (Bion) and Messenger (Harpin).

Due to the phasing out of efficient nematicides from the market (Caillaud et al., 2008; Dervan and Sogut, 2010; 2011; Macleod, 2011), it is anticipated that the damage caused by plant parasitic nematodes on economically important crops, such as potato, will significantly increase and this will result in major crop losses. In the absence of efficient management strategies against plant parasitic nematodes, and considering the lack of efficient alternatives, exploiting plant resistance appears to be the most attractive and environmentally friendly strategy.

Priming, which mostly involves the induction of SAR, has been demonstrated to be effective against *Meloidogyne javanica* and *Rotylenchulus reniformis* on Pineapple (Chinnasri et al., 2006), *Meloidogyne* spp. on grapevine (Owen et al., 2002), *Meloidogyne* spp. and *Heterodera* spp. on cereal (Oka and Cohen, 2001) and RKN on tomato (Oka et al., 1999). In these examples, the induction of SAR through the use of priming agents was shown to decrease the number of infective stages that penetrated into host roots and therefore the number of females in the roots. This translated to significantly lower galling indices and less egg production thus lower parasitic nematode inoculum levels in the soil.

For chemicals to be regarded as potential priming agents it is important to ensure that they have no biocidal effects on the target pathogen. The results obtained in this study, therefore, eliminate the possibility that the tested resistance inducing chemicals may have directly on the J2s in the soil outside plant tissue. It is also an indication that the effect of the priming agents on RKN was through increased plant defense mechanism rather than direct toxicity on nematodes. These results were in correlation with those obtained by Oka et al., (1999) and Chinnasri et al., (2006). Both research groups used similar chemicals and found that these chemicals did not have any biocidal effects on the targeted plant parasitic nematodes used in the experiments.

The low infection rates in experiments of chapter three were normal as with *Meloidogyne* spp. one generation of the same species cultured under the same conditions can have variable levels of virulence compared to the previous or next generation (Professor Drikie Fourie, Personal communication). *M. incognita* inoculum that was used in previous experiments (Chapter 2) was much more aggressive whereby even plants that were inoculated with 200 freshly hatched J2s were heavily infested. The population of *M. incognita* obtained for use in experiments of this study was unfortunately not as aggressive and this was indicated by the low average number of the RKN populations (females and J2s) that were extracted from untreated plants. Nonetheless, the effect of different priming agents at different concentrations was still observed.

When testing the efficiency of BABA and other SAR-inducing chemicals against RKN, Oka et al., (1999) observed necrotic lesions on the roots of tomato plants. When the same chemicals were applied on the foliage, plants developed chlorosis on small leaves. Oka and Cohen (2001) also observed phytotoxic lesions on cereal after applying BABA against RKN. The phytotoxic action of priming agents on plants was also highlighted by Heil et. al. (2000), working on wheat. They demonstrated that acibenzolar (Bion) can significantly decrease seed production. The literature strongly suggests that resistance inducing chemicals, at most times, have some form of phytotoxic effect on plants. The results obtained in this study were, thus, contrary to what has mostly been observed by other researchers. There were no signs of phytotoxocity on plants in terms of spots or discolouration of the foliage or roots. This might have been due to the duration of the greenhouse trials. It is possible that the necrotic lesions might have developed had the experiments been conducted for more than eight weeks. Another possibility is that the chemicals used might simply have not adverse effects on potato at the tested concentrations. The results

obtained suggest that BABA, Bion and Messenger can potentially be used as priming agents against RKN infection in potato since they do not have adverse effects on the host but more work such as field trials need to be conducted to obtain a true reflection of the effect of these priming agents on host plants in a natural environment.

Following the life cycle of RKN, the J2s hatch from the eggs and infect the roots of compatible hosts in the soil. In the roots they migrate towards the root tip where they enter the vascular cylinder. In the vascular cylinder they transform parenchyma cells into nutrient sinks from where they feed as the females enter the reproduction stages. In all the treatments, in the three independent trials conducted, *M. incognita* J2s were able to infect potato roots even though it was at varying degrees. The observed infection rates of J2s, together with the fact that all the resistance inducer chemicals used in this study did not have any direct biocidal effect on the J2s, suggests that plant protection was triggered post infection with the first few J2s in host root tissue. It is known that primed plants, unlike those in which resistance is constitutively expressed, efficiently manufacture pathogenesis-related compounds upon infection. It has been shown that infection of primed plants often leads to thickening of the host epidermal tissue through the production of pappilae and lignification of cell walls thereby preventing further infections. Callose deposition and lignification of plant cell walls has also been shown to be induced by priming agents (Stitcher et. a., 1997; Siegrist et.al., 2000; Zimmerli et. a., 2000). This might have been the case in this study whereby the first few J2s to penetrate host root tissue might have triggered the rapid thickening and lignification of the host cell walls of primed plants thus significantly decreasing further infection by other J2s. Mild infection by root knot nematode can induce resistance in plants that have not been treated with resistance inducers (Ogallo and McClure, 1995). In the experiments conducted here, it is possible that plant treatment with 20mM BABA might have amplified the rate at which resistance mechanisms were activated in potato plants against further infection by *M. incognita* J2s. This suggested mechanism of action is supported by the significantly lower numbers of RKN that were extracted from plants that were treated with 20mM BABA compared to other chemicals.

After successfully penetrating host roots, RKN J2s enter the vascular cylinder where they can physiologically modify parenchyma cells to become multinucleate. This leads to an increase in size and these cells become nutrient sinks from which the RKN feed and reproduce. Inside the host roots the development of RKN J2s into females is therefore important for the production of inoculum, in the form of eggs, which hatch into the next generation of J2s. Results obtained in this study indicated that none of the resistance inducer chemical was able to reduce the development of RKN J2s into adult females. This result was contrary to what was found by Oka and Cohen (2001) when they observed a decrease in the development of *Meloidogyne* spp. in primed cereal plants.

Root galls develop as a result of the physiological modification of parenchyma cells in the vascular cylinder of host roots. Feeding females modify parenchyma cells to become multinucleate and enlarge in size. The enlarged cells are later used as nutrient sinks by *Meloidogyne* females as they reproduce. It is known that the higher *Meloidogyne* inoculum load there is in the soil the higher the galling index. This is due to larger numbers of J2s which infect the roots and therefore result in severe galling of host roots. In the experiment conducted, plants treated with 20mM BABA had the lowest average galling index compared to plants in other treatments. Since BABA reduced the number of J2s that penetrated host roots, it is then not surprising that 20mM BABA-treated plants had a significantly lower average galling indices compared to other plants in different treatments and untreated controls. Therefore, when analysing the number of RKN in the roots and galling indices, we can link the decrease in the number of J2s that initially penetrated into potato roots to lower galling indices. Furthermore, these results are in correlation with what was found by Oka et. al. (1999) when they demonstrated that lower J2 numbers in the roots resulted in lower galling indices. These results indicated that the mode of action that BABA used to decrease galling indices of infected plants was through reduction of J2s that initially infect the plants. This, in turn, led to fewer females in the host root tissue and therefore significantly lower galling indices.

The number of J2s in host roots that developed into mature females is an indication of the anticipated levels of egg-inoculum that can be produced for the next generation. The number of females in the host roots is dependent on the initial number of J2s that managed to penetrate and successfully infect host roots. The difference in the number of eggs extracted from each

treatment can be linked to previous discussed results whereby *M. incognita* J2 infection rates were significantly lower in BABA-treated plants. This led to lower female numbers which in turn resulted in significantly lower egg-numbers extracted. A similar observation was also made by Oka et. al. (1999). In their experiments they demonstrated that significantly lower egg-numbers were extracted from BABA-treated tomato plants compared to plants treated with other resistance inducing chemicals and untreated plants. These results were in correlation with those obtained by Chinnasri et. al. (2006). They found that BABA- and Bion-treated plants decreased egg production in pineapple significantly. The reduction of initial infection rates of *Meloidogyne* spp. by BABA, therefore, has a potential ripple effect that can result in the decrease of inoculum in the soil as it also affects egg production. This is likely to have an effect on RKN numbers of the next generations since *Meloidogyne* spp. require a compatible host to reproduce.

The results obtained from this study further suggest that priming, using 20mM BABA, can also potentially reduce RKN-assisted *Pcb* infection of potato roots. This was supported by the bacterial isolation data whereby the concentration of *Pcb*_mCherry isolated from primed plants inoculated with both pathogens was significantly lower than that of their untreated counterparts. Also, the amount of *Pcb*_mCherry in roots of primed plants inoculated with both pathogens was not significantly different from that of primed and non-primed plants inoculated with *Pcb*_mCherry alone. These result served as an indication that 20mM BABA neither has any direct adverse effects on the bacteria nor the ability to prime plants against *Pcb* infection but rather reduces *Pcb* infection by reducing *M. incognita* infection rates. Based on these results we suggest that the reduction of *Pcb* amounts in potato roots was as a result of the reduced number of wounds/entry sites on the roots of primed plants as a direct outcome from the reduction of *M. incognita* infection rates. These results also implied that in plants treated with 20mM BABA, RKN J2 infection does not significantly increase the amount of *Pcb*_mCherry that enters host root tissue.

Experiments conducted in this study consisted only of glasshouse trials and observations. Future experiments that incorporate molecular supporting data to these observations will be undertaken as part of a future project. Bbased on previous research from the literature, BABA, Bion and Messenger have been shown to induce pathogenesis-related reactions more efficiently in plants under pathogen attack. BABA was shown to induce the timely and fast production of

pathogenesis-related proteins such as β-1,3-glucanase and chitinase, which are essential in the protection of pepper plants against *Phytophthora capsici* (Sunwoo et al., 1996; Hwang et al., 1997). Furthermore, BABA has been shown to induce systemic accumulation of other signalling molecules such as salicylic acid and the expression of the PR-1a gene in tobacco response to tobacco mosaic virus (Siegrist et al., 2000). In another study, Bion has been shown to increase the activity of β-1,3-glucanase in the roots against *Meloidogyne* spp. in grapevine (Owen et al., 2002). It has also been demonstrated that, Harpin, the active ingredient of Messenger, can induce SAR against *Ralstonia solanacearum* in tomato and tobacco mosaic virus in tobacco (Wei and Beer, 1996). The results obtained strongly suggest that BABA, Bion and Messenger are priming agents that can potentially be used to induce SAR in potato against *Meloidogyne* spp. infection, with BABA being the most efficient.

3.4 Conclusion

The agricultural sectors in many countries, with pressure from environmental lobbyists, have banned the use of Temik and other effective nematicides against parasitic nematodes on crops. This will, without a doubt, put pressure on crop production as many farmers will experience significant crop and financial losses due to root knot nematode damage. This study was focused on determining the efficacy of three commonly used SAR inducers against *M. incognita* infection in potato. This study provided some insight into the potential of BABA as a priming agent that can be used as one of the strategies to combat root knot nematode infection in integrated management systems on potatoes. In this study BABA was able to significantly induce resistance in potato, subsequently reducing the infection rate of *M. incognita* J2s. These experiments were performed in the glasshouse under controlled conditions, hence, they will need further validation either through field experiments or gene expression analysis. Nonetheless, the results obtained in the study suggest that BABA has a potential to significantly decrease root knot nematode infection in potato. Hence, future planned experiments in the research group include the analysis of transcriptomic profiles of potato plants primed with BABA and challenged with RKN.

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CHAPTER FOUR

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER FOUR

General discussion and conclusions

In the life cycles of RKN and SRE, possible areas of overlap were identified. As part of their life cycle, J2s of *Meloidogyne* spp. penetrate host roots and enter the vascular cylinder, therefore creating possible entry points for opportunistic pathogenic bacteria. Once inside the roots, J2s develop into females and these become sedentary and start to feed and reproduce. Some *Pectobacterium* spp., such as *Pcb*, are opportunistic pathogens that can only penetrate the host roots through natural openings such as lenticels or wounds formed by various agents such as RKN (Dropkin, 1969; Agrios, 2005; Nabhan et. al., 2012). As part of their life cycle, *Pectobacterium* spp. enter host roots, colonise and subsequently cause rot using cell wall degrading enzymes (Barras et. al., 1994; Rowe et. al., 1995; Roberts et. al., 2007). As the host tissue continues to decay and disintegrate, SRE escape into the soil where they colonise roots of healthy roots.

From the life cycles of the two pathogen groups, it is clear that at some point both pathogens need to be inside host roots where, if they are both present, share minute spaces. In the rhizosphere where root knot nematodes and *Pectobacterium* spp. are prevalent, the two pathogen groups share the same environment and that is another area, apart from the roots, where interactions can occur. Based on the identified possible overlaps in the life cycles of *Pectobacterium* spp. and RKN it was necessary to first whether there was a physical interaction between the two groups of pathogens. The first objective of this study was therefore to determine whether there was an interaction between *Pectobacterium* and *Meloidogyne* spp. that could result in *Meloidogyne* spp. being able to disseminate *Pectobacterium* spp. The results obtained in this study strongly demonstrate that *Pcb* can adhere on to *Meloidogyne incognita* J2s at 4, 28 and 37°C, but optimally at 28°C. There were no significant differences in the amount of *Pcb* that was associated with *M. incognita* J2s over time. Dissemination assay results also strongly suggested that *M. incognita* J2s can disseminate *Pcb in vitro*. This, however, still needs

to be demonstrated in the natural environment. These results imply that there is a physical interaction between *Pcb* and *M. incognita* J2s. Furthermore, our findings suggest that in the environment, *M. incognita* J2s as the mobile and infective stages of *Meloidogyne* spp., can mobilise and disseminate *Pcb* inoculum faster than normal. This implies that *Pcb* will be able to invade new spaces in the environment and therefore have access to a variety of healthy hosts within a short space of time. Pathogen movement in the environment plays an important role in survival and therefore this interaction between *Pcb* and *Meloidogyne* J2s can possibly be a factor that is pivotal in the success of *Pcb* as a soft rot and blackleg-causing agent and an important pathogen in the potato industry.

Meloidogyne spp., as part of their interactions with other pathogens, are known to have the ability to increase the severity and incidence of disease caused by secondary pathogens (Inagaki and Powell, 1969; Mayol and Bergesen, 1970; Hallmann et. al., 1998). Both RKN and *Pectobacterium* spp. are found in the rhizospheres of their hosts and are both capable of causing important diseases in potato. Hence, we hypothesised that these two pathogens are likely to interact with each other forming a synergistic relationship. Hence, to test this hypothesis, the second objective of this study was undertaken. The effect of co-infections of RKN and *Pectobacterium* spp. on the incidence and severity of disease caused by *Pectobacterium* spp. on potato was thus evaluated. The results obtained from this study indicated that there is synergistic interaction between the *M. incognita* and *Pcb*. Plants that were co-inoculated with *M. incognita* and *Pcb* were more severely diseased than those that were inoculated with *Pcb* alone. Plants that were inoculated with *M. incognita* alone were as healthy as uninoculated plants whereas *Pcb* alone-inoculated plants showed signs of wilt that were significantly less severe compared to those observed on co-inoculated plants. This strongly suggested that *M. incognita* infection of potato plants significantly increased the amount of *Pcb* that entered potato roots thus resulting in those plants being more diseased than those inoculated with individual pathogens. This result was confirmed with plate counts and confocal microscopy where significantly higher numbers of *Pcb* cells were visualised in mother tuber tissue of plants inoculated with both pathogens. Based on these observations, we therefore suggest that wounds on host roots created by *M. incognita* J2s served as entry points that were used by *Pcb* to enter potato roots. The pivotal role of *M. incognita*-induced wounds in the interaction between *Pcb* and *M. incognita* was confirmed with results obtained from the study where plants were co-inoculated with the same concentration of

Pcb but different *M. incognita* numbers (200 and 6000). Plants inoculated with 6000 *M.* incognita J2s had more *Pcb* cells in their roots compared to those inoculated with 200 *M. incognita* J2s. This suggested that there was an increase in the rate of *Pcb* infection in potato roots with increasing numbers of RKN J2s in the soil. In the same experiment, plants that were inoculated with *Pcb* alone had significantly lower *Pcb* concentrations in their roots. Since these experiments were performed in glasshouse conditions, we cannot be certain that the same would happen in the natural environment. Hence to fully establish the economic impact of these interactions it is crucial that field trials are conducted to complete these glasshouse experiments.

Meloidogyne spp. have been shown to have the ability to break the tolerance of their hosts to microorganisms which were previously not known to cause disease. This phenomenon was demonstrated by Marley and Hillocks (1994) using *Cajunus cajun* that displayed significantly higher disease severity when inoculated with *M. incognita* or *M. javanica* and *Fusarium udum* as compared to *F. udum* alone. *Solanum tuberosum* cv BP1 has, in previous greenhouse experiments, shown high levels of tolerance against *Pcb* stem infections compared to the commonly used cv Mondial (Khubheka, Unpublished). Information pertaining to RKN ability to break the tolerance of *S. tuberosum* cv BP1 to *Pectobacterium* spp. is currently unknown. This information is important to potato producers, especially those producing cultivars that are more tolerant to *Pectobacterium* spp. The third objective of this section of the study was therefore to determine the potential of RKN to break the tolerance of cv BP1 to *Pectobacterium* spp. stem infections. Under glasshouse conditions, *M. incognita* could not break the tolerance of BP1 plants to *Pcb*-induced blackleg. Nonetheless, it is important to note that in the field, under natural conditions, where there is an interplay of many other factors, a different result might be observed. Also, in glasshouse experiments it is possible that if conditions such as temperature, humidity, etc are varied, there may be different outcomes to the ones observed here. Nevertheless, under the greenhouse conditions and with the pathogen species used, at their respective concentrations, no tolerance-breakage was observed.

Nematicides have for years been used to control RKN and other plant parasitic nematodes and, as a result of their high efficacy, very little research has gone towards understanding the threat of RKN. Due to the gradual phasing-out of nematicides such as Temik and Methyl Bromide (Rother and Jacobs, unknown year; Giannakou et. al., 2002; Macleod, 2011), management of these organisms will be difficult especially if there are no effective alternatives. Using plant resistance to combat pathogen attacks has been shown to be an important commodity in crops like tomato, cucumber and pepper (Walters et. al., 1997; Thies and Fery, 1998) where resistant cultivars are available. Since the use of Temik, Methyl Bromide and other effective nematicides on potatoes and other crops has been terminated, plant resistance remains one of the most practical strategies to use in management of RKN and other plant parasitic nematodes.

Resistance in plants can be enhanced by constitutive gene expression of resistance genes or through priming. Comparing the two methods of resistance induction, priming stands out as the most attractive alternative to chemical control to combat parasitic nematode infections (Heil, 2002). The aim of the third chapter of this study was therefore to determine whether priming can be used as an alternative to chemical control in potato against RKN infections. The first objective of this section of the study was to determine which priming agent amongst DL-β-aminobutyric acid (BABA), Benzothiadiazole (Bion) and Messenger can potentially induce resistance more efficiently in potato plants against RKN infection and the concentration at which that priming agent is more effective. This was measured by assessing root galling indices of different treatments, egg production and the number of J2s and females in the roots. The second objective was to determine whether priming against RKN, using the most effective resistance chemical inducer amongst the three, can decrease *Pcb* infection in potato roots. This was assessed by determining the amount of *Pcb* in roots of primed and non-primed plants inoculated with *M. incognita* J2s and *Pcb* and those inoculated with *Pcb* alone.

Amongst the three priming agents that were tested against RKN infection, BABA was the most efficient, especially at 20mM. This was based on the significant reduction of root galling index, egg production and the number of J2s that managed to penetrate into the host roots of BABA-treated plants. When compared to other priming agents and the water control, none was able to reduce the percentage of J2s that develop into females once inside the host roots. 20mM BABA was also, as a result of *M. incognita* J2s infection reduction, able to indirectly reduce *Pcb*

infection in potato roots. Based on the results obtained 20mM BABA has the potential to be used as an alternative to banned chemicals in the management of RKN. The concentration of *Pcb* in roots of primed plants inoculated with both pathogens was significantly lower than that of their untreated counterparts. The amounts of *Pcb* in primed and non-primed plants inoculated with *Pcb* alone were not significantly different. This was an indication that 20mM BABA is potentially effective in resistance induction against *M. incognita* but not against *Pcb*. Therefore BABA treatment should be combined with other management strategies that can target pathogenic bacteria in order to manage this kind of an interaction especially in situations whereby RKN inoculum is significantly high and therefore a significant number of J2s penetrates the host roots.

In conclusion, using mCherry-tagged *Pcb*, we have developed a system that can be used to study the interaction between plant parasitic nematodes and other phytopathogens. The details and application of this tool have now been published in *Letters in Applied Microbiology*. Moving forward we will need to determine whether interactions that have been detected in a glass-house environment using the same system can also be detected in the natural environment. We have also shown that BABA can potentially be used as one of the alternative chemicals for root knot nematode management in integrated management strategies. Future work includes determining whether this is a practical strategy in the field where many other factors such as temperature fluctuations, moisture and nutritional status of the soil, amongst others, come into play. Moreover, gene expression profiling of resistance mechanisms induced in potato plants by the use of BABA require further investigation.

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RESEARCH OUTPUTS

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1. Accepted

Mongae, A., Kubheka, G.C. and Moleleki, L.N. (2012) 'The use of fluorescent reporter protein tagging to study the interaction between Root-Knot Nematodes and Soft Rot Enterobacteriaceae.' *Letters in Applied Microbiology*

ABSTRACT

The study of plant parasitic nematodes such as *Meloidogyne spp.* and their interactions with phytopathogenic bacteria remains underexplored. One of the challenges towards establishing such interactions is the dependence on symptom development as a measure of interaction. In this study, mCherry was employed as a reporter protein to investigate the interaction between the soft rot *Enterobacteriaceae* (SRE) *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pcb*) and root knot nematode (*M. incognita*). *Pectobacterium carotovorum* subsp. *brasiliensis* was transformed with pMP7604 generating *Pcb_mCherry* strain. This strain was shown to attach to the surface coat of *M. incognita* J2 at the optimum temperature of 28°C. This suggests that RKN juveniles may play a role in disseminating *Pcb* in soils that are heavily infested with *Pcb*. The presence of RKN juveniles was shown to play a role in introducing *Pcb_mCherry* into potato tubers potentially acting as a source of latent tuber infections.

2. Manuscript in preparation

Mongae, A. O., Kubheka, G. C. and Moleleki, L. N. (2013) 'The effect of priming on combined soft rot *Enterobacteriaceae* (SRE) and root knot nematode (RKN) infection of *Solanum tuberosum*.' *Crop Protection*.

Other publications

4. Accepted

Moleleki, L.N., Onkendi, E.N., Mongae, A. and Kubheka, G.C. (2012) 'Characterisation of *Pectobacterium wasabiae* causing blackleg and soft rot diseases in South Africa.' *European Journal of Plant Pathology* DOI 10.1007/s10658-012-0084-4

ABSTRACT

Pectolytic bacteria were isolated from potato tubers and stems showing tuber soft rot and blackleg symptoms. Approximately half (52%) of the isolates could grow at both 27 and 37°C while another half (48 %) failed to grow at 37 °C. All isolates could be amplified with primers specific to the pectate lyase (pel) gene. Carbon utilisation profiles could not conclusively identify these isolates. PCR amplification using primers specific for *Pectobacterium carotovorum* subsp. *brasiliensis* was positive for all isolates that grew at 37°C. However, the group that did not grow at 37°C failed to amplify with *P. atrosepticum* specific primers. To characterise this group of isolates, the intergenic transcribed spacer region (ITS) was amplified and PCR products digested with two restriction enzymes (RsaI and CfoI) to generate ITS-PCR RFLP profiles. The profiles of these new isolates were compared to those of the type strains of other pectolytic bacteria. Profiles of five of the selected atypical strains generated with the enzyme CfoI appeared to be most similar to those of *P. wasabiae* type strain. Phylogenetic analysis using concatenated partial gene sequences of housekeeping genes mdh and gapA clustered these isolates together with those of *P.*

wasabiae reference strains thus confirming their identity. These strains were virulent on potato tubers and stems but did not elicit hypersensitive response on tobacco plants. This is the first report of *P. wasabiae* causing soft rot and blackleg of potatoes in South Africa.

5. Accepted

van der Nest, M. A., Steenkamp, E. T., Slippers, B**.,** Mongae, A. O., van Zyl, K., Stenlid, J. Wingfield, M. J. and Wingfield, B. D. (2011) Gene expression associated with vegetative incompatibility in *Amylostereum areolatum*. Fungal Genetics and Biology 43: 1034-1043

ABSTRACT

In filamentous fungi, vegetative compatibility among individuals of the same species is determined by the genes encoded at the heterokaryon incompatibility (het) loci. The hyphae of genetically similar individuals that share the same allelic specificities at their het loci are able to fuse and intermingle, while different allelic specificities at the het loci result in cell death of the interacting hyphae. In this study, suppression subtractive hybridization (SSH) followed by pyrosequencing and quantitative reverse transcription PCR were used to identify genes that are selectively expressed when vegetatively incompatible individuals of Amylostereum areolatum interact. The SSH library contained genes associated with various cellular processes, including cell-cell adhesion, stress and defence responses, as well as cell death. Some of the transcripts encoded proteins that were previously implicated in the stress and defence responses associated with vegetative incompatibility. Other transcripts encoded proteins known to be associated with programmed cell death, but have not previously been linked with vegetative incompatibility. Results of this study have considerably increased our knowledge of the processes underlying vegetative incompatibility in Basidiomycetes in general and *A. areolatum* in particular.