Interspecific competition between the potato cyst nematode species *Globodera pallida* and *G. rostochiensis*

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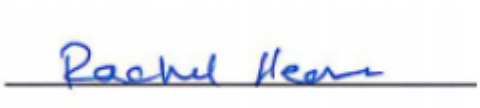
Declaration

This work has not been previously accepted in substance for any degree and is not being concurrently submitted in candidature for any degree. This thesis is the result of my own independent work/investigation, except where otherwise stated.



Rachel Hearne

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Abstract

The two potato cyst nematode species, *Globodera pallida* and *G. rostochiensis,* are among the most important pests of potato. PCN are difficult to manage, while the two species respond differently to the main control methods. An increase in the incidence of *G. pallid*a had been reported and is generally attributed to greater effectiveness of control measures against *G. rostochiensis*. The status of PCN in Ireland was studied using PCR. The results demonstrated qPCR to be an efficient means of high-throughput PCN sampling, being able to accurately identify both species in mixed-species populations. Species discrimination using qPCR revealed an increase in the incidence of *G. pallida* in Ireland in the absence of *G. pallida*-selective control measures. The population dynamics of *G. pallida* and *G. rostochiensis* in Ireland were studied in mixed- and single-species competition assays *in vivo*. *G. pallida* proved to be the more successful species, with greater multiplication in mixed- than single-species populations, with *G. rostochiensis* showing the opposite. This effect was similarly observed in staggered inoculation trials and population proportion trials. It was hypothesised that the greater *G. pallida* competitiveness could be attributed to its later hatch. *G. pallida* exhibited a later peak in hatching activity and more prolonged hatch, relative to *G. rostochiensis*. *G. rostochiensis* hatch was significantly reduced in mixed-species hatching assays. *G. pallida* hatch was significantly higher when hatch was induced in potato root leachates containing *G. rostochiensis*-specific compounds, indicating that *G. pallida* hatch is stimulated upon perception of *G. rostochiensis*–derived compounds. Rhizotron studies revealed that root damage, caused by feeding of the early-hatching *G. rostochiensis*, resulted in increased lateral root proliferation and significantly increased *G. pallida* multiplication. Split-root trials indicated a significant *G. pallida*-induced ISR effect. *G. rostochiensis* multiplication was significantly reduced in split-root rhizotrons when *G. pallida* colonised roots before or after *G. rostochiensis* infection.

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Abbreviations

a.s.	Active substance
AFBI	Agri-Food and Biosciences Institute
AHDB	Agriculture and Horticulture Development Board
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
avr	avirulence
BCA	Biological control agent
CN	Cyst nematode
cPCR	Conventional PCR
C_t	Critical threshold
d	Days
DAFM	Department of Agriculture, Food and Marine
DAMPs	Damage-associated molecular patterns
DEFRA	Department for Environment, Food & Rural Affairs
DD	Degree days
DDC	Accumulated day degrees
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dpi	Days post infection/inoculation
dsDNA	Double stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunological assay
EPPO	European Plant Protection Organization
ET	Economic threshold
ET	Ethylene
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
FAO	Food and Agriculture Organization
FLN	Free living nematode
FRET	Fluorescent resonance energy transfer
FVO	Food and Veterinary Office of the European Commission
GM	Genetically modified
GMNR	Genetically modified nematode-resistant

Gp	*G. pallida*
Gr	*G. rostochiensis*
GR&R	Gauge repeatability and reproducibility
GUS	β-glucuronidase
h	Hours
HF	Hatching factors
HFEF	High frequency electric field
HI	Hatch inhibitor
HR	Hypersensitive response
HRGP	Hydroxyproline rich glycoproteins
HS	Hatch stimulants
HTS	High-throughput screening
IFA	Irish Farmers Association
IPM	Integrated pest management
ISR	Induced systemic resistance
ITS	Internal transcribed spacer
J1	First-stage juvenile nematode(s)
J2	Second-stage juvenile nematode(s)
J3	Third-stage juvenile nematode(s)
J4	Fourth-stage juvenile nematode(s)
JA	Jasmonic acid
LNA	Locked nucleic acid
LPS	Lipopolysaccharides
LRR	Leucine rich repeats
LSU	Large subunit
MAMPs	Microbe associated molecular patterns
MAPK	Mitogen-activated protein kinases
MAS	Marker assisted selection
MGB	Minor groove binder
mRNA	Messenger RNA
N	Population size
NB-LRR	Nucleotide-binding site, Leucine-rich repeat
PAMPs	Pathogen associated molecular patterns
PCN	Potato cyst nematode(s)
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction-restriction fragment length

P_f	Final population
P_f/P_i	Multiplication (final population/initial population)
PGPR	Plant growth promoting rhizobacteria
P_i	Initial population
PI	Proteinase inhibitor
PPN	Plant parasitic nematode
PR	Pathogenesis-related protein
PRR	Pathogen- or pattern-recognition receptors
PTI	PAMP triggered immunity
qPCR	Quantitative PCR
QTL	Quantitative trait loci
R	Resistance gene
R	Reproductive rate (final population/initial population)
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RKN	Root-knot nematode
RM ANOVA	Repeated measures ANOVA
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RPI	Relative population increase
rpm	Rotations per minute
rRNA	Ribosomal RNA
s	Seconds
SA	Salicylic acid
SAR	Systemic acquired resistance
SASA	Science and Advice for Scottish Agriculture
SDS	Sequence detection system
siRNA	Short interference RNA
ssDNA	Single stranded DNA
SSU	Small subunit
TBE	Tris Borate EDTA
TF	Transcription factors
TRV	Tobacco rattle virus

Research objectives

- To evaluate the efficacy and reproducibility of molecular PCN diagnostic techniques.
- To investigate the extent of PCN infestation in Ireland, particularly the incidence of *G. pallida*.
- To evaluate the nature of interspecific competition between *G. pallida* and *G. rostochiensis*.
- To assess the effect of inoculation timing on PCN multiplication in single- and mixed-species populations.
- To determine whether *G. pallida* competitiveness was associated with its delayed hatch.
- To evaluate the effect of infestation density on PCN multiplication in single- and mixed-species populations.
- To elucidate differences in PCN hatching mechanisms in relation to different biotic and abiotic parameters.
- To investigate whether ISR was elicited upon PCN infestation and to measure the impact of systemic resistance on nematode multiplication in split root studies.
- To analyse PCN-induced changes in root architecture using rhizotrons and split-root rhizotrons to perform biometric root analyses.
- To assess differential species responses to ISR and changes in root architecture.
- To evaluate the effect of PCN-induced root morphogenesis on multiplication rate.

Chapter 1 Literature review

1.1 Introduction

Nematodes represent one of the most abundant and diverse phyla in the animal kingdom, comprising nearly 20,000 species that exist in an extensive range of habitats. Plant parasitic nematodes (PPN) are important agronomic pests that reduce global crop yields by 10% annually at an estimated cost of more than $100 billion (Chitwood, 2003; Atkinson *et al.*, 2004; McCarter, 2009). Potato cyst nematodes (PCN) are the most economically important pest of potato (*Solanum tuberosum tuberosum* L.), one of the world's most widely cultivated food crops (FAO, 2008). As obligate sedentary endoparasites, they sustain a prolonged biotrophic relationship with the host and inflict extensive root tissue damage during invasion (Jones & Perry, 2004). The lack of adequate control measures for such specialist pests leads to considerable agronomic losses; similarly, conventional control measures add significantly to the cost of potato production. The incidence of PCN is gradually reaching epidemic proportions in intensive potato-growing regions (Minnis *et al.*, 2002). As a result, there is an unprecedented need to control PCN infestation. Thus, further understanding of the nature of PCN populations is essential to afford future sustainability of the potato crop.

1.2 PCN species

PCN was first detected near Kühn in Germany in 1881 and was initially identified as a new species of *Heterodera schachtii* Schmidt (Schmidt, 1871). Due to differences from *H. schachtii* in cyst morphology and juvenile size, PCN was recognised as a unique species and subsequently named *H. rostochiensis* Wollenweber (Wollenweber, 1923); a second species, *H. pallida* Stone (Stone, 1973) was later identified. Subtle morphometric discrepancies among species within *Heterodera* spp., particularly the globular morphology of PCN cysts, led to the designation of a new genus, *Globodera*, which includes most endoparasitic cyst nematodes of solanaceous crops (Table 1.1; Skarbilovich, 1959). The sibling species *Globodera rostochiensis* (Wollenweber) Beherens (Wollenweber, 1923; Behrens, 1975) and *Globodera pallida* (Stone) Behrens (Stone, 1973; Behrens, 1975) are now recognised as among the most sophisticated and economically important potato pests. An

atypical *Globodera* nematode population was discovered in Idaho in 2008 (Skantar *et al.*, 2011). Subsequent characterisation of the nematode identified a new species of PCN, *Globodera ellingtonae* Handoo in 2012 (Handoo *et al.*, 2012; Zasada *et al.*, 2013).

Table 1.1 Classification and taxonomy of PCN (Turner *et al.*, 2006; Chronis *et al.*, 2014).

Phylum	Nematoda
Class	Chromadorea
Sub-class	Chromadoria
Order	Rhabditida
Sub-order	Tylenchina
Infra-order	Tylenomorpha
Super family	Tylenchoidea
Family	Hoploaimidae
Subfamily	Heteroderinae
Genus	*Globodera*
Species	*Globodera rostochiensis*
	Globodera pallida
	Globodera ellingtonae

1.2.1 PCN Pathotypes

Pathotypes are sub-groups of PCN that exhibit differential virulence to host cultivars, carrying PCN resistance genes and are classified according to their ability to multiply on resistant cultivars. Various different pathotypes of PCN may exist in pest populations, contributing to difficulties in PCN identification and management. As a result, classification schemes have been developed to distinguish and characterise PCN pathotypes. The International Pathotype Scheme, introduced by Kort *et al.* (1977), is the most internationally recognised classification scheme, which identifies five *G. rostochiensis* pathotypes (Ro1, Ro2, Ro3, Ro4, and Ro5) and three *G. pallida* pathotypes (Pa1, Pa2 and Pa3). The Latin American Scheme (Canto Saenz & De Scurrah, 1977) identifies four *G. rostochiensis* pathotypes (R_1A, R_1B, R_2A, R_3A) and six *G. pallida* pathotypes (P_1A, P_1B, P_2A, P_3A, P_4A, and P_5A; Turner and Rowe, 2013). In the European pathotype scheme, *G. rostochiensis* Ro1/4, Ro2, Ro3 and Ro5 are differentiated by the qualitative *H1* resistance

gene (*R*), as the *H2* locus distinguishes *G. pallida* pathotypes Pa1 from Pa2/3 (Hockland *et al.*, 2012). *G. rostochiensis* Ro2, Ro3, and Ro5, and *G. pallida* Pa2 and Pa3 are differentiated based on quantitative resistance from a number of resistance genes (Hockland *et al.*, 2012).

The classification of pathotypes is rather inconsistent and certain pathotype designations are ambiguous. Furthermore, the current classification schemes are not entirely representative of the extensive genetic diversity evidenced in introduced European PCN populations and the endemic South American populations (Grenier *et al.*, 2001). Environmental factors and increasing population heterogeneity contribute significantly to variation within the pathotype groups. As a result, modern pathotyping now recognises races and sub-pathotypes or virulence groups (Turner & Rowe, 2013).

Pathotypes are generally characterised using molecular analyses, namely immunological assays and polymerase chain reaction (PCR) techniques (§ 1.2.4.2), rather than by multiplication on resistant differential cultivars. Accurate identification of PCN pathotypes, facilitated by a definitive pathotype scheme, is necessary to provide practical information regarding PCN virulence characteristics, which is an essential determinant of the most appropriate control option (Manduric *et al.*, 2003).

1.2.2 PCN detection and identification

PCN are soil-borne pests which exist in the form of cysts and vermiform second-stage juveniles (J2) or as cysts/females attached to infected potato root systems (§ 1.2.3). Cysts are approximately 500 μm in diameter and assume a globose, spheroid shape (Turner & Rowe, 2013). PCN populations multiply in consecutive years and it can often take several years after the initial introduction for PCN to establish a sufficient, detectable population size to inflict visible crop injury and yield losses (Trudgill *et al.*, 2003). PCN infestation is often difficult to diagnose in the field. Plant injury is mainly restricted to the root system and concurrent non-specific haulm symptoms are noticeable during the later stages of plant development.

Early detection and regular inspection is imperative to manage PCN infestation levels and thereby, to minimize the need for subsequent pest control measures and incurred production costs. PCN are mainly introduced to new fields as cysts present in contaminated soil; infestations are randomly distributed and occur in patches. Regular PCN detection surveys, in-soil population modelling and accurate pest identification are necessary for adequate pest management (Turner, 1993). Furthermore *G. rostochiensis* and *G. pallida* are frequently present in mixed populations; therefore, precise species detection is often necessary (Minnis *et al.*, 2004).

1.2.3 PCN morphology

PCN exhibit sexual dimorphism with a sedentary female stage (which develops into a cyst) and a vermiform male and juvenile stage. Mature females are visible to the naked eye and can be seen as minute white or yellow spheres on the root surface. Cysts constitute the cutinised remains of the female nematodes and each cyst contains approximately 300-500 eggs; an individual egg encloses an infective second-stage juvenile (Jones *et al.*, 2003). Juveniles are approximately 500 µm in length, motile and annulated, and have a vermiform morphology with tapered anterior and posterior ends.

1.2.4 Species discrimination

Species identification is possible by observation of female chromogenesis at an appropriate stage of development. The cysts of *G. rostochiensis* females are initially white in colour when they develop on potato roots and subsequently transform through a temporary yellow colour phase before turning brown upon cyst maturity (§ 1.3.3). *G. pallida* females have a prolonged white developmental cyst stage with no intermediate yellow stage before turning brown. As a result, synonyms for *G. rostochiensis* and *G. pallida* include golden PCN and white (pallid) PCN, respectively. Both adult and juvenile PCN life stages are usually present in infested soil samples (Ebrahimi *et al.*, 2014); consequently, both cyst and juvenile morphological characteristics serve as valid species indicators in PCN identification.

1.2.4.1 Morphological characteristics

Although the sibling species appear similar, subtle morphological and morphometric differences between *G. rostochiensis* and *G. pallida* are evident. Specialist microscopic examination of J2, developing females and PCN cysts can be employed for precise species identification. PCN cysts exhibit a zigzag pattern of ridges on the cuticular surface and a distinct d-layer is apparent (Zunke & Eisenback, 1998). Cuticular and perineal area characteristics also facilitate species identification (Table 1.2.). PCN cysts may be distinguished by inspection of the perineal area, which comprises the vulval basin and anus, to determine Granek's ratio (the distance from the anus to the edge of the vulval basin, divided by vulval basin diameter) and the number of cuticular ridges between the vulva and anus (EPPO, 2004). PCN may be distinguished by morphometric measurements of the male and second-stage juvenile characteristics, such as, body length, true tail length and hyaline tail length, stylet length and shape (Manduric *et al.*, 2003).

Table 1.2 Range (mean) values of measurements of *Globodera rostochiensis* and *G. pallida*, used in morphological identification and species differentiation (Baldwin, 1992; EPPO, 2004).

Cyst		
	Number of cuticular ridges between anus and vulval basin	**Granek's ratio**
G. rostochiensis	16–31 (> 14)	1.3–9.5 (> 3)
G. pallida	8–20 (< 14)	1.2–3.5 (< 3)
J2 Stylet		
	Shape of anterior surface of knob	**Length (µm)**
G. rostochiensis	rounded	19–23 (21.8)
G. pallida	pointed	22–24 (23.8)

However, the identification and quantification of PCN based on cyst and juvenile morphology and morphometric characteristics is often technically difficult, laborious, inaccurate and time consuming (Stone, 1985; Trudgill, 1985). Biochemical and molecular diagnostic techniques are more efficient and are more generally employed in species discrimination.

1.2.4.2 Biochemical and molecular diagnostics

Various biochemical diagnostic techniques have been used in species identification and pathotype characterisation. Biochemical protein analyses, such as isozyme analysis using 2D-gel electrophoresis, isoelectric focusing (IEF) and immunological methods, namely ELISA, have been successful in PCN species identification (Fullaondo *et al.*, 1999; Turner *et al.*, 2006). DNA-based diagnostic methods have revolutionised species discrimination and pathotyping, providing a rapid and accurate means of species identification, particularly in mixed-species populations. PCR technology, using species-specific primers determined from sequence differences in the internal transcribed spacer (ITS) region of the rRNA gene, provides a precise and sensitive diagnostic technique that can accurately detect and distinguish between *G. pallida* and *G. rostochiensis* (Bulman & Marshall, 1997; Reid *et al.*, 2015).

PCR is an efficient method for PCN population quantification; multiplex-PCR is employed in the determination of species composition and population densities (Fleming *et al.*, 1998). PCR-RFLP (restriction fragment length polymorphisms) and similar sequencing methods also facilitate species and pathotype discrimination by analysing inter- and intraspecific variation in the ITS region (Szalanski *et al.*, 1997; Powers, 2004). Similarly, analyses of the ITS-rRNA and RAPD techniques using repetitive DNA probes are also effective taxonomic tools. Real-time quantitative PCR (qPCR) diagnostic tests facilitate rapid, high-throughput screening (HTS) and speciation of nematodes (Chapter 2; Kenyon *et al.*, 2010; Reid *et al.*, 2015). Furthermore, the genome of *G. pallida* has been recently sequenced (Cotton *et al.*, 2014); such advancements in PCN genomics may allow more feasible diagnostic testing in the future.

1.2.5 Host range

PCN are obligate specialist plant parasites; hosts are restricted to members of the Solanaceae family. *Solanum tuberosum tuberosum* is the primary host; however, other solanaceous crops, including aubergine (*Solanum melongena* L.) and tomato (*Solanum*

lycopersicum L.) are also suitable hosts. The host range extends to solanaceous weeds such as *Solanum dulcamara* L.*, Solanum capsicastrum* L. and *Atropa belladonna* L. (Evans & Rowe, 1998). PCN populations can build-up in the soil if suitable weed hosts are present to facilitate parasitism and PCN multiplication (Baldwin & Mundo-Ocampo, 1991).

1.2.6 Origin

G. rostochiensis and *G. pallida* are indigenous to the Andean Cordillera; it is generally accepted that PCN originated from the Peruvian highlands in co-evolution with its unique host genus *Solanum* (Canto Saenz & De Scurrah, 1977; Stone, 1985; Plantard *et al.*, 2008). Potatoes were first introduced to Europe from South America and were extensively distributed and domesticated by early 1600. PCN dispersal coincided with the spread of the potato crop; PCN was passively introduced into Europe from South America in the mid-19th Century via cysts adhering to potato tubers or dispersed in PCN-contaminated soil and guano fertilizer bags which had previously held potato tubers. PCN was first recorded on mainland Europe in 1881 near Kühn in Germany and later discovered in England in 1917 and Ireland in 1922 (Carroll, 1933).

The initial PCN population build-up was relatively slow, considering the limitations of international trade and the primitive agricultural methods employed during this era. The Irish Famine (*c.* 1845) caused by late blight (*Phytophthora infestans*) infection of the potato crop provided the impetus for an intensive potato breeding programme. Consequently, potato seed importation from South America accelerated as different varieties were sought in an attempt to breed disease resistant potato cultivars. Increased potato consumption, subsequent intensified potato cultivation and the distribution of seed tubers *circa* 1910 for the commercialisation of the potato crop inevitably contributed to the establishment and distribution of PCN in Europe.

1.2.7 Distribution

PCN is ubiquitous in South America, where it is endemic, and is prevalent in most of the major potato-growing areas of the world (Fig 1.1; Appendix I-II) including, Europe, Central, South, and North America; and the Pacific basin (Turner & Evans, 1998). PCN is mainly found in temperate regions, but it also occurs in the coastal and upland areas of the tropics. Various pathotypes and races of the two PCN species co-exist within these regions (Jatala *et al.*, 1979). Limited introductions of *G. rostochiensis and G. pallida* have occurred in North America (Hafez *et al.*, 2007) and Australia (Hodda & Cook, 2009); in many instances, the detection of recently introduced PCN has led to intensive decontamination regimes where PCN has been successfully eradicated and regions have been declared free of PCN infestation.

In most regions *G. rostochiensis* and *G. pallida* coexist; globally, *G. rostochiensis* is the more widely distributed species and is generally the predominant species in endemic and introduced PCN populations (Canto Saenz & De Scurrah, 1977). The relative distribution of species and pathotypes is generally a reflection of the original PCN introduction (Zaheer *et al.*, 1993). However, a recent transition in species composition has been recorded in Europe where *G. pallida* is now emerging as the more prevalent species (Minnis *et al.*, 2002; Trudgill *et al.*, 2003; FVO, 2013).

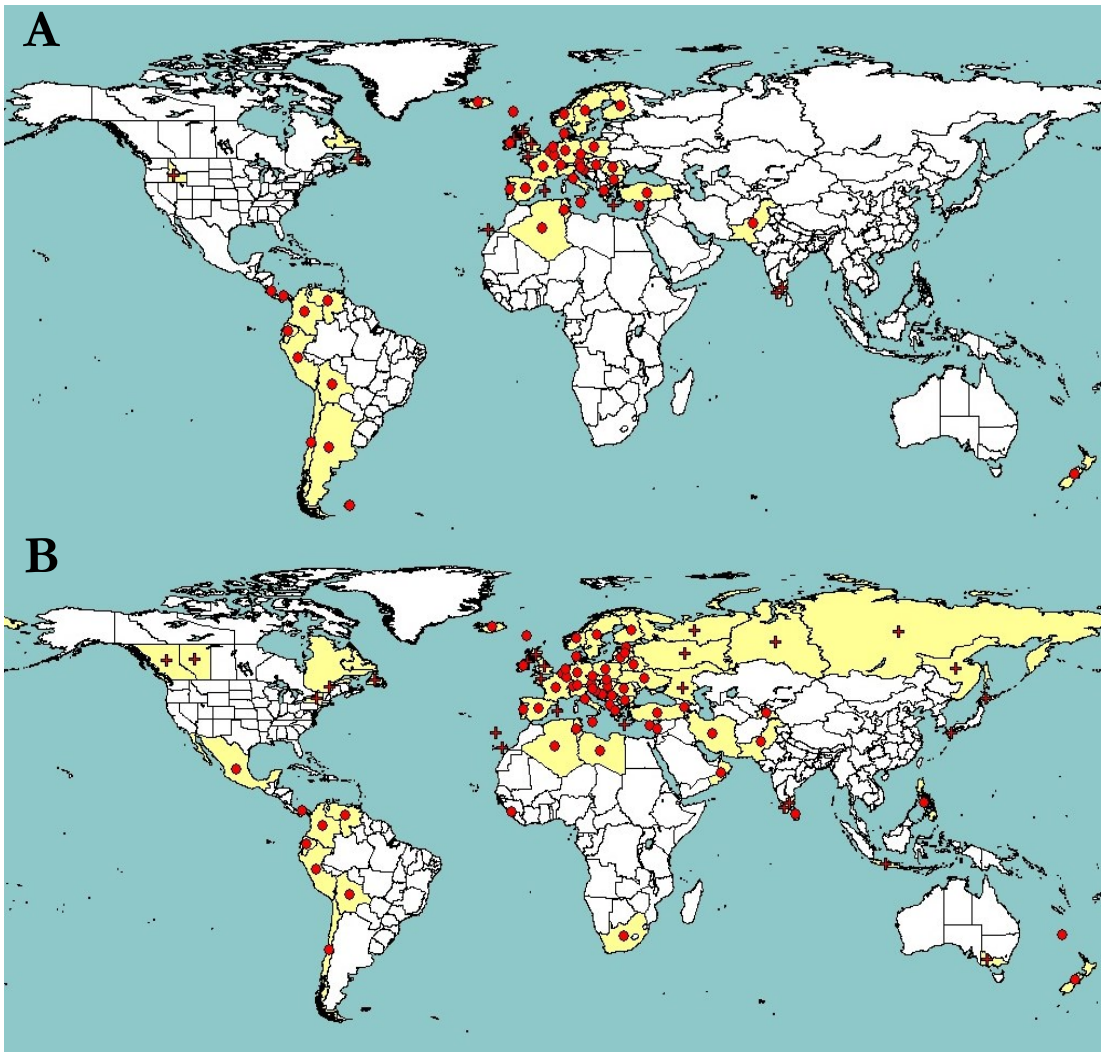

Figure 1.1 Distribution of (a) *G. pallida* and (b) *G. rostochiensis* on national (●) record (+) subnational records (EPPO, 2014).

1.2.8 Dispersal

As a soil-borne pest, PCN is mainly introduced to a new environment as cysts present in contaminated soil. PCN dissemination is facilitated by the minute size of the cyst (approx. 500 μm in diameter) and the resistant nature of PCN cysts. The cyst is a durable structure composed of collagen, which provides tensile strength, and affords protection to the enclosed eggs from adverse environmental conditions. Encysted eggs are dormant and may persist in the environment for up to 30 years (Winslow & Willis, 1972; Perry, 1989). Motile J2 actively disperse in the soil when locating a suitable host; similarly, males migrate when searching for a mate. PCN migrate in soil via a film of water; the extent of in-soil motility is less than 1 m (EPPO, 2004).

PCN populations are highly aggregated and randomly distributed within fields. Infestation densities are greatest at the infestation focus; successive infestations emanate from the source of the infestation focus and naturally assume an elliptical distribution pattern due to migrating juveniles and passive cyst dispersal. The direction of the elliptical distribution is generally dependent on the direction of cultivation within the field (Been & Schomaker, 2000). The introduction of a few cysts to a field can lead to progressive increases in PCN infestation densities if a suitable host is grown in successive years (Trudgill *et al.*, 2003). Agricultural practices are the main cause of localized cyst dispersal; land cultivation facilitates the growth of the primary infestation focus and the establishment of secondary foci in infested fields. PCN cysts are easily dislodged from host plant roots at harvest, and are passively dispersed by natural means via biological vectors and environmental factors including wind and water. Cysts are generally transported via soil movement and extensively distributed by anthropogenic means, particularly via contaminated soil attached to agricultural machinery and equipment, footwear, seed tubers and plant roots (EPPO, 2013).

The exportation and importation of infected seed tubers has significantly contributed to the widespread dispersal of PCN cysts. Consequently, PCN has spread internationally into the major potato-producing regions of the world via the potato trade. Because of its infectivity, persistence, wide dispersal and economic consequences, PCN is designated as an EPPO A1 quarantine pest and is subject to stringent legislative governing its dispersal and prevention of new infestations in uncontaminated areas (§ 1.7.1; Council Directive 2007/33/EC, Article 15).

1.3 PCN life-cycle

PCN is an obligate specialist parasite with a narrow host range (§ 1.2.5); in order to overcome such limitations and maximise infectivity and reproductive success, PCN has evolved a synchronised life cycle and a sophisticated biotrophic relationship with its host. Pest-host synchrony is achieved by dormancy of the invasive parasite stage; J2 remain

dormant until stimulated to hatch by host-specific hatching chemicals (Perry, 1989). Encysted eggs are protected from environmental extremes to enhance pest persistence in the environment; as a result, PCN eggs can remain viable in the soil for several decades but hatch rapidly in response to host stimuli (Blair *et al.*, 1999).

1.3.1 Host plant detection

Host detection is largely mediated by host root leachates that are synthesised at the root cap (§ 1.4.3; Perry, 1986). Potato root leachates (PRL) directly affect PCN eggs by altering eggshell permeability resulting in hatch (§ 1.4). When stimulated to hatch under favourable environmental conditions, J2 emerge from the cysts and locate the host via anterior amphids, the principal chemosensory receptors that are highly sensitive to variable PRL concentrations (Jones & Perry, 2004). J2 migrate chemotactically through the soil in response to PRL concentration gradients; the rate of mobility and the distance travelled is dependent on the content of hatching chemicals in PRL, which varies with host age and genotype, and edaphic factors (Devine & Jones, 2003). The concentration of carbon dioxide in the soil is also proposed to influence nematode orientation by guiding the juveniles to an appropriate root depth (Dusenbery, 1987).

1.3.2 Host plant infection

After locating a host root, second-stage juveniles penetrate the root epidermis and invade the root behind the root tip in the zone of elongation and at points of lateral root emergence (Abad *et al.*, 2003). The J2 migrate intracellularly through the roots, using the stylet, a hollow, protractible piercing and feeding structure, to perforate and cut through successive cells. J2 inject digestive secretions, containing enzymes such as cellulases (§ 1.3.2.1), via the stylet to degrade structural cell components and to facilitate root penetration and migration (Hussey & Grundler, 1998). Migration within the root is orientated by an electrical potential, particularly the lower redox potential created at the root's surface (Perry, 1996).

After invasion, J2 migrate through the cortical cells in the direction of the vascular cylinder (Bohlmann & Sobczak, 2014). J2 recognise differential root tissues and cell surfaces to aid orientation to the vascular bundle, where a permanent feeding site is established (Perry, 1996; Gheysen & Jones, 2013). Plant cellular signalling is inferred in juvenile orientation through the roots and the selection of a feeding cell (Perry, 1996). J2 settle in the inner cortex opposite a phloem cell, where they become sedentary and induce host cellular modifications in the parasitized cell (Goverse & Bird, 2011). This results in a specialized feeding/transfer cell structure known as a syncytium (Gheysen & Fenoll, 2002; Gheysen & Jones, 2013; Gardner *et al.*, 2015)

1.3.2.1 Parasitism by PCN

Parasitic secretions elicit a series of complex biochemical changes in host cell physiology, morphology, function and gene expression, culminating in syncytium induction (Williamson & Hussey, 1996; Haegeman *et al.*, 2012; Gardner *et al.*, 2015). After syncytium formation, juvenile development occurs entirely within the roots of the host plant; PCN locomotory musculature degenerates as the J2 become sedentary biotrophic parasites (Prior *et al.*, 2001). PCN parasitism secretions are produced in the oesophageal glands (Fig. 1.2), which comprise one dorsal gland and two subventral glands (Davis *et al.*, 2000). The subventral glands are highly transcriptionally active, especially with regard to genes encoding cell wall-modifying proteins, including cellulolytic enzymes, that upregulate pectate lyase, pectinase and cellulase biosynthesis (Popeijus *et al.*, 2000; Jones *et al.*, 2003).

Proteins derived from the subventral glands play a crucial role in nematode parasitism and are most active during the early stages of the J2 life cycle, particularly during host invasion, migration and feeding site induction (Jones *et al.*, 2004). The dorsal gland is a single cell that is relatively active and grows progressively throughout the PCN life cycle, particularly in the later stages of parasitism, and during syncytium development and maintenance (Jones & Perry, 2004).

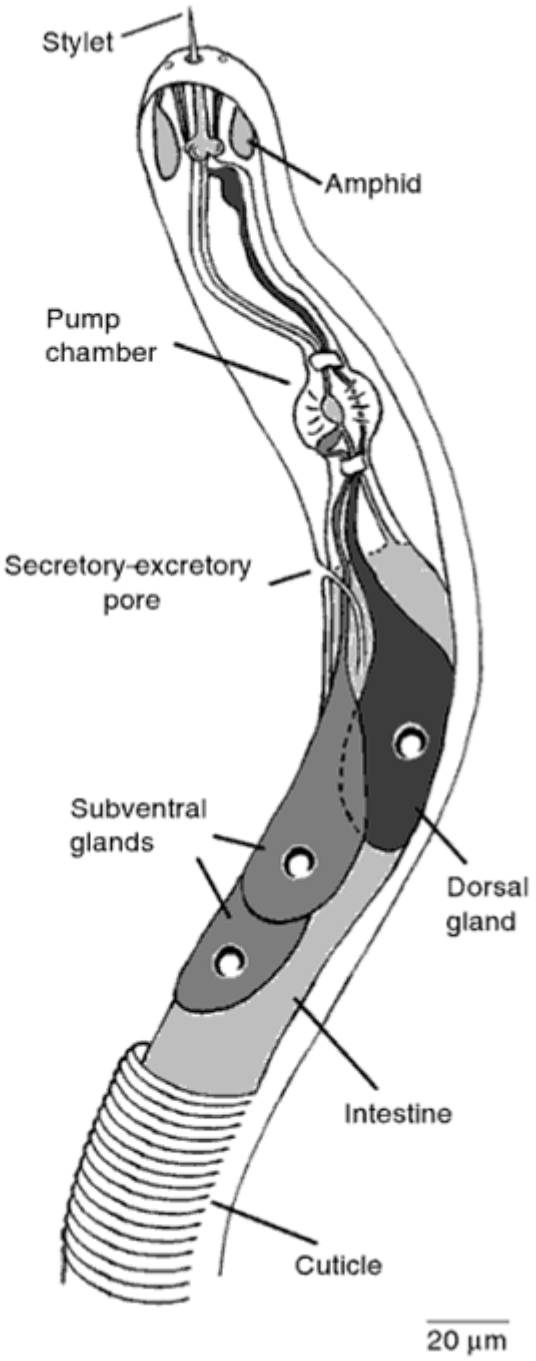

Figure 1.2 Diagram of the anterior anatomy of a second stage juvenile (Gheysen & Jones, 2013)

PCN secretions are likely to be recognized by the host as avirulence factors (Chen *et al.*, 2015; Mei *et al.*, 2015), which may result in the elicitation of host defences in resistant plants (§ 5.1.1). As endoparasites, juveniles must contest an array of physical and chemical host defences; consequently, PCN secretions deliver effector molecules to evade recognition by the host plant and to down-regulate host defences (Smant *et al.*, 1998; Prior *et al.*, 2001; Mitchum *et al.*, 2013; Thorpe *et al.*, 2014; Hewezi, 2015).

1.3.2.2 Feeding Mechanisms

J2 produce tiny perforations in the cell wall of the designated feeding cell using the stylet. Nutrients are withdrawn from the cytosol via a perforation created at the interface of the plasma membrane and the stylet (Williamson & Hussey, 1996). During the feeding process, PCN stimulate complex changes in host cell physiology, metabolism and gene expression that ultimately transform parasitized root cells into elaborate syncytial feeding cells (Davis *et al.*, 2008).

1.3.2.3 Syncytia

Syncytia are large, multinucleate transfer cells (Fig. 1.3) that serve as a permanent source of nutrients during the sedentary, biotrophic stage of parasitism (Williamson & Hussey, 1996; Duncan *et al.*, 1997). After stylet perforation of the cell wall, dorsal gland secretions instigate nuclear enlargement and manipulate host cell signalling to divert cell contents towards the nucleus to initiate feeding cell induction (Gheysen & Fenoll, 2002). Syncytium formation involves structural cellular transformations, including cell wall breakdown by gradual plasmodesmata extension to neighbouring cells and fusion of the protoplasts of the initial syncytial cell and neighbouring cells (Duncan *et al.*, 1997; Jones & Perry, 2004).

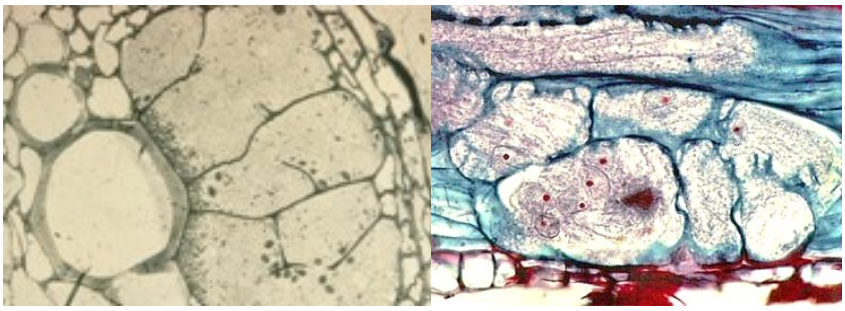

Figure 1.3 The multinucleate syncytia structure in root tissue (D'Arcy *et al.*, 2001).

Progressive cell wall dissolution contributes to syncytium expansion in the direction of the inner vascular bundle, particularly along the stele and xylem vessels (Goverse *et al.*, 2000b). Cells adjacent to the initial syncytial cell, namely phloem parenchyma and endodermal cells, eventually coalesce to form a multinucleate syncytium. Cell nuclei become enlarged and the cytoplasm condenses and becomes rich in cell organelles due to

fusion with adjacent cells. The syncytium cell wall protrudes towards the vascular bundle, and cell membranes are extensively invaginated to increase cell surface area for efficient nutrient absorption (Jones & Perry, 2004). The cell walls are condensed to withstand the increasing internal osmotic pressure which can often reach levels of 9,000 to 10,000 hPa (Jones & Northcote, 1972). The central vacuoles of the converging cells are replaced by several smaller secondary vacuoles; the resultant hypertrophic multinuclear syncytium can contain up to 250 cells (Hussey & Grundler, 1998).

1.3.2.4 Genes expressed in syncytia

Syncytium induction and maintenance is dependent on continuous stimulation and PCN-induced molecular signalling involving complex changes in host gene expression to modulate cell physiology and metabolism (§ 1.3.2.3; Williamson & Gleason, 2003). Specific compounds and transcription factors (TF) in juvenile secretions interact with plant cell receptors and elicit signal transduction cascades to modulate host gene expression and phytohormone synthesis (Williamson & Hussey, 1996; Akhkha *et al.*, 2004). Syncytium induction and maintenance is largely mediated by auxin, which has a prominent role in cell cycling (Goverse *et al.*, 2000b). Host transcription is altered to upregulate genes involved in cell metabolism, cell cycling, water transport and osmoregulation (Williamson & Gleason, 2003). Similarly, genes involved in host defences are suppressed to optimize parasitism and to facilitate syncytium formation (Smant & Jones, 2011).

PCN initiate host cell cycle promoters to induce shorter, more frequent cell cycles and thereby enhance DNA amplification via continuous endoreduplication to maintain a metabolically active syncytium (Goverse *et al.*, 2000a; Williamson & Gleason, 2003). Extensive changes in cell wall architecture occur during syncytium development, which is facilitated by the upregulation of host genes encoding cell wall-degrading enzymes i.e. endoglucanase and polygalacturonase (Williamson & Gleason, 2003). Changes in gene expression are also evident at the phenotypic level; the proliferation of lateral roots has

been attributed to syncytia establishment within the vicinity of pericycle cells and concurrent elevations in auxin levels (Goverse *et al.*, 2000b; Gardner *et al.*, 2015; Hewezi, 2015). Host stress responses to syncytial activity include upregulation of effectors involved in cell osmoregulation. *Lea* (late-embryogenesis abundant) genes and ARSK promoters, which serve as osmoprotectants by eliciting osmotic stress signals in response to elevated osmotic pressure, are particularly abundant in syncytia (Lilley *et al.*, 2004).

1.3.3 Development and reproduction

PCN maintain a metabolically highly active state for several weeks, feeding continuously via syncytia and progressing through a series of moults. J2 develop into third- (J3) and fourth-stage juveniles (J4) and feed until development to a sexually mature adult is complete (Jones & Perry, 2004). Adult PCN exhibit sexual dimorphism; males are motile and vermiform, while the sedentary females remain attached to the host roots where they develop a spheroid cyst morphology. Gender is determined by the third moult; male frequency increases under conditions of intraspecific competition and poor nutrition (Williamson & Hussey, 1996).

J3 males initially swell and pass through a sedentary developmental stage and eventually revert to the vermiform state. J4 males elongate to approximately 1 mm within the cuticle retained from the third-juvenile stage and regain motility before leaving the root (Jones & Perry, 2004). Adult males leave the roots to mate and die shortly thereafter. Sedentary J4 females feed continuously and embryogenesis occurs after fertilization. As females enlarge and increase in reproductive capacity, the body cavity is almost entirely occupied by the reproductive system and developing eggs. Under optimal conditions, females can produce up to 600 eggs (Whitehead, 1992). Females swell progressively and eventually rupture the outer root tissue; however, they remain attached to the root system and continue to extract nutrients via the syncytium until they die (Fig. 1.4).

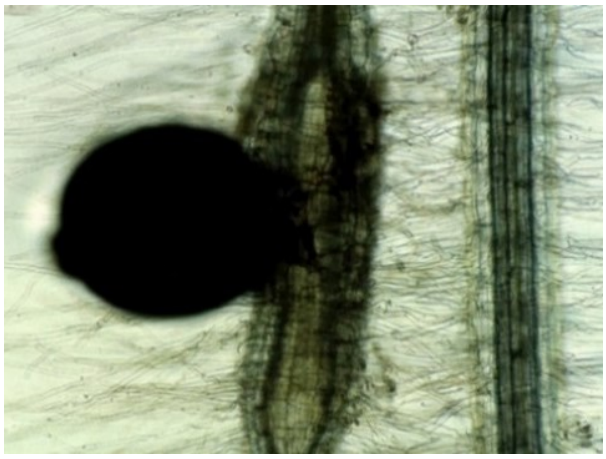

Figure 1.4 *G. rostochiensis* female emerging from a host root, attached to a syncytium (D'Arcy *et al.*, 2001).

Reproductive success is reflected by cyst size, which can range from between 300 μm to 600 μm. PCN reproduce sexually; females emit sex pheromones to attract males. The response to sex pheromones is generally species-specific; however, *G. pallida* exhibits interspecific responsiveness to pheromones from both PCN species relative to *G. rostochiensis,* which only responds to *G. rostochiensis* pheromones (Riga *et al.*, 1997). After fertilization, embryonic development occurs; each egg encloses a J1, which undergoes one moult within the egg to form the infective J2, which remain dormant until hatching (Cook & Noel, 2002).

As the female develops, cyst chromogenesis occurs. Initially *G. rostochiensis* females exhibit a white cyst phase lasting approximately four to six weeks before turning golden yellow and then brown upon maturity. *G. pallida* females produce white cysts before turning brown upon maturity, unlike *G. rostochiensis,* there is no intermediate yellow cyst stage. Mature females die shortly after fertilization as the internal organs degenerate during embryogenesis. The cuticular remains of the female nematode undergo polyphenol oxidase-mediated tanning to form the mature brown cyst. The resultant protective cyst encloses approximately 300 to 600 unhatched eggs (Cook & Noel, 2002).

1.3.4 Intrinsic developmental and physiological differences between species

G. rostochiensis and *G. pallida* exhibit distinct physiological differences throughout their life cycle. The two species have different rates of embryogenesis, utilisation of energy reserves and variable hatching responses (Robinson *et al.*, 1987). *G. pallida* has a faster rate of post-embryonic development; consequently the rate of *G. pallida* female development is faster than that of *G. rostochiensis* (Perry *et al.*, 2002). This also has survival implications as eggshell structure and permeability is reinforced in concert with embryonic development, which enhances structural integrity and resistance (Perry, 1997). Thus, the more rapid *G. pallida* embryonic development, concomitant eggshell fortification and faster rate of cyst development may enhance *G. pallida* persistence in the environment (Perry *et al.*, 2002).

G. pallida juveniles exhibit slower rate of lipid utilization in comparison to *G. rostochiensis*. The average half-life of lipid content in *G. pallida* and *G. rostochiensis* juveniles is 22 d and 15 d, respectively (Perry, 1996). Juvenile infectivity and persistence in the soil is correlated with the availability of neutral lipid reserves and the rate of lipid utilisation (Robinson *et al.*, 1987; Perry *et al.*, 2002). Reduced lipid reserves suggests a loss of infectivity in later juvenile stages, this has implications for *G. rostochiensis* juveniles as their reduced lipid reserves indicate a more urgent requirement to locate and infect a suitable host prior to depletion of lipid reserves. The larger lipid reserves in *G. pallida* juveniles confers a competitive advantage and enhances juvenile persistence in the soil by prolonging the invasion period, thereby increasing the probability of successful host invasion (Robinson *et al.*, 1987).

1.3.5 Dormancy

Pest-host synchrony is achieved through dormancy of the infective parasite stage and the longevity and resistant nature of the nematode resting structure (Perry, 1989). Dormancy is a state of arrested development and PCN exhibit two distinct states of dormancy, namely diapause and quiescence. The different states of dormancy optimise PCN-host synchrony, enhance pest persistence in the absence of a suitable host and maximise

infectivity in the presence of a host. PCN dormancy is highly adaptive and is intuitively terminated under certain suitable conditions to ensure successful host infection (Jones *et al.*, 1998).

1.3.5.1 Diapause

Diapause is a temporary prolonged state of arrested J2 development, that is initiated in response to predictable, cyclical events, particularly seasonal temperature fluctuations. Diapause in PCN comprises facultative and obligate dormancy. Obligate diapause is the initial stage of PCN dormancy, occurring only once in the nematode's life cycle, immediately after cyst formation (Jones *et al.*, 1998). Obligate diapause is neuro-hormonally-mediated, the event is initiated and regulated by endogenous factors (§ 1.4), and relieved by exogenous factors, i.e. host stimuli, after a prolonged period of time when certain environmental conditions are satisfied (Jones *et al.*, 1998). The duration of obligate diapause is often affected by environmental parameters and host physiology, i.e., temperature, photoperiod and host signalling perceived by the developing female (Hominick, 1986).

Facultative diapause succeeds obligate diapause and is initiated in the second and in successive growing seasons by external factors, specifically temperature and photoperiod, and may occur several times throughout the pest life cycle (Jones & Perry, 2004). Facultative diapause is terminated by exogenous factors, particularly in spring when certain environmental conditions, such as elevated soil temperatures and moisture, are conducive for hatch, and is immediately succeeded by quiescence (Devine & Jones, 2001a).

1.3.5.2 Quiescence

Quiescence is a temporary reversible state of suppressed metabolism, which is initiated by unpredictable adverse exogenous factors, namely, host absence, unfavourable temperature and moisture conditions or low nutrient levels (Jones *et al.*, 1998). Unlike

diapause, quiescent dormancy is terminated immediately when the environmental stress is alleviated. A variety of environmental conditions may incite quiescence. The most frequent form of quiescence is anhydrobiosis, which is stimulated by desiccation (Jones *et al.*, 1998). In extreme cases of prolonged quiescence, the metabolic rate may fall below detectable levels in a form of quiescence known as anabiosis. The termination of quiescence and subsequent hatching is contingent upon favourable environmental conditions and exposure to exogenous triggers such as host-specific hatching factors (§ 1.4.3) present in PRL (Jones *et al.*, 1998).

1.4 Hatching

Prior to hatching, dormant J2 are retained within encysted eggs and remain in a state of partial dehydration (67% water content), until stimulated to hatch by host stimuli under suitable conditions (Clarke *et al.*, 1978). The PCN eggshell consists of three structural layers; the external and internal vitelline lipid layers and a rigid chitinous middle layer that provides tensile strength (Jones *et al.*, 1998). Each egg encloses a single J2 suspended in perivitelline fluid, comprised of a 0.34 M trehalose solution (Perry, 1989). The high osmolality of the perivitelline fluid preserves the enclosed J2 in a semi-dehydrated state and imposes a high turgor pressure within the egg, which suppresses J2 metabolism and hatch (Clarke & Hennessy, 1984; Perry, 1986). The internal lipid layer is the primary permeability barrier and comprises two or three lipoprotein membranes. Oxygen is freely diffusible across the membranes, although water access is regulated to retain turgidity and J2 dehydration (Perry *et al.*, 2013).

1.4.1 Hatch stimulation

PCN hatch is initiated by host-specific hatch-stimulating chemicals known as hatching factors (HF), present in the potato root leachates (PRL). Exposure to PRL induces a series of physiological and metabolic changes and eventually culminates in J2 eclosion (Fig. 1.5; Perry, 1996). Host-specific chemicals stimulate ion exchange, resulting in Ca^{2+} displacement (Perry *et al.*, 2002). This leads to destabilization of the lipoprotein membrane

and consequently affects permeability, as the inner lipid layer transforms from a selectively permeable membrane to a fully permeable one (Perry, 1989).

The change in eggshell permeability is a necessary precursor to PCN hatch and occurs within 24 h of PRL stimulation (Perry & Beane, 1982). A fully permeable inner lipoprotein layer results in the release of trehalose from the perivitelline fluid, consequently reducing osmotic pressure in the eggshell and leading to an influx of water and subsequent rehydration and activation of the dormant J2 (Perry, 1989).

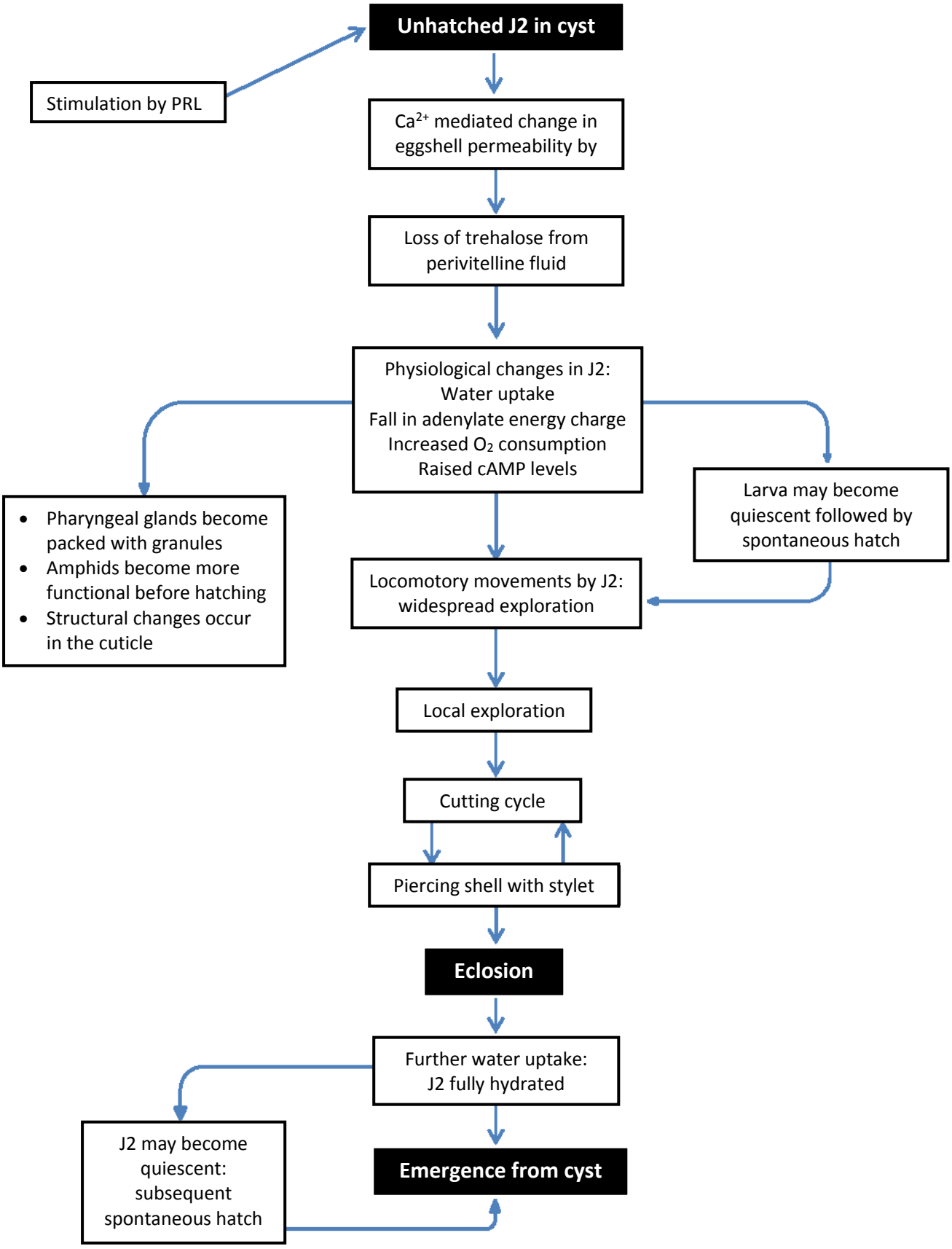

Figure 1.5 Hatching process of *G. rostochiensis* (modified from Jones *et al.*, 1998).

Once active, the J2 perforate the rigid eggshell, using the stylet to cut its way through the layers, emerge from the egg and exit the cyst via the head and vulval apertures. PCN enter the rhizosphere to search for a suitable host (§ 1.3.1). The majority of hatched juveniles emerge within two weeks of PRL stimulation; successful host detection and infection is generally attributed to rapid juvenile hatch (Perry, 1989).

1.4.2 Physiological response to PRL

PRL has two principal actions: (a) the physical alteration of eggshell permeability to initiate hatch, and (b) the stimulation of J2 metabolism. Exposure to PRL induces a series of physiological and behavioural responses in the unhatched J2, including changes in gene expression and alterations of the cuticle and amphidial ultrastructure, which stimulate J2 sensory perception (Jones *et al.*, 1998). After hatching, the metabolically active juveniles rapidly increase their consumption of lipids, water and oxygen, reduce their adenylate energy content and increase cAMP levels (Atkinson & Ballantyne, 1977). The dorsal and ventral glands respond to PRL-induced hatching and subsequent rehydration by stimulating nematode secretions, representing the transition of the J2 to the parasitic phase of the life cycle (Perry, 1989).

Hatched J2 have a limited infective period, surviving for less than two weeks in the absence of a host and must successfully locate and infect a suitable host before exhaustion of lipid reserves (Robinson *et al.,* 1987). As PCN have a narrow host range, they have evolved to hatch specifically in response to host stimuli to ensure large-scale, efficient hatch in the presence and proximity of a suitable host to maximise infection success (Perry, 1989). Optimum PRL production is confined to a short period in the host's life cycle; maximum PRL production typically occurs two weeks after planting (Perry, 2002).

J2 exhibit greater metabolic activity and increased mobility in the presence of PRL and exhibit positive chemokinesis and chemotaxis to host stimuli (Clarke & Hennessy, 1984; Blair *et al.*, 1999). PRL-induced hatch may sensitise J2 and upregulate sensory receptors

in juvenile chemosensory organs, namely the amphids, to refine the chemical perception to PRL gradients and thereby enhance J2 orientation and in-soil migration towards the host (Robinson *et al.*, 1987; Devine & Jones, 2003). Nematodes are also known to display chemotactic responses to CO_2 and thermotactic responses to metabolic heat produced by host roots and associated rhizobacteria (Prot, 1980; Dusenbery, 1987).

1.4.3 Hatching factors

PCN hatch is initiated by PRL, which contains multiple hatching factors (HF), namely the two glycoalkaloids, α-chaconine and α-solanine (Byrne *et al.*, 1998; Būda & Čepulytė-Rakauskienė, 2015), and numerous different terpenoids (Devine & Jones, 2000a). More than 10 structurally different HF, with similar chemical profiles and a molecular weight of 530.5 Da, have been identified using mass spectrometry (Byrne *et al.*, 2001; Devine & Jones, 2001b). HF have complex chemical structures and are difficult to synthesise. To date, total synthesis of the HF solanoeclepin-A has been achieved (Tanino *et al.*, 2011).

HF are either species-specific, species-selective or species non-selective (Chapter 4; Byrne *et al.,* 1998, 2001). Several classes of hatching chemicals exist, namely HF, hatch inhibitors (HI) and hatch stimulants (HS). Hatch inhibitors are abundant in early produced PRL and thereby inhibit hatch until later stages of root development. HS are more plentiful in PRL (§ 1.4.6) produced later in the host growth cycle (Byrne *et al.*, 2001).

The response of PCN eggs to HF is highly specific, certain HF may initiate hatch at low concentrations, less than 2 x 10^{-8} M *in vitro* (Devine & Jones, 2000b). Similarly, a limited exposure time is required to induce hatch, which has been observed after five minutes' exposure to PRL (Perry & Beane, 1982). Certain HF are active at low concentrations and concentration-dependent responses to PRL are critical in modulating the timing and rate of hatch (Devine *et al.*, 2001a). Hatching chemical profiles of PRL exhibit complex interactions between HF, HS and HI, with differential HF compositions among potato

varieties (Byrne *et al.*, 2001; Devine & Jones, 2001b). Consequently, *G. rostochiensis* and *G. pallida* exhibit different hatching responses to different cultivars (Devine & Jones, 2001b).

1.4.4 Hatching dynamics

PCN usually hatch over a 6- to 8-week period depending on the host variety, growing season, physiological host characteristics and environmental conditions (Trudgill *et al.*, 1996). Host-specific stimuli triggers large-scale PCN hatch to achieve mass infection under opportune conditions (Devine *et al.*, 1996). HF-mediated hatch can account for up to 90% of hatch; a fraction of J2, approximately 5% of the total, hatch spontaneously in the absence of a suitable host (Devine *et al.*, 1999).

In general, the percentage of hatch in a mixed-generation population rarely exceeds 80%, a certain proportion of the J2 remain dormant and hatch in subsequent years. The carry-over of unhatched viable eggs is density dependent and remains relatively constant (approximately 20%) from year to year (Jones and Perry, 1978; Turner, 1996). This adaptation enhances the genetic variation of populations in subsequent hatching years and ensures pest persistence by permitting a future source of inoculum.

There are distinct intrinsic hatching differences between both species (Chapter 4). *G. pallida* exhibits delayed hatching mechanism, a lower level of spontaneous hatch in the field and prolonged hatching period combined with a later annual peak in hatching activity compared with *G. rostochiensis* (Robinson *et al.*, 1987; Turner & Evans, 1998; Devine & Jones, 2001a). In field trials, the level of *G. rostochiensis* hatch in the absence of a host (i.e. spontaneous hatch) is significantly greater than that of *G. pallida,* particularly during the early part of the season. Later in the season, however, both PCN species exhibit a similar degree of hatch. The different hatching responses between *G. rostochiensis* and *G. pallida* (Chapter 4) can be attributed to differential optimum hatching temperatures, species-specific HF preferences, host physiology and environmental adaptations (Ryan & Devine, 2005).

1.4.5 Abiotic factors affecting hatch

As poikilothermic organisms, nematode behaviour and activity is greatly influenced by abiotic factors. Environmental parameters, namely temperature, pH, CO_2, organic matter, moisture and aeration have a significant effect on PCN hatch. Nematodes do not emerge from quiescence until environmental conditions are favourable (Ingham *et al.*, 2015). As such, environmental factors can have a major influence on PCN population dynamics, interspecific competition and species selection.

The rate and extent of viable PCN hatch is correlated with the availability, mobility and concentration of HF in the soil (Devine & Jones, 2001b). Thus, soil type and edaphic physicochemical properties may affect hatch. Coarse-textured soils, particularly those at soil field capacity, favour PCN multiplication. A low soil cation exchange capacity and optimum moisture facilitates J2 migration and host location (Turner & Rowe, 2013). Individual HFs in PRL have varied polarities and affinities to certain soil types, which consequently affects their dispersal in soil.

There is a negative correlation between HF mobility and the organic matter content of the soil, which is primarily due to the high binding affinity of HFs to organic materials (Devine & Jones, 2001b). PRL HFs are highly diffusible and mobile in soil and may induce hatching up to 80 cm from the potato root (Turner *et al.*, 2006; Turner & Rowe, 2013). The optimum soil depth for hatching activity for both species of PCN is 10-20 cm; however, *G. pallida* exhibits greater hatching activity below 20-30 cm in comparison to *G. rostochiensis,* suggesting greater vertical mobility of *G. pallida*-selective hatching factors (Devine & Jones, 2003).

1.4.6 Biotic factors affecting hatch

PRL chemical properties are related largely to host physiology, development and cultivar. The chemical profile of PRL changes quantitatively and qualitatively in concert with plant development. Plant age has an impact on the relative proportion of HF in PRL,

particularly the ratio of HI:HS. HI are generally more abundant in early-produced PRL and HS are prevalent in later-produced PRL (Byrne *et al.*, 2001). Host physiological characteristics, including vigour, root growth dynamics, root physiology and architecture, may also modify hatching behaviour (Byrne *et al.*, 2001). Larger root systems produce more PRL and thus produce greater quantities of HF. Root branching pattern, as opposed to root system size, is also more closely associated with greater quantitative production of HF due to the increased number of root tips at the sites of PRL production (Rawsthorne & Brodie, 1986). Soil biology, particularly, plant-microbe interactions (§ 4.1), indirectly influence PCN hatch by modulating the synthesis and composition of host root leachates (Ryan *et al.*, 2000).

1.4.7 The effect of micro-organisms on PCN hatch

Microbes are proposed to alter the qualitative and quantitative composition of hatching chemicals in PRL, suggesting a tritrophic (host-microbe-PCN) interaction (Ryan *et al.*, 2003; Ryan & Jones, 2003). Soil-microbe communities, particularly arbuscular mycorrhizal fungi (AMF) and plant growth promoting rhizobacteria (PGPR), such as *Bacillus* spp., have a significant impact on host root dynamics. Indeed, HF production is partially mediated by root-associated micro-organisms (Ryan and Jones, 2004). PRL from conventionally grown potato plants contains more HF activity and subsequently induces higher hatch relative to PRL from aseptically grown plants (Ryan & Jones, 2004).

1.5 Population dynamics

PCN soil populations consist of the most recent generation (new cysts produced from the previous season) and population carry-over from previous years. Population dynamics are largely regulated by inoculum density, pest virulence, host genotype, host resources and environmental factors (Trudgill *et al.*, 1996). There is a negative correlation between PCN multiplication rate and the initial population density (Fig. 1.6). Multiplication rates are significantly reduced at high population densities due to competition for limiting resources, namely, root space and nutrition (Trudgill *et al.*, 1996).

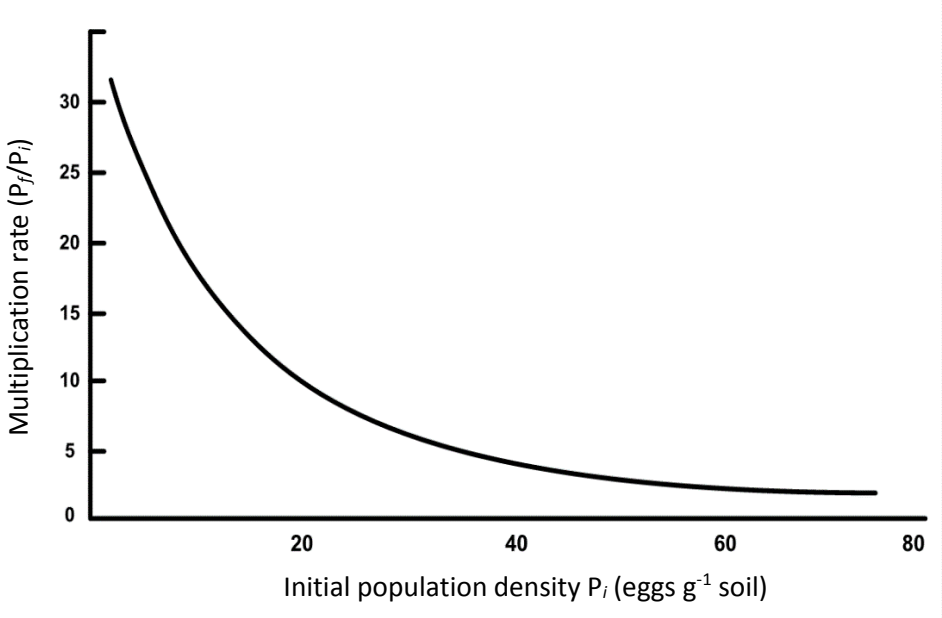

Figure 1.6 The effect of population density on nematode multiplication (Trudgill *et al.*, 1996).

The initial PCN population build-up is relatively slow: infestation can remain undetected until a large enough population has accrued to inflict noticeable economic injury (Baker *et al.*, 2012). Once a population has established, however, it is relatively persistent and can multiply in successive years if a host crop is grown, rendering it difficult to eradicate (Turner, 1996). The PCN multiplication rate (P_f/P_i) is density dependent and is directly proportional to the number of eggs in the soil at the time of planting (P_i). As such, the increase in PCN populations is greatest when the initial population inoculum is low (La Mondia & Brodie, 1986; Trudgill *et al.*, 2014).

PCN require a certain area of root space to establish and maintain syncytia for successful completion of the parasite's life cycle. At higher levels of PCN infestation, the surface area available for syncytium development is reduced and host root photosynthate reserves are continuously depleted by feeding nematodes. This leads to nutritional stress and a subsequent decline in female fecundity and a reduced number of females. Sex is determined by nutrient availability and at high infestation densities, the greater female nutritional demand selects for males, resulting in a higher male-to-female ratio. Consequently, the population size of the second generation is directly proportional to the

level of nutrition attained by the first generation, which relates to the volume of root surface area and the quality and quantity of nutrients available for female development (Jones & Perry, 1978).

1.5.1 Factors affecting PCN population dynamics

PCN populations are intrinsically regulated by the initial population inoculum (P_i), inter- and intraspecific competition and environmental conditions (Fig. 1.7). Population dynamics are also related to pest virulence (according to species and pathotypes) and the pest's ability to infect the host and to establish a syncytium. Similarly, PCN reproductive capacity is a prime determinant of population success and is largely influenced by host genotype characteristics, namely, host susceptibility, resistance or tolerance. Environmental factors such as temperature, pH and physicochemical soil properties also affect PCN populations (Hockland, 2002). Differences in temperatures can greatly influence populations (§ 4.1.3). Climate affects egg viability and thus affects pest persistence in the environment. A loss in egg viability due to aging is logarithmically dependent on temperature (Turner, 1996).

1.5.2 Population decline

In the absence of host crops, PCN populations decline naturally due to spontaneous hatch, a loss in egg viability resulting from natural aging, attack by other organisms and unfavourable environmental factors. The rate of decay is greatest in the first year, often exceeding 50% (Turner, 1996). *G. rostochiensis* displays a faster in-soil decline rate than *G. pallida* (Turner, 1996; Devine & Jones, 2001a). In-egg mortality, due to partial loss of trehalose or to increased juvenile sensitivity to abiotic and biotic stress, generally accounts for 20% loss in egg viability and 80% loss is attributed to spontaneous hatch (Turner, 1996; Turner & Evans, 1998).

The decline rate is density independent and remains relatively constant, *c.* 30%, in consecutive years, and the annual decrease in nematode populations declines

exponentially (Trudgill *et al.*, 2003). The rate of decline has been extensively studied as an important aspect of pest management and relevance to population modelling. Jones and Perry (1978), devised an equation to model PCN populations:

$$P_f = (aP_i)/[1 + (a - 1)P_i/E_1]$$

where P_f denotes the final nematode population density, P_i the initial population density, *a* represents the number of surviving offspring per female, and E_1 is the equilibrium population density, a reflection of the population carrying capacity. Due to the relatively slow decline rate, PCN may remain viable in the soil for up to 30 years (Reid, 2009), thus long rotations are necessary for adequate population regression (Hockland *et al.*, 2012; Palomares-Rius *et al.*, 2014)

1.6 Effect of PCN infection on the host

PCN infection is mainly confined to the root system. Progressive intracellular migration by the J2 causes extensive cellular damage, while subsequent syncytium expansion leads to a rupturing of the root cortex, incurring irreparable cell damage and root necrosis (Barker & Koenning, 1998; Back *et al.*, 2002). Prolonged biotrophic parasitism disrupts host metabolism, having a detrimental effect on plant fitness. The structural root damage incurred in severe PCN infestations facilitates concomitant infection by opportunistic pathogens; for example the incidence of soil-borne pathogens, particularly *Verticillium dahliae* and *Rhizoctonia solani,* may increase in concert with PCN invasion (Haydock & Evans, 1998; Back *et al.*, 2006).

Heavy PCN infection impairs root growth and primary root functions, such as water absorption and nutrient uptake, are also impaired (De Ruijter & Haverkort, 1999). Low PCN infestation densities may be tolerated. In low PCN infestations, cellular wound responses elicited at the site of the syncytium may initiate compensatory lateral root growth and consequently may improve root architecture, enhance nutrient- and water-use efficiency and ultimately improve yield (§ 5.1.2). PCN-induced lateral root proliferation is attributed to higher levels of auxins expressed at syncytium sites (Goverse *et al.*, 2000a).

In addition to mechanical injury, PCN also induce detrimental host metabolic and physiological changes, causing an adverse effect on plant hormone regulation (Gheysen & Mitchum, 2011). Host metabolism and physiology are significantly disturbed due to resource deprivation, altered gene expression involved in syncytium induction and the elicitation of host defences in response to wounding (Matthews *et al.*, 2013; Thorpe *et al.*, 2014).

1.6.1 Symptoms of PCN infestation

1.6.1.1 Field and haulm symptoms

As PCN is a soil-borne pest, infestation usually spreads from a primary infestation focus resulting in an elliptical distribution (§ 1.2.7). Thus, infested plants are evident in isolated patches, which enlarge in successive years if a host is regularly grown on the infested site. Crop damage can vary from small patches of plants exhibiting poor growth to complete crop failure (EPPO, 2004).

Symptoms of PCN infection are not specific and are often symptomatic of several other diseases and of abiotic stresses; as a result PCN infection is often misdiagnosed (EPPO, 2004). The main effects of PCN damage are not apparent until later in the growing season when the crop canopy is more developed. The most apparent haulm symptoms include stunting and premature senescence. Foliage appears chlorotic, and severely wilted due to a chronic deficiency of macronutrients, inefficient nutrient utilization and poor water uptake (Lilley *et al.*, 2005).

1.6.1.2 Root and tuber symptoms

PCN infection and intracellular migration can lead to changes in root architecture PCN-infected roots are smaller and are discoloured due to extensive wounding and cell necrosis. Plants are less vigorous due to root system impairment and are rendered more susceptible to environmental stresses, namely temperature, moisture and UV stresses. Tuber

symptoms include reduced size and increased pitting, although these effects do not necessarily impact yield, they can affect marketability (Turner & Evans, 1998).

1.6.1.3 Yield effects

PCN infestation can contribute to a global potato yield loss of 10% annually. The extent of yield loss is directly proportional to the number of invading nematodes; yield loss may occur at infestation densities as low as 5 eggs g^{-1} soil (Trudgill *et al.*, 1996). Yield loss estimates are variable, under light infestations, approximately 2.2 t ha^{-1} is lost for every 20 eggs g^{-1} (Brown, 1969). The prediction of yield loss is related to the initial PCN inoculum densities as reflected in yield modelling equations. The model by Seinhorst (1970) describes a curvilinear relationship between crop growth and the initial population density; where Y = yield, m = minimal yield, P = PCN population, T = tolerable PCN population density and z = constant.

$$Y = m + (1 - m)z^{P-T}$$

The economic loss implicated by modelling equations reflects the direct yield loss. Indirect economic losses are imposed by the expense of PCN control and increased susceptibility to secondary invasion by pathogens. The level of yield loss (Fig. 1.7) is correlated with the type of cultivar infected (i.e. the level of tolerance and resistance of the planted cultivar), agricultural practice, concomitant pathogen invasion and environmental conditions (Trudgill & Phillips, 1994). The economic threshold (ET), the population density at which the value of damage incurred equates to the cost of control (Abd-Elgawad & Askary, 2015), is contingent on soil type, pathotype, growth conditions and cultivar, accordingly. In the UK, a typical PCN population has an ET of 2.7 eggs g $soil^{-1}$ (Trudgill *et al.,* (1996).

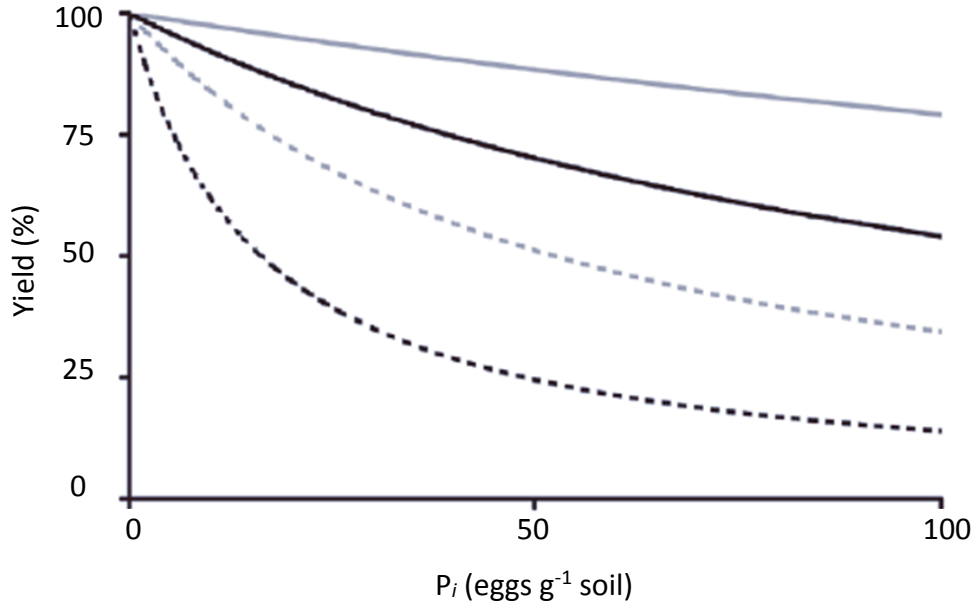

Figure 1.7 Effect of increasing *G. pallida* P_i on the relative yields of an intolerant ('Pentland Dell' - black lines) and a tolerant ('Cara' -grey line) cultivar in a loamy (dotted line) or a sandy (solid line) soil (Trudgill & Phillips, 1997).

1.7 PCN management

PCN is a difficult pest to control due to the ease of cyst dispersal, the sophisticated pest-host interaction and synchronization of the pest-host life cycle. Furthermore, PCN exists in various life stages, namely infective juveniles, sedentary females and cysts. The host roots afford protection to the infective J2 and females during endoparasitism. Similarly, the durable and resistant nature of the cyst permits longevity and persistence in the environment and protects the dormant eggs from desiccation, natural enemies and granular nematicides. PCN is most effectively controlled by Integrated Pest Management (IPM), which utilises several different complementary control strategies to maximise efficiency (Turner & Subbotin, 2013). The main IPM components in PCN management include the combined use of crop rotation, nematicides and resistant cultivars. No single strategy affords complete control of PCN in soil, nor in infected roots. Therefore, each component should be used in conjunction with other control methods to enhance crop protection.

Nematicidal control is the most effective method of PCN eradication. However, the expense and the environmental consequences of nematicides reduces their use in crop protection (Rousselle-Bourgeois & Mugniery, 1995). Effective PCN management requires a keen understanding of PCN biology; the success of any control measure is contingent on the efficacy of application, i.e. correct timing and synchrony with the pest life cycle, and compatibility with other elements of IPM.

In general, control of *G. pallida* has proven less successful than that of *G. rostochiensis* (Trudgill *et al.*, 2003). This is primarily due to the lack of major gene resistance in cultivars conferring complete resistance to *G. pallida*, the greater heterogeneity of *G. pallida* pathotypes and the difficulties encountered in breeding resistant cultivars (Armstrong *et al.*, 2000; Hockland, 2002). Other elements of IPM (rotation and nematicides) work less effectively against *G. pallida* than against *G. rostochiensis* which has led to selection for *G. pallida*. Consequently, *G. pallida* is now the dominant PCN species in the UK and mainland Europe (Minnis *et al.*, 2002; Trudgill *et al.*, 2003)

1.7.1 Legislative control

PCN can be a devastating pest of potatoes in temperate regions if infestation is not adequately managed. PCN is classified as an A1 quarantine pest in the EU and is subject to stringent quarantine law and regulatory protocols. Many countries have imposed a quarantine ban on PCN and have implemented legal restrictions on the movement of infested soil and seed tubers to prevent pest dissemination.

The EU Directive 69/465/EEC was introduced to protect potato cultivation from PCN. During the past 30 years, there have been significant developments in PCN epidemiology, identification, distribution and population dynamics. In light of such advances, the 1969 PCN Directive was replaced by the new European Council Directive 2007/33/EC. The main objective of the new directive is to determine the distribution of PCN in the EU and to curtail its dispersal. The directive requires all member states to conduct a thorough

official investigation for the presence of PCN on all land designated for seed production. In the event of PCN detection, land is officially scheduled and cannot be used for seed potato production until it is declared PCN-free and descheduled. Export and import consignments must be declared PCN free, which may require official documentation declaring compliance with the Directive (2007/33/EC). Ware potato production on scheduled land is permitted subject to the implementation of a PCN control programme, as stipulated in Article 9(2). Regulatory procedures in agricultural practice are imposed; crop rotation in particular is mandatory in certain EU states. The directive also requires an annual survey of at least 0.5% of the ware potato production area to determine the incidence and distribution of PCN in member states (2007/33/EC, Annex III, Section II).

1.7.2 Integrated pest management

IPM involves the combined use of crop rotation, resistant cultivars and nematicides and is an integral part of PCN management.. Resistant cultivars can confer up to 85% control (Phillips & Trudgill, 1998; Sobczak *et al*., 2005), and crop rotation can confer up to 50% control in the first year. Nematicides can reduce PCN populations by more than 90% (§ 2.1.4; Kerry *et al*, 2002) and are most effective at reducing yield loss rather than controlling nematode populations by killing individuals that are surplus to the crop carrying capacity. As such, the integrated use of host resistance, chemical and cultural control in PCN management schemes is the most effective means of supressing PCN populations to mitigate PCN multiplication in successive generations (Minnis *et al*., 2004).

1.7.2.1 Crop rotation

Crop rotation is an effective and practical means of PCN control that exploits the natural PCN population decline (§ 1.5.3) due to spontaneous hatch and a loss of egg viability in the absence of a host crop. (Whitehead *et al*., 1980; Turner, 1996; Whitehead, 1997). The length of rotation required for effective PCN reduction depends on the level of PCN infestation and the rate of population decline (§ 1.5.3). Other factors affecting rotation

efficacy include environmental conditions, namely temperature, pH and moisture; in general, higher rates of spontaneous hatch occurs in drier climates (Turner, 1996; Whitehead *et al.,* 1998). Regular land cultivation can accelerate the PCN decline rate, so that cultivation of non-host crops may enhance PCN control via crop rotation (Turner, 1996). A theoretical annual population decline of 30 % was proposed by Hancock (1988), indicating that a minimum 7-year rotation period is needed to reduce PCN densities below the ET (Whitehead *et al.,* 1998).

Potatoes represent a substantial cash crop and potato farmers invest considerable capital in potato specific items such as harvesters and cold stores. As crop rotation often involves the cultivation of a less profitable crop, a potential economic loss is incurred. Consequently, most agronomic systems implement shorter three- to four-year rotations. *G. pallida* and *G. rostochiensis* decline at different rates (§ 1.5.2), with *G. pallida* having a slower decline rate in comparison to *G. rostochiensis* (Turner, 1996). As a result, crop rotation may be less effective against *G. pallida*, which requires longer rotation periods for adequate control.

1.7.2.1.1 Trap cropping

Trap cropping involves the deliberate planting of a suitable host or non-host crop to stimulate PCN hatch and permit J2 emergence and invasion. Host trap crops are grown for approximately five to seven weeks and are subsequently removed and destroyed before PCN multiplication can occur (Suszkiw, 2013). This technique limits nematode reproduction by destroying the plants before female maturity is reached. The efficacy of trap cropping is dependent on the length of time that the crop remains in the soil to stimulate a sufficient degree of hatch, without allowing hatched individuals to complete their life cycle (Scholte, 2000). Optimum juvenile activity and physiological development occurs above basal temperatures of 3.9°C and 6.2°C for *G. pallida* and *G. rostochiensis*, respectively (Mugniery, 1978). Consequently, a measurement of accumulated day degrees (DDC) is used to deduce the stage of PCN development and to determine the optimum

date for crop removal and destruction. Soil temperature monitoring is critical and cultivar, planting date and destruction dates must be highly coordinated to avoid PCN multiplication (Scholte, 2000). Failure to synchronise the destruction date and timing of PCN emergence/establishment may facilitate PCN multiplication (on host trap crops) and exacerbate the pest problem (Scholte & Vos, 2000; Timmermans *et al.*, 2007).

Trap cropping with non-host trap crops can mitigate the risk of PCN multiplication and has many benefits as an alternative PCN control strategy. Correct implementation of trap crops using wild, resistant potato species, such as *Solanum sisymbriifolium* L. can achieve up to 80% reduction in *G. pallida* and *G. rostochiensis* populations (Haydock & Evans, 1998; Dias *et al.*, 2012). The main limitations of using wild potato species as trap crops include the expense of seed tubers and cultivation costs for no commercial end product (Ryan & Devine, 2005).

1.7.2.2 Chemical control

The cyst and eggshell afford considerable protection to the unhatched J2; the infective juveniles within the host root system are similarly protected. As such, the migratory juveniles present in the rhizosphere are highly susceptible to nematicidal control. The current nematicide market is worth $1 billion and is expected to reach $1.34 billion by 2020, with an average annual growth rate of 3.2% (Zouhar *et al.*, 2010; Anonymous, 2015). Currently 16% of the total potato growing area in Europe is treated with nematicides, signifying the importance of nematicides in PCN management.

1.7.2.2.1 Granular nematicides

Non-fumigant, granular nematicides are applied and incorporated into the soil immediately prior to potato planting. Granular nematicides include organophosphates, namely carbamates such as aldicarb and oxamyl (Table 1.3). These chemicals are non-phytotoxic and are often referred to as nematistats, as their mode of action involves disrupting host metabolism, feeding and movement of the juveniles. Such nematicides are

only effective against vermiform, motile nematodes and act as neurotoxins, which inhibit cholinesterase and affect the nervous system. This leads to nematode disorientation and paralysis of the hatched J2, inhibiting host detection and root invasion.

Nematicide products are among the most toxic agrochemicals, which incur vertebrate toxicity and contaminate groundwater systems. Nematicide use is highly regulated (Regulation 1107/2009/EC; Council Directive 2009/128/EC) and several products have been withdrawn due to environmental and health implications. However, some nematicides have subsequently been reintroduced due to the lack of effective alternative control measures (Whitehead, 1992). Aldicarb (marketed as 'Temik' by Bayer) was withdrawn in the EU and the US in 2008 and 2010, respectively, but has been recently been reregistered with a lower toxicity profile as a new product 'Meymik'. Nematicide residues in fresh produce are a concern for consumers and alternative control methods are highly sought after.

The efficacy of nematicides is dependent on environmental parameters and edaphic conditions such as soil moisture content, temperature and the soil binding affinity which may impede nematicide diffusion and in-soil biodegradation (Whitehead, 1997). Nematicides typically have half-lives in soil of 2-3 weeks (Deliopoulos *et al.*, 2010; Haydock *et al.*, 2012); concentrations in soil can decline to ineffective levels by the time of *G. pallida* J2 emergence due to its prolonged hatch and the later peak in hatching activity (Hockland, 2010). As such, nematicides may need to be applied twice per season to achieve adequate levels of *G. pallida* control (Whitehead, 1997).

Table 1.3 A summary of commercially available nematicides in the USA and EU.

Product	Manufacturer	Active Substance (Class)	Formulation	Mode of action
Avicta	Syngenta	Abamectin (Avermectin)	Liquid	Ingested toxin
Temik [1,2] **Meymik** [3]	Bayer AgLogic	Aldicarb (Carbamate)	Granular	Anticholinesterase (Nematistat)
Furadan [1,2]	FMC	Carbofuran (carbamate)	Granular	Anticholinesterase (Nematistat)
Mocap	Bayer	Ethoprophos (Organophosphate)	Granular	Anticholinesterase (Nematistat)
Nemacur	Bayer	Fenamiphos (Organophosphate)	Liquid	Anticholinesterase (Nematistat)
Nemathorin	Syngenta	Fosthiazate (Organophosphate)	Granular	Anticholinesterase (Nematistat)
Counter [2]	Amvac	Terbufos (Organophosphate)	Granular	Anticholinesterase (Nematistat)
Nimitz	Amvac	Fluensulfone (Fluoroalkenyl)	Liquid	Anticholinesterase (Nematistat)
Rugby[2]	FMC	Cadusafos (Organophosphate)	Granular	Anticholinesterase (Nematistat)
Vydate	DuPont	Oxamyl (Carbamate)	Granular	Anticholinesterase (Nematistat)
Velum-Total	Bayer	Imidacloprid (Neonicotinoid)	Liquid	Multiple MOA (Nematistat)
Movento	Bayer	Spirotetramat (Keto-enol)	Liquid	Lipid Biosynthesis Inhibitor
Basamid	Certis	Dazomet (Unclassified)	Granular fumigant	Sterilant
Metam 510 **Vapam-HL**	Certis Amvac	Metam-sodium (Dithiocarbamate)	Liquid fumigant	Sterilant
Telone II [2]	DowAgro	1,3 dichloropropene (Halogenated organic)	Liquid fumigant	Sterilant

[1] Banned in the US, [2] Banned in the EU, [3] Approved in the US

1.7.2.2.2 Fumigants

Fumigant nematicides generate gases that percolate through the soil to eliminate PCN e.g. methyl bromide, methyl isothiocyanate, 1,3-dichloropropene (Telone II) and chloropicrin. The liquid fumigant Telone II (1,3-dichloropropene) is the most extensively applied fumigant nematicide, although methyl bromide is considered to be the most effective fumigant available. Fumigants are extremely phytotoxic and are only applied before the potato crop is grown. Unlike granular nematicides, fumigants affect both species of PCN equally; its toxicity is also effective against both the dormant and active pest stage.

The efficacy of fumigants depends on edaphic conditions such as soil moisture content, aeration and temperature, which affect nematicide diffusion through the soil profile and the efficacy of application. Successful application with optimum percolation through the soil can effectively kill up to 90% of PCN eggs (Haydock & Evans, 1998; Kerry *et al.*, 2002). Field trials conducted with 1,3-dichloropropene achieved 60% PCN mortality in the top 25 to 30 cm of the profile. However, potato roots and cysts can extend to soil depths greater than 80 cm. Often polythene sealants are applied to the area in concert with the nematicide to reduce the escape of the gas, although this method is extremely expensive for the management of PCN in the field. In optimum conditions, a 90% reduction in PCN populations may be achieved. Fumigants are non-specific, soil sterilants that affect non-target organisms and are extremely hazardous. The considerable expense of the nematicide and its application is a main limitation of this control method. Telone II was banned in the EU in 2008 and it is heavily restricted in the US (Table 1.3).

1.7.2.3 Host resistance

Host resistance is the ability of a host genotype to impede pest invasion and feeding, nematode development and population increase. Resistance is conferred by the transfer of resistance genes to a susceptible genotype to confer complete (monogenic) or partial resistance (polygenic). Natural PCN resistance genes are generally derived from wild

potato species and unimproved land races. Initial resistance screening in *Solanum* spp. identified resistance in *S. tuberosum* ssp. *andigena* L., *S. multidissectum* L. and *S. vernei* L. (Toyota *et al.*, 2008).

Major-gene or vertical resistance is usually conferred by specific resistance genes (*R*), which confer a high level of race-specific resistance to a specific pest. Major gene resistance is a qualitative form of resistance as cultivars are either completely resistant or susceptible; there are no intermediate levels of resistance. Major gene resistance to *G. rostochiensis* (Ro1 and Ro5) was conferred by the *H1* gene, derived from *S. tuberosum* ssp. *andigena* (Ellenby, 1952). This was exploited in the first commercial cultivar expressing complete resistance *G. rostochiensis*, 'Maris Piper'.

Polygenic resistance is conferred by several quantitative trait loci (QTL), which provide a certain degree of resistance to all PCN pathotypes. Polygenic resistance is not race-specific and generally confers partial resistance, which is the predominant form of resistance available against *G. pallida*. The *H2* QTL derived from *S. multidissectum* (Dunnett, 1961) affords partial resistance to *G. pallida* Pa2/3 (Grenier *et al.*, 2001). A polygenic form of resistance derived from *Solanum tuberosum* ssp. *andigena*, referred to as *H3* resistance (Howard *et al.*, 1970; Dale & Phillips, 1982) is a promising source of a high level of partial resistance to *G. pallida* Pa2/3 (Bryan *et al.*, 2004; Tan *et al.*, 2009).

1.7.2.3.1 Mechanisms of resistance

Host *R*-genes display a high degree of specificity for a particular pathogen (Atkinson *et al.*, 2003). *R*-mediated resistance entails a gene-for-gene interaction, involving the recognition of a specific *avr* gene product by a corresponding host *R*-protein. The resultant incompatible interaction between the *avr* and *R*-proteins elicits a defence response (Chapter 5). Non-specific resistance can also be expressed by the recognition of essential *avr* proteins by several *R*-proteins, which may elicit generic local defence responses and subsequent systemic responses (§ 5.1.1.3).

R-gene completely resistant varieties usually reduce PCN multiplication by impeding nematode invasion, preventing syncytium induction or attenuating syncytium development by up-regulating cellular physical and chemical defence responses (Jung *et al.*, 1998; Fuller *et al.*, 2008). Juveniles that infect the root system of resistant varieties generally exhibit slower invasion rates and leave resistant host roots shortly after infection (Forrest *et al.*, 1986). The mechanism of resistance derived from the *H1* gene includes feeding cell attenuation, by walling-off, and reduced nutrient availability (Sobczak *et al.*, 2005).

Upon recognition of *G. rostochiensis avr* gene products, *H1*-mediated resistance initiates the hypersensitive response (HR) upon syncytium formation (§ 5.1.1). The HR results in the formation of a necrotic layer around the developing syncytium, separating the syncytium from the vascular tissue, and thereby interfering with the transfer of photosynthate to the developing nematode (Sobczak *et al.*, 2005). Host resistance is conferred by syncytium attenuation, resulting in nutritional stress and a significant reduction in female development and fecundity. This is evident as a loss of egg viability and a reduced number of eggs per cyst (Mullin & Brodie, 1988; Toyota *et al.*, 2008). In partially resistant cultivars, the root system is affected by J2-induced cell necrosis incurred by intracellular migration and HR elicitation. Syncytia appear intact; however, females are adversely affected and multiplication is reduced. Thus, partially resistant varieties affect PCN multiplication by disturbing sex ratios and selecting for male-dominated populations (Castelli *et al.*, 2005).

1.7.2.3.2 Resistance genes

G. rostochiensis and *G. pallida* pathotypes are distinguished by their ability to multiply on *Solanum* clones containing different *R*-genes and QTL (Phillips & Blok, 2008). Natural nematode *R*-genes are members of a gene family characterized by a nucleotide-binding leucine-rich repeats (NB-LRR; Zhang *et al.*, 2013). Indigenous *Solanum* clones from South America, namely *S. gourlayi* L.*, S. sparsipilum* L.*, S. kurtzianum, S. vernei* and *S. acaule* L. are the main targets for isolating nematode resistance genes. Several genetic markers

associated with *R*-genes are sought via marker assisted selection (MAS) in breeding resistant varieties (Gebhardt, 2013; Sudha *et al.*, 2016). Several *R*-genes and QTLs conferring resistance to PCN have been identified and cloned (Table 1.4). *H1, GroV1, Gpa2* and *Gro1-4* confer complete resistance to *G. rostochiensis* pathotypes. The most successful *R*-gene, the *H1* gene derived from *S. tuberosum* ssp. *andigena,* confers a high level of durable resistance to *G. rostochiensis* Ro1 and Ro4 (Gebhardt *et al.*, 1993; Brodie *et al.*, 2000).

Partial resistance to *G. pallida* has been derived from *S. vernei, S. andigena, S. sucrenese* L.*, S. spegazzinii, S. tarijense* L.*, S. sparsipilum and S. oplocense* L. Numerous QTLs conferring partial PCN resistance have been identified (*Gro1.4*, *Gpa4*, *Gpa*, *Gpa5*, *Gpr1*, *Gpa6, Gro1.2, Gro1.3*). The *Gpa2* gene conferring partial resistance to *G. pallida* has been isolated and cloned from *Solanum tuberosum* ssp. *andigena* and has significant potential in engineering PCN resistant varieties (Moloney *et al.*, 2010). The combination of two QTLs can give additive resistance levels. *Solanum* clones expressing both *Gpa5* and *Gpa6* loci afford additive resistance to *Globodera* spp. Similarly, co-expression of $GpaV^{s}_{spl}$ and $GpaXI^{s}_{spl}$ confer a high level of resistance to *G. pallida* (Caromel *et al.*, 2005).

Table 1.4 Natural resistance genes to PCN, that have been isolated and cloned, modified from Tomczak *et al.* (2009).

Gene	Chromosome	Source variety	Species	Resistance	Reference
Gpa	V	*S. spegazzinii*	*G. pallida*	QTL	Kreike *et al.* (1994)
Gpa2	XII	*S. tuberosum ssp. andigena*	*G. pallida*	SD	van der Voort *et al.* (1997)
Gpa4	IV	*S. tuberosum ssp. andigena*	*G. pallida*	QTL	Bradshaw *et al.* (1998)
Gpa5	V	*Solanum spp.*	*G. pallida*	QTL	van der Vossen *et al.* (2000)
Gpa6	IX	*S. vernei*	*G. pallida*	QTL	van der Voort *et al.* (2000)
GpaIV$^{s}_{adg}$	IV	*S. tuberosum ssp. andigena*	*G. pallida*	QTL	Caromel *et al.* (2005) Moloney *et al.* (2010)
GpaM1	V	*S. spegazzinnii*	*G. pallida*	QTL	Caromel *et al.* (2003)
GpaM2	VI	*S. spegazzinnii*	*G. pallida*	QTL	Caromel *et al.* (2003)
GpaM3	XII	*S. spegazzinnii*	*G. pallida*	QTL	Caromel *et al.* (2003)
GpaV$^{s}_{spl}$	V	*S. sparsipilum*	*G. pallida*	QTL	Caromel *et al.* (2005)
GpaXI$^{s}_{spl}$	XI	*S. sparsipilum*	*G. pallida*	QTL	Caromel *et al.* (2003)
GpaXI$^{t}_{tar}$	11	*S. tarijense*	*G. pallida*	QTL	Tan *et al.* (2009)
Gro1	VII	*S. spegazzinii*	*G. rostochiensis*	QTL	Barone *et al.* (1990) Paal *et al.* (2004)
Gro1.2	X	*S. spegazzinii*	*G. rostochiensis*	QTL	Kreike *et al.* (1996)
Gro1.3	XI	*S. spegazzinii*	*G. rostochiensis*	QTL	Kreike *et al.* (1996)
Gro1.4	III	*S. spegazzinii*	*G. rostochiensis*	QTL	Kreike *et al. (*1996)
GroV1	V	*S. vernei*	*G. rostochiensis*	SD	Jacobs *et al.* (1996)
Grp1	V	*Solanum spp.*	*G. rostochiensis, G. pallida*	QTL	van der Voort *et al.* (1998)
H1	V	*S. tuberosum ssp. andigena*	*G. rostochiensis*	SD	Kreike *et al.* (1993) Bakker *et al.* (2004)
HeroA	11	*Lycopersicon pimpernellifolium*	*G. rostochiensis*	SD	Ernst *et al.* (2002)

1.7.2.4 Host tolerance

Host tolerance refers to the extent to which the host crop may withstand PCN infection without any significant loss to crop yield (Trudgill & Cotes, 1983). Plant characteristics that confer tolerance include molecular and physiological responses that potentially reduce the effects of PCN infection. Physical traits, such as an extensive root system (e.g. cv. 'Cara'), may promote mineral and water uptake and thereby increase plant vigour. Other plant characteristics that confer tolerance generally include late maturity, delayed tuber initiation coupled with increased vigour and large haulm to maximise light interception and to achieve higher photosynthetic rates (Trudgill & Phillips, 1994). Tolerant varieties can potentially increase the PCN multiplication rate as infected plants continue to provide nutrients and facilitate nematode development (Trudgill & Phillips, 1997).

1.7.3 Novel control

New approaches to nematode control are imperative for future sustainable PCN management systems; advances in technology and resources have significantly contributed to the development of novel control strategies. Genetic engineering for host resistance, deployment of semiochemicals, biological control agents (BCA) and the manipulation of rhizosphere microbial communities are the key areas of research in the future integrated control of PCN. The main targets of novel and transgenic resistance involve chemodisruption, anti-invasion and migration, syncytia attenuation and anti-nematode feeding and development strategies (Atkinson *et al.*, 2003; Kandoth & Mitchum, 2013). Similarly, down-regulation of nematode effectors and host genes functional in syncytium induction and maintenance via antisense inhibition or suppression may afford resistance to PCN (Lilley *et al.*, 1999; Urwin *et al.*, 2001; Tamilarasan & Rajam, 2013).

RNA interference (RNAi) has been extensively researched as a means of PCN resistance (Lilley *et al.*, 2012; Dutta *et al.*, 2013; Niu *et al.*, 2015). Nematode parasitism genes are the

principle targets of gene silencing by RNAi; genes encoding essential cellulases, β-1,4, endoglucanase, were targeted and silenced in *G. rostochiensis* and successfully incurred a reduction in J2 infecting (Chen *et al.*, 2005). RNAi silencing of genes encoding neuropeptide, such as *Gp-flp-12* in *G. pallida*, successfully inhibited neuromuscular function and migration and reduced J2 infection (Dalzell *et al.*, 2010; Atkinson *et al.*, 2013).

Genetically modified nematode-resistant (GMNR) potatoes secreting the chemodisruptive peptide (nAChRbp) exhibited significant nematode resistance to *G. pallida* by inhibiting chemoreception (Green *et al.*, 2012). Furthermore, the use of syncytium specific promoters to induce local necrogenic resistance to inhibit PCN development has significant potential in GM-mediated PCN resistance (Siddique *et al.*, 2011; Ali *et al.*, 2013; Wiśniewska *et al.*, 2013). Similarly, induction of proteinase inhibitors (PI) may suppress female fecundity and affect the male to female ratio and thereby reduce subsequent population size (Urwin *et al.*, 2000; Atkinson *et al.*, 2003; Atkinson *et al.*, 2012).

Semiochemicals can be deployed to disrupt J2 perception and incur disorientation (Perry, 1994; Hiltpold & Turlings, 2012), having the same mode of action as commercial chemical nematistats, without the detrimental environmental consequences. Biological control is relatively underexploited, despite the availability and relative success rate of biocontrol products (López-Lima *et al.*, 2013). Nematode BCA include predatory nematode trapping fungi (Hyde *et al.*, 2014), endoparasitic fungi (Larsen, 2000; Jacobs *et al.*, 2003), ovicidal egg-parasitizing fungi (Morton *et al.*, 2004) and nematophagous bacteria and associated metabolites. In addition, plant growth-promoting rhizobacteria can affect nematode infectivity by the production of anti-nematode metabolites or by inducing systemic resistance in plants (Siddiqui, 2006).

Alternative agricultural practices, such as deep-ploughing, are also highly effective in the management of PCN. Deep ploughing prior to potato planting, entails incorporating PCN cysts deeper into soil with the intention of isolating them from the vicinity of host

roots (Castelli *et al.*, 2003). Juveniles that hatch at a distance from host plant roots have a lower probability of locating and infecting the host. Juvenile mortality increases with increasing migration distances due to depleted lipid reserves and increased vulnerability to biotic and abiotic stresses in the rhizosphere (Whitehead & Nichols, 1992). Similarly, hatch induction is reduced in cysts located at a distance from host roots due to the gradual decline of HF concentrations because of PRL diffusion and leaching throughout the soil profile (§ 1.4.5).

Other alternative control practices may involve physical control techniques including solarisation and high frequency electrical fields (HFEF). Soil solarisation has also proven effective in the control of PCN. La Mondia and Brodie (1984) reported a 96-99% decline in *G. rostochiensis* populations within the top 10 cm of soil. Biofumigation with green manures rich in volatile organic compounds, namely isothiocyanate-producing glucosinolates of *Brassica* spp., has also proven successful in the suppression of PCN multiplication (Lord *et al.*, 2011; Ngala *et al.*, 2015).

1.8 Research aims

Research will primarily focus on the comparative ecology of *G. pallida* and *G. rostochiensis* to establish the nature of competition between these sibling species. The project aims to determine how *G. pallida* out-competes *G. rostochiensis* in mixed-species populations and to deduce whether this greater *G. pallida* competitiveness is related to its delayed hatch. The PCN-host interaction will be studied to investigate the effect of PCN infection on the potato root system to assess the differential host physiological responses to *G. rostochiensis* and *G. pallida* infection.

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Chapter 2 PCN in Ireland and validation of diagnostic techniques

Abstract

Population surveys in the UK and mainland Europe have confirmed the increasing incidence of *G. pallida*. This appears to be a result of the use of control measures, which tend to be more effective against *G. rostochiensis* than *G. pallida,* leading to selection pressure for *G. pallida*. Previous studies indicate that *G. rostochiensis* is the dominant species in Ireland. PCN control strategies in Ireland are generally restricted to crop rotation. However, anecdotal evidence suggests that *G. pallida* may be becoming more prevalent despite the lack of selection pressure for this species. Results from a national PCN soil sampling survey in 2010-2011 revealed that frequencies of both *G pallida* and *G. rostochiensis* have increased. Few studies have investigated the relative proportion of *G. pallida* and *G. rostochiensis* in PCN-infested soil, which is mainly due to difficulties encountered in species discrimination in pooled samples. In this study, PCN samples were analysed using both conventional endpoint PCR and real-time TaqMan probe-based qPCR methods. The qPCR method proved more efficient and consistent and provided a higher degree of reproducible quantitative analysis relative to conventional PCR test methods. The current EPPO standards for PCN detection and the applicability of high throughput screening methods for PCN identification and species discrimination are reviewed.

2.1. Introduction

Potatoes are the second most economically important horticultural crop grown in Ireland after mushrooms (DAFM, 2015); as such, PCN infestation poses a significant threat to the industry. In Ireland, PCN is commonly known as the potato eelworm and is primarily a concern in the highly regulated seed potato production. However, the threat of PCN to the ware industry has gained considerable recognition in recent years, in light of the introduction of the EU Council Directive 2007/33/EC on the Control of Potato Cyst Nematodes. *G. rostochiensis* was the first PCN species reported in Ireland in 1922 (Carroll, 1933) and it remains the most prevalent species (DAFM, 2011). However, the incidence of *G. pallida* infestation in both single- and mixed-species populations is increasing (Griffin *et al.*, 2015), although a detailed nationwide survey of the frequencies of the two species has not yet been published.

In recent years there has been a marked increase in the prevalence of *G. pallida* in mainland Europe and in the UK; consequently, it is now the dominant species in these regions (Minnis *et al.*, 2002; Taylor & Hockland, 2010; FVO, 2013). Although the level of *G. pallida* infestation in Ireland is not comparable to that of the UK or mainland Europe, it would appear that *G. pallida* is gaining in importance (D. Murphy, DAFM), although there is no hard supporting evidence. The research presented in this chapter investigates the incidence of both PCN species in Ireland and the challenges of PCN diagnostic testing and species identification. Comparisons of conventional and real-time PCR methods for species discrimination and quantification and the optimization of PCR techniques will be made.

2.1.1. PCN detection and speciation

Difficulties in achieving accurate detection, identification and quantification of PCN are the main reasons for the lack of PCN population data and inadequate pest monitoring at a national level. Species identification is not stipulated in the EU regulations (Council Directive, 2007/33/EC) nor in the Statutory Instruments (S.I. 359/2011) for pre-planting

seed certification or ware surveys. Although many ware surveys entail speciation, Article 6(2) of the legislation merely calls for 'testing for the presence' of PCN (§ 2.1.3). Considering the emerging prevalence of *G. pallida* in PCN populations, accurate species identification is fundamental to the implementation of appropriate and effective PCN control measures.

Morphometric-based species identification is laborious, time consuming and does not offer a high degree of precision. Molecular techniques, particularly PCR, have proven to be the most efficient and rapid means of PCN detection and are instrumental in species identification (§1.2.2; Ibrahim *et al.*, 2001; van de Vossenberg *et al.*, 2014).

2.1.1.1. Polymerase chain reaction (PCR)

PCR is a highly sensitive analytical technique capable of amplifying a specific sequence of DNA or RNA to generate a large copy number for detection and analysis. In a PCR reaction, a target sequence of double-stranded DNA (dsDNA) is denatured to single-stranded DNA (ssDNA). Specific oligonucleotide sequences (primers) anneal to the target sequence on the template strand, Taq polymerase attaches to the forward and reverse primer and extends each primer in the 5' to 3' direction, by binding free nucleotides to complementary bases on the template strand. This results in a 2-fold amplification of the specific DNA fragment between the primers (Fig. 2.1). PCR reactions typically generate more than one billion template copies. End-point PCR detects amplification at the final plateau phase of the PCR reaction and requires post-PCR analyses via gel electrophoresis and UV visualisation.

PCR analyses feature prominently in the literature (Bulman & Marshall, 1997; Szalanski *et al.*, 1997; Fleming *et al.*, 1998; Fullaondo *et al.*, 1999; Vejl *et al.*, 2002). The method published by Bulman and Marshall (1997) is the recognized PCR method in the EPPO standards PM 7/40 (3) as a diagnostic protocol for *G. pallida* and *G. rostochiensis* (EPPO, 2013). The method utilizes a universal forward primer targeting the small ribosomal

subunit (SSU) of 18S rRNA (White *et al.*, 1990) and two species-specific reverse primers targeting the ITS1 region. Numerous modifications have been developed to optimize multiplex PCR reactions for the identification of species in mixed populations (Pylypenko *et al.*, 2005; Quader *et al.*, 2008; van de Vossenberg *et al.*, 2014). Other PCR-based diagnostic methods including RAPD-PCR (Thiery *et al.*, 1997; Bendezu *et al.*, 1998) and PCR-RFLP (Thiery & Mugniery, 1996; Szalanski *et al.*, 1997; Širca *et al.*, 2011) have been used for the successful PCN identification and quantification. The ITS PCR-RFLP tests of Thiery and Mugniery (1996) and Fleming *et al.* (2000) are also recognized as standard PM 7/40 (3) diagnostic protocols (EPPO, 2013).

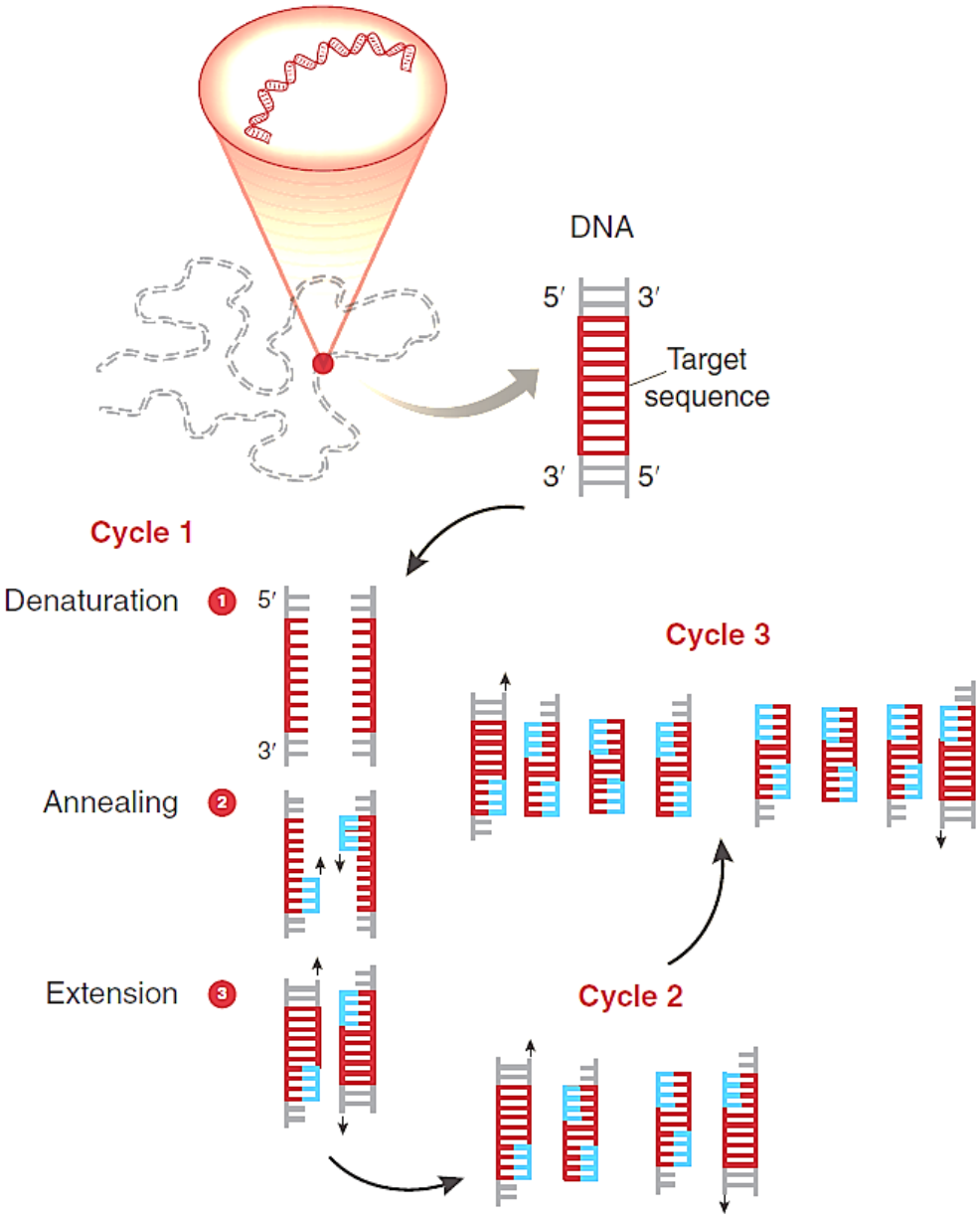

Figure 2.1 Schematic representation of a PCR reaction (Garibayan & Avashia, 2013).

2.1.1.2. Real-time PCR

Real-time PCR technology offers greater sensitivity and specificity compared to conventional endpoint PCR. Samples do not require further processing post-PCR i.e. product separation by gel electrophoresis and robust quantifiable data is collated in 'real-time' with progressive cell cycles. Numerous fluorescent chemistries are available for real-time PCR, namely probe-based assays or DNA binding dyes to measure amplicon fluorescence (Fig. 2.2A) or melting peak analysis (Fig. 2.2B).

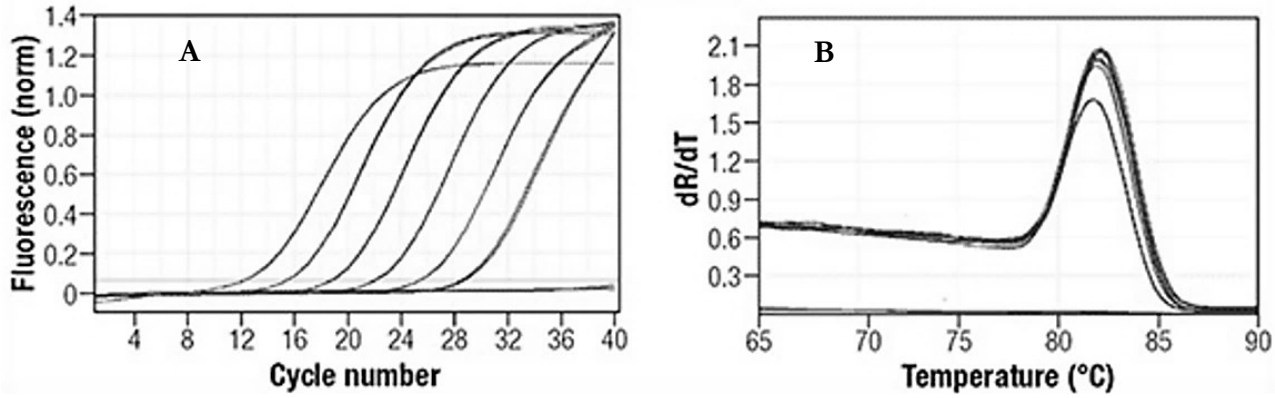

Figure 2.2 Quantitative PCR data measuring (A) fluorescence and (B) melting peak analysis (ThermoFisher, 2015).

Quantitative (q)PCR chemistries facilitate the detection of PCR amplification during the early cycles of the reaction; real-time measurements and early detection is a distinct advantage over conventional (c)PCR. Detection and quantification of DNA is achieved by measuring the fluorescence emitted by the amplification products. Assays utilizing sequence-specific, dual-labelled probes such as TaqMan®, Minor Groove Binding (MGB), HybProbes, Scorpions® or locked nucleic acid (LNA®) are efficient, quantifiable methods of nematode detection (Jianjun *et al.*, 2009; Waeyenberge *et al.*, 2010; Yu *et al.*, 2011; Beniers *et al.*, 2014; Reid *et al.*, 2015). Probes can be labelled with different dyes allowing multiplex assays. The hydrolysis probes are cleaved during extension releasing the reporter fluorophores from a quencher. Fluorescence is measured after light excitation resulting in fluorescent resonance energy transfer (FRET) at the end of each PCR reaction cycle. The emitted FRET signal fluorescence is proportional to the amount of product formed.

TaqMan-based PCR tests by Madani *et al.* (2011) and Nakhla *et al.* (2010) are recognized detection protocols in the EPPO diagnostic standard PM 7/40 (3) (EPPO, 2013).

SYBR® Green is a fluorescent dye that binds to the minor groove of dsDNA; as the dye binds, the intensity of the fluorescent emissions increases. As such, fluorescence increases in proportion to the amplicon produced and the quantitative data is a function of fluorescence versus reaction cycle. Alternatively, melting peak analysis of the amplicon may be used for detection or quantitative measurement (Bates *et al.*, 2002). Relative or absolute quantification analyses based on reference standards and critical threshold (C_t) values provide accurate enumeration data (Schmittgen & Livak, 2008). A SYBR-green assay based on LSU rDNA sequences developed by Clear Detections and Wageningen University are the standard EPPO real-time PCR diagnostic method (BLGG, 2009; EPPO, 2013).

qPCR analyses provide rapid and conclusive quantitative data; indeed, real-time PCR assays have replaced most molecular assays for the quantitative detection of PCN (Toyota *et al.*, 2008; Petter & Suffert, 2010; van den Berg *et al.*, 2012). Smaller sample sizes, reduced reagent requirements and automation facilitate high-throughput sampling (Valasek & Repa, 2005; Reid *et al.*, 2015). The risk of cross contamination is also significantly reduced, largely due to automation and the lack of post PCR processing (Toyota *et al.*, 2008). The recent changes in the EU Directive (2007/33/EC) necessitate a more frequent and extensive level of soil sampling. Thus, a high-throughput method for the detection and speciation of PCN is necessary to facilitate the intensified level of sampling (Reid *et al.*, 2015).

2.1.2. The current status of PCN

Prior to the introduction of resistant potato cultivars expressing the *H1 R* gene conferring resistance to *G. rostochiensis*, PCN populations across Europe were dominated by *G. rostochiensis*. However, there has been an apparent transition in species composition and

G. pallida is now the most prevalent species in potato production areas (Minnis *et al.*, 2002; Taylor & Hockland, 2010; FVO, 2013). The change in PCN population structure is apparent in the UK, where the overall incidence of PCN has increased from 42% to over 64% infested land between 1992 and 2002 (Hancock, 1996; Minnis *et al.*, 2002). Minnis *et al.*, (2002) reported an increase in pure *G. pallida* populations from 1992 (54%) to 2002 (67%), whereas the proportion of mixed populations had decreased to 25% (Fig. 2.3). *G. pallida* essentially occurred in 92% of the land surveyed, compared with 33% for *G. rostochiensis* (Fig. 2.3; Minnis *et al.,* 2002). A more recent UK study reported similar results, with pure *G. pallida* populations representing 62%, *G. rostochiensis* 29% and 8% of samples being mixed-PCN populations (Taylor & Hockland, 2010).

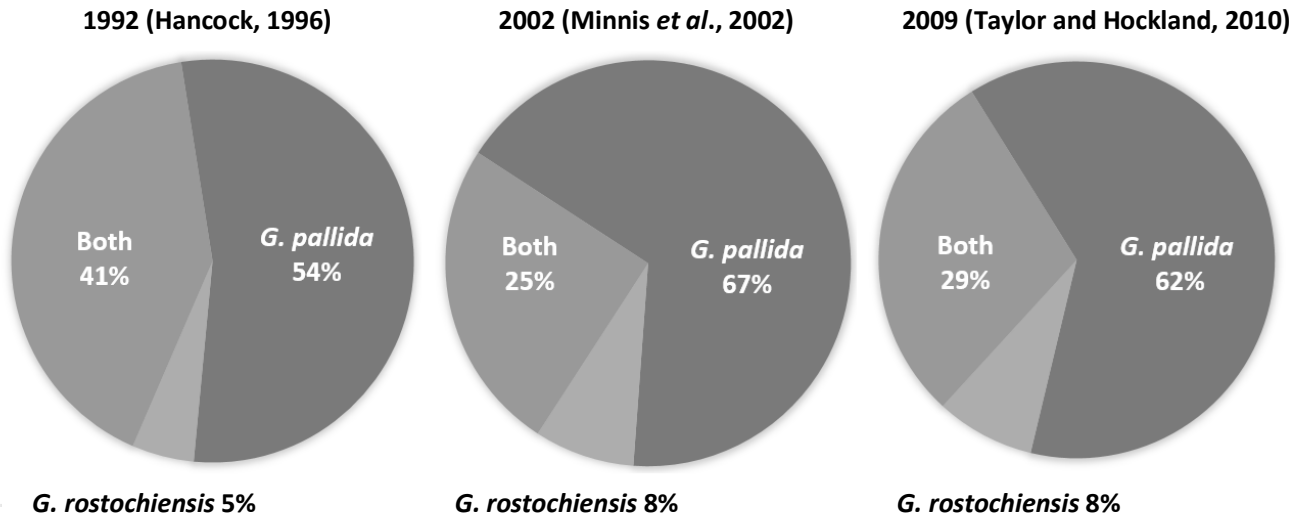

Figure 2.3 The relative proportions of *G. pallida* and *G. rostochiensis* in the UK between 1992 and 2009.

Results from the Netherlands also show selection for *G. pallida*. An audit carried out by the EU Food and Veterinary Office (FVO) in the Netherlands in 2000 identified *G. pallida* in 80% of PCN-infested samples, *G. rostochiensis* comprised 15% and 5% were mixed populations. PCN infestation in the Netherlands declined from 90% in 2000 to 60% in 2011; however, the proportion of *G. pallida* increased (FVO, 2013). Results from annual PCN surveys carried out in fields with both ware and starch potatoes in 2010-2011 revealed that 87% of PCN-infested samples contained pure *G. pallida*, pure *G. rostochiensis* constituted 7% and 6 % of samples had both species (FVO, 2013). These data from the

UK and the Netherlands confirm the apparent dominance of *G. pallida* and highlight the critical need for effective *G. pallida* control measures.

This transition in species composition reflects the greater control of *G. rostochiensis* and the less effective control of *G. pallida* (§ 1.7.2). Conventional PCN management schemes employ nematicides, resistant varieties and crop rotation and appear quite effective in the control of *G. rostochiensis* but are less effective against *G. pallida.* As a result, the control measures appear to select for *G. pallida*-dominant populations. Current rotations have an insufficient duration to accommodate the prolonged decline rate of *G. pallida.* Furthermore, nematicides with a short half-life degrade to ineffective levels by the time of *G. pallida* juvenile emergence and only 8% of planted areas in the UK use *G. pallida* resistant varieties, which only confer partial resistance (Haydock & Evans, 1998; Trudgill *et al.*, 2003). In addition, there is a greater degree of heterogeneity among *G. pallida* populations and pathotypes. The extent of genetic variance in *G. pallida* populations is evident in the range of virulence, which ranges between 4 - 90%, on partially resistant clones derived from *Solanum vernei* (Blok *et al.*, 1997; Phillips & Trudgill, 1998). Consequently, the greater *G. pallida* heterogeneity appears to select for increased virulence (Blok *et al.*, 2000; Hockland *et al.*, 2012).

There are very little data available on PCN in Ireland. However, *G. rostochiensis* is the dominant species. cPCR analyses of 163 PCN-positive samples from ware and seed land in Ireland in 2009-2010 revealed that 8% of PCN-positive samples were mixed populations, 8% were pure *G. pallida* and 84% were pure *G. rostochiensis* (DAFM, 2011).

2.1.3. Phytosanitary regulations

PCN pose a significant threat to the potato industry and are subject to stringent phytosanitary regulations accordingly (§ 1.7.1). The Council Directive 2007/33/EC on the control of potato cyst nematodes was transposed into Irish law in July 2011 (S.I. No. 359/2011). The directive consolidates PCN sampling methods and requirements.

Previously, few surveys of PCN populations were conducted within Europe. Article 9(2) of the directive requires member states to survey a minimum of 0.5% of land used for planting potatoes other than land intended for seed production, although the detection methods are not specified. This survey of ware acreage will facilitate future assessment of the incidence and distribution of PCN in EU potato-growing regions. The directive does not call for species discrimination in statutory soil samples of ware and seed production area. However, Article 11 stipulates species identification and pathotyping in the event of PCN overcoming varietal resistance or a change in the effectiveness of a PCN resistant variety.

2.1.4. PCN control

The change in species composition, particularly the increasing prevalence of *G. pallida,* is generally attributed to the successful control of *G. rostochiensis*, compared to *G. pallida* by conventional control measures (§ 2.1.2). Conventional PCN control strategies, which integrate the use of nematicides, resistant varieties and crop rotation, are generally effective in the management of *G. rostochiensis* but do not confer the same degree of control against *G. pallida* (§ 1.7.2).

Article 9(2) of the Directive permits ware potato growth in PCN infested land provided an official control programme aimed at PCN suppression is implemented. Prior to this introduction, Ireland did not have a standardized national PCN management plan. Resistant cultivars and nematicides are similarly underutilized and rarely advertised as control measures. PCN, however, is gaining recognition as 10-15% of Irish land sampled is infested with PCN (Griffin *et al.*, 2015). Current PCN management guidelines outlined by Teagasc, the Agriculture and Food Development Authority in Ireland include:

- Efforts to minimise the dispersal and spread of PCN.
- Implementation of longer rotations and introduce new land into rotation.
- Use of certified seed.
- Promotion of PCN resistant varieties.

In effect, good agricultural practice, namely crop rotation, is the primary form of control practiced in Ireland for PCN management.

2.1.4.1. Crop rotation

PCN can remain dormant in the soil for up to 30 years; however, most juveniles emerge within the first six years and PCN population levels decline considerably after several years in the absence of a host crop (§ 1.7.2.1). A theoretical annual population decline of 30% was proposed by Hancock (1988), inferring a minimum seven-year rotation period to reduce PCN densities below the economic threshold (Smith *et al.*, 1997). However, crop rotation may involve the sub-optimal use of land, if land is planted with a less profitable crop. This may consequently impose economic deficits and most potato growers implement shorter rotations accordingly. In the EU, four-year rotations are mandatory for seed certification. In Ireland, a minimum of six years is recommended for pre-basic grades and four years for ware production (D. Murphy, DAFM, pers. comm.), although anecdotal reports indicate that two- to three-year rotations are common.

Crop rotation confers a different level of control of the two PCN species (§ 1.7.2.1). *G. rostochiensis* is more vulnerable to crop rotation control as it has a faster decline rate in soil relative to *G. pallida*. (§ 1.5.2). Therefore, longer rotation periods are necessary for adequate *G. pallida* control. In Ireland, 6-year rotations are recommended; whereas, shorter two to four-year rotations are implemented in the UK and Europe. These, shorter rotations could select for *G. pallida* due to the insufficient duration to accommodate the prolonged decline rate of *G. pallida* (Whitehead, 1997). In longer rotations, the disparity in decline rates between the two species is negligible as *G. pallida* population levels decline to sufficiently low levels negating selection pressure.

2.1.5. Nematicide use in Ireland

Although nematicides are an integral element of PCN control in the UK and Europe, they are not extensively utilised in Irish agricultural systems. This may be due to the

considerable expense of nematicides, which is estimated at €247 ha^{-1} (DAFM, 2011). Similarly, there is also a negative commercial impact attached to PCN infestation, owing to the Annex I designation of the pest. As a result, many potato growers will not acknowledge PCN infestation; hence, it is difficult to ascertain the extent of nematicide usage for PCN control in Ireland (D. Murphy, DAFM, pers. comm.). The vast majority of potato growers use nematicides for the reduction of wireworm that are vectors for tuber diseases particularly spraing, which is associated with tobacco rattle virus (TRV).

Several of the active substances (a.s.) used to control PCN have been withdrawn by the EU due to environmental concerns, most recently Temik® (§ 1.7.5). Following implementation of the European Directive (2007/33/EC), three nematicide products are currently registered for use in Ireland, namely, Vydate® (a.s. oxamyl), Nemathorin® (a.s. fosthiazate) and Mocap® (a.s. ethoprophos). Nemathorin is mainly marketed in Ireland to control wireworm and spraing in Ireland (B. Cotter, Syngenta pers. comm.). Mocap is relatively unavailable in Ireland due to distribution difficulties (C. Maughan, Whelehan Crop Protection, pers. comm.). Although Vydate has been recently re-introduced to the register of permissible pesticides for use in Ireland, it is not commercially available and global supply shortages are not expected to be replenished until 2017 (FWI, 2015). Fumigants are not a viable option in Ireland as there are currently no agricultural contractors offering fumigation services. In effect, the market for nematicides in Ireland is relatively unexploited owing to the expense of and the lack of availability of nematicides, which further reflects the insignificance of nematicides in Irish potato production.

2.1.6. Resistant cultivars

Resistant cultivars can potentially reduce *G. rostochiensis* populations by 80% (Whitehead & Westerdijk, 1987) and as a result, the widespread deployment of *H1*- and *H2*-containing cultivars has inadvertently selected for *G. pallida*. Varieties expressing partial resistance to *G. pallida* are few in number; only three such varieties are present on the register of varieties in Ireland (Table 2.1). Ware potato production in Ireland is primarily for the

domestic market; therefore, the types of varieties grown generally reflect consumer preferences, marketability and profitability.

In Ireland, consumers exhibit preferences towards older potato varieties such as 'Golden Wonder' and 'British Queen', that evolved from older lineages and were primarily bred for pathogen resistance. Maincrop varieties account for over 70% of total production with the balance being made up of early and processing variety production (Fig. 2.4). The ware potato market in is dominated by four potato varieties; 'British Queen' (second early) and the maincrop varieties; 'Rooster', 'Kerr's Pink' and the very late maincrop 'Golden 'Wonder'. These varieties do not have resistance to either *G. rostochiensis* or *G. pallida*. Indeed, the use of resistant cultivars against PCN is relatively limited in Ireland (Table 2.1; Fig. 2.5). Most varieties with PCN resistance are processing varieties or grown as seed for export and occupy a small proportion of the total potato production area in Ireland. *G. rostochiensis*-resistant cultivars collectively account for approximately 18% of the total production area (Table 2.1).

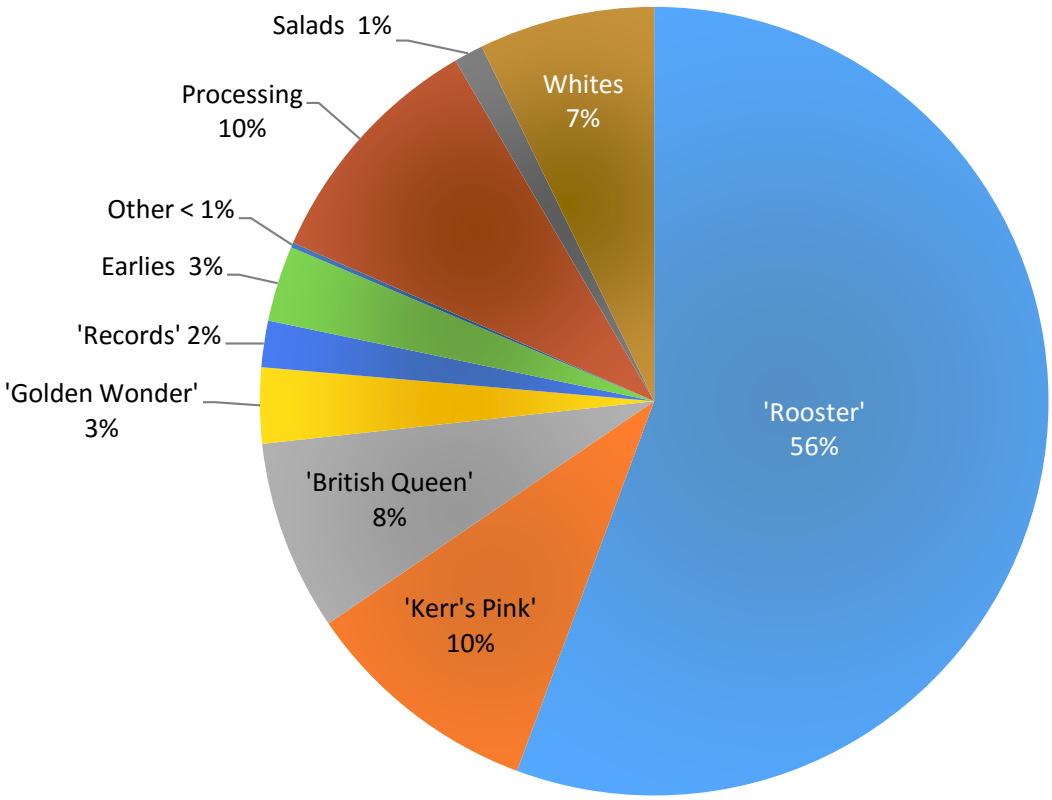

Figure 2.4 Types of potato varieties grown in Ireland represented as a proportion of the total potato growth area (DAFM, 2011).

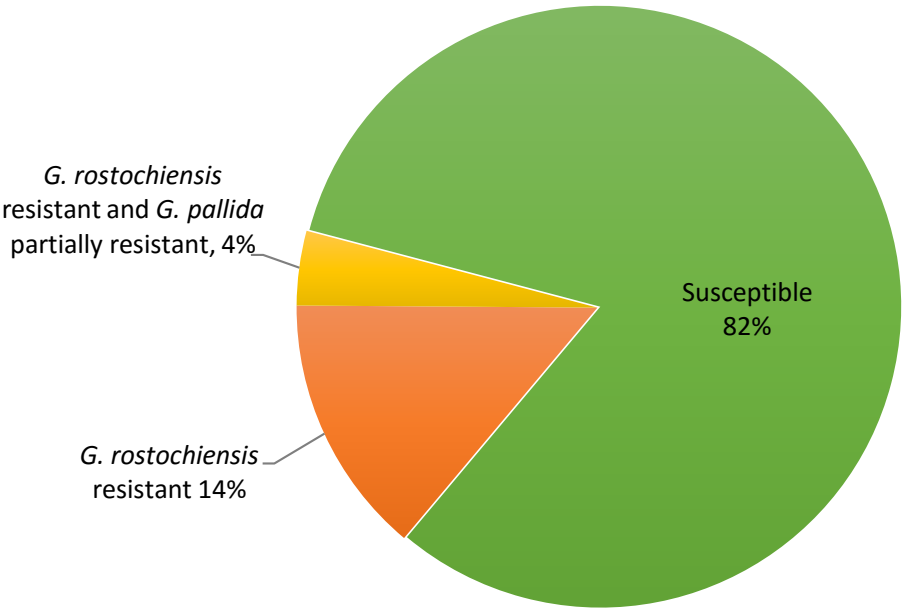

Figure 2.5 Breakdown of PCN resistant cultivars used in Ireland represented as a proportion of PCN infested land (DAFM, 2011).

Table 2.1 Types of potato varieties planted in Ireland (DAFM, 2011).

			Resistance status*		
Cultivar	**Area (ha)**	**% ware area**	**Gr**	**Gp**	**Susceptible**
Rooster	13040.56	55.72			✓
Kerr's Pink	2307.96	9.86			✓
Queens	1836.07	7.85			✓
Golden Wonder	718.55	3.07			✓
Maris Piper	638.32	2.73	r		
Cultra	582.16	2.49	r		
Lady Jo	564.83	2.41			✓
Lady Rosetta	546.85	2.34	r		
Sassy	530.17	2.27	r	pr	
Record	455.71	1.95			✓
Lady Claire	417.20	1.78	r	pr	
Home Guard	316.43	1.35			✓
Premier	281.13	1.20	r		
Maris Peer	236.20	1.01			✓
Markies	174.58	0.75	r		
Ramus	101.41	0.43	r	pr	
Estima	83.44	0.36			✓
Marfona	64.18	0.27			✓
Osprey	64.18	0.27			✓
Cabaret	59.05	0.25	r		
Casablanca	55.20	0.24	r		
Wilja	53.92	0.23			✓
Mozart	51.35	0.22	r		
Karlena	50.06	0.21			✓
Navan	43.65	0.19	r		
Sunrise	25.67	0.11			✓
Melody	23.11	0.10	r		
Courage	19.26	0.08	r		
Fianna	19.26	0.08	r		
Hunter	19.26	0.08			✓
Daisy	8.99	0.04	r		
San Piper	8.99	0.04			✓
Cara	5.13	0.02	r		✓
	23402.82		**4.5%**	**2.2%**	**95.2%**

*r = resistant, pr = partially resistant

2.1.7. PCN infestation in Ireland

To date, there are very little data available on the status of PCN infestation in Ireland; most of the national records pertain to the seed potato area, which requires statutory PCN testing. Sampling records obtained from the Department of Agriculture, Fisheries and Marine (DAFM) between 2009 and 2010 revealed that 11% of ware land and approximately 2% of the seed land tested positive for PCN (DAFM, 2011). Recent figures show an increase in PCN incidence; an average of 7% of seed samples and 17% of ware samples tested positive for PCN between 2010 and 2013 (Fig. 2.6).

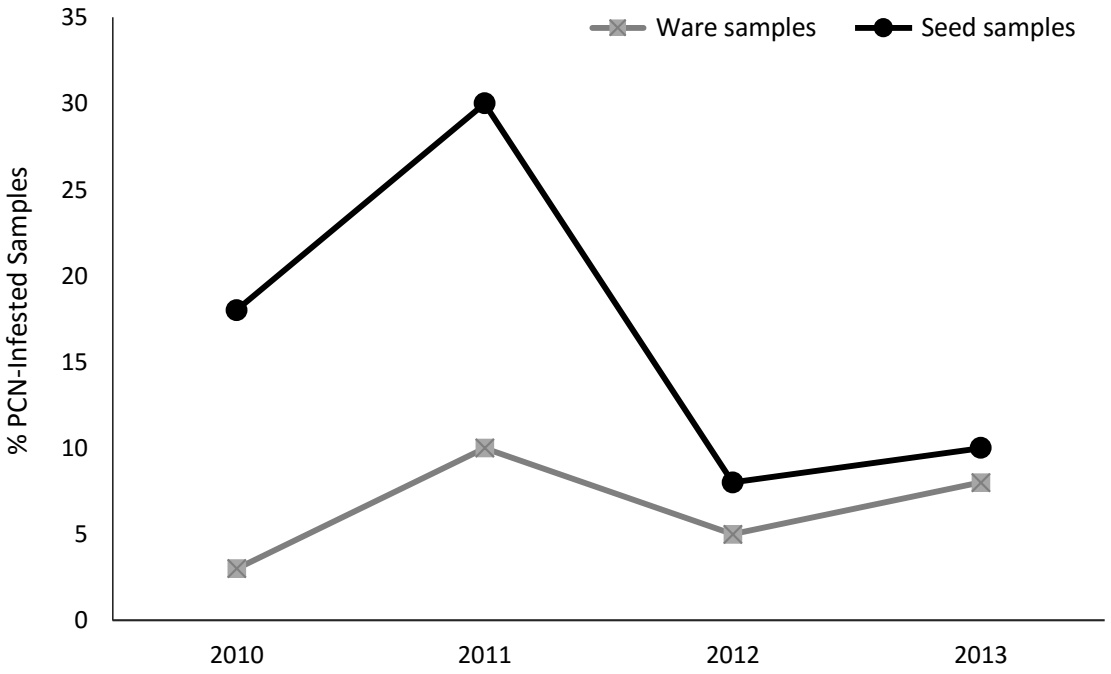

Figure 2.6 PCN infestation levels in ware and seed potato produce in Ireland between 2010 and 2013. From Rigney (2015).

A national PCN survey in 2002 (Rigney, 2015) revealed that 95% of PCN-infested samples contained *G. rostochiensis,* of which 84% were pure *G. rostochiensis* populations (Fig. 2.7). *G. pallida w*as present in 16% of infested samples, of which 5% were pure *G. pallida* and 11% were mixed-species populations. The statutory PCN sampling of seed production areas merely test for the presence of PCN in soil samples and does not discriminate between species. As a result, very few statistics are available for the relative proportion of PCN species in infested samples.

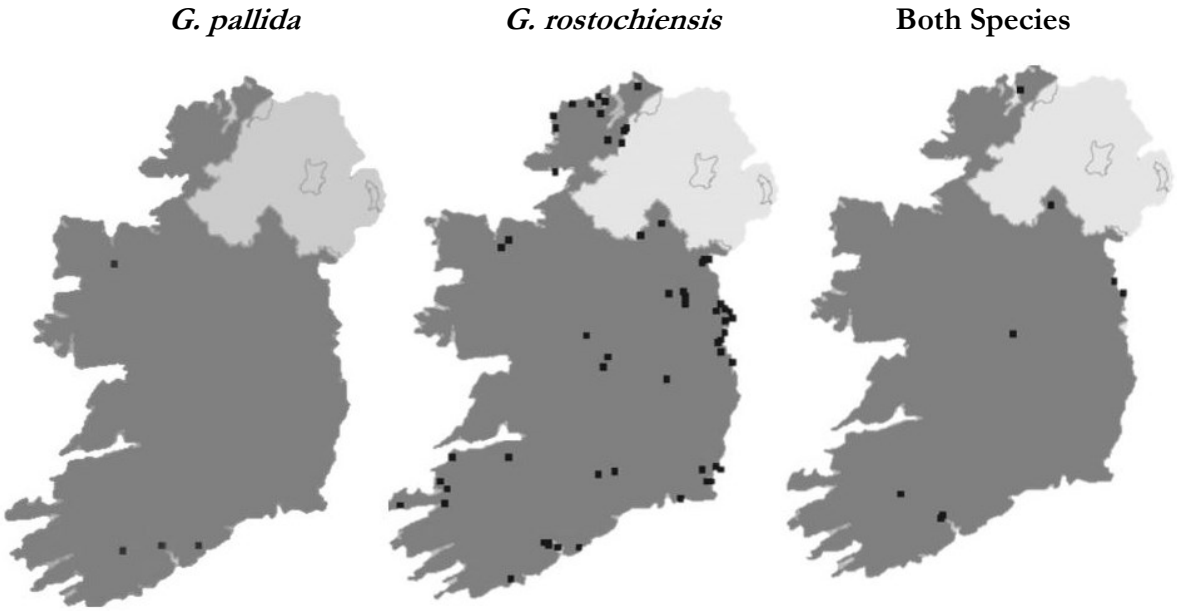

Figure 2.7 A national survey of PCN infestation in Ireland in 2002 (Rigney, 2015).

2.2. Aims

This chapter aims to assess the relative proportions of *G. pallida* and *G. rostochiensis* in Ireland from PCN-infested soil samples. The efficacy of conventional and real-time PCR methods in particular, are compared for species discrimination and quantification.

2.3. Materials and Methods

2.3.1. Nematode populations

PCN cyst samples was supplied by Teagasc, Oakpark Research Centre, Co Carlow. In total, 140 PCN cyst samples were analysed. The samples were acquired from statutory PCN soil samples from ware and seed production areas and were submitted in 2010/11 to the Department of Agriculture, Fisheries & Marine to determine the presence of PCN.

2.3.2. DNA isolation (Reid *et al.*, 2010; Kaczmarek, 2014)

Genomic DNA was extracted from isolated cysts using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Crawley, UK). Cysts were transferred to 2 ml screw-cap microtubes containing two 5 mm metal beads (Qiagen, Crawley, UK) and subjected to homogenization by bead beating at maximum speed for 30 s in a mini-bead beater (Mini-

Beadbeater-24, Biospec Products, OK, USA). After dry beating, an aliquot of 180 µl ATL buffer (Qiagen, Crawley, UK) was added to each sample and the sample was re-homogenised in the bead beater for 30 s at maximum speed (Reid *et al.*, 2010). Samples were denatured by the addition of 20 µl proteinase K, vortexed for 15 s and incubated at 56ºC for 2 h in a rotating incubator (New Brunswick Scientific, Edison). Samples were subsequently vortexed for 15s and centrifuged at 8000 *g* for 1 min. The lysate was transferred to DNeasy spin-columns for purification of total DNA according to the manufacturer's guidelines. DNA was eluted twice with 200 µl AE (Qiagen, Crawley, UK) elution buffer. The concentration of the eluted DNA was quantified using a NanoDrop 1000c spectrophotometer (ThermoFisher, Wilmington, DE, USA).

2.3.3. cPCR (Bulman and Marshall, 1997)

Extracted nematode DNA was analysed in a multiplex PCR reaction according to the methods of Bulman and Marshall (1997). Target nematode sequences were amplified with a universal reverse primer ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG; White *et al.*, 1990), the *G. rostochiensis*-specific primer PITSr3 (AGCGCAGACATGCCGCAA-3') and *G. pallida*-specific primer PITSp4 (5'- ACAACAGCAATCGTCGAG-3').

DNA was amplified in 25 µl PCR reactions comprising 1X GoTaq PCR reagent (Promega Madison, WI, USA), 1.5 mM $MgCl_2$, 0.2 µM of each dNTP, 0.4 µM of each primer ITS5, PITSR3, PITSP4 (Invitrogen, Paisley, UK), 1 U HotStart Taq DNA Polymerase (Promega Madison, WI, USA) and 1 µl template DNA at 2 ng μl^{-1}. Amplification was performed in a Master Gradient Thermocycler (Eppendorf, Hamburg, Germany). The applied thermal profile consisted of an initial denaturation step at 94°C (3 min.), followed by 40 cycles of 94 °C (45 s), 60 °C (60 s), 72 °C (45 s) and a final extension step at 72 °C for 5 min. DNA extracted from purified *G. pallida* or *G. rostochiensis* cysts were used as positive controls. A negative template control was included in each reaction.

PCR products were separated by gel electrophoresis on 2% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen, Paisley, UK). A 1 KB DNA ladder (Sigma-Aldrich, Saint Louis, MO, USA) was loaded for molecular weight reference. The gel was run in 0.5% TBE buffer (Sigma Aldrich, Arklow, Ireland) at 80 V for 1 hour and visualised under UV light using a G:Box transilluminator (Syngene, Cambridge, UK). Images were acquired using GeneSnap (Syngene, Cambridge, UK) and PCR products were analysed with GeneTools image analysis software (Syngene, Cambridge, UK).

2.3.4. qPCR (Reid *et al.*, 2010)

PCN primers and probes used in qPCR reactions were designed in the ITS1 region (Kenyon *et al.*, 2010; Reid *et al.*, 2010). Simplex qPCR reactions were conducted using *G. pallida*-specific MGB® probe 5'-6FAM-CCGCTATGTTTGGGC-3' and the *G. rostochiensis*-specific MGB® probe 5'-VIC-CCGCTGTGTATKGGC-3' labelled with VIC™ (ThermoFisher, Foster City, USA). The primers included the universal PCN forward primer 5'-CGTTTGTTGTTGACGGACAYA-3' and the universal PCN reverse primer 5-GGCGCTGTCCRTACATTGTTG-3' (Eurogentec, Seraing, Belgium).

Reactions comprised 15.0 µl Fast Blue™ 2X qPCR Probe MasterMix (Eurogentec, Seraing, Belgium), 1.25 µl each primer (at 5 pmol μl^{-1}), 1 µl *G. pallida*-specific probe and (at 5 pmol μl^{-1}), 1 µL of the *G. rostochiensis*-specific probe, 6.25 µl sterile deionised water and 5 µl template DNA at 1 ng μl^{-1}. Triplicate 10 µl aliquots of this PCR reaction mixture were analysed on a MicroAmp® 364-well PCR plate (ThermoFisher, Foster City, USA). The PCR plate was sealed with MicroAmp® optical adhesive film (ThermoFisher, Foster City, USA) and was centrifuged at 1500 *g* for 3 min prior to amplification.

Amplification was performed in an ABI 7900HT real-time PCR machine (ThermoFisher, Foster City, USA) run in standard 9600 emulation mode with the following cycling conditions; 60°C for 2 min, 95°C for 10 min followed by 40 cycles of 95° for 15 s and 60°C for 1 min (Reid *et al.*, 2010). Reactions determined absolute quantification and

involved a final dissociation step. A standard curve for each species was constructed using serial dilutions of known quantities of nematode DNA to achieve a curve in the range of 100 ng – 10 pg DNA. Reactions included a positive control for *G. rostochiensis* and *G. pallida*, a negative no-template control and an exogenous qPCR positive control (Eurogentec, Seraing, Belgium). PCR amplification was analysed with Sequence Detection Software (SDS) V 2.4 (ThermoFisher, Foster City, USA).

2.3.5. Comparison of real-time and conventional PCR detection methods

Cysts of single-generation populations of *G. pallida* Pa2/3 and *G. rostochiensis* Ro1 (Northern Ireland populations) were used in the isolation of genomic DNA used in validation and PCR efficiency tests as described in § 2.3.2.

2.3.5.1. Method consistency

qPCR and cPCR results were collated and the results from each sample were individually compared to assess detection consistency between the two methods. Data was represented in binary format with 1 denoting an agreement in detection and 0 representing an inconsistent outcome.

2.3.5.2. PCR efficiency

PCR efficiencies were calculated by amplifying serial dilutions of template DNA in qPCR and cPCR reactions (§ 2.3.4). DNA from 100 *G. rostochiensis* cysts was extracted and diluted as described in § 2.4.3. Ten replicate aliquots were established and triplicates of each were analysed (n = 30). Serial dilutions of 1:10 were performed on each sample and a standard curve was generated from the data. The line of the best fit was analysed using SDS 2.4 for qPCR assays and GeneTools for cPCR assays. Efficiency (E) was calculated with the following equation $E = 10^{(-1/slope)}$. Percentage efficiency was recorded for cPCR and qPCR assays and compared.

2.3.5.3. PCR validation

In order to gauge repeatability and reproducibility (GR&R), a repeated-measures test was performed on template DNA from a 100-cyst sample of *G. rostochiensis*. Nucleic acid extraction was performed as described in § 2.3.2. Five aliquots of template DNA were analysed in a simplex cPCR and qPCR reactions as described in § 2.3.3 and § 2.3.4, respectively. Samples were analysed in duplicate to test repeatability and tests were repeated twice to determine reproducibility (n = 20).

2.3.6. Statistical analysis

The detection of PCN was represented binomially as either positive (1) or negative (0) for the presence of *G. pallida*, *G. rostochiensis* or both species. Data were analysed using non-parametric tests. Differences in cPCR and qPCR results for the detection of *G. pallida* and *G. rostochiensis* were analysed by a Kruskal-Wallis test. PCR efficiency data were also analysed with the Kruskal-Wallis test. The Chi-squared was used to compare detection consistency between cPCR and qPCR assays. The degree of consistency within each test method was analysed by the Wilcoxon signed rank test. The PCR validation assay dataset was normally distributed and was analysed by a three-way ANOVA. Data from cPCR and qPCR assays were analysed with 2-way repeated-measures parametric ANOVAs to gauge repeatability and reproducibility (GR&R) for each method.

The relative proportion of *G. pallida* and *G. rostochiensis* in Irish PCN populations was analysed by a Kruskal-Wallis test. A Chi-square test was used to analyse changes in the incidence of *G. pallida* and *G. rostochiensis* over time using Irish, UK and Dutch PCN data presented in § 2.1.2. All multiple comparisons were performed using the Tukey test. Significance was defined as $P < 0.05$. Statistical tests were performed using SigmaPlot V.12.5. (Systat Software, Inc. Erkrath, Germany).

2.4. Results

2.4.1. cPCR results

PCR products were visualized against a 1Kb DNA ladder; *G. pallida* was detected as a band at 256 bp and *G. rostochiensis* at 434 bp (Fig. 2.9). cPCR assays revealed *G. rostochiensis* as the predominant species, which was detected in 83% of samples and *G. pallida* was detected in 66% of samples. In total, 49% of samples contained both *G. pallida* and *G. rostochiensis* (Fig. 2.8), while pure *G. pallida* and pure *G. rostochiensis* populations comprised 17% and 34 %, respectively.

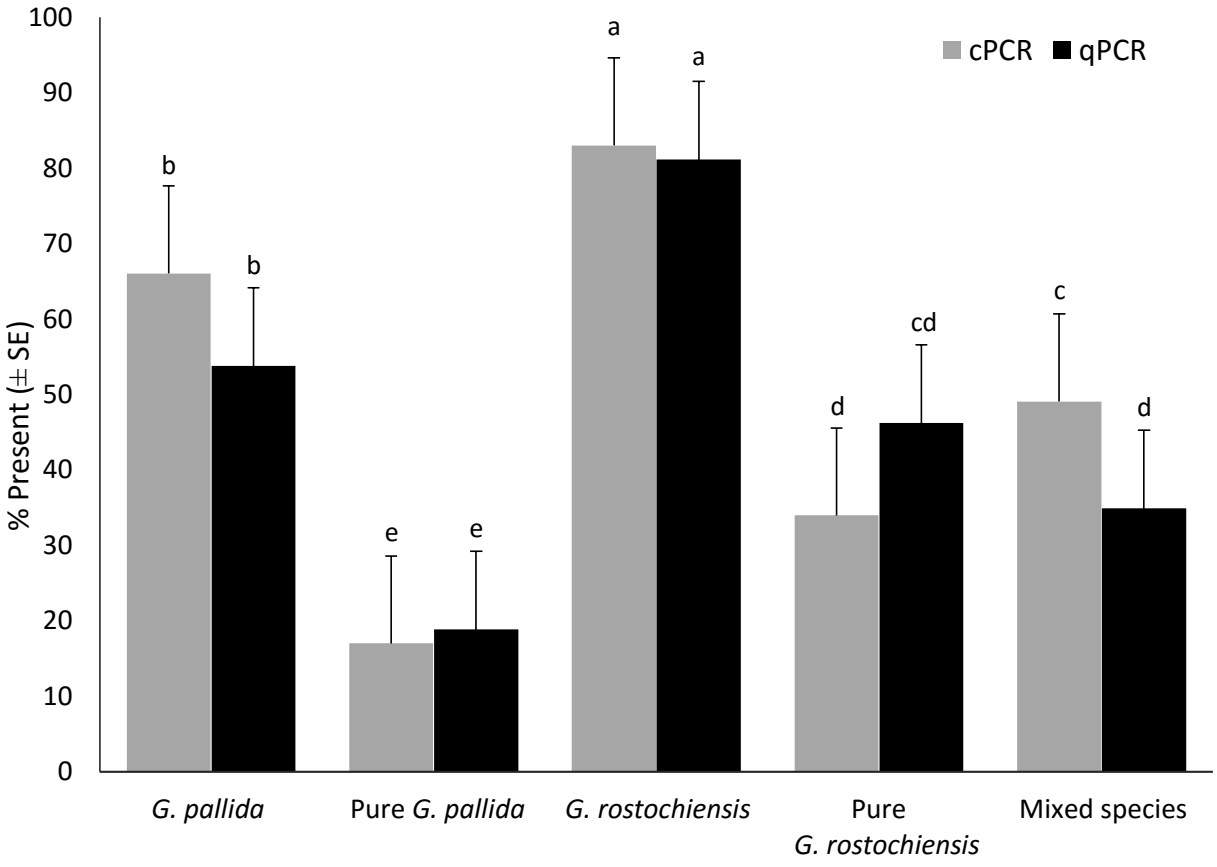

Figure 2.8 Species proportion of PCN in Ireland determined by cPCR and qPCR methods. Samples with a common letter are not significantly different ($P > 0.05$), using the Tukey Test.

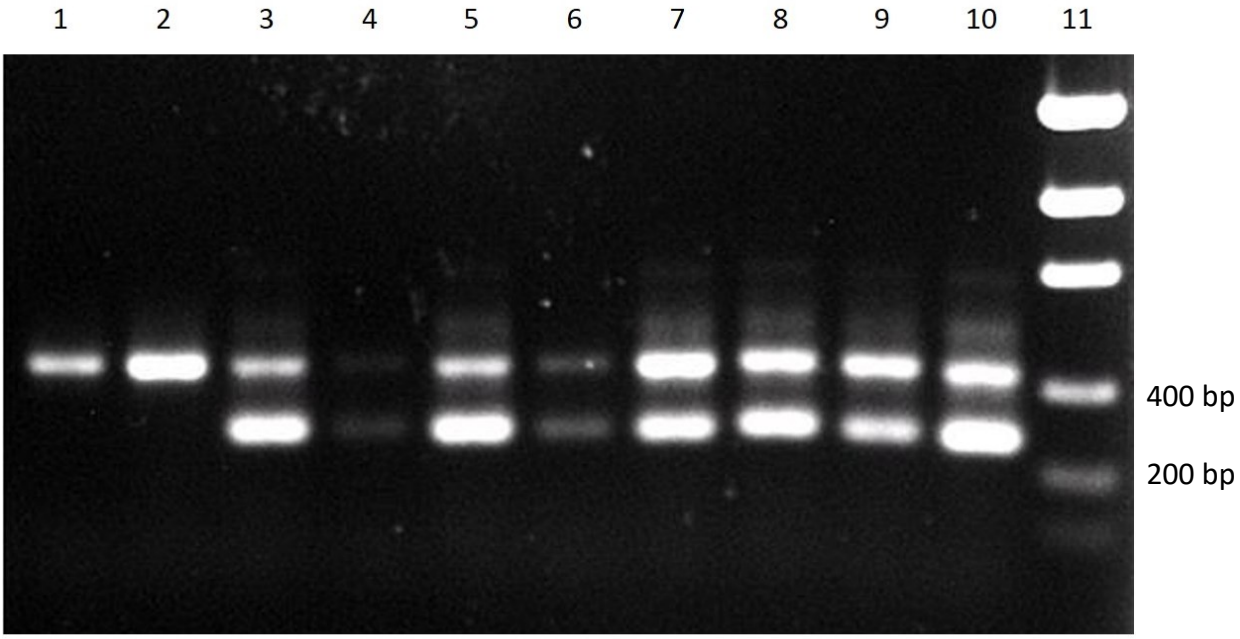

Figure 2.9 cPCR analysis of PCN populations from different samples. *Globodera pallida*-specific bands appeared at 256 bp and *G. rostochiensis* at 434 bp; lane 11 contains a 1 Kb DNA ladder.

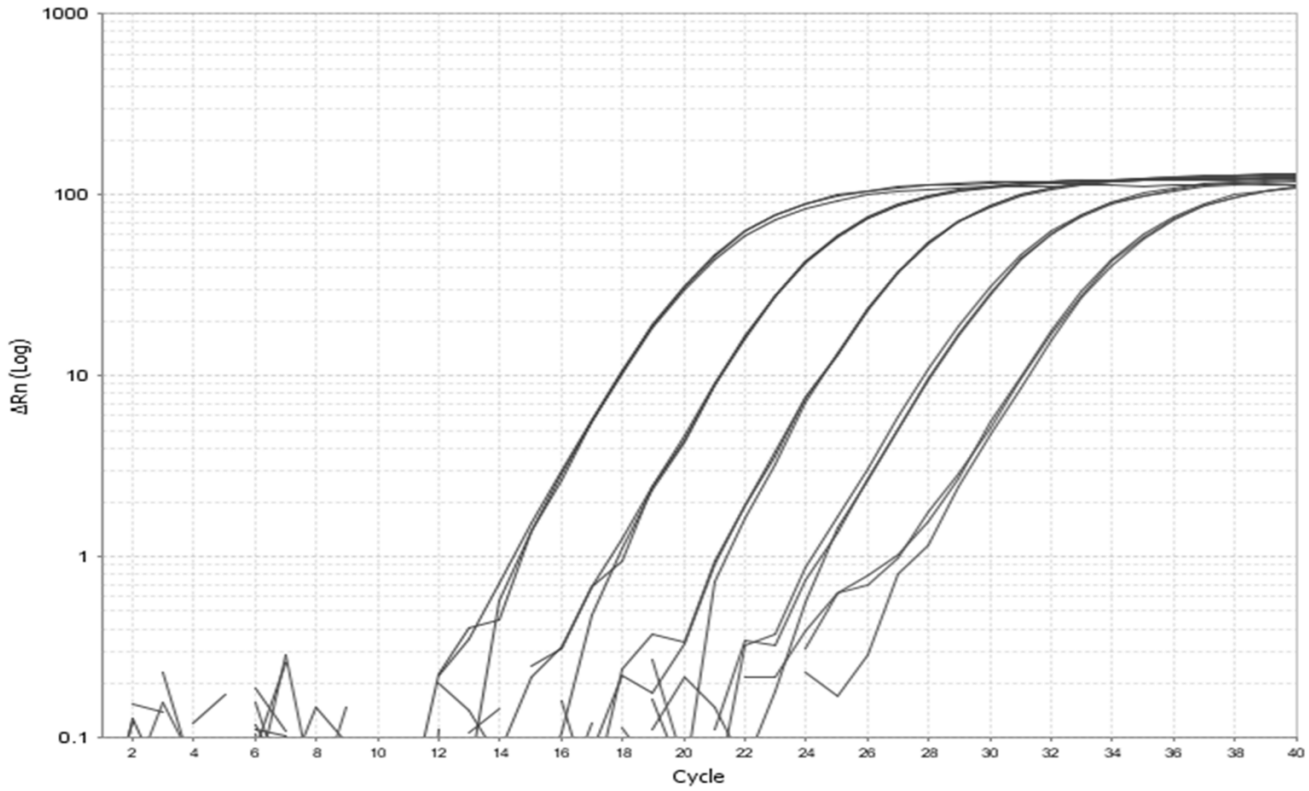

Figure 2.10 Detection of *G. rostochiensis* using ExpressionSuite Software 1.04 (ThermoFisher, Carlsbad, CA, USA). The amplification plot depicts 1:10 serial dilutions of 100 *G. rostochiensis* cysts.

2.4.1.1. qPCR results

qPCR assays (Fig. 2.10), 35% of samples detected both *G. pallida* and *G. rostochiensis* (Fig. 2.8). *G. rostochiensis* was the predominant species and was detected in 81% of the samples, while *G. pallida* was identified in 54% (Fig. 2.8). Pure *G. pallida* populations represented 19% and pure *G. rostochiensis* populations comprised 46%, with 35% representing mixed-species samples.

2.4.2. Comparison of c PCR and qPCR analyses

2.4.2.1. Method consistency

qPCR and cPCR values were compared and revealed significant differences in detection consistency ($\chi^2_{(2)} = 374.54$; $P < 0.001$). *G. rostochiensis* was detected in 83% of samples by cPCR and 81% by qPCR. Detection of *G. rostochiensis* by both cPCR and qPCR was consistent in 96 samples with an overall consistency of 69%, *G. pallida* was detected in 92 samples tested by cPCR and in 76 samples by qPCR (Fig. 2.11). There was a consistency level of 56% between qPCR and cPCR methods for *G. pallida* detection methods.

There was a significant difference between comparability levels ($H_{(1)}= 8.142$; $P < 0.01$). The lowest level was evidenced in mixed-species population where consistency in the concomitant detection of *G. pallida* and *G. rostochiensis* was 50%. The degree of consistency within samples analysed by the Wilcoxon signed rank test revealed a significant difference between qPCR and cPCR methods ($Z_{(1)}= -2.14$; $P < 0.05$) for *G. pallida* detection. No significant difference between test methods was observed for the detection of *G. rostochiensis* ($Z_{(1)} = 1.13$; $P > 0.05$).

2.4.2.2. PCR efficiency

PCR efficiency was assessed by the slope of the standard curve (Fig. 2.14). Analysis of the data using non-parametric Kruskal-Wallis test revealed a significant difference between qPCR and cPCR efficiencies ($H_{(1)}$= 13.26; $P < 0.001$). Conventional PCR efficiencies averaged (mean ± SE) 96.2% ± 0.43 (Fig. 2.12). The average level of qPCR efficiency was 99.05% ± 0.18 (Fig. 2.13).

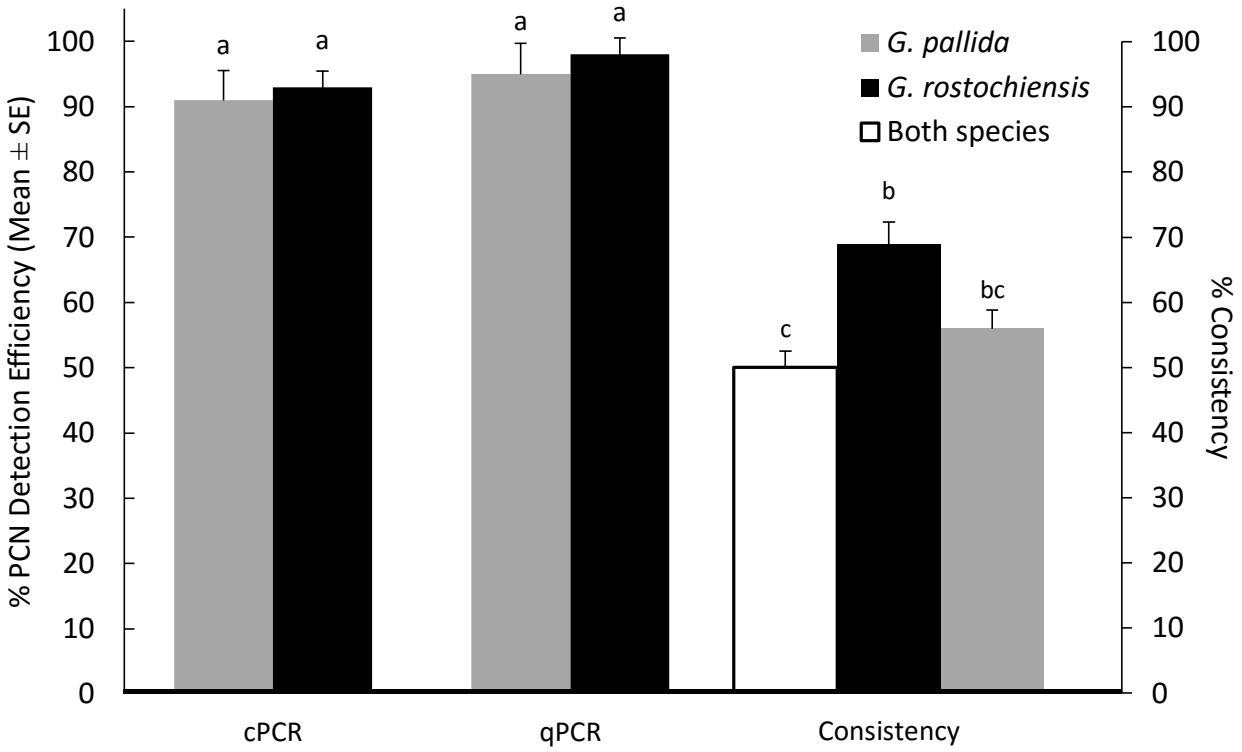

Figure 2.11 Level of consistency and efficiency in the detection of *G. pallida* and *G. rostochiensis* by cPCR and qPCR. Samples with a common letter are not significantly different ($P > 0.05$) using the Tukey test.

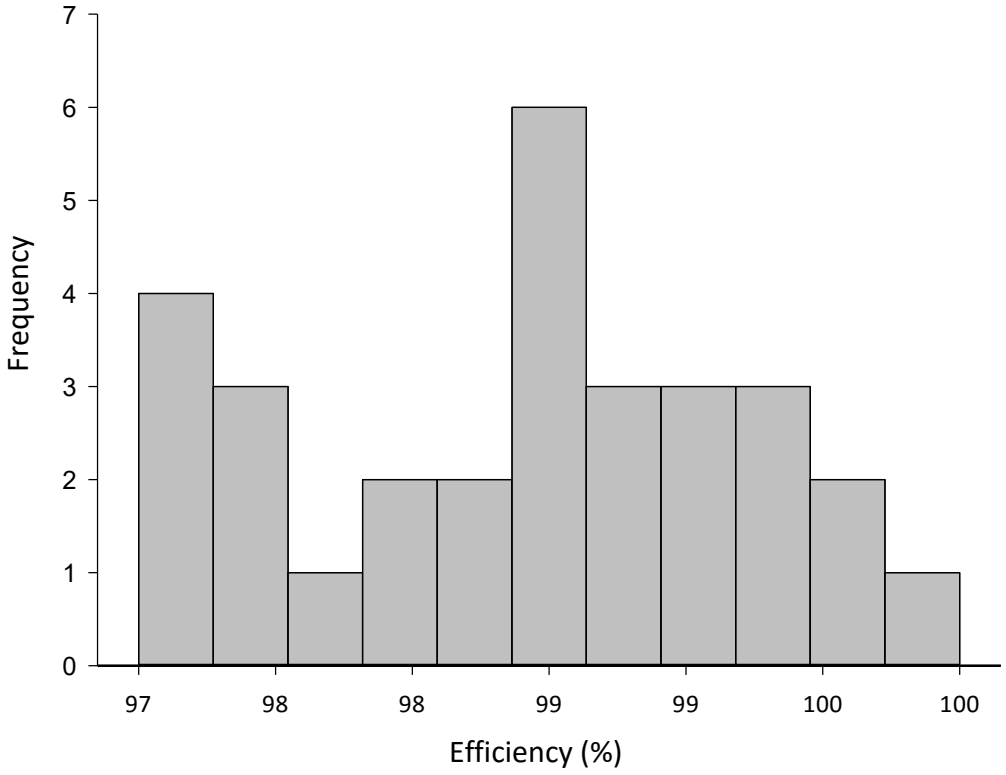

Figure 2.12 Frequency distribution of cPCR efficiency.

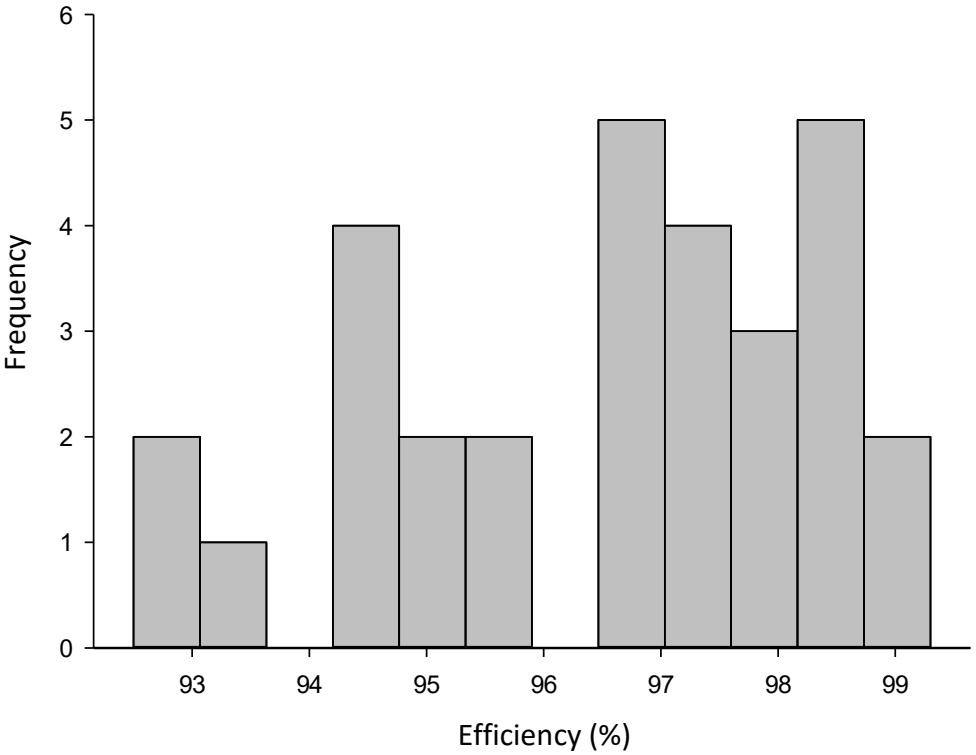

Figure 2.13 Frequency distribution of qPCR efficiency.

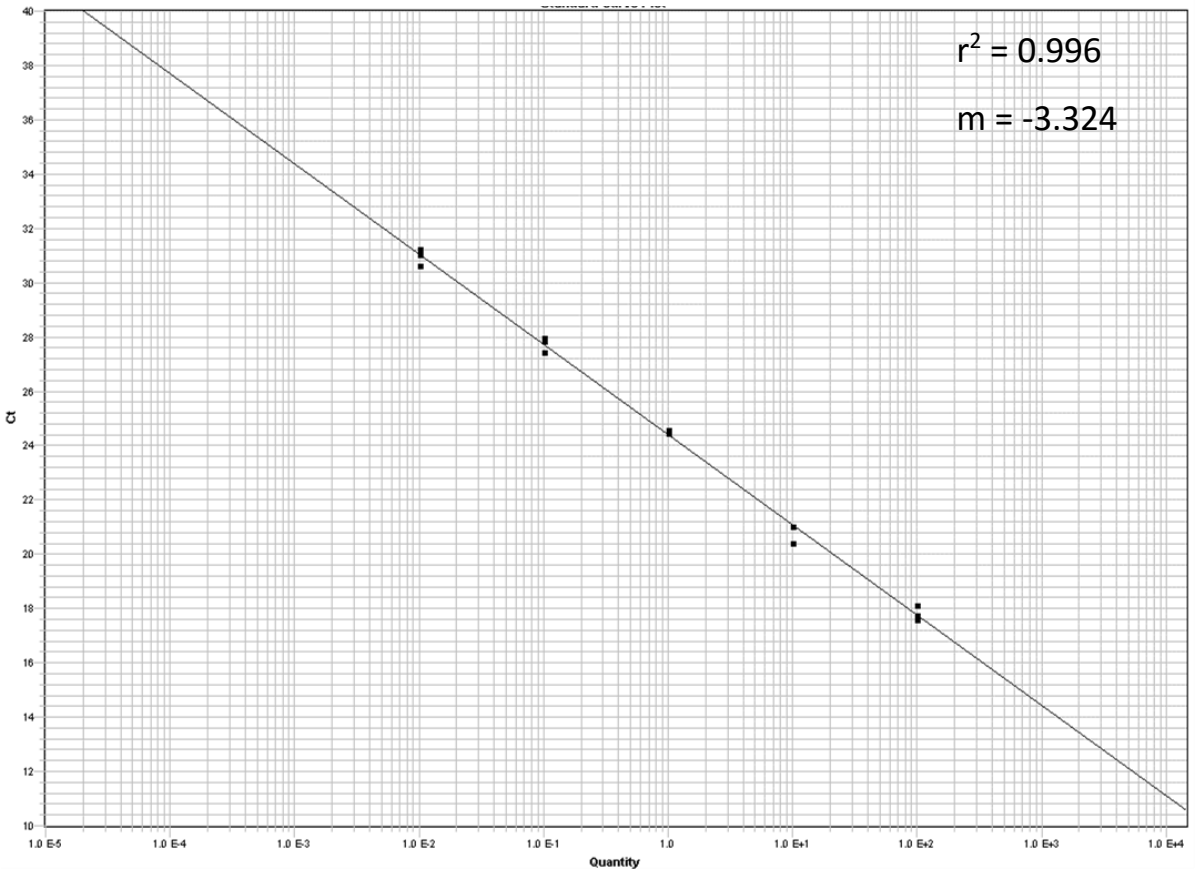

Figure 2.14 Standard curve of 1:10 serial dilutions of known quantities of *G. rostochiensis* DNA plotted as C_t value vs. quantity (*G. rostochiensis* eggs).

2.4.2.3. Validation of PCR methods

Three-way ANOVA tested repeat assays and the degree of variance within each assay (Table 2.2). There appeared to be a difference between cPCR and qPCR test methods; however, the result was not significant ($F_{(1,32)} = 4.09$; $P = 0.052$). There was a significant difference between replicate samples ($F_{(1,32)} = 8.34$; $P < 0.001$). However, there was no significant difference between repeated tests ($F_{(1,32)} = 0.937$; $P > 0.05$). The two-way repeated measures ANOVA (Table 2.3) revealed this significant difference in cPCR assays ($F_{(1,4)} = 12.98$; $P < 0.05$). No significant differences were noted for qPCR assays (Table 2.4; $F_{(1,4)} = 0.19$; $P > 0.05$).

The total GR&R for cPCR was 35.54% with a repeatability and reproducibility of 87% and 76% respectively. qPCR scored a GR&R value of 21.35%, with 98.4% repeatability

and 99.2% reproducibility. qPCR assays revealed a higher degree of test sensitivity and produced results with more precision and accuracy relative to cPCR tests (Fig. 2.15). Although test methods did not differ significantly (P = 0.052), there was a significant degree of variation in cPCR tests (Fig. 2.15) that reduced reproducibility and reliability.

Table 2.2 Three-way ANOVA comparing cPCR and qPCR assays.

Source of Variation	DF	SS	MS	F	*P*
Method	1	52.67	52.67	4.087	0.052
Test	1	107.52	107.52	8.343	<0.001
Repeat	1	12.08	12.08	0.937	>0.05
Method x Test	1	39.84	39.84	3.091	>0.05
Method x Repeat	1	7.85	7.85	0.609	>0.05
Test x Repeat	1	2.01	2.01	0.156	>0.05
Method x Test x Repeat	1	2.53	2.53	0.196	>0.05
Residual	32	412.39	12.89		
Total	39	636.88	16.33		

Table 2.3 Two-way RM ANOVA cPCR summary table.

Source of Variation	DF	SS	MS	F	*P*
Test	1	139.15	139.15	12.98	<0.05
Repeat	1	19.73	19.73	0.91	>0.05
Test x Repeat	1	4.52	4.52	0.50	>0.05
Residual	4	36.29	9.07		
Total	19	339.08	17.85		

Table 2.4 Two-way RM ANOVA qPCR summary table.

Source of Variation	DF	SS	MS	F	*P*
Test	1	8.23	8.23	0.19	>0.05
Repeat	1	0.23	0.23	0.35	>0.05
Test x Repeat	1	0.02	0.02	0.10	>0.05
Residual	4	0.62	0.16		
Total	19	245.23	12.91		

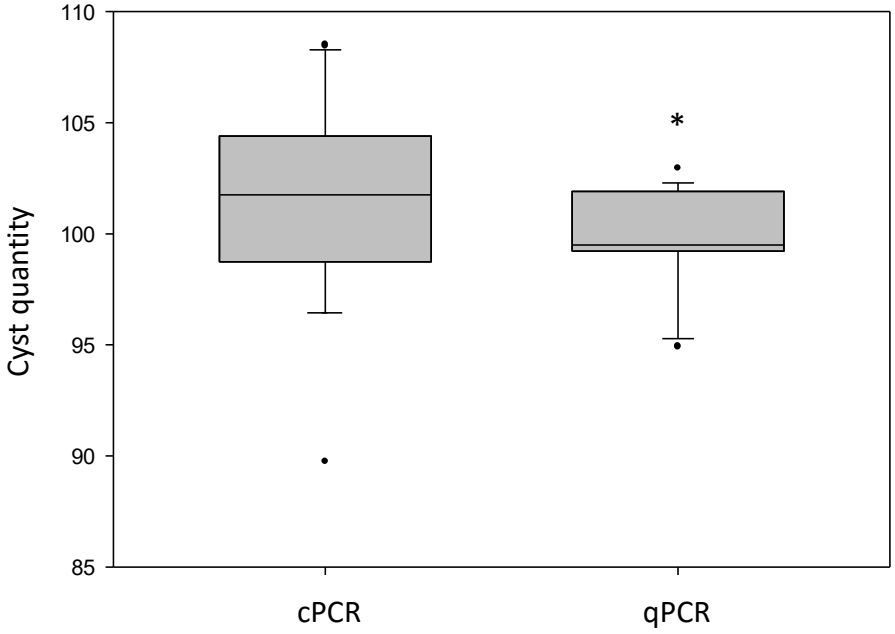

Figure 2.15 Quantitative analysis of PCN samples analysed by cPCR and qPCR. The range is denoted by (●). An asterisk denotes a significant difference between samples ($P < 0.05$) using the Tukey test.

2.4.3. Relative proportion of *G. rostochiensis* and *G. pallida* in Irish populations

Simplex qPCR assays were performed to validate the findings of earlier cPCR and qPCR. Inconclusive results were omitted. The results concluded that *G. rostochiensis* was present in 82% of PCN-infested samples; pure *G. rostochiensis* populations represented 36% (Fig. 2.16). *G. pallida* was present in 64% of the total samples, while 18% were pure *G. pallida* populations and 46% contained both species. There was a significant difference in species proportions ($H_{(1)}$= 34.09; $P < 0.001$), namely between pure *G. pallida* and pure *G. rostochiensis* populations ($H_{(1)} = 25.24$; $P < 0.001$) and between *G. pallida* and mixed-species populations ($H_{(1)} = 29.17$; $P < 0.001$). No difference was observed between pure-*G. rostochiensis* and mixed-species populations ($H_{(1)} = 0.16$; $P > 0.05$).

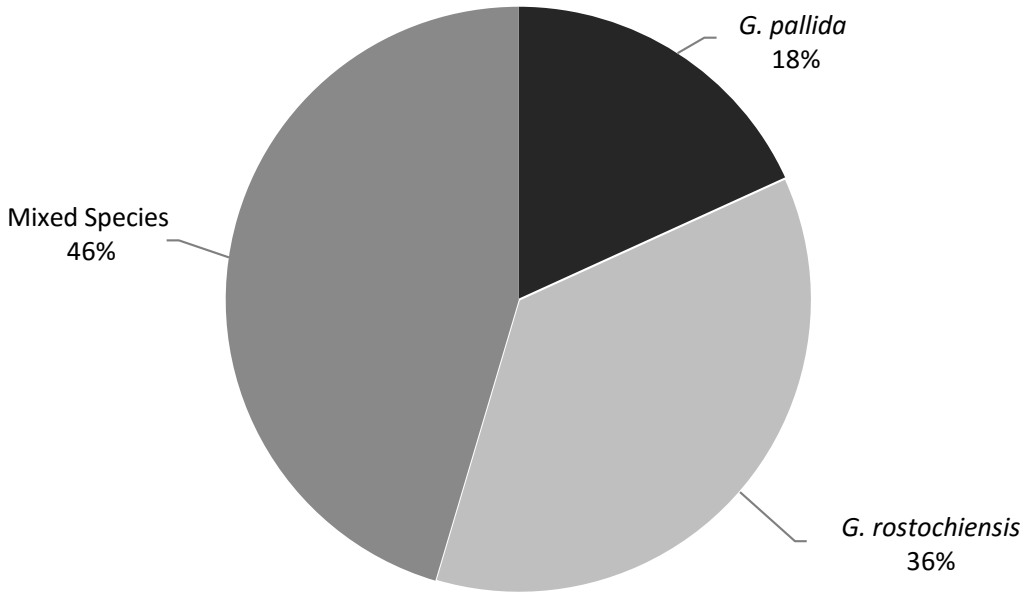

Figure 2.16 The relative proportion of *G. pallida* and *G. rostochiensis* in Irish PCN populations in 2011 using qPCR analyses.

2.4.4. Changes in the frequency of PCN species over time

Chi-squared analysis revealed a significant change ($\chi^2_{(4)} = 72.8$; $P < 0.001$) in the incidence of PCN in Ireland between 2002 (Rigney, 2015) and 2011 (this study). No significant difference ($\chi^2_{(4)} = 6.621$; $P > 0.05$) was detected in the UK between 1992 and 2009 (Hancock, 1996; Minnis *et al.*, 2002; Taylor and Hockland, 2010). Similarly, there was no significant difference in the Netherlands ($\chi^2_{(2)} = 3.29$; $P > 0.05$) between 2000 and 2010 (FVO, 2013).

2.5. Discussion

2.5.1. PCN diagnostic tools

Effective PCN management strategies are vital to the potato industry in Europe; producers and agricultural advisors require informed knowledge in order to deploy appropriate PCN control measures. An effective PCN management system requires species identification and quantification of the relative densities of *G. pallida* and *G. rostochiensis* in PCN infestations. Developing molecular diagnostic tools to facilitate the

understanding of plant-nematode interactions and population dynamics is critical to the success of PCN control.

PCR diagnostic techniques have facilitated the detection and population analysis of complex nematode communities. In this study both PCR assays afforded adequate detection and speciation of *Globodera* in PCN-infested soil samples. Although qPCR was significantly more efficient than cPCR for the identification of PCN, no treatment was statistically different in the detection of PCN. qPCR proved to be the more robust assay, with a PCN detection level of 99% relative to 95% in cPCR. The sensitivity of the qPCR assays was comparable to those in studies by Papayiannis *et al.* (2013), who reported detection sensitivities of 94% for *G. pallida* and 97% for *G. rostochiensis*.

Consistency between PCR methods was lowest in mixed-species populations and multiplex assays appeared to lack the sensitivity to simultaneously detect *G. pallida* and *G. rostochiensis*. Multiplex cPCR assays adequately detect PCN and are a viable option for PCN detection (Ibrahim *et al.*, 2001); however, the post-PCR analysis is its main limitation. Therefore, it would appear that multiplex qPCR methods are the best suited for high-throughput PPN screening (Goto *et al.*, 2011; Hu *et al.*, 2011). Indeed, a multiplex TaqMan qPCR-based molecular screening system for high-throughput PCN detection is currently operated at Science and Advice for Scottish Agriculture (SASA) and Department for Environment, Food & Rural Affairs (DEFRA) in the UK. The system also uses an automated cyst extraction process from soil samples and is capable of analysing up to 18,000 samples a year using automated DNA extraction and PCR (Reid *et al.*, 2015).

PCN population quantification techniques also require a critical assessment and further optimization. In this study, the total GR&R for qPCR assays was quite high at 21.35%. Ideally, the degree of variation should not exceed 10%; thus, further assay optimization is required. Furthermore, the studies presented analysed samples in duplicates, which provides insufficient data for proper assessment of PCR capabilities and defects. As such,

future GR&R studies should combine a greater number of triplicate samples to assess assay robustness. PCR assays are highly sensitive and are capable of detecting and amplifying low quantities of DNA. However, if the copy number is high, the level of efficiency subsides. This is further complicated by genomic DNA extraction. It is impossible to obtain pure isolates of nematodes from soil samples, foreign DNA is inevitable and highly variable in soil samples. As such, this may have an inhibitory effect on qPCR quantification efficiencies.

Efficient target sequence amplification is highly contingent on primer specificity and on minimal primer competition and cross-interference. In both PCR methods, *G. rostochiensis*-specific primers appeared more sensitive than *G. pallida*-specific primers, which was also reported in the studies of Papayiannis *et al.* (2013). Simplex PCR assays are generally more accurate and sensitive than multiplex assays in the detection and speciation of *Globodera* than multiplex reactions. Interference and competition between primers are most apparent in multiplex reactions. Multiplex assays were often flawed by primer dimers, the detection of foreign DNA and subsequent amplification of non-specific bands in post-PCR, gel-electrophoresis analysis. This has implications for the false positive or false negative detection of PCN.

2.5.1.1. Alternative PCN diagnostic methods

Other diagnostic technologies can include DNA microarrays that can discriminate species by targeting phylogenetic markers i.e. 18S rRNA gene. A microarray consists of a chip integrated with differential DNA probes arranged in a specific surface pattern. Species identification is possible by analysing complementary sequences to detect marker genes in isolates. Microarray analysis has been used extensively for PPN gene expression analysis, particularly the detection and characterisation of nematode parasitism genes (Elling *et al.*, 2009; Thorpe *et al.*, 2014). However, the technology remains underutilized for nematode identification and diagnostics (Castagnone-Sereno *et al.*, 2011; Escobar *et al.*, 2011).

One of the principal advantages of microarrays over PCR is the ability to perform numerous simultaneous species-specific tests at reduced costs. Various microarray platforms have been developed and commercialised to account for high-throughput pathogen detection. Customisation of such platforms utilising PCN-specific probes could have potential as a rapid molecular diagnostic tool for PCN detection. Francois *et al.* (2006) developed an array for the detection of *Meloidogyne chitwoodi* and demonstrated the potential for DNA chip technology for the simultaneous detection of PPN. Nevertheless, PCR diagnostic tests remain the gold standard for PCN speciation (You *et al.*, 2006; Madani *et al.*, 2008; Waeyenberge *et al.*, 2010).

2.5.2. PCN in Ireland

Species discrimination of PCN cysts confirmed *G. rostochiensis* as the predominant species in Ireland. *G. rostochiensis* was present in over 80% of samples. *G. pallida* also occurred in most of the PCN-infested samples (64%), namely in mixed-species populations (46%). The samples analysed in this study were from soil samples in which ware potatoes were produced. However, the data obtained from the national PCN census combined both seed and ware PCN samples. Seed production is subject to strict phytosanitary regulations therefore new introductions of PCN are less likely than in ware production areas. Similarly, 100% of seed area is tested relative to 0.5% of ware land, which may infer greater PCN detection probability in seed land, which would reflect the higher proportions of PCN detected in seed land (Fig. 2.5). These factors may account for the disparity between the results obtained by cPCR and qPCR assays in this study and those reported by DAFM (2011) and Rigney (2015). The data presented in this chapter report considerably higher values for *G. pallida* 18% relative to 8% in the national survey, in which speciation was performed using the EPPO standards i.e. cPCR (D. Murphy, pers. comm.). This may further account for the perceived differences in results.

The results from this study and Chi-squared analyses propose that the incidence of *G. pallida* populations in Ireland is increasing, particularly with respect to mixed-species

populations. This observation appears to be consistent with the PCN population trends evidenced in mainland Europe and the UK, although no further increase in *G. pallida* has been reported through published surveys and populations in the UK and Netherlands appear to have plateaued. The transition from *G. rostochiensis*- dominant populations to *G. pallida*-dominant populations is largely credited with the adoption of *G. rostochiensis*-suppressive control methods. In Ireland, PCN control measures are not implemented to the same extent as in other regions of intensive potato production (§ 2.1). Consequently, alternative explanations for the increasing incidence of *G. pallida* require investigation.

2.5.2.1. Approaches to PCN management and surveillance in Ireland

PCN is a prominent threat to the potato industry in Ireland and the need to adopt PCN control measures may be necessitated in the future. To address this problem, Irish agricultural authorities must employ a proactive approach towards PCN management. Increased agricultural awareness, effective sampling regimes and the development of PPN diagnostic facilities are critical for effective pest management. The regulations have intensified PCN sampling and aimed to harmonize field testing and PCN detection methods (S.I. No. 359/2011). The EPPO standards on nematode detection and diagnostics provide comprehensive guidelines for appropriate diagnostic procedures (EPPO, 2013). However, this study revealed a considerable degree of variation between diagnostic test methods and assay sensitivity. Furthermore, most molecular diagnostic protocols pertain to single cyst or juvenile identification. However, the isolation of nematode juveniles or cysts from soil samples is extremely laborious and is unsuitable for high-throughput screening. This highlights the necessity for standardised diagnostic protocols with regular validation to ensure quality and consistency in PCN diagnostic testing.

2.6. Conclusions

PCN poses a significant threat to the Irish potato industry and complacency regarding the "potato eelworm" has significant repercussions. At an endemic level, PCN management

is difficult and costly to implement. Therefore, efforts to mitigate the dissemination and establishment of the pest must be strengthened. A preventative rather than a reactive approach to PCN infestation is critical to maintain populations below the economic threshold. Increased awareness and implementation of PCN management strategies and regular PCN sampling are necessary to curtail further infestation and help preserve the Irish potato industry. This research proposes that the incidence of *G. pallida* in Ireland in both single- and mixed-species populations has increased in the absence of *G. pallida*-selective control measure. As such, intrinsic pest characteristics and species competitiveness may be accountable for an increase prevalence of *G. pallida* in Ireland.

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Chapter 3 Competition between *G. pallida* and *G. rostochiensis*

Abstract

This chapter investigates the effects of *in vivo* interspecific competition between *G. pallida* and *G. rostochiensis* under variable population conditions. PCN-susceptible varieties were inoculated with single- and mixed-species populations of *G. pallida* and *G. rostochiensis*. The results indicated a significant ($P < 0.001$) difference in species multiplication between single- and mixed-species populations. *G. pallida* multiplication rates were higher in mixed- relative to single-species populations, but not significantly so. However, *G. rostochiensis* multiplication in mixed-species populations was significantly ($P < 0.001$) lower than in single-species populations. The results indicate the possible inhibition of *G. rostochiensis* multiplication due to the presence of its antagonist *G. pallida*. The success of *G. pallida* was particularly clear in the density-dependent trials, with a significantly higher increase in multiplication at low infestation densities relative to *G. rostochiensis*. The results indicate that *G. pallida* has a negative effect on *G. rostochiensis* populations and proves to be the more successful when in competition with *G. rostochiensis*. A staggered inoculation trial was performed to assess whether the greater competitiveness of *G. pallida* was attributed to its later hatch. *G. pallida* was the dominant species in the time-dependent trial; however, the effect of inoculation timing on the multiplication rate was not significant ($P > 0.05$).

3.1. Introduction

Globodera rostochiensis has a larger distribution area than *G. pallida* and it is an established pest in most intensive potato growing regions (Appendix I - II). Recent national PCN surveys confirmed *G. rostochiensis* as the dominant species in Ireland (Chapter 2). The incidence of *G. pallida* is increasing despite the relative non-use of *G. pallida*-selective control measures. As such, there must be alternative reasons for the increased incidence of *G. pallida*.

The population dynamics of *G. rostochiensis* on both susceptible and resistant varieties have been extensively studied (Storey, 1982; La Mondia & Brodie, 1986; Salazar & Ritter, 1992). However, given the relative success of *G. rostochiensis* control, most studies in the past two decades have focused primarily on *G. pallida* (Phillips *et al.*, 1991; Halford *et al.*, 1995; Trudgill *et al.*, 2003; 2014). Few studies have researched the relative multiplication of both species in mixed PCN populations (Marshall, 1989; Den Nijs, 1992c; Schans, 1993). To date, there has been little research published on the direct competition between *Globodera pallida* and *G. rostochiensis*, despite the apparent threat of these species to the potato industry. Regional variance and the nature of agro-ecosystems most probably affect the composition of PCN populations, which demands an adequate understanding of nematode population dynamics to achieve effective IPM (Alonso *et al.*, 2011).

In their native Andean habitat, both *G. pallida* and *G. rostochiensis* coexist, with a great diversity of races (Jatala *et al.*, 1979). PCN occur in temperate and tropical climates. Depending on climate, *G. pallida* and *G. rostochiensis* complete one or two generations per year (Greco *et al.*, 1988; Kaczmarek *et al.*, 2014). The two species have different thermal hatching optima, which will affect population success according to geographical distribution. *G. pallida* has an optimum hatching temperature, that is 2 ºC lower than that of *G. rostochiensis* which may give it an advantage in colder temperate climates (Moxnes & Hausken, 2007). PCN populations are intrinsically regulated by intra- and interspecific competition for limited resources (Trudgill, 1986; Ettema, 1998), with host root area

availability being the main determinant affecting PCN multiplication. PCN populations exhibit a frequency-dependent response (Fig. 3.1); the final population (P_f) is intrinsically related to the initial population density (P_i). The multiplication rate (P_f/P_i) is negatively correlated with the initial population density.

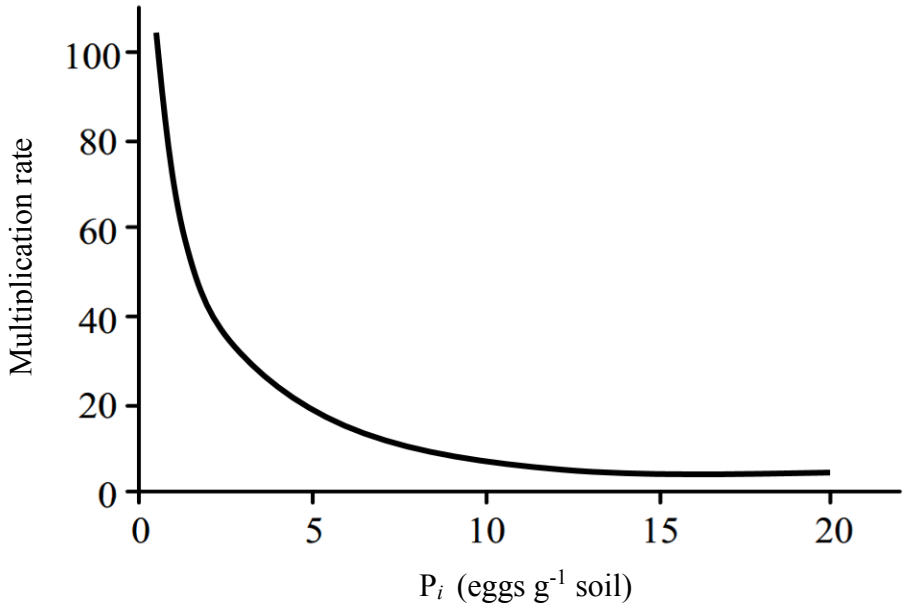


Figure 3.1 Relationship between initial population density (P_i) and multiplication rate (P_f/P_i), modified from Evans *et al.* (2003).

Theoretically, *G. rostochiensis* should be the more dominant species, considering its numerous physiological advantages. *G. rostochiensis* hatches earlier and therefore colonises the host first before *G. pallida* emerges, and therefore can occupy prime feeding sites (Marshall, 1989; Den Nijs & Lock, 1992). *G. rostochiensis* has greater J2 mobility, thereby reducing in-soil residency time and mortality (Robinson *et al.*, 1987). *G. rostochiensis* also exhibits less specificity for hatching factors relative to *G. pallida* (Byrne *et al.*, 2001). However, *G. rostochiensis* is less persistent in the absence of a host plant due to a high degree of spontaneous hatch, which may be accountable for its greater decline rate in soil (Den Nijs and Lock, 1992). Previous studies on the direct competition between *G. pallida* and *G. rostochiensis* reveal contradicting results. Marshall (1986, 1989) found *G. rostochiensis* to be the more competitive species in pot experiments in New Zealand conducted in both glasshouses and outdoors. Conversely, Den Nijs (1993) in the Netherlands and Ryan *et*

al., (2005) and Lettice (2014) in Ireland found *G. pallida* to be the more competitive species in pot trials outdooors. The effect of the *G. pallida* and *G. rostochiensis* interaction is not consistent between studies, which may be partially due to experimental conditions. As such, this study aims to determine the more competitive PCN species.

G. rostochiensis encounters little interspecific competition initially as it colonises host roots before *G. pallida*. Therefore, at high *G. rostochiensis* P_i and low *G. pallida* P_i, it is susceptible to intense intraspecific competition (Marshall, 1993). *G. pallida* experiences minimal intraspecific and only competes interspecifically with *G. rostochiensis* for feeding sites (Den Nijs & Lock, 1992). *G. pallida* generally hatches several days after *G. rostochiensis* so that, by the time of *G. pallida* juvenile emergence, the host root system is more developed (Stanton & Sartori, 1990; Salazar & Ritter, 1993). Furthermore, there are potentially more lateral roots available due to previous *G. rostochiensis* infection to accommodate the later-infesting *G. pallida* juveniles (Widdowson *et al*., 1958). Consequently, at low infestation densities *G. pallida* multiplication is relatively unaffected by the predominance of *G. rostochiensis*. In mixed-species populations, *G. pallida* is rarely eliminated from populations (Marshall, 1989); however, it is subject to intraspecific competition as the number of females that can establish on host roots is limited at high infestation densities (Marshall, 1986; Marshall, 1989; Trudgill *et al*., 1996).

The exact stage at which *G. pallida* exerts greater competitiveness over *G. rostochiensis* remains elusive. The developmental differences between the sibling species will invariably affect competition, particularly the faster rate of development and the more efficient use of energy reserves by *G. pallida* (Webley & Jones, 1981; Robinson *et al*., 1987). Furthermore, species competitiveness may also be related to greater virulence (Hockland *et al*., 2012) and host physiological responses (Phillips & Trudgill, 1998b). *G. pallida* competitiveness may be related to the later hatch of this species (Stanton & Sartori, 1990), *G. pallida* exhibits greater selectiveness and dependence on HF, requiring a higher minimum HF threshold concentration to stimulate hatch, which may contribute to its

delayed hatch (Ryan *et al.*, 1999; Byrne *et al.*, 2001; Devine & Jones, 2003a). In addition, both species of PCN exhibit quantitatively different responses to individual HFs ("species-selectivity").

The experiments in this chapter are designed to investigate whether either *G. pallida* or *G. rostochiensis* is the more competitive species in mixed-species populations and to assess plausible hypotheses as to why one species outcompetes the other. Research investigating the relative competitiveness of *G. pallida* and *G. rostochiensis* in pure and mixed-species populations revealed that *G. pallida* significantly outcompetes *G. rostochiensis* in the mixed-species populations (Lettice, 2014). *G. pallida* multiplication in mixed-species populations was considerably greater than that of *G. rostochiensis*. However, the effect was not reflected in single-species populations. *G. pallida* multiplication was 15% higher in mixed-species populations relative to pure *G. pallida* populations.

On the other hand, *G. rostochiensis* exhibited much lower multiplication in mixed-species populations relative to single-species populations (Ryan *et al.*, 2005). Conversely, *G. rostochiensis* multiplication was reduced by 66% in mixed-species populations, inferring that *G. pallida* is the more successful species in mixed populations (Ryan *et al.*, 2005). Furthermore, it is postulated that *G. pallida* competitiveness was related to its delayed hatch, greater dependence on HF and the higher HF threshold concentration required to instigate hatch. Studies investigating the timing of PCN infestation revealed that *G. pallida* multiplication was significantly reduced when *G. pallida* cysts were applied before those of *G. rostochiensis* (Ryan *et al.,* 2005). This further supports the hypothesis that the greater *G. pallida* competitiveness was in part, due to its later hatch relative to *G. rostochiensis*.

Few publications have evaluated the direct competition between *G. pallida* and *G. rostochiensis*, owning to technical difficulties in differentiating cysts or juveniles of the two species in mixed-species populations (§ 1.2.4). Population dynamics and interspecific interactions between *G. pallida* and *G. rostochiensis* require accurate methods of

identification and quantification to assess the relative proportions of both species in mixed populations. As conveyed in Chapter 2, technical advances in molecular diagnostic methods have made species discrimination and quantification more feasible (Chapter 2; Bates *et al.*, 2002; Kenyon *et al.*, 2010).

3.2. Aims

The aims of the experiments described in this chapter included:

- To investigate the degree of competition between *G. pallida* and *G. rostochiensis* in single- and mixed-species populations in the absence of control measures.
- To determine if this interaction is causally associated with the timing of hatch.
- To investigate the manipulation of hatching times on competition.
- To evaluate the effect of infestation rates on PCN competition.

3.3. Materials and Methods

3.3.1. PCN

Single-generation *G. pallida* pathotype Pa 2/3 and *G. rostochiensis* pathotype (Ro 1) cysts, generously supplied by Dr. Colin Fleming, Agri-Food and Biosciences Institute, Belfast, Northern Ireland, were used throughout the experiment. The cysts were cultured on non-resistant potato varieties outside in pots containing a sandy soil. Cysts were pre-soaked for one week in distilled water, on Whatman No.5 filter paper in closed Petri dishes at 20 ºC. Prior to experimentation, cysts were enclosed in a 5 × 5 cm nylon (200 μm) mesh envelopes to facilitate recovery of the initial inoculum and to allow for ease of identification and quantification of first-generation nematodes (Devine & Jones, 2001). *In vitro* hatching assays were conducted before each trial to ascertain egg viability and hatching efficiency.

3.3.2. PCN viability test

Three individual samples, each of ten cysts, were selected from each PCN population. Cyst samples were pre-soaked for one week (§ 3.3.1), then mechanically crushed with a

polypropylene homogeniser (Sigma Aldrich, Arklow) in 200 µl dH_2O in a 1.5 ml microtube. The egg suspension was centrifuged at 2500 *g* and the supernatant removed. The eggs were re-suspended in 400 µl 0.05% (w/v) aqueous Meldola's blue solution (Shepherd, 1962) and incubated for five days at 20 ºC. The stain solution was removed by centrifugation 1500 *g* and the eggs were re-suspended in 400 µl dH_2O for 24 h to remove excess stain (Twomey *et al.*, 1995). The suspension was vortexed and three individual 20 µl aliquots were extracted for analysis. Samples were observed under a light microscope at 400x magnification. The percentage of non-viable eggs was calculated by counting the number of stained (non-viable) and unstained (viable) eggs. The average number of viable eggs per cyst was used to determine inoculum infestation rates per treatment.

3.3.3. Soil

A silty loam topsoil was acquired from a field that had been free of potato production for at least 35 years. Several randomly selected soil samples were elutriated through a Wye Washer elutriator to detect PCN cysts (Winfield *et al.*, 1987). To ascertain whether any viable eggs or undetected cysts were present in the soil, tubers of the PCN-susceptible variety 'Golden Wonder' were grown in pots containing this soil. Soil samples were tested prior to experimentation and no PCN cysts were detected after samples were processed, signifying the absence of viable PCN in the soil.

3.3.4. Intra- and interspecific competition

3.3.4.1. Planting material

Four PCN-susceptible *Solanum tuberosum* varieties of different maturity classes were used in each experiment namely;

- **Second early**: 'British Queen'
- **Maincrop:** 'Kerr's Pink', 'Rooster'
- **Very late maincrop:** 'Golden Wonder'

Three different treatments, each with ten replicates per treatment for each variety, were established (n = 120). Certified seed tubers were chitted at 16 ºC in light for three weeks

prior to planting. Seed tubers were planted in 25 cm diameter pots containing soil inoculated with PCN cyst (§3.3.1). The pots were placed a plunge pit (inner dimensions = 520 x 450 x 35 cm); pots were aligned in a grid format, allowing sufficient space for canopy development, and were partially embedded in standard potting compost, which acted as a buffer to achieve temperature stability. The plants were grown from early April to late September to permit adequate PCN multiplication. After shoot emergence, both systemic and contact fungicides were applied regularly throughout the growing season to prevent blight infection.

3.3.4.2. Inoculation

Prior to planting, pre-soaked cysts were sealed in nylon mesh envelopes (§ 3.3.1) and placed in pots at a depth of 15 cm from the soil surface, which approximated to 10 cm from the tuber. Three treatments were established: pure *G. pallida*, pure *G. rostochiensis* and mixed species *G. pallida* and *G. rostochiensis* in a 50:50 ratio. Negative controls were established in pots without a host plant.

- **Treatment 1:** 5 eggs g^{-1} soil *G. pallida*
- **Treatment 2:** 5 eggs g^{-1} soil *G. rostochiensis*
- **Treatment 3:** 2.5 eggs g^{-1} soil *G. pallida* : 2.5 eggs g^{-1} soil *G. rostochiensis*

3.3.4.3. Cyst Extraction and purification

Soil samples were air dried in an oven at 20 ^{o}C for 48 h before separation to optimize cyst extraction. Whole soil samples were crushed and filtered through a 19.0 mm sieve. Cysts were recovered using a Wye Washer elutriator (Winfield *et al.*, 1987). The float was collected in a 355 µm aperture sieve nested over a 250 µm sieve. The float was further elutriated in a 250 ml beaker and cysts were isolated on filter paper (Whatman No. 1, Ø = 240 mm). The recovered cysts were air dried and passed through a 1000 µm sieve and subsequently separated from debris by acetone extraction (van Bezooijen, 2006). Cysts were counted and stored in 1.5 ml microtubes at 4 ^{o}C prior to DNA extraction.

3.3.5. Timing of PCN inoculation

3.3.5.1. PCN

See section 3.3.1. 'Golden Wonder' seed was unavailable in the year this trial was performed and was therefore omitted from this and subsequent multivariety trials.

3.3.5.2. Planting material

Four potato varieties were used in this study namely: 'British Queen', 'Kerr's Pink', 'Rooster' and 'Golden Wonder' (§ 3.3.4.1.) Plants were grown outdoors in 25-cm pots contained in a plunge pit (§ 3.3.4.1) from mid-April to late September. Two sections of polypropylene tubing (20 cm length; 7 mm diameter; Reagecon, Shannon, Ireland) were incorporated into the soil at the time of planting to facilitate direct J2 application to the root area. The tubes were sealed at the surface to prevent desiccation and inoculum degradation; no soil was contained within the tubing.

3.3.5.3. Inoculation

PCN cysts were pre-soaked as described in § 3.3.1. To eliminate the hatching delay between species, an inoculum of hatched juveniles of either PCN species was directly administered to the rhizosphere at a rate of 5 eggs g^{-1} soil. Pre-soaked cysts of *G. pallida* and *G. rostochiensis* were suspended in 200 µl PRL at 0.1 mg ml^{-1} (§ 3.3.6) for 8 d to induce hatch, as determined by a time-dependent hatching assay (§ 4.2.2). The juvenile/PRL solution was diluted with dH_2O to a final volume of 1 ml and administered to the roots via the tubing via a pipette. Juveniles of each species were applied at a rate of 2.5 eggs g^{-1} soil to give a final mixed-population of 5 eggs g^{-1} soil (1:1 ratio). The tubes were rinsed with 500 µl dH_2O after application of the J2s to ensure efficient inoculum delivery. Inoculation times were staggered; three treatments were established:

- **Treatment 1:** Gp at I_0/ Gr at I_0;
- **Treatment 2:** Gp at I_1/ Gr at I_0;
- **Treatment 3:** Gp at I_0/ Gr at I_1.

The first inoculum (I_0) and the simultaneous inoculation (I_0) were applied to the roots 14 d after shoot emergence (0 d). The second inoculum (I_1) was applied 7 days post inoculation (dpi). Ten replicates of each treatment were prepared. Negative controls were included. At the end of the growing season, cysts were extracted as described in § 3.3.4.3.

3.3.6. Potato root leachate production

Potato root leachate was produced by the method used by Ryan and Jones (2003). Sterile potato (*Solanum tuberosum* cv. 'Golden Wonder') plantlets were aseptically propagated in sterile plastic tissue culture tubs (Fig. 3.2; Wilsanco Plastics Ltd., Dungannon, Northern Ireland) via nodal culture on half-strength Murashige & Skoog (M&S) culture medium: 2.21 g l^{-1} M&S basal salts, 15 g l^{-1} sucrose, 100 µg l^{-1} kinetin, 200 µg l^{-1} gibberellic acid, 6 g l^{-1} agar, pH 5.8 (Murashige & Skoog, 1962). Plantlets were grown in a growth room with a photosynthetic photon flux rate of 300 µmol m^{-2} s^{-1}, under a 16-h day at 22 ± 2 °C, relative humidity: day, 55%; night, 100% (Lettice and Jones, 2016). After 4 weeks, each plantlet was removed and rinsed in sterile dH_2O to remove any adhering medium. The plantlet was secured in a sterile sand medium on a polypropylene membrane LifeRaft (Sigma-Aldrich, Arklow, Ireland) and placed in a sterile Magenta container (Sigma-Aldrich, Arklow, Ireland).

Each Magenta tub contained 40 ml sterile nutrient medium (Phostrogen, Bayer CropScience Limited, Cambridge, UK) N-P-K 14-10-27, diluted to a final concentration of 1.7 g l^{-1} N-P-K 24-17-46. This nutrient solution was supplied to the plant by capillary action and absorption through the membrane raft. The Magenta vessel was capped with a modified lid with a 10 mm diameter hole covered with a Suncap closure (Sigma-Aldrich, Arklow, Ireland) to permit sterile gas exchange (Lettice and Jones, 2016). After 4 weeks of growth, the microplant was removed and the liquid was harvested, filtered through a Whatman No.2 filter-paper and concentrated to 10% of the original volume by rotary evaporation at 55 °C.

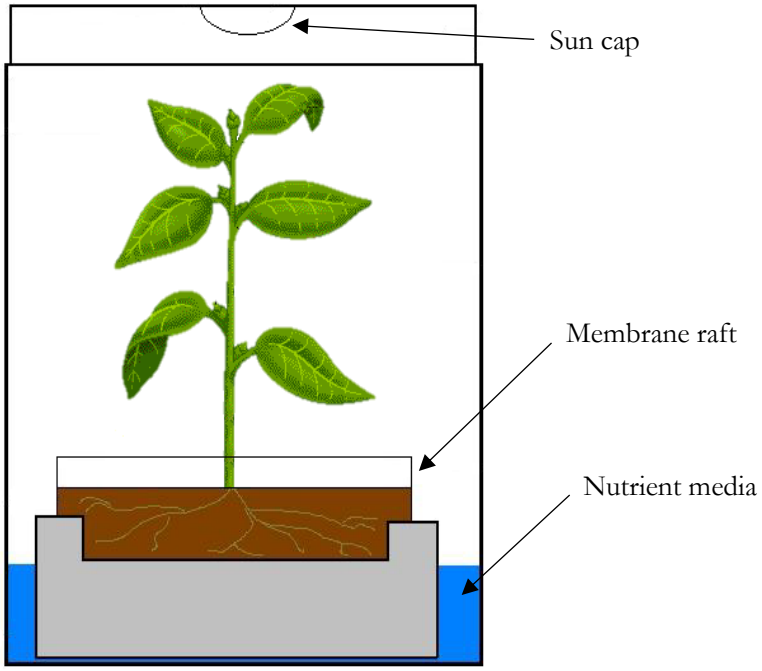


Figure 3.2 Microplant system for the production of sterile PRL modified from Lettice and Jones (2016).

3.3.7. Population proportion

3.3.7.1. Planting material

Three potato varieties were used: 'British Queen', 'Kerr's Pink' and 'Rooster'. Chitted seed tubers (§ 3.3.4.1) were planted after the soil had been inoculated with PCN cysts in conditions described in § 3.3.4.1.

3.3.7.2. Inoculation

Pre-soaked PCN cysts (§ 3.3.1) were used as inoculum with different proportions of *G. pallida* and *G. rostochiensis*. Prior to planting, cysts were sealed in nylon sachets (§ 3.3.1) and placed in pots at a depth of 15 cm from the soil surface. Mixed populations of both species were inoculated with the densities of *G. rostochiensis* : *G. pallida* cysts; 0:100, 5:95, 20:80, 25:75, 50:50, 75:25, 80:20, 95:5, 100:0. Negative controls with no PCN cysts were also included.

3.3.7.3. Cyst Extraction

See section 3.3.4.3

3.3.8. DNA extraction

Subsamples were used for DNA extraction. DNA was extracted as described in § 2.3.2.

3.3.9. DNA analyses

Nucleic acid concentration and purity was determined by spectrophotometry at 260 nm and 260/280 nm, respectively, using a NanoDrop 1000c spectrophotometer (ThermoFisher, Wilmington, USA). Isolates that did not achieve a ratio of >1.7 were re-purified by silica column-based DNA purification. DNA was extracted from a known number of *G. pallida* and *G. rostochiensis* eggs to use as standards for absolute quantification. Five serial 10-fold dilutions of DNA standards were used to achieve a standard curve in the range of 100 ng – 10 pg DNA. Standards were stored in 20 µl aliquots at -20 ºC to minimize repetitive thawing and freezing and thus to prevent DNA degradation.

3.3.10. Primers and probes

Universal PCN primers and species-specific probes designed in the ITS1 region were used in qPCR reactions (Kenyon *et al.*, 2010; Reid *et al.*, 2010). Simplex qPCR reactions were conducted using TaqMan® MGB® probes labelled with FAM™ fluorescent dyes with the sequence 5'-6FAM-CCG CTA TGT TTG GGC-3' for the *G. pallida*-specific probe and 5'-6FAM-CGT TTG TTG TTG ACG GAC AYA-3' for the *G. rostochiensis*-specific probe (ThermoFisher, Foster City, USA). Primers comprised the universal PCN forward primer (5'-CGTTTGTTGTTGACGGACAYA-3') and reverse primer (5'-GGC GCT GTC CRT ACA TTG TTG-3'; Eurogentec, Seraing, Belgium). Primer validation and optimization experiments were conducted prior to qPCR assays. Validation assays confirmed species-specificity for each probe, with no cross-reaction.

3.3.11. Quantitative real-time PCR

Simplex qPCR reactions were performed using ABI Prism 7900 Sequence detection system (ThermoFisher, Foster City, USA). Reactions consisted of 15.0 µl Takyon®. 2X qPCR Probe MasterMix (Eurogentec, Seraing, Belgium), 1.25 µl each primer (5 pmol µl-

^{1}), 1 µl *G. pallida*-specific probe (5 pmol µl^{-1}), 1 µl *G. rostochiensis* specific probe (5 pmol µl^{-1}), 6.25 µl sterile molecular grade water (Sigma Aldrich, Arklow, Ireland) and 5.0 µl template DNA (1 µg µl^{-1}). PCR amplifications were performed in triplicate. Aliquots (10 µl) of the PCR reaction mixture were analysed in MicroAmp 384-Well Reaction Plates (Life Technologies, Paisley, Scotland). Standard curves for both species were established from DNA standards with known quantities of nematode DNA (§ 2.3.4). All reactions were run in 9600 emulation mode with the following cycling conditions, 60°C for 2 min, 95°C for 10 min followed by 40 cycles of 95° for 15 s and 60°C for 60 s and involved a final dissociation step (Kenyon *et al.*, 2010). qPCR reactions provided absolute quantification using the standard curve method (§ 2.3.4).

Reactions included a positive control for *G. rostochiensis* and *G. pallida*, a negative no-template control and an exogenous qPCR positive control (Eurogentec, Seraing, Belgium). PCR amplification was analysed with SDS 2.4 software (ThermoFisher, Foster City, USA). Baseline values were manually adjusted: a minimum baseline of three cycles was assigned to eliminate background noise in the early amplification cycles and the end baseline was selected as one cycle before the earliest amplification signal. The critical threshold (C_t) was assigned at the beginning of the logarithmic phase of PCR amplification and the difference in the C_t values of the control and experimental samples were used to determine gene expression in each sample. Amplification efficiencies were calculated by the equation $E_{exp} = 10^{(-1/m)-1}$, where m is the slope of the linear regression of C_t values versus log [DNA] concentration. An efficiency of 100 +/- 10% was accepted.

3.3.12. Statistical analysis

All statistical analyses were carried out using SigmaPlot V12.5 (SYSstat Software Inc. Erkrath, Germany). All datasets were checked for normality and homogeneity of variance; data presented graphically represent the mean values of untransformed data. PCN multiplication or reproductive rate (R) was calculated as follows: $R = P_f / P_i$, where P_i = initial density and P_f = final density (Den Nijs, 1992c). In mixed-species competition

analysis, the relative population increase (RPI) of each species was expressed. The total RPI for each species in a mixed population was calculated as follows:

$$\text{RPI} = \frac{\text{R species A}}{\text{R species A + R species B}}$$

Both the R and RPI were calculated for each species. The R value provided details of single-species multiplication in single- and/or mixed-species populations, whereas the RPI value was indicative of the individual species multiplication within a mixed-species population as a function of the total PCN population.

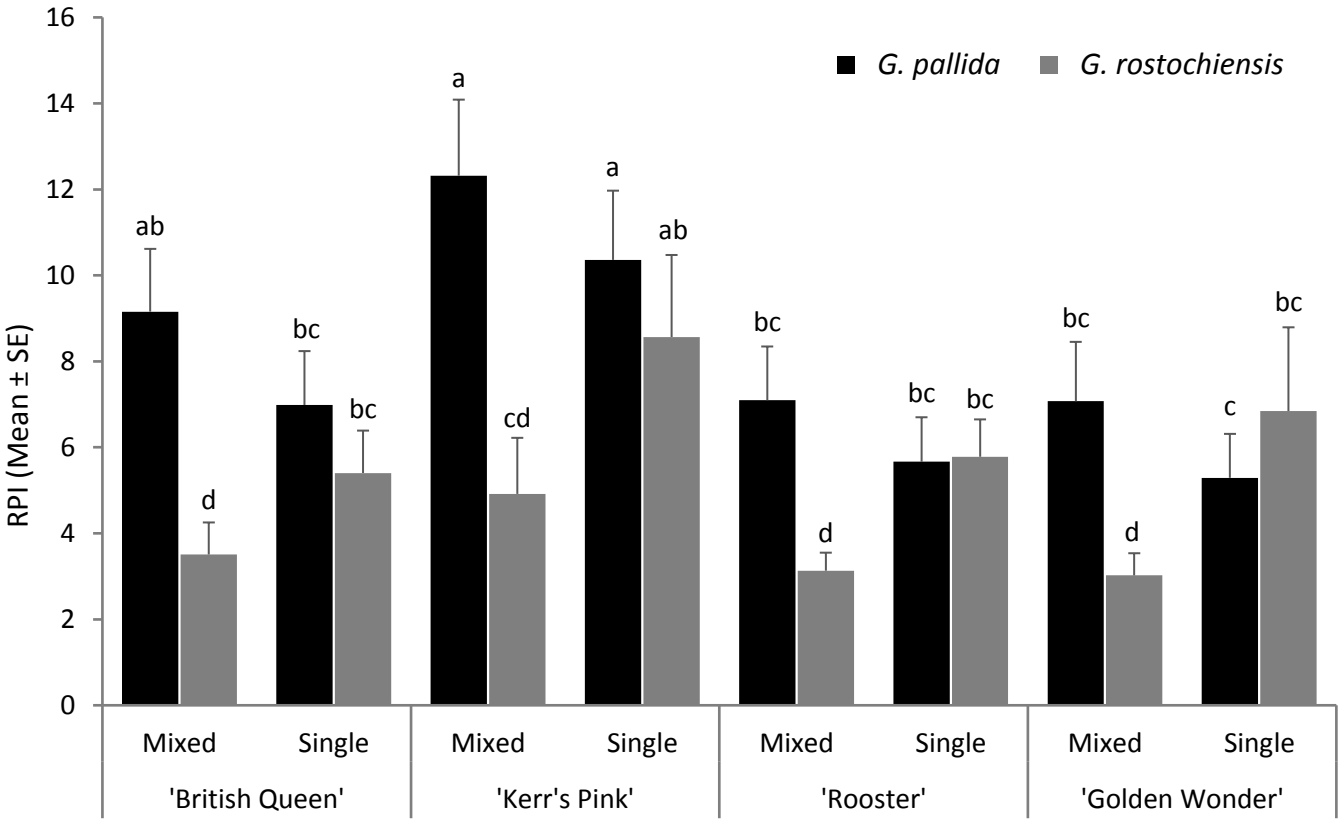
Inter- and intraspecific competition assays analysed RPI with 3-way interaction ANOVA followed by a multiple comparison Tukey test. Time-dependent data were normalised by $\log_{10}$ transformation and analysed by ANOVAs. Three-way ANOVA investigated the difference between the order of J2 infestation i.e. Gr at I_0 / Gp at I_0; Gr at I_1 / Gp at I_0; Gr at I_s / Gp at I_s, and differences in the intervals between inoculation e.g. 0 d, and 7 d. Infestation density data were analysed using 3-way ANOVA after $\log_{10}$ transformation. *G. pallida* and *G. rostochiensis* were individually analysed using 2-way ANOVA; *post hoc* comparative analyses were performed using the Tukey test. The test was repeated on each variety to analyse the differences between species multiplication. Infestation density ANOVAs were performed on both R and RPI data to measure individual species populations and cumulative mixed-species populations. Linear, quadratic and cubic regressions were performed on the final population density (P_f) as a function of initial infestation density (P_i).

3.4. Results

3.4.1. Intra-and interspecies competition

Competition assays revealed a significant difference in multiplication between species (Fig. 3.3; $F_{(1,144)} = 27.01$; $P < 0.001$) and between varieties ($F_{(3,144)} = 5.10$; $P < 0.001$). There

was no significant difference between populations ($F_{(1,144)} = 1.90$; $P > 0.05$); but there was a significant interaction between species and population ($F_{(1,144)} = 16.37$; $P < 0.001$).

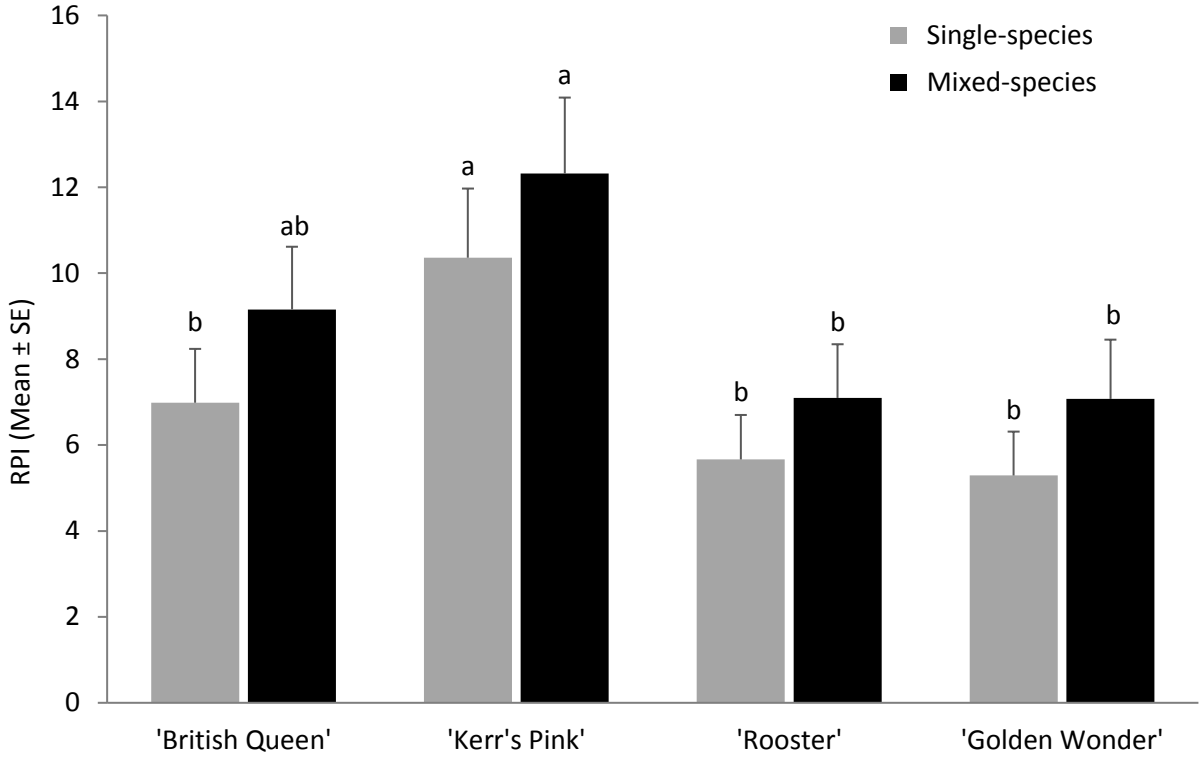


Source of Variation	DF	SS	MS	F	*P*
Variety	3	1.14	0.38	5.10	<0.001
Population	1	0.14	0.14	1.90	>0.05
Species	1	2.01	2.01	27.01	<0.001
Variety x Population	3	0.02	0.01	0.08	>0.05
Variety x Species	3	0.21	0.07	0.93	>0.05
Population x Species	1	1.22	1.22	16.37	<0.001
Variety x Population x Species	3	0.01	0.003	0.04	>0.05
Residual	144	10.72	0.07		
Total	159	15.47	0.10		

Figure 3.3 RPI of *G. rostochiensis* and *G. pallida* in mixed- and single-species populations on PCN susceptible varieties. Samples with a common letter are not significantly different ($P > 0.05$) using the Tukey test.

3.4.1.1. *G. pallida* inter-and intraspecific competition

G. pallida mixed- and single-species populations were not significantly different despite the greater multiplication evidenced in mixed-species population ($F_{(1, 72)} = 3.65$; $P = 0.06$). There was a significant difference between varieties ($F_{(3, 72)} = 5.64$; $P < 0.01$); however, there was no significant difference between populations within any variety (Fig. 3.4). No significant interaction between population and variety was observed ($F_{(3, 72)} = 0.05$; $P > 0.05$). *G. pallida* had a higher RPI in the maincrop 'Kerr's Pink', followed by the early variety 'British Queen'.

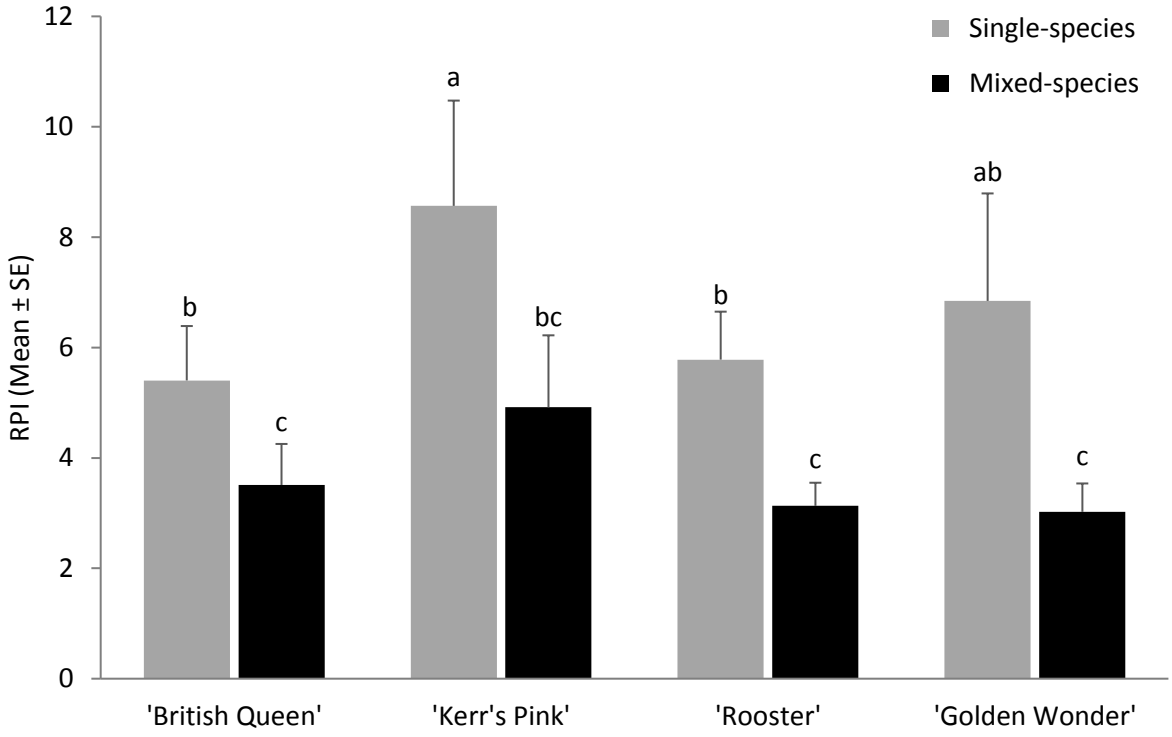


Source of Variation	DF	SS	MS	F	*P*
Variety	3	1.09	0.36	5.64	<0.001
Population	1	0.24	0.24	3.65	0.06
Interaction	3	0.01	0.01	0.05	>0.05
Residual	72	4.64	0.06		
Total	79	5.98	0.08		

Figure 3.4 The effect of inter-and intraspecific competition on *G. pallida* multiplication on PCN susceptible varieties. Samples with a common letter are not significantly different ($P > 0.05$) using the Tukey test.

3.4.1.2. *G. rostochiensis* intra-and interspecific competition

G. rostochiensis multiplication was significantly ($P < 0.001$) lower in mixed-species populations than in single-species populations (Fig. 3.5). The greatest multiplication was evident in single-species populations, particularly in the late maincrop variety 'Kerr's Pink' and the very late maincrop 'Golden Wonder'. There was a significant difference between populations ($F_{(1, 72)} = 12.92$; $P < 0.001$), but not between varieties ($F_{(3, 72)} = 1.01$; $P > 0.05$). There was no significant interaction between variety and population ($F_{(3, 72)} = 0.06$; $P > 0.05$).



Source of Variation	DF	SS	MS	F	*P*
Variety	3	0.26	0.09	1.01	>0.05
Population	1	1.10	1.10	12.92	<0.001
Interaction	3	0.02	0.01	0.06	>0.05
Residual	72	6.11	0.08		
Total	79	7.48	0.09		

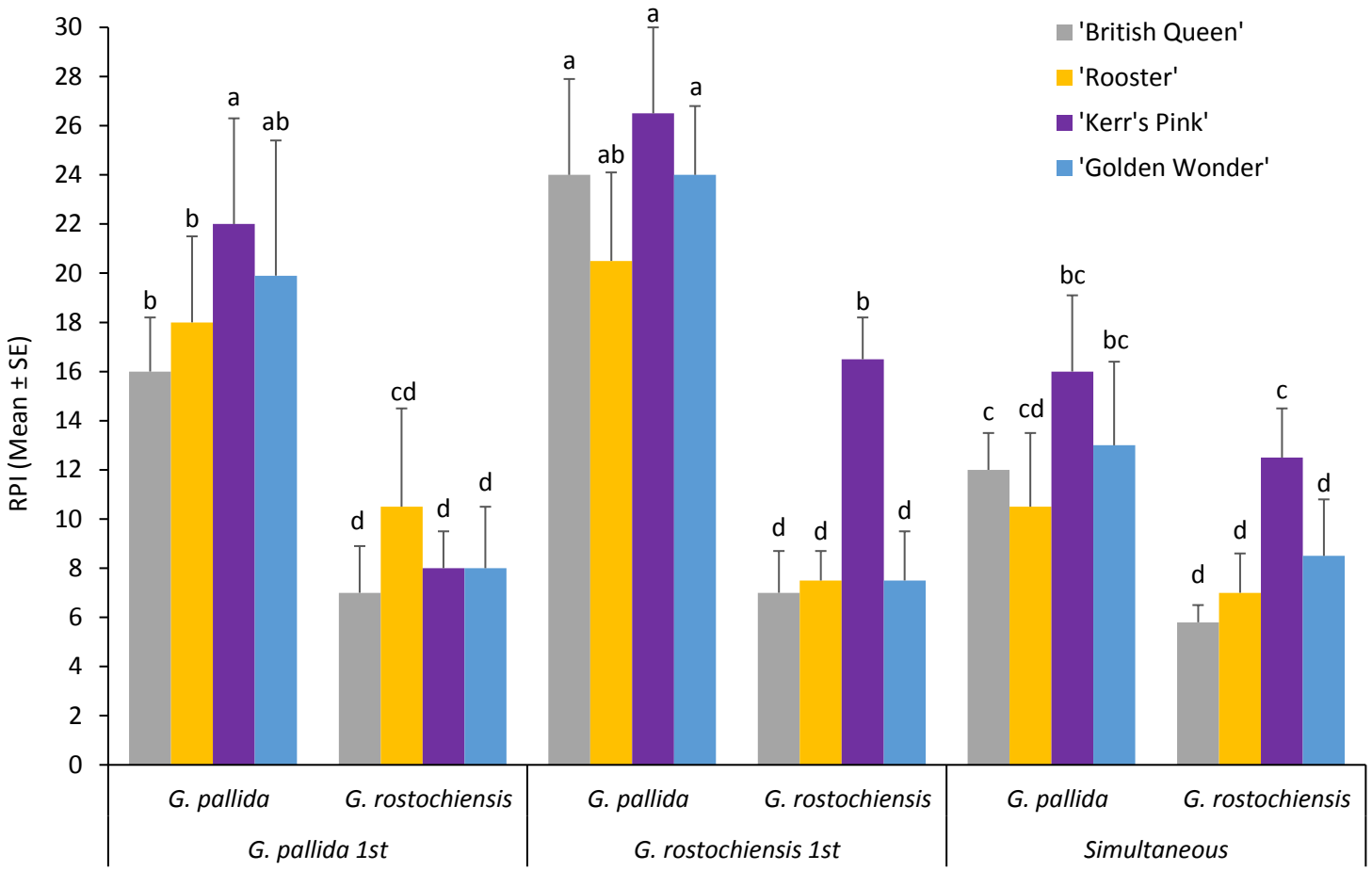
Figure 3.5 The effect of inter- and intraspecific competition on *G. rostochiensis* multiplication on PCN susceptible varieties. Samples with a common letter are not significantly different ($P > 0.05$) using the Tukey test.

3.4.2. Timing of PCN inoculation

Staggered inoculation had a significant effect on PCN multiplication (Fig. 3.6; $F_{(2, 217)} = 3.14$; $P < 0.05$) and a significant difference in multiplication was observed between the two species ($F_{(1, 217)} = 65.72$; $P < 0.001$). *G. pallida* was the more successful species in all treatments and achieved significantly higher multiplication ($P < 0.05$) than *G. rostochiensis* when applied in advance of, simultaneously with or following *G. rostochiensis*-inoculation (Fig. 3.6). Comparative analyses between inoculation times within each species revealed that the only significant difference occurred in *G. pallida* ($P < 0.001$) between I_1 (7 dpi) and simultaneous inoculation (I_0), with average RPI of 11.55 and 7.83, respectively. As such, *G. pallida* multiplication was significantly reduced when it was applied concomitantly with *G. rostochiensis* (Fig. 3.7).

There was a significant difference between varieties (Fig. 3.7; $F_{(3, 217)} = 4.48$; $P < 0.001$), but no significant interaction between species and variety was observed ($F_{(3, 217)} = 1.45$; $P > 0.05$). *Post hoc* assays revealed a significant difference between 'Golden Wonder' and 'Kerr's Pink' within I_0 ($P < 0.05$) and between 'British Queen' and 'Kerr's Pink' in I_s ($P < 0.001$). A significant interaction was observed between variety and treatment ($F_{(6, 217)} = 2.24$; $P < 0.01$), and between time and species ($F_{(2, 217)} = 3.03$; $P < 0.01$). There was no significant interaction between all three main effects ($F_{(6, 217)} = 1.50$; $P > 0.05$).

Early inoculation did not have a significant effect on *G. pallida* multiplication ($P > 0.05$); *Post hoc* assays also revealed a significant difference ($P < 0.05$) between varieties when the two species were inoculated simultaneously (Gp at I_0/ Gr at I_0), particularly between 'Kerr's Pink' and 'British Queen'. Interestingly, 'Kerr's Pink' appeared to be most susceptible to inundative (concomitant) inoculation. However, *G. rostochiensis* multiplication was least affected by time intervals between infestations and the only significant difference was evident in 'British Queen' (Fig. 3.7).



Source of Variation	DF	SS	MS	F	*P*
Treatment	2	0.33	0.17	3.14	<0.05
Variety	3	0.71	0.24	4.48	<0.01
Species	1	3.48	3.48	65.72	<0.001
Treatment x Variety	6	0.71	0.12	2.24	<0.05
Treatment x Species	2	0.39	0.19	3.63	<0.05
Variety x Species	3	0.23	0.08	1.45	>0.05
Treatment x Variety x Species	6	0.48	0.08	1.50	>0.05
Residual	217	11.50	0.05		
Total	240	17.81	0.07		

Figure 3.6 The effect of staggered inoculation on *G. pallida* and *G. rostochiensis* multiplication. Samples with a common letter are not significantly different ($P > 0.05$) using the Tukey test.

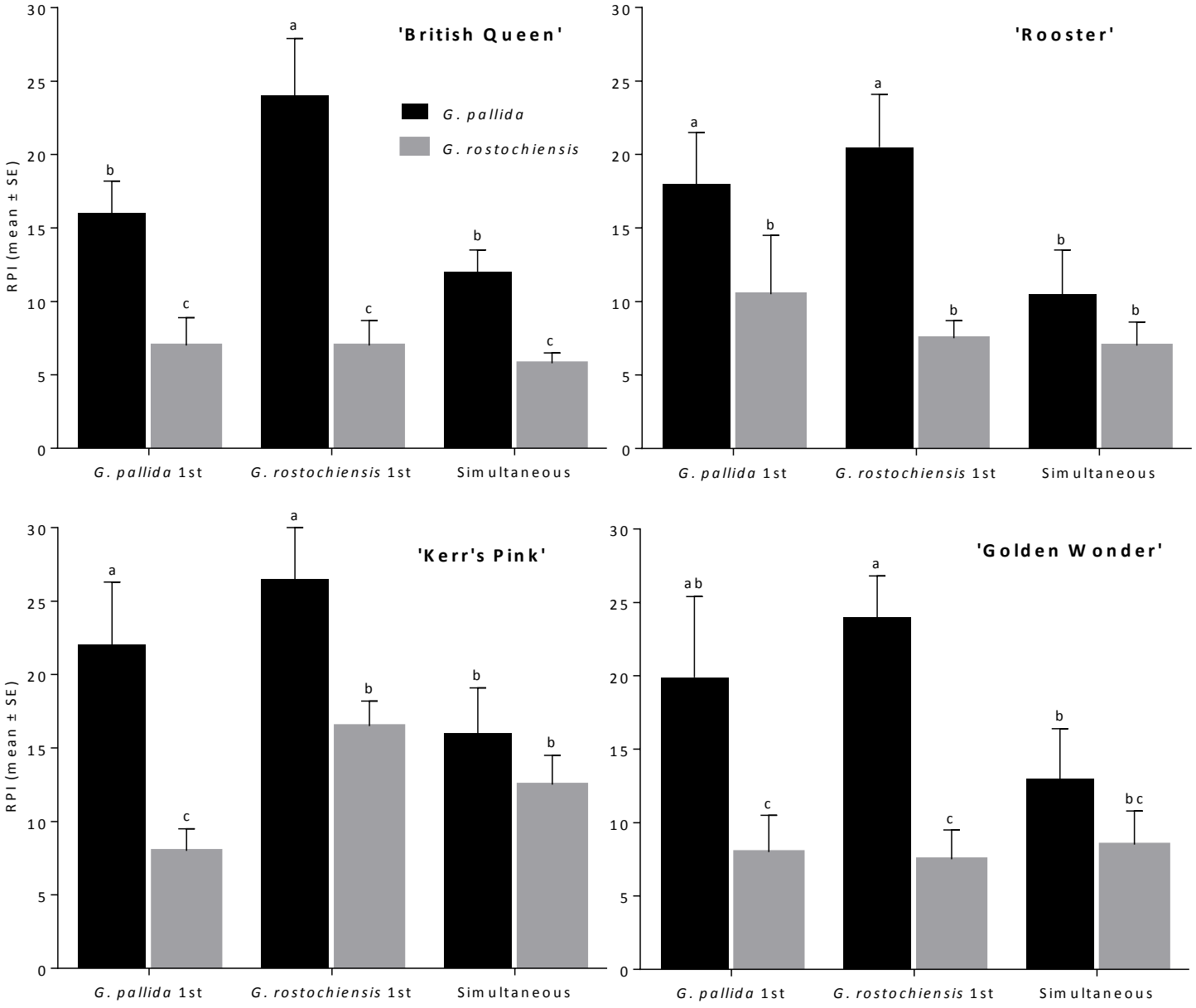


Figure 3.7 The effect of inoculation times between *G. pallida* and *G. rostochiensis* infestation on multiplication. Samples with a common letter are not significantly different ($P > 0.05$) using the Tukey test.

Inoculation interval had a significant effect on PCN multiplication (Fig. 3.7; $F_{(1, 225)} = 5.247$; $P < 0.05$), with significant differences between both species ($F_{(1, 225)} = 44.058$; $P < 0.001$) and varieties ($F_{(3, 225)} = 6.235$; $P < 0.001$). Significant interactions were observed between interval and variety ($F_{(3, 225)} = 2.751$; $P < 0.05$), and between interval and species ($F_{(1, 225)} = 6.459$; $P < 0.01$), but no significant interaction between species and variety ($F_{(3, 227)} = 0.860$; $P > 0.05$) or between all three effects ($F_{(3, 225)} = 0.331$; $P > 0.05$).

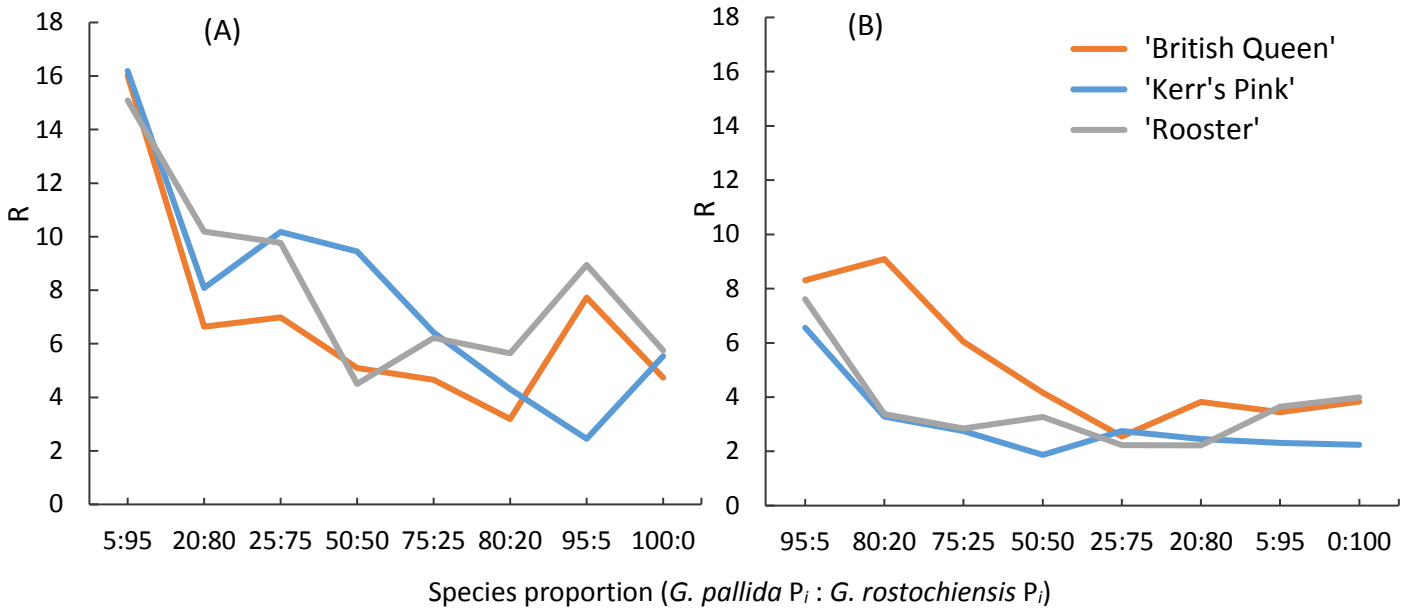
Table 3.1 The effect of interval between G. *pallida* and *G. rostochiensis* multiplication on PCN susceptible varieties.

Source of Variation	DF	SS	MS	F	*P*
Interval	1	0.286	0.286	5.247	<0.05
Variety	3	1.020	0.340	6.235	<0.001
Species	1	2.402	2.402	44.058	<0.001
Interval x Variety	3	0.450	0.150	2.751	<0.05
Interval x Species	1	0.352	0.352	6.459	<0.01
Variety x Species	3	0.141	0.0469	0.860	>0.05
Interval x Variety x Species	3	0.0541	0.0180	0.331	>0.05
Residual	225	12.266	0.0545		
Total	240	17.809	0.0742		

3.4.3. Population proportion

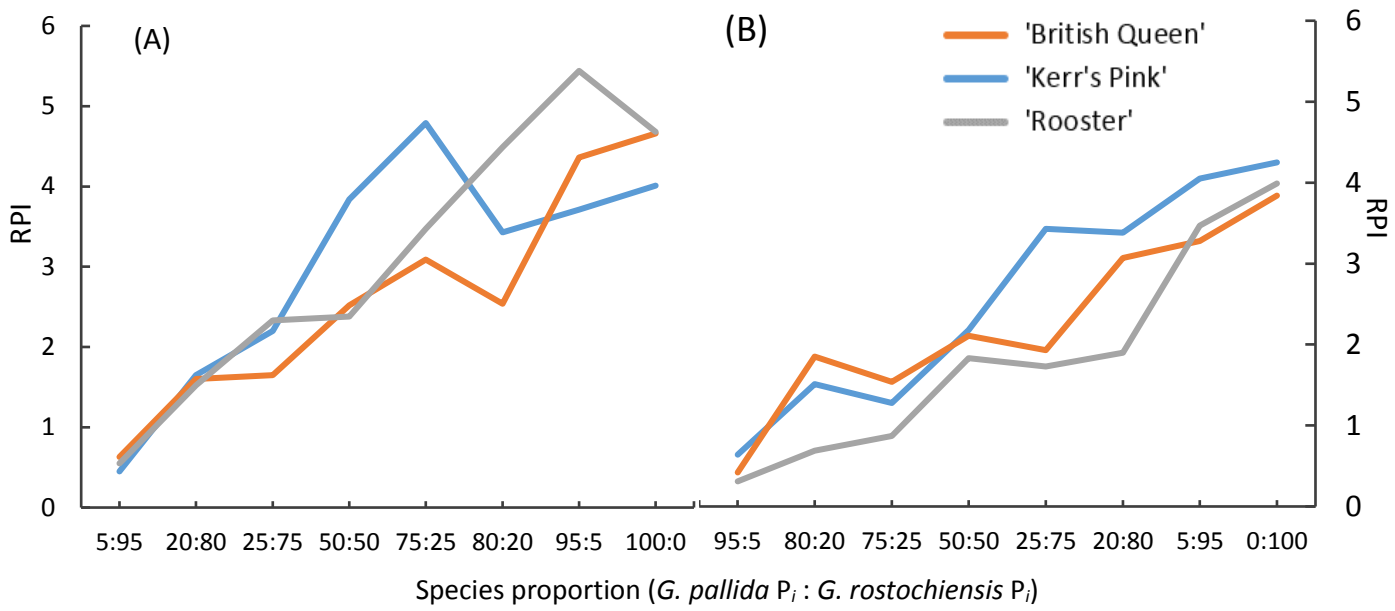
Population proportion had a significant effect on PCN multiplication (Fig. 3.8; $F_{(7, 432)} = 14.10$; $P < 0.001$). There was a significant difference between PCN species ($F_{(1, 432)} = 93.11$; $P < 0.001$); however, no significant interaction was evident between infestation density and species ($F_{(7, 432)} = 0.99$; $P > 0.05$). Variety did not have a significant effect on multiplication ($F_{(2, 432)} = 2.88$; $P = 0.057$). However, there was a significant interaction between variety and species ($F_{(2, 432)} = 7.28$; $P < 0.001$), but no significant interaction between variety and population proportion ($F_{(14, 432)} = 1.08$; $P > 0.05$). Similarly, there was no significant interaction between variety, population proportion and species ($F_{(14, 432)} = 0.96$; $P > 0.05$).

The RPI ANOVA revealed significant differences between population proportion (Fig. 3.9; $F_{(7, 432)} = 36.06$; $P < 0.001$) and between species ($F_{(1, 432)} = 44.75$; $P < 0.001$), but not between varieties ($F_{(2, 432)} = 2.06$; $P > 0.05$). However, there was a significant interaction between variety and species ($F_{(2, 432)} = 4.37$; $P < 0.01$). There was no significant interaction between variety and population proportion ($F_{(2, 432)} = 1.12$; $P > 0.05$) or between species and population proportion ($F_{(14, 432)} = 0.94$; $P > 0.05$). Similarly no interaction between all three variables was evident ($F_{(14, 432)} = 0.99$; $P > 0.05$).



Source of Variation	DF	SS	MS	F	*P*
Variety	2	0.77	0.39	2.88	0.057
Density	7	13.18	1.88	14.10	<0.001
Species	1	12.44	12.44	93.11	<0.001
Variety x Density	14	2.02	0.14	1.08	>0.05
Variety x Species	2	1.95	0.97	7.28	<0.001
Density x Species	7	0.92	0.13	0.99	>0.05
Variety x Density x Species	14	1.80	0.13	0.96	>0.05
Residual	432	57.70	0.13		
Total	479	90.77	0.19		

Figure 3.8 The effect of population density (proportion of *G. pallida* P_i : *G. rostochiensis* P_i) on the multiplication rate (R) of *G. pallida* (A) and *G. rostochiensis* (B).



Source of Variation	DF	SS	MS	F	*P*
Variety	2	0.54	0.27	2.06	>0.05
Density	7	33.29	4.76	36.06	<0.001
Species	1	5.90	5.90	44.75	<0.001
Variety x Density	14	2.07	0.15	1.12	>0.05
Variety x Species	2	1.15	0.58	4.37	<0.01
Density x Species	7	0.87	0.12	0.94	>0.05
Variety x Density x Species	14	1.82	0.13	0.99	>0.05
Residual	432	56.98	0.13		
Total	479	102.63	0.21		

Figure 3.9 The effect of population proportion (*G. pallida* P_i : *G. rostochiensis* P_i) on the relative population increase (RPI) of *G. pallida* (A) and *G. rostochiensis* (B).

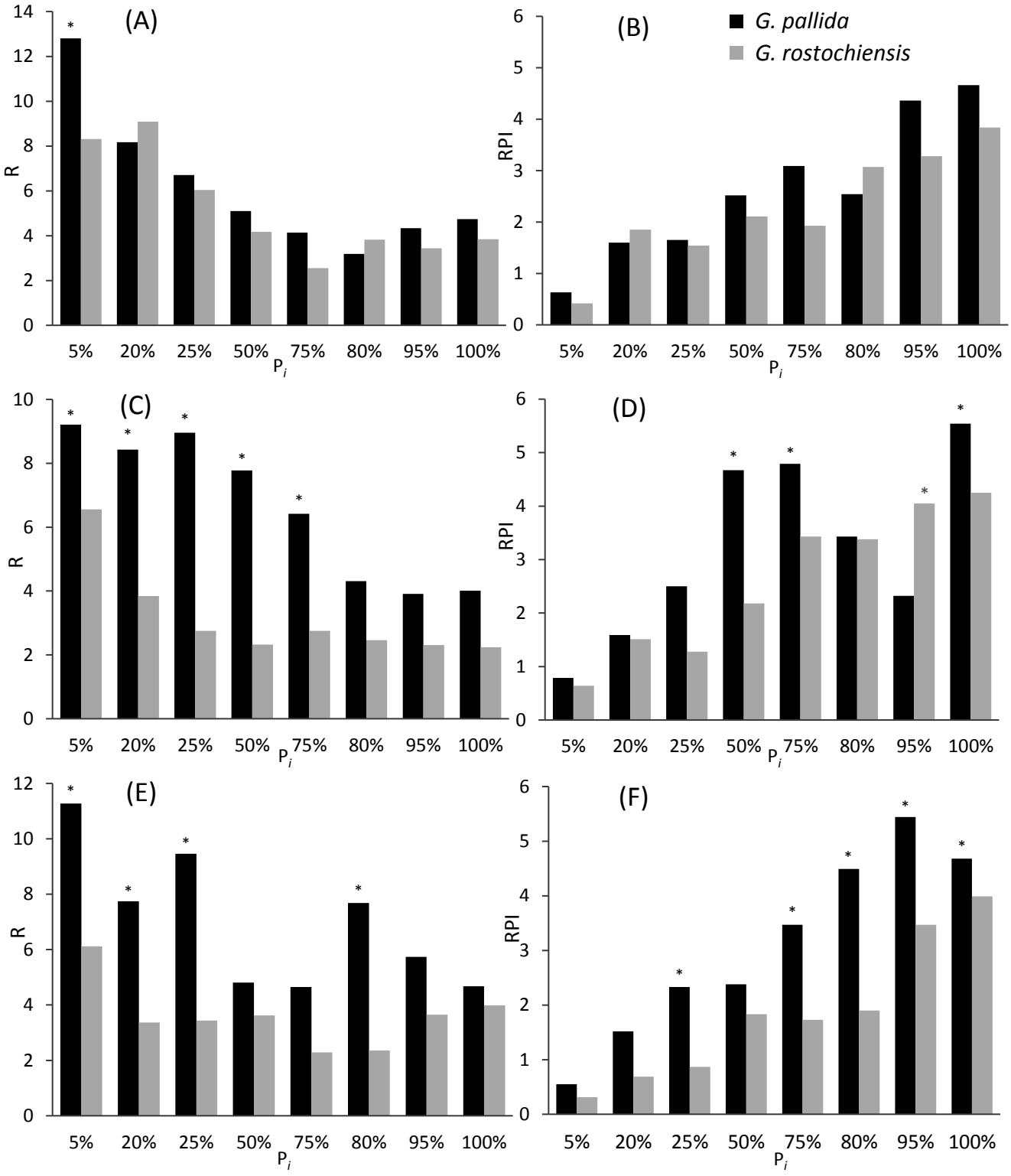


Figure 3.10 The effect of initial population density (% P_i) on the multiplication rate (R) and relative population increase (RPI) of *G. pallida* and *G. rostochiensis* in 'British Queen' (A and B), 'Kerr's Pink' (C and D) and 'Rooster' (E and F). An asterisk denotes a significant difference between species ($P < 0.05$) using the Tukey test.

Although there were no significant differences between variety for R ($P = 0.057$) and RPI ($P > 0.05$), significant interactions between species and variety for both R ($P < 0.001$) and RPI ($P < 0.01$) were evident. Significant differences ($P < 0.01$) between species multiplication at different population proportions were evident within 'Kerr's Pink' (Fig. 3.10C) and 'Rooster' (Fig. 3.10E), but the effect was not apparent in 'British Queen' (Fig. 3.10A). *G. pallida* exhibited greater multiplication rates than *G. rostochiensis* at most population proportions, particularly between 5 - 25%. 'Kerr's Pink' showed the greatest variation in multiplication and *G. pallida* had significantly higher multiplication than *G. rostochiensis* between 5% and 75% (Fig. 3.10C).

The data would suggest that the level of competition is more prominent at the lower population proportions of *G. pallida,* particularly on the late maincrop varieties 'Kerr's Pink' (Fig. 3.10C) and 'Rooster' (Fig. 3.10E). The levels of *G. pallida* and *G. rostochiensis* multiplication on 'British Queen' were quite similar, the only significant difference ($P < 0.05$) being evident at 5% (Fig. 3.10A); however, this was not reflected in the RPI data (Fig. 3.10B). *G. rostochiensis* had significantly lower RPI values when present at higher proportions in 'Rooster' but not 'Kerr's Pink' (Fig. 3.10D) or 'British Queen' (Fig. 3.10B). There was no significant difference in R ($P > 0.05$) when each species represented 100% (i.e. single-species populations), although *G. pallida* had significantly ($P < 0.05$) higher RPI than *G. rostochiensis* at 100% in 'Kerr's Pink' and 'Rooster but not 'British Queen'.

Overall, *G. pallida* expressed higher multiplication than *G. rostochiensis* at different initial population proportions (Fig. 3.11). with significantly higher multiplication in the maincrop varieties 'Kerr's Pink' and 'Rooster', although there was no significant difference between *G. pallida* and *G. rostochiensis* in the early variety 'British Queen'. Upon analysis of the species RPI as a function of the entire PCN population, there was no significant difference between species multiplication on 'British Queen' or Kerr's Pink' However, *G. rostochiensis* RPI was significantly lower ($P < 0.05$) than that of *G. pallida* on 'Rooster' (Fig. 3.11E).

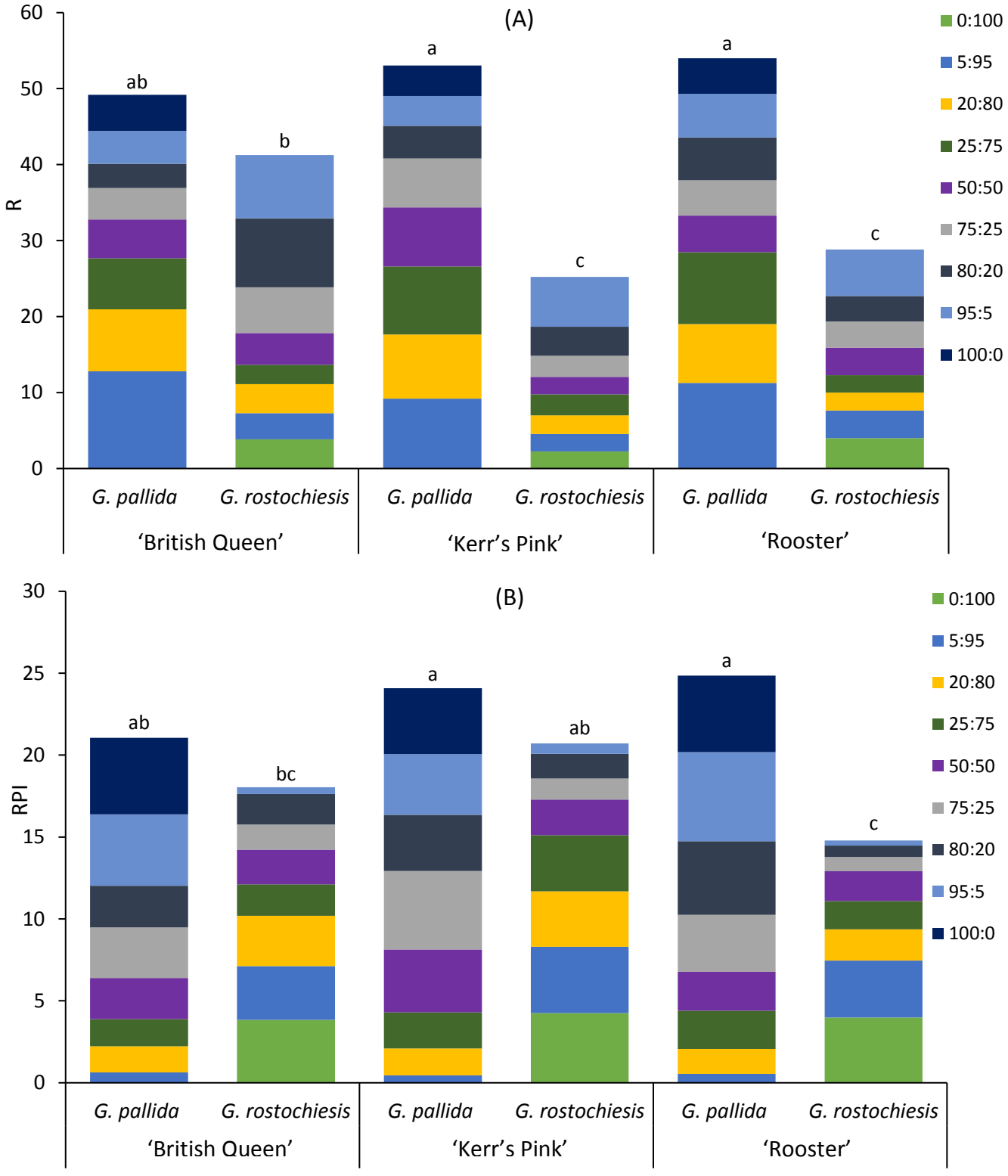


Figure 3.11 The cumulative multiplication rate (A) and relative population increase (B) of *G. pallida* and *G. rostochiensis* and the relative proportions of each species in mixed-species populations. Samples with a common letter are not significantly different ($P > 0.05$) using the Tukey test.

Polynomial regression analysis of each species revealed similar multiplication responses within each variety. Both species displayed quadratic curve conformations for on 'Kerr's Pink' and both species revealed cubic relationships on 'British Queen' and 'Rooster' (Fig. 3.14; Table 3.2). In 'Kerr's Pink', *G. pallida* exhibited a significant initial increase in P_f followed by a gradual decline with increasing P_i on 'Kerr's Pink' (Fig. 3.14; Table 3.2), whereas *G. rostochiensis* produced a linear increase ($r = 0.624$; $P < 0.001$).

The population increase curve of *G. pallida* conformed to an S-shaped curve on 'British Queen' ($r = 0.717$; $P < 0.001$) and 'Rooster' ($r = 0.720$; $P < 0.001$). *G. rostochiensis* also showed a sigmoidal population increase with a considerable degree of variation on both 'British Queen' ($r = 0.584$; $P < 0.001$) and 'Rooster' ($r = 0.638$; $P < 0.001$). *G. rostochiensis* had a much lower rate of population increase at lower infestation densities in comparison to *G. pallida*. This is particularly evident in 'Rooster' where the initial population increase between 5 - 25% was relatively low in comparison to 'Kerr's Pink' and 'British Queen". A plateau at 50 - 80% was followed by a sharp increase in P_f at higher infestation densities. *G. rostochiensis* exhibited a deflection when populations were in equilibrium (50:50) in both 'British Queen' and 'Rooster'. *G. pallida* also exhibited a pronounced deflection in 'British Queen' and to a lesser extent in 'Rooster'.

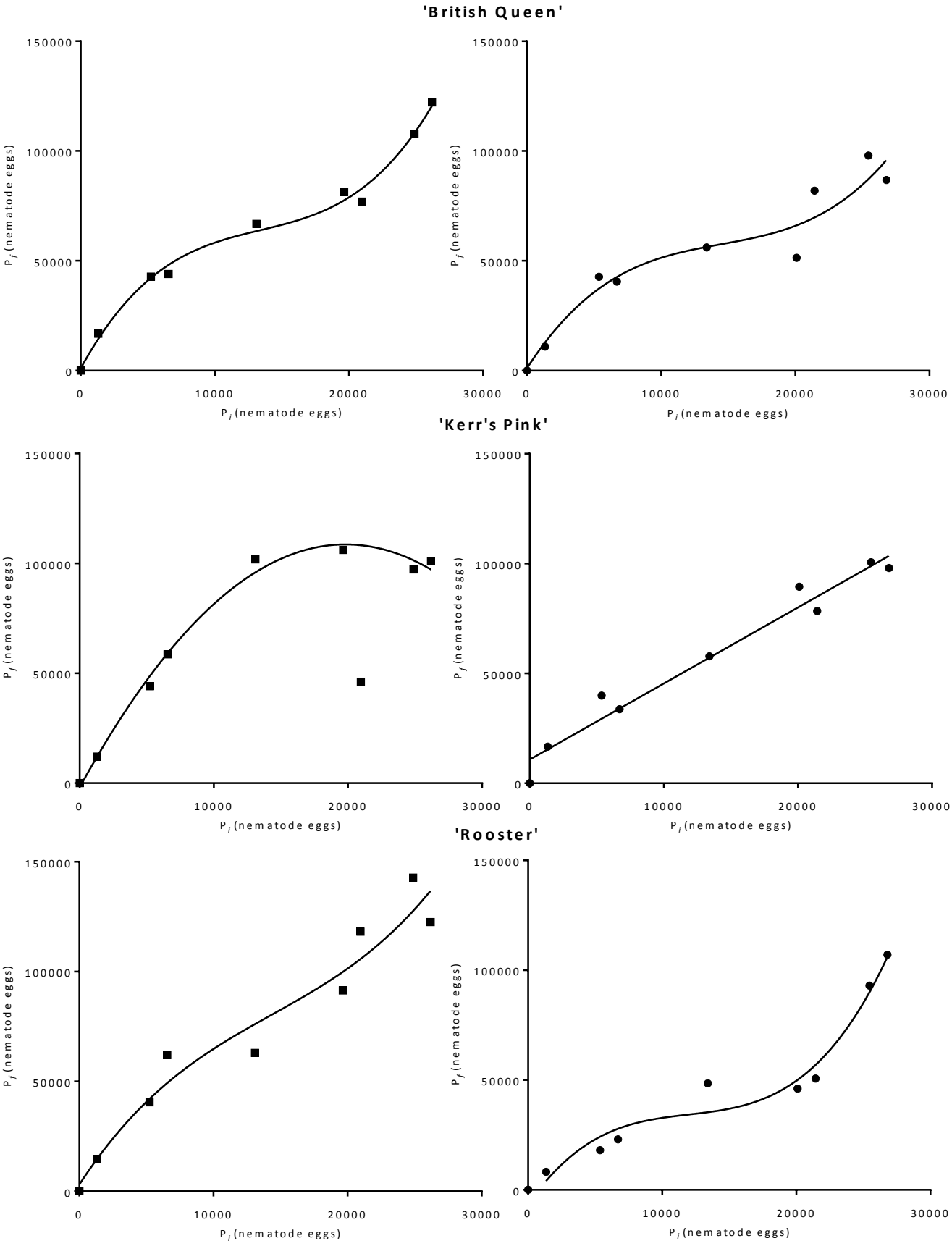


Figure 3.12 The relationship between initial population density (P*i*) and final population density (P*f*) of *G. pallida* (■) and *G. rostochiensis* (●) in 'Kerr's Pink', 'British Queen' and 'Rooster'.

Table 3.2 Regression summary table of the relationship between initial and final population density.

G. pallida	**Regression Equation**	**r**	**Adjusted r^2**	**F**	***P***
'British Queen'	$y = 4.03 + 0.0001x - 9.93e^{-9}x^2 + 2.19e^{-13}x^3$	0.707	0.481	25.36	<0.001
'Kerr's Pink'	$y = 4.10 + 8.9e^{-5}x - 2.34e^{-9}x^2$	0.624	0.374	24.56	<0.001
'Rooster'	$y = 3.99 + 0.0001x - 8.45e^{-9}x^2 + 1.72e^{-13}x^3$	0.720	0.518	27.20	<0.001
G. rostochiensis	**Regression Equation**	**r**	**Adjusted r^2**	**F**	***P***
'British Queen'	$y = 3.89 + 0.0001x - 8.21e^{-9}x^2 + 1.84e^{-13}x^3$	0.584	0.315	13.11	<0.001
'Kerr's Pink'	$y = 4.21 + 2.88e^{-5}x$	0.624	0.389	49.78	<0.001
'Rooster'	$y = 3.65 + 0.0001x - 7.7e^{-9}x^2 + 1.8e^{-13}x^3$	0.638	0.383	17.37	<0.001

3.5. Discussion

Competition is a key aspect of population ecology and plant parasitic nematodes compete intra- and interspecifically for host resources, namely space and food, and intraspecifically for mates. In PCN, the ultimate effect of competition on a population translates as a reduction in abundance, fecundity and reduced fitness contribution to the next generation. This series of experiments suggest an overwhelming dominance of *G. pallida* in mixed-species populations in the absence of PCN control measures. *G. pallida* successfully outcompeted *G. rostochiensis* in the interspecific competition assays, as well as the time-dependent and population proportion trials.

Interactions between closely related species are proposed to either enhance or inhibit nematode multiplication and thereby heighten interspecific competition (Karssen *et al.*, 2013). In this case it would appear that *G. pallida* inhibits *G. rostochiensis* via interspecific antagonism, as previously reported in published studies (Table 3.3). Marshall (1986), reported *G. rostochiensis* as the principal antagonist in interspecific competition in mixed-species populations in New Zealand. Regional variances in PCN populations and pathotypes coupled with different climatic and soil conditions may account for *G. rostochiensis* antagonism.

Table 3.3 Summary of interactions between *G. pallida* and *G. rostochiensis*. Modified from Eisenback (1993).

Dominant species	Competition	Reference
G. pallida	Interspecific antagonism	Lettice (2014)
G. pallida	Interspecific antagonism	Kaczmarek (2014)
G. pallida	Interspecific antagonism	Ryan *et al.* (2005)
G. pallida	Interspecific antagonism	Den Nijs (1992b)
G. rostochiensis	Interspecific antagonism	Marshall (1986)
G. pallida	Intraspecific antagonism	Seinhorst and Oostrom (1989)
G. pallida	Intraspecific antagonism	Seinhorst (1986)
G. pallida	Interspecific antagonism	Parrot *et al.* (1975)

The multi-variety competition experiments infer greater competitiveness of *G. pallida* in mixed-species populations relative to intraspecific populations, mainly due to suppression of *G. rostochiensis* or minimal interspecific competition. *G. pallia* multiplication was slightly higher in mixed- relative to single-species populations. *G. pallida* RPI in mixed- and single-species populations and the cumulative RPI were not significantly different. Furthermore, there was no significant difference ($P > 0.05$) in multiplication between *G. pallida* populations within any variety. These data suggest that *G. rostochiensis* populations are largely suppressed by *G. pallida*. *G. rostochiensis* RPI appeared even further reduced in mixed-species populations, which concurs with previous studies on *G. rostochiensis* inhibition due to the presence of *G. pallida* (Ryan *et al.*, 2005).

The studies inferred that maturity class can significantly influence PCN multiplication. *G. rostochiensis* performed better on the late maincrop varieties 'Kerr's Pink' and the very late maincrop 'Golden Wonder' in single-species populations. *G. rostochiensis* exhibited an affinity for later eluting hatching factors and later maturing maincrop varieties (Byrne *et al.*, 2001; Devine & Jones, 2003b). Conversely, *G. pallida* performed better than *G. rostochiensis* on early maturing varieties (Lettice, 2014).

G. pallida dominance in mixed-species populations was apparent in the time-dependent assays. It was postulated that *G. pallida* competiveness was causally associated with its delayed hatch (Ryan *et al.*, 2005). The staggered inoculation trials manipulated the timing of *G. pallida* hatch and the order of J2 emergence and infestation. *G. pallida* successfully outcompeted *G. rostochiensis* when inoculated before or 7 d after *G. rostochiensis*. However, the effect was less apparent upon simultaneous inoculation of both species, which is supported by the findings of Ryan *et al.*, (2005) and Lettice (2014).

Concomitant application of *G. pallida* and *G. rostochiensis* would have the highest level of both intra- and interspecific competition due to the high density of juveniles of both species inundating the roots at one time. In high nematode infestations, several thousand J2 emerge simultaneously to locate and infect a potential host root. The juvenile and egg suspension method of application employed in this study simulates an inundative release of nematodes. This instigates extreme competition for feeding sites and resources. Moreover, J2 perception of other competitors can instigate population stimulation or inhibition (Eisenback, 1993). This effect is a plausible reason for the heightened competition evident in the mixed-species and time-dependent trials.

It is proposed that the early-hatching *G. rostochiensis* J2 induce morphological root changes (§ 5.1.2) leading to excessive root branching (Widdowson *et al.*, 1958), potentially creating more root tips and a greater root surface area for the later-colonising *G. pallida* juveniles. However, there was no apparent difference in multiplication between inoculation times I_0 (first application) and I_1 (second application) within either species. The only significant difference between inoculation times was evident in *G. pallida*, which exhibited significantly lower multiplication in I_s (simultaneous application) compared to other application times.

G. rostochiensis exhibited little variation in multiplication across all treatments, indeed the only notable difference in multiplication between varieties occurred in 'Kerr's Pink'.

Differences in variety may be attributed to root morphology and tolerance. The time-dependent trials revealed significant differences between varieties and an interaction between variety and timing. Further studies are required to determine the exact cause of this effect. It is most probable that temporal changes and varietal differences in physiology, induced resistance and PCN-induced root proliferation contribute to this effect (Chapter 5). Differential virulence on different cultivars will also affect multiplication (Phillips *et al.*, 2006).

PCN exhibit both scramble and contest competition (Van den Berg *et al.,* 2006). Scramble competition (exploitation) occurs when a population competes equally for finite resources. This may result in reduced fecundity as the number of feeding sites decline with increased population density. Contest (interference) competition occurs when the finite resources are unequally partitioned between species; this is evident when prime feeding sites are restricted due to already established females.

Either form of competition may exist temporally throughout the infective and sedentary biotrophic parasitic stages of the PCN life cycle and competition is similarly modulated by host physiology, i.e. resource availability. Initially, scramble competition is the main determinant of population success during early infestation. However, contest competition will become more intense as resources are exhausted, particularly by the later time of *G. pallida* emergence. This would suggest that interference competition is necessary to displace *G. rostochiensis* populations. As such, it would appear that *G. rostochiensis* is more susceptible to contest competition with *G. pallida*. Such antagonism would have a significant effect on the male-to-female ratio of nematodes and consequently influence multiplication and species proportions in successive generations.

PCN population dynamics have been described by numerous models integrating many population variables, namely residual population, fecundity, virulence, varietal resistance and competition. According to the earliest models of Seinhorst (1966), at low infestation

densities, multiplication is limited only by nutrient availability and competition is negligible when resources are adequate. Conversely, at high population densities nematode multiplication is limited by competition for resources, namely root tip density and surface area for colonisation and nutrition. As such, plant growth declines due to PCN infestation and a decline in resources ensues. As a result, there is a negative correlation between resource availability and nematode multiplication, which can exacerbate intra- and interspecific competition. PCN population growth models typically conform to a logistic growth curve with substantial initial population increase at low P_i (Evans *et al.*, 2003). Population growth gradually stabilizes, reaching an asymptote signifying the population carrying capacity, followed by population decline with increasing P_i.

In this study, the population proportion trials exhibited quadratic and cubic polynomial regression growth. However, the study had a narrow experimental range from 0 – 100 cysts which equates to a maximum of 5 eggs g soil^{-1}. These initial infestation densities are relatively low in comparison to those described in population models (Seinhorst, 1970; Trudgill, 1986; Phillips *et al.*, 1991). For instance, Evans *et al.* (2003) described an infestation density range between 0 - 2500 eggs g soil^{-1}. Moxnes and Hausken (2007), proposed that competition is not a limiting factor until PCN densities exceed a threshold of 1,500 eggs g^{-1} soil. Therefore, the range of initial infestation densities represented in this study typically reflect those defined in the exponential growth phase of logistic PCN growth models. (Phillips *et al.*, 1991; Phillips & Trudgill, 1998a; Moxnes & Hausken, 2007; Trudgill *et al.*, 2014). Consequently, the PCN growth curves presented in this study represent low PCN population densities (Elston *et al.*, 1991; Trudgill & Phillips, 1997). Nevertheless, the trials confirmed the greater competitiveness of *G. pallida* in mixed-species populations and revealed a greater degree of competitiveness at lower infestation densities, which was most evident in 'Kerr's Pink' and 'Rooster'.

G. pallida competiveness subsided at higher infestation densities which was most apparent in 'British Queen' and 'Rooster'. Although variety did not have a significant effect on *G. pallida* R or RPI, both values in 'British Queen' were considerably lower than those evident in 'Kerr's Pink' and 'Rooster'. Furthermore, there was no significant difference between *G. pallida* and *G. rostochiensis* R and RPI in the early variety 'British Queen' and both species exhibited similar multiplication curves in this variety. The differential responses to variety may have been impacted by planting date. The population proportion dependent trials were planted relatively late in the season (May), which may have affected growth in the early variety and favoured the late-maincrop varieties, such as 'Kerr's Pink' and 'Rooster'.

Competition is strongest among species that are similar in terms of physiology, pathogenicity and host demands (Ettema, 1998). As sibling species, *G. pallida* and *G. rostochiensis* occupy the same ecological niche and therefore, both species cannot coexist in equilibrium (Parrot *et al.*, 1975; Eisenback, 1993). Kort and Bakker (1980) proposed that both species may temporally coincide at various population proportions, but one species will eventually dominate, leading to species displacement. A natural soil community is characterised by a sustainable level of competition and predation that facilitates community equilibrium. Niche partitioning mechanisms may reduce the extent of interspecific competition and thereby permit coexistence of both species (Al-Naimi *et al.*, 2005; Duyck *et al.*, 2012). In essence, the intrinsic differences between species may enhance *G. pallida* dominance whilst permitting *G. rostochiensis* persistence in mixed-species populations.

Niche adaptations include soil temperature optima, variations in physiology, virulence, metabolism and hatching mechanisms (Chapter 4). Such adaptations include the lower hatching optimum and lower rate of lipid utilization of *G. pallida* (Robinson *et al.*, 1987). Furthermore *G. pallida* has a faster rate of embryogenesis and female development at lower temperature optima. Webley and Jones (1981) proposed that these factors would

favour *G. pallida* during colder temperatures of the spring period and early harvesting would select for *G. pallida*.

The most significant interactions and intense competition occur when juveniles infest the same root. Root surface exploration and recognition of competing juveniles forces nematodes to compete and successfully penetrate the host root, leading to female and syncytium establishment or maturation into males. Alternatively, such high levels of concomitant J2 infestation may force juveniles to locate an alternative root tip as a result of the 'contest' interaction. The slower and prolonged rate of *G. pallida* hatch may significantly reduce intraspecific competition as root infestation is less inundated relative to the shorter and faster rate of *G. rostochiensis* hatch. Den Nijs (1992a) proposed that mating competition between species can negatively impact reproductive output. Interference competition between males to mate with females may interrupt intraspecific mating due to an excess influx of males of the other species. This potentially results in cross fertilization between species that may result in sterile or deformed hybrids (Den Nijs, 1992b; Eisenback, 1993).

3.5.1. Critical evaluation and future research recommendations

This series of competition assays supports the hypothesis of *G. pallida* antagonism and *G. rostochiensis* inhibition in mixed-species populations (Den Nijs, 1992b; Ryan *et al.*, 2005) However, further studies are required to characterise fully PCN interactions and population dynamics. Research entailing analysis of population fitness could help elucidate the greater competitiveness of *G. pallida*. Female development, the rate of embryogenesis and female fecundity are important variables in population dynamics and are recommended for future multi-variety studies. A comparative analysis of the number of hatched J2, infectivity rate, infection success rate and the male-to-female ratio of *G. pallida* and *G. rostochiensis* in mixed- and single-species populations would similarly provide a comprehensive understanding of PCN populations. Evaluation of the effect of inundative and augmentative J2 inoculation on the root system may provide an insight

into the mechanisms of intra- and interspecific competition in PCN infestation. Future population proportion trials should include intra- and interspecific analyses on root systems to examine the behavioural responses of nematodes in each scenario. Interspecies communication/perception and plant-nematode interactions will be discussed further in Chapter 4 and 5 respectively.

Plant vigour is an important determinant of PCN population success. However, yield and growth parameters were not included in this study. Ideally, yield and root biomass data should be correlated with PCN multiplication to provide a thorough account of PCN infestation and relative success on multi-variety host root systems. Nematode populations can be difficult to manipulate, particularly in population proportion trials. As a result, experiments in nematology are complicated by variation in soil populations and environmental constraints (van den Berg *et al.*, 2006). Nematode populations are governed by numerous biotic and abiotic factors. Multivariate analysis of nematode populations is therefore imperative in future studies to efficiently evaluate population dynamics.

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Chapter 4 Role of intrinsic hatch characteristics of *G. pallida* and *G. rostochiensis* in interspecific competition

Abstract

In mixed-species PCN populations, *G. pallida* outcompeted *G. rostochiensis in vivo* in terms of multiplication rate. The research described in this chapter was designed to determine whether hatching characteristics of *G. pallida* could contribute to its greater competitiveness. A series of *in vitro* hatching bioassays were conducted to assess differential hatching responses of the two species under various abiotic and biotic hatching conditions. The overall hatching efficiency of *G. pallida* was significantly greater than that of *G. rostochiensis*. The abiotic assays assessed the time course of hatching responses and the temperature-dependent hatching responses of each species. *G. pallida* had a lower hatching optimum temperature relative to *G. rostochiensis*. There was a significant difference between mixed- and single-species hatching assays, particularly the marked decline in *G. rostochiensis* hatch in mixed-species populations. Species-specific chemoreception of hetero- and homospecific compounds on PCN hatch was also investigated. The results suggested the inhibition of *G. rostochiensis* hatch in the presence of compounds released during *G. pallida* hatch and the stimulation of *G. pallida* hatch in the presence of *G. rostochiensis*-derived compounds. The results suggest that the hatching characteristics of *G. pallida* may contribute to the greater competitiveness of this species.

4.1. Introduction

Hatching is a crucial element of the PCN life cycle. Successful J2 hatch is critical to effective PCN infestation and host parasitism. Hatching represents the termination of quiescence and is mainly initiated by host-derived chemical cues and is regulated by numerous biotic factors and abiotic factors (§ 1.4.6). The two sibling species *G. pallida* and *G. rostochiensis* have similar lifecycles (Turner & Evans, 1998). Furthermore, both species share the same habitat and often coexist in field populations and are therefore exposed to similar environmental conditions and host physiologies. Despite the similar life-cycles and ecologies of the two species, *G. rostochiensis* and *G. pallida* are differentiated by 70% of their polypeptides (Bakker & Bouwman-Smits, 1988). This reflects a vast degree of interspecific variation between the two PCN species (Bendezu *et al.*, 1998; Manduric & Andersson, 2003; Plantard *et al.*, 2008) and the two species exhibit different eco-physiological adaptations (Table 4.1).

4.1.1. Intrinsic hatching differences

The primary differences in hatch between the two PCN species include the rate of hatch, timing of hatch and differences in temperature optima. HF-specificity and physiological and metabolic differences in J2 also contribute to interspecific variation in PCN hatch. Lipid reserves and the rate of consumption also vary between the two species. *G. pallida,* having greater lipid reserves and a more efficient rate of consumption, has greater in soil juvenile longevity relative to *G. rostochiensis* (Robinson *et al.*, 1987). *G. pallida* exhibits a delayed hatching process and a prolonged hatching period, resulting in a later annual peak in hatching activity than that of *G. rostochiensis* (Robinson *et al.,* 1987; Turner & Evans, 1998; Devine & Jones, 2001b). Indeed, the greater HF concentration required to stimulate *G. pallida* hatch is proposed as part of the reason for its delayed hatch (Den Nijs & Lock, 1992).

Spontaneous hatch occurs in the absence of a host crop. Depending on soil type and temperature, spontaneous hatch approximates to 20% and 30% annually for *G. pallida*

and *G. rostochiensis,* respectively (Hockland, 2002). The spontaneous hatch of *G. rostochiensis* is significantly higher and earlier than that of *G. pallida* early in the season (Ryan & Devine, 2005). Later in the season, however, both PCN species exhibit a similar degree of hatch (Ryan & Devine, 2005). The slower and later hatch of the more conservative *G. pallida* may also account for its slower decline rate during crop rotation (Den Nijs & Lock, 1992; Turner, 1996; Byrne *et al.*, 2001).

Table 4.1 Physiological and hatching differences between *G. pallida* and *G. rostochiensis.*

G. rostochiensis	***G. pallida***	**Reference**
Higher in-field spontaneous hatch	Lower in-field spontaneous hatch	Devine *et al.* (1999); Hockland (2002)
Faster spontaneous hatch	Slower spontaneous hatch	Ryan and Devine (2005)
Rapid initial hatch	Prolonged hatch	Robinson *et al.* (1987); Salazar and Ritter (1993)
Early hatch	Later hatch	Stanton and Sartori (1990)
Specificity for later-eluted HF	Specificity for early-eluted HF	Byrne *et al.* (2001)
Faster decline rate in soil	Slower decline rate in soil	Whitehead (1995); Turner (1996)
Higher hatch optimum temperature	Lower hatching optimum temperature	Franco (1979)
Reduced lipid reserves	Slower rate of lipid utilization	Robinson *et al.* (1987)
Slower embryogenesis and post-embryonic development	Faster embryogenesis and post-embryonic development	Perry *et al.* (2002)
Shorter developmental thermal time	Longer developmental thermal time	Robinson *et al.* (1987); Ebrahimi *et al.* (2014)
Higher basal developmental temperature	Lower basal developmental temperature	Mugniery (1978)
Reduced HF-selectivity	Greater HF-selectivity	Byrne *et al.* (2001)

4.1.2. Species-selective responses to HF

G. pallida appears to have a more PRL-dependent hatching response, as illustrated by its lower spontaneous hatch rate (Devine *et al.*, 1999; Hockland, 2002). Therefore, the more specific and conservative *G. pallida* hatch response may account for its greater hatching efficiency, its delayed and more prolonged hatch and its slower decline rate in soil (Den Nijs & Lock, 1992; Byrne *et al.*, 2001). Conversely, the higher level of spontaneous hatch and in-egg mortality exhibited by *G. rostochiensis* may be attributed to its lack of HF specificity (Devine & Jones, 2001b).

G. rostochiensis and *G. pallida* exhibit different hatching behaviour in response to PRL and exhibit different preferences and selectivity to individual HFs. PCN response to HF concentration gradients often reflects species-sensitivity to PRL (Devine & Jones, 2001a) and the respective HF concentration thresholds required for hatch stimulation (Rawsthorne & Brodie, 1986). The degree of HF-sensitisation affects J2 chemotactic responses; PRL-hatched *G. pallida* J2 are attracted to different HF fractions than those of *G. rostochiensis*. On the other hand, water-hatched *G. pallida* and *G. rostochiensis* J2 are attracted to common HF fractions (Devine & Jones, 2000a). Variation in the hatching responses to HF is also evident between species and pathotypes (Byrne *et al.*, 2001). As such, the temporal expression of species-selective HF can greatly affect PCN hatch and subsequently influence population dynamics (Devine & Jones, 2001b).

Conventionally and aseptically grown plants exhibit similar HF profiles for early-eluting HF, which are most active towards *G. rostochiensis*. Conversely, there are marked differences in the late-eluting HF profiles, with extra HF present in the PRL of conventional potato plants compared to aseptically-grown plants (Ryan *et al.*, 2003). Moreover, colonisation of potato roots with mycorrhizal fungi or rhizobacteria increases the *in vitro* hatch of *G. pallida* but not *G. rostochiensis* (Ryan *et al.*, 2000; Ryan & Jones, 2004). This suggests microbial-mediated production of HFs contributing to either greater HF quantities to satisfy the greater *G. pallida* hatch threshold or the production of *G. pallida*-

selective HF and HS (Ryan & Jones, 2003). Mycorrhization has been show to increase root biomass and consequently to augment PRL production and enhance PCN multiplication on plants due to greater colonization area (Ryan *et al.*, 2003). AMF-inoculation of potato roots also eliminated the time delay between *G. rostochiensis* and *G. pallida* hatch by stimulating the earlier production of *G. pallida*-selective HF (Ryan *et al.*, 2000).

4.1.3. Temperature

Temperature, coupled with relative humidity, directly influences PCN infectivity, longevity, development and reproduction. Host metabolic processes and induced responses to abiotic stresses may inadvertently affect hatch by altering PRL production and potentially modifying the different proportions of HFs in PRL. Furthermore, temperature can modulate the production of root leachates (Badri & Vivanco, 2009). The persistence of PRL in soil may also be compromised by temperature; organic compounds, such as hatching chemicals, are subject to decay by microbial activity in the rhizosphere, which is also regulated by temperature (Saraf *et al.*, 2014).

Nematodes exhibit different temperature optima for different stages of the life cycle, i.e. hatching, feeding, cyst development, reproduction, induction of dormancy and survival. The PCN life cycle is complete within a period of 38-48 days depending on soil temperature and host development (Chitwood & Buhrer, 1945). Dormancy is terminated when soil temperatures are favourable for PCN hatch, which is generally above 10 °C (Ferris, 1957). Facultative diapause ranges from 3 to 12 months and can be terminated if cysts are preserved below 4 °C for more than 3 months (Chitwood and Buhrer, 1945). In-soil temperature is the primary abiotic factor affecting hatch and is a critical environmental cue for the termination of obligate diapause and quiescence (Ebrahimi *et al.*, 2014).

The optimal hatching temperature of PCN has been extensively studied (Franco, 1979; Robinson *et al.*, 1987; Greco *et al.*, 1988; Stanton & Sartori, 1990). *G. pallida* is better

adapted to lower temperatures with an optimum temperature range of 10 to 20°C and maximal hatching at 18°C (Franco, 1979). *G. rostochiensis* is adapted to higher temperatures, with multiplication rates increasing above 20°C (Franco, 1979). Robinson *et al.* (1987) reported *in vitro* hatching temperature optima of 16°C and 20°C in *G. pallida* and *G. rostochiensis,* respectively, but concluded that the activity of hatched juveniles of both species had similar optimum temperatures.

Temperature significantly influences the rate and duration of female development and embryogenesis in PCN. Mugniery (1978) recorded a post-embryonic development temperature optimum of 9.5°C and 11.5°C for *G. pallida* and *G. rostochiensis,* respectively and basal temperatures of 3.9°C and 272 degree days (DD) for *G. pallida* and 6.2°C and 204 DD for *G. rostochiensis*. Studies by Ebrahimi *et al.* (2014) confirmed the basal lower temperature preference of *G. pallida,* but recorded a longer thermal time for both species. The basal temperature of *G. pallida* was determined to be 4°C, while thermal time was calculated at 450 DD, whereas *G. rostochiensis* required 398 DD and a basal temperature of 6°C to complete its life cycle (Ebrahimi *et al.,* 2014).

Host physiology and host responses to environmental variables such as temperature and photoperiod significantly impact the rate of female development (Hominick, 1986). Thermal stress to the host may reduce female fecundity and increase the male-to-female ratio and consequently affect nematode populations. Extremely high soil temperatures (above 30°C) adversely affect egg viability and significantly increase the nematode decline rate (Greco *et al.*, 1988; Turner & Evans, 1998).

PCN is a widely distributed pest in the potato-growing areas and has adapted to tropical and temperate climates, which are characterised by a wide range of soil temperatures. PCN populations in these climatic regions have adapted accordingly. Inter-regional variations in optimal hatching temperature thresholds have been observed among PCN populations. Hatching activity at lower temperatures has been documented in PCN

populations at higher latitudes, with Finnish populations of *G. rostochiensis* successfully hatching at 4°C (Stoyonov & Tilikkala, 1995). Temperatures decline with increasing soil depth; consequently, the lower *G. pallida* hatching optima may enable it to adaptively hatch at wider range of soil depths and thereby enhance hatching efficiency (Barker & Koenning, 1998). Soil temperatures in Ireland during the PCN hatching period (March to May) range between 8 and 13 °C (Fig. 4.1).

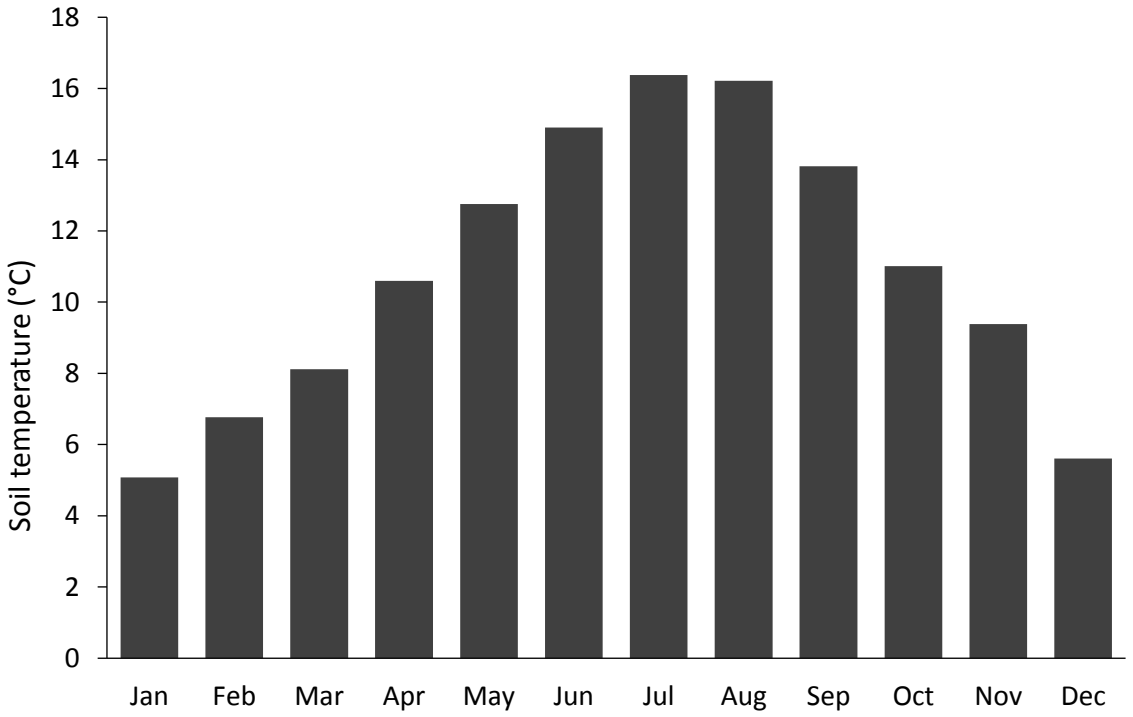


Figure 4.1 Average monthly soil temperatures in Ireland between 2009 and 2012 (Met Eireann, 2012).

4.2. Aims and objectives

The research in this chapter aimed to examine the differential effects of abiotic and biotic conditions on *G. pallida* and *G. rostochiensis* hatching dynamics and their possible role in determining interspecific competitiveness. The effect of temperature on PCN hatch will be examined and the temporal differences in PCN hatching are studied to assess whether the reported prolonged hatch of *G. pallida* is a strategic advantage over the rapid initial hatch of *G. rostochiensis*. Interspecific hatching differences will be monitored in density-dependent population proportion and mixed- and single-species populations *in vitro*. Putative interspecific signalling and communication is investigated via PRL exchange

hatching assays to assess PCN hatching responses to *G. pallida*- and *G. rostochiensis*-sensitised PRL.

4.3. Materials and Methods

4.3.1. Nematodes

Single-generation *G. pallida* pathotype Pa 2/3 and *G. rostochiensis* pathotype (Ro 1) cysts, generously supplied by Dr. Colin Fleming, Agri-Food and Biosciences Institute, Belfast, Northern Ireland, were used throughout the experiment (§ 3.3.1). PCN cysts were pre-soaked for one week in Petri dishes lined with filter paper (Whatman no. 5, Ø = 90 mm) moistened with 5 ml distilled water.

4.3.2. Sterile PRL

PRL was isolated from potato plants as described in § 3.3.6. Sterile PRL from 'Golden Wonder' potato plants, diluted to a final concentration of 0.17 g l^{-1}, was used in all experiments. Sterile distilled water was used as a control in all assays.

4.3.3. Egg viability

Viability tests were performed as described in § 3.3.2. The percentage of non-viable eggs was estimated by removing 20 µl aliquots from each sample and counting the numbers of stained (non-viable) and non-stained (viable) eggs. Only completely stained eggs were considered non-viable. Three replicates were analysed from each of three samples. A crush test was used to count the total number of eggs per sample to deduce the number of eggs per cyst. The average weight of individual cysts was calculated by counting 15 replicate samples, each of 100 cysts, and determining the average cyst weight.

4.3.4. Hatching assays

Five pre-soaked PCN cysts were placed in a single well of a microtitre plate (Nunc, ThermoFisher, Dublin, Ireland) containing a 100 µl aliquot of PRL test solution (i.e. 20 µl per cyst). The microplate containing cysts was placed in a humidity chamber, which

was constructed from a polypropylene box (Sarstedt, Wexford, Ireland) with a perforated lid, the box being lined with moistened tissue paper. The cysts were incubated in the dark at 20 ºC for one week. The PRL was subsequently removed and cysts were rinsed twice in sterile distilled water. The cysts were subsequently soaked in 100 µl Meldola's Blue stain (Sigma Aldrich, Wicklow, Ireland) solution (0.05 % w/v) for one week Thereafter, cysts were washed in dH_2O and soaked for 24 h. The cysts were then placed in a 1.5 ml microtube (Sarstedt, Wexford, Ireland) containing 200 µl dH_2O. Cysts were mechanically crushed using a polypropylene homogeniser (Sigma Aldrich, Wicklow, Ireland) to free the enclosed eggs (Twomey *et al.*, 1995; Byrne *et al.*, 1998). The solution was vortexed and three 20 µl subsamples were extracted for analysis. Samples were observed under a light microscope at 400 x magnification; the number of viable unhatched eggs (unstained eggs), non-viable (stained eggs) and hatched eggs were counted. Hatching activity was deduced according to the formula (Byrne, 1997):

$$\% \text{ viable hatch} = \frac{(\text{number of hatched eggs} \times 100)}{(\text{number of hatched eggs} + \text{number of unhatched viable eggs})}$$

4.3.5. The effect of temperature on hatching

Hatching assays were established as described in § 4.3.4. Hatching assay plates were contained in humidity chambers (§ 4.3.4) and placed in incubators at set temperatures (8, 10, 12, 14, 16, 18, 20, 22, 24, 26 ºC) for 15 d.

4.3.6. Time course of hatch

Hatching assays were performed as described in § 4.3.4. After one week, cysts from three replicate wells were randomly selected from the bioassay unit on alternate days for a 26-day period to assess hatching activity over time. Hatching activity in each replicate was analysed in triplicate.

4.3.7. Single- and mixed-species hatching assays

Hatching assays were established and analysed as described in § 4.3.4. For mixed-species hatching assays, five cysts of each species were added to the same microtitre well containing 200 µl PRL. A permeable nylon mesh barrier (200 µm mesh size) was secured within each well to separate the cysts of the two species and to facilitate species discrimination for hatching analyses.

4.3.8. Hatch responses to inter- and intraspecific PRL

Single-species nematode hatching assays were established and analysed (§ 4.3.4). After one week, the PRL from *G. pallida* and *G. rostochiensis* treatments was collected and used to stimulate hatch of either the same or different PCN species. Intraspecific PRL hatching assays consisted of (a) *G. pallida* cysts in *G. pallida* PRL and (b) *G. rostochiensis* cysts in *G. rostochiensis* PRL. Interspecific PRL hatching assays included (a) *G. pallida* cysts in *G. rostochiensis* PRL and (b) *G. rostochiensis* cysts in *G. pallida* PRL. Treatments were maintained in a humidity chamber in darkness at 20 °C and analysed (§ 4.3.4).

4.3.9. Density-dependent hatching responses

Hatching assays were established in unsealed 1.5 ml microtubes, consisting of a permeable 200 µm-mesh partition, secured to separate cysts of either species and to establish different densities (Fig 4.2).

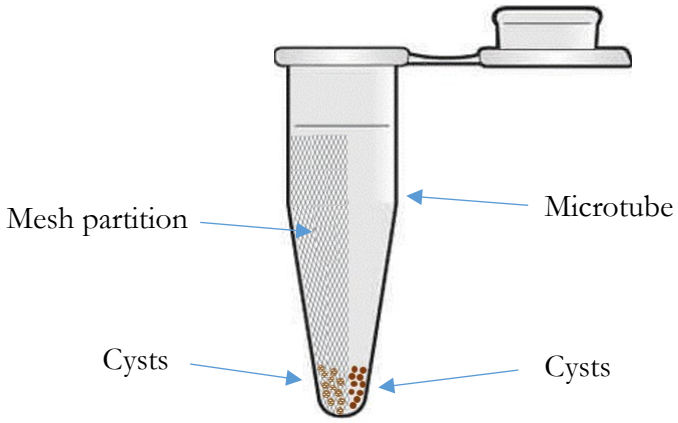


Figure 4.2 Modified 1.5 ml microtube with a 300 µm mesh partition to separate *G. pallida* and *G. rostochiensis* cysts and permit PRL exchange.

A preliminary hatching assay was performed to test the hatching environment and to ensure that test conditions comparable to the microtitre plate hatching assays. Two different population sizes (N) were established: small population (N = 100 cysts) and large populations (N = 1000). A microtube for the small population contained 200 µl PRL, while the one for the large population contained 400 µl PRL. Treatments were maintained and analysed as described in § 4.3.4. Within each population size, different population proportions of *G. pallida* and *G. rostostochiensis* were established, namely:

Small population	**Large population**
G. pallida : G. rostochiensis	*G. pallida : G. rostochiensis*
100 : 0	0 : 1000
5 : 95	100 : 900
10 : 90	250 : 750
25 : 75	500 : 500
50 : 50	750 : 250
75 : 25	900 : 100
90 : 10	1000 : 0
95 : 5	
0 : 100	

4.3.10. Statistical analysis

All statistical analysis was carried out using SigmaPlot V 12.5 (Systat Software Inc., Erkrath, Germany). All datasets were checked for normality and homogeneity of variance, and normalised by transformation where necessary. Data presented graphically represent the mean values of untransformed data. Population viability characteristics were compared using a two-tailed t-test. All other datasets were analysed with ANOVA followed by *post hoc* multiple comparison analyses using the Tukey test. Second order polynomial regressions were performed to assess relationships between temperature or time and hatching.

4.4. Results

4.4.1. Population characteristics

Egg viability was expressed as the proportion of unstained eggs within a cyst. Single generation cysts of *G. pallida* Pa 2/3 and *G. rostochiensis* Ro 1 cysts, produced under identical conditions, revealed small differences in overall biomass, female fecundity and hatching efficiency (Fig. 4.3). Biomass was measured as overall weight per cyst (Fig. 4.3). *G. rostochiensis* had a slightly higher biomass (mean ± SE = 0.0652 mg ± 0.0011) compared to *G. pallida* (0.0620 mg ± 0.0028); however, there was no significant difference between species ($t_{(28)}$ = 1.048; $P > 0.05$; Fig. 4.3).

Biomass data did not positively correlate with total egg count per cyst. *G. pallida* had a higher egg count relative to *G. rostochiensis*, with averages of 376 and 306 eggs per cyst respectively (Fig. 4.3); however, there was no significant difference in egg count between populations ($t_{(16)}$ = 1.49; $P > 0.05$). There was a significant difference in hatching efficiency between the two species ($t_{(16)}$ = 2.287; $P < 0.05$), *G. pallida* exhibited a higher (mean ± SE) degree of viable egg hatch (80.55 % ± 2.37) relative to *G. rostochiensis* (68.53 % ± 4.69). *G. pallida* had a significantly ($t_{(16)}$ = 2.42; $P < 0.05$) higher proportion of viable eggs (88.23 % ± 1.69) relative to *G. rostochiensis* (81.14 % ± 2.38)

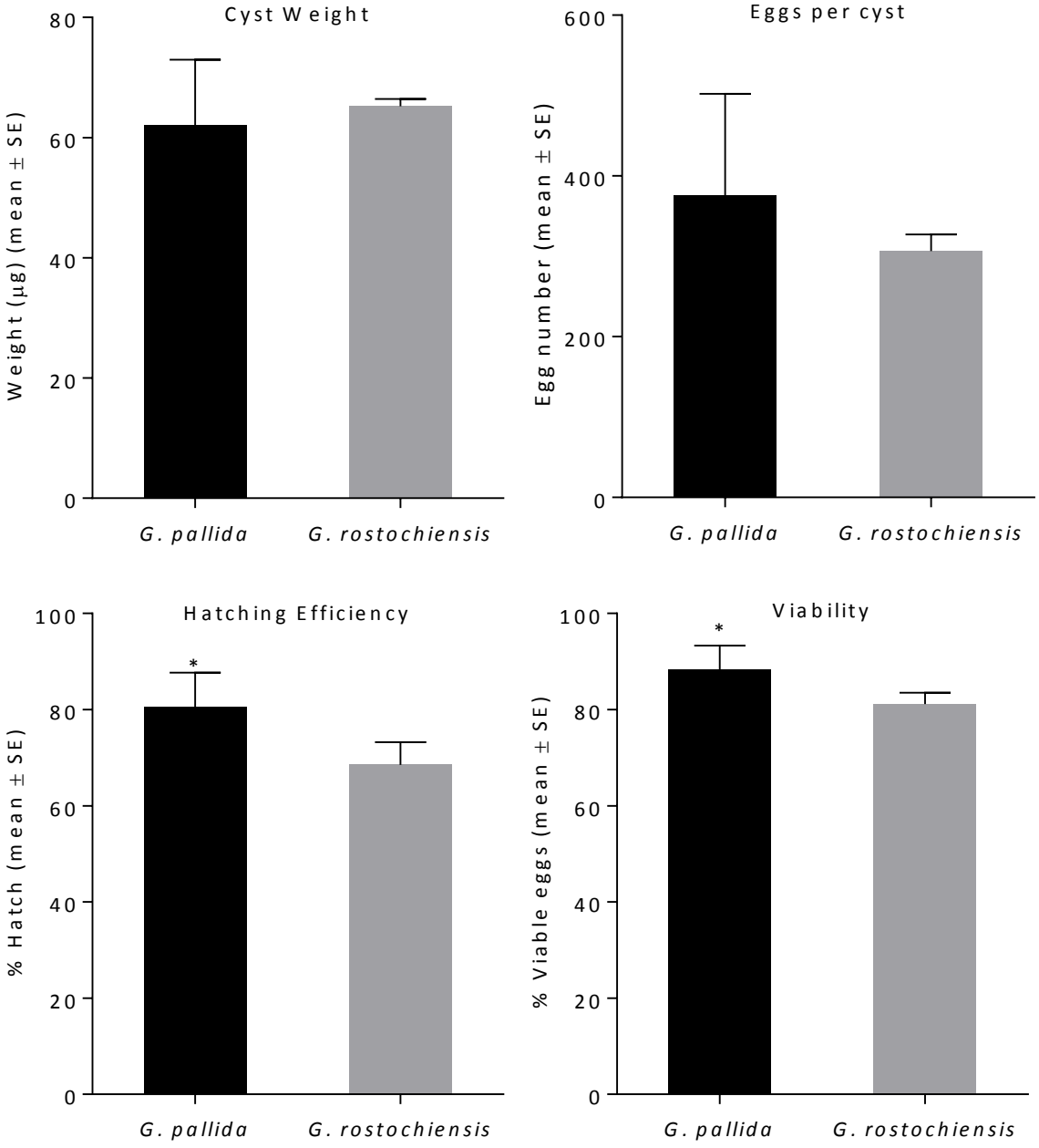
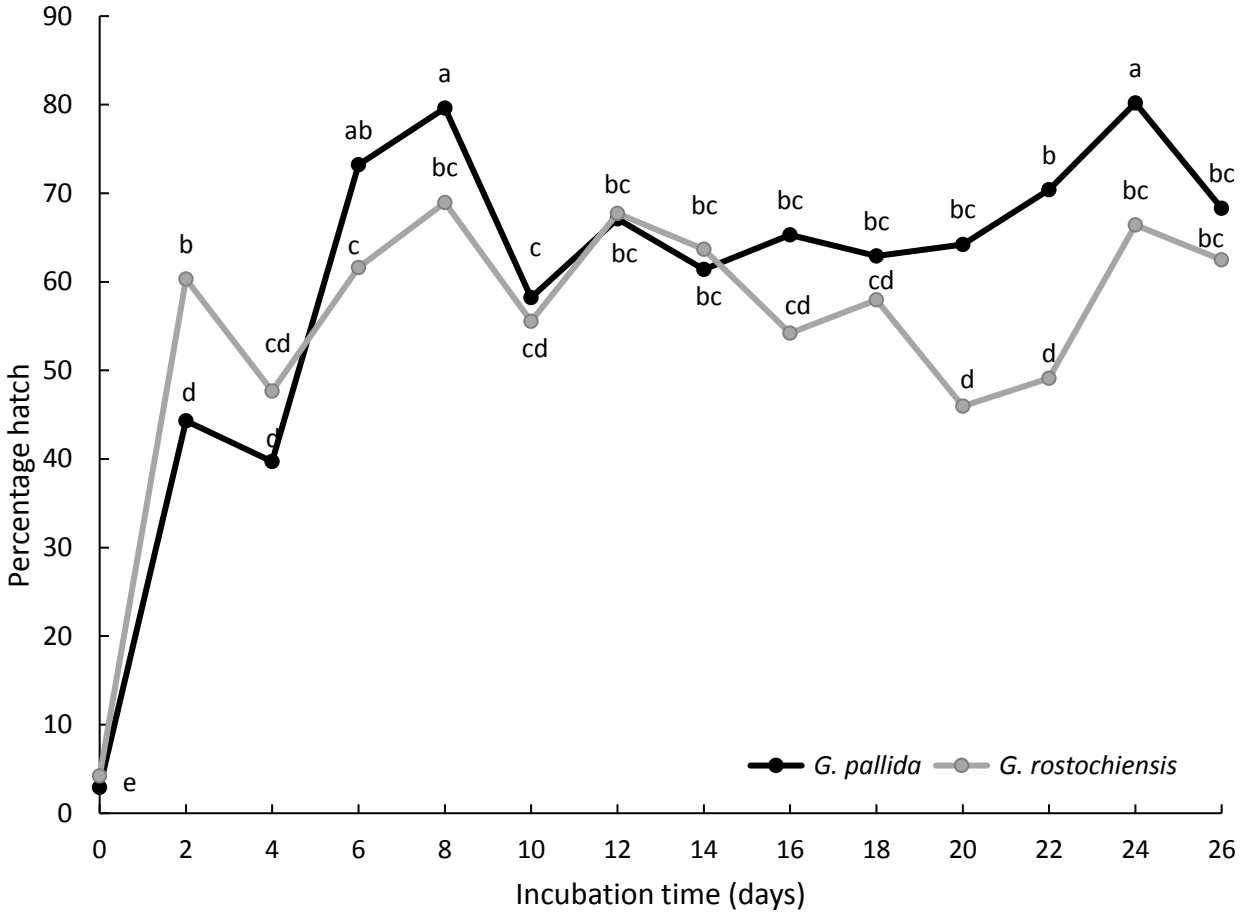


Figure 4.3 Differential PCN species characteristics. Samples with an asterisk are significantly different ($P < 0.05$) using the Tukey test.

4.4.2. Timing of PCN hatch

The time course of hatching (Fig. 4.4) revealed a significant difference between *G. pallida* and *G. rostochiensis* hatch ($F_{(1, 224)} = 18.68$; $P < 0.001$). *G. pallida* had an average cumulative hatch of 59.63% over 26 days relative to *G. rostochiensis* with an overall hatching average of 54.4%.

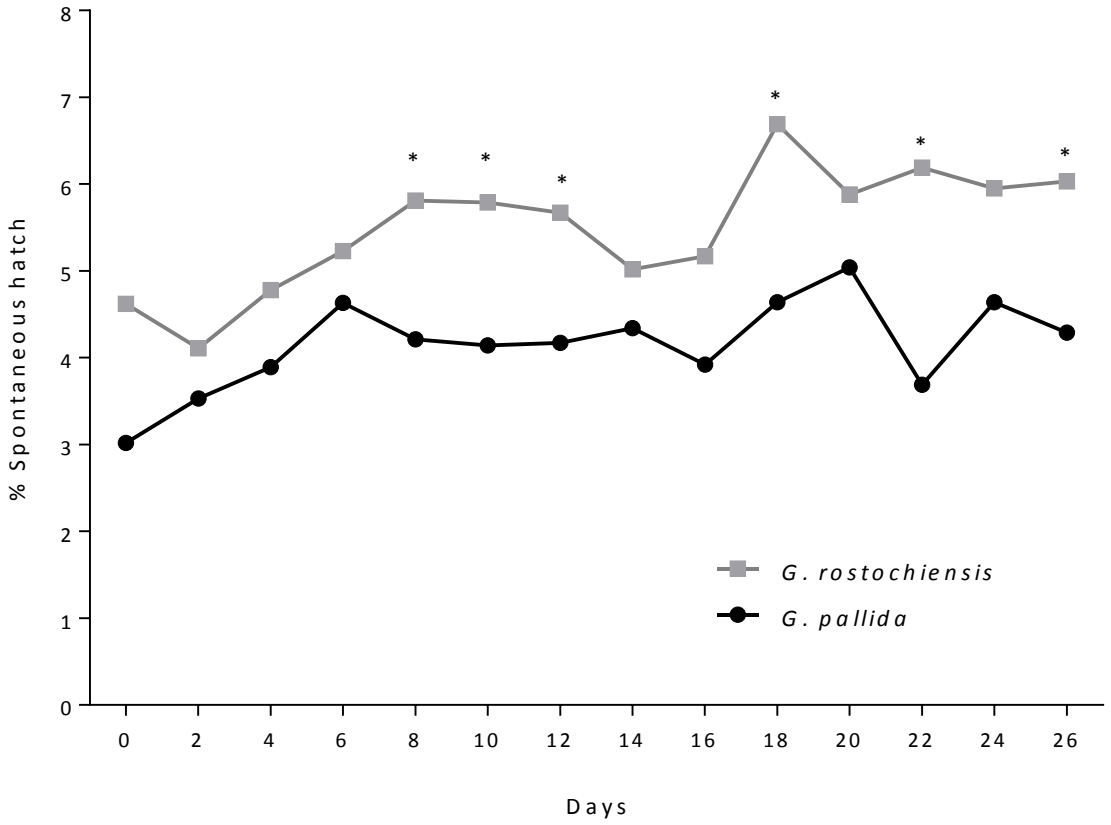


Source of Variation	DF	SS	MS	F	*P*
Species	1	1664.7	1664.7	18.68	<0.001
Time	13	71418.0	5493.7	61.64	<0.001
Interaction	13	6164.0	474.2	5.32	<0.001
Residual	224	19965.0	89.1		
Total	251	99211.7	395.3		

Figure 4.4 The effect of timing on PCN hatch *in vitro*. Samples with a common letter are not significantly different ($P > 0.05$) using the Tukey test.

An interaction between time and species was observed ($F_{(13, 224)} = 5.32$; $P < 0.001$). *G. rostochiensis* had a significantly greater rate of hatch at 2 d compared with *G. pallida*, but by 6 d, hatch of *G. pallida* was significantly greater than that of *G. rostochiensis*. *G. rostochiensis* exhibited bimodal hatching activity with a peak at 8 d, followed by a fluctuating levels of hatch and a peak at 24 d. *G. pallida* also displayed bimodal hatch, both hatching peaks coincided with *G. rostochiensis* maxima at 8 and 24 d. *G. pallida* hatch was also significantly

higher than that of *G. rostochiensis* at days 20 to 24, before hatch of the two species becoming similar at 26 d *G. pallida,* however, had a more consistent and higher level of hatch from 7 d onwards, with no significant variation in hatching activity between 8 and 18 d. Regression analysis revealed a significant logarithmic relationship for the response of *G. pallida* hatch in response to time (R^2 = 0. 701; $F_{(1, 124)}$ = 291.22; $P < 0.001$). *G. rostochiensis* also exhibited a significant relationship (R^2 = 0. 572; $F_{(1, 124)}$ = 165.88; $P < 0.001$). A significant difference in spontaneous hatch was observed between species (Fig. 4.5; $P < 0.001$).



Source of Variation	DF	SS	MS	F	*P*
Species	1	63.56	63.56	12.29	<0.001
Time	13	113.95	8.77	1.70	0.06
Interaction	13	13.10	1.01	0.20	>0.05
Residual	224	1158.11	5.17		
Total	251	1348.72	5.37		

Figure 4.5 The effect of timing on the spontaneous hatch of PCN *in vitro.* An asterisk denotes a significant difference ($P > 0.05$) between species using the Tukey test.

4.4.3. Temperature

Temperature had a significant influence on hatch ($F_{(8, 161)} = 13.91$; $P < 0.001$). *G. pallida* exhibited the greatest degree of hatch, with maximum hatching activity between 14 ºC and 16 ºC. *G. pallida* hatch was relatively consistent within this range but declined significantly at 22 ºC (Fig. 4.6; 4.7). *G. rostochiensis* displayed maximum hatching activity at 14 ºC and 22 ºC with lowest hatching activity at 8 ºC and a significant decline at 24 ºC.

Overall there was no significant difference between species ($F_{(1, 161)} = 0.001$; $P > 0.05$). The only significant difference between species hatch response was evident at 22 ºC. However, there was a significant interaction between species and temperature ($F_{(8, 144)} = 2.34$; $P < 0.05$). *G pallida* exhibited a hatching preference at lower temperatures; conversely, *G. rostochiensis* had an affinity towards higher temperatures. The regression curves infer a broader hatching temperature range in *G. rostochiensis* relative to *G. pallida* (Fig. 4.6).

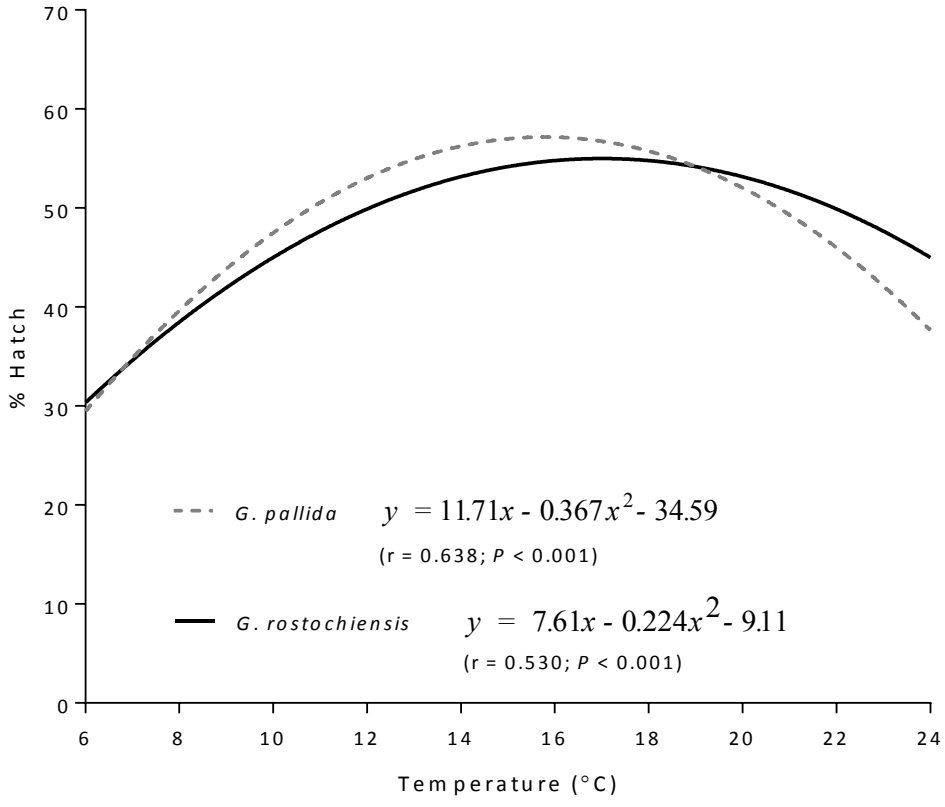
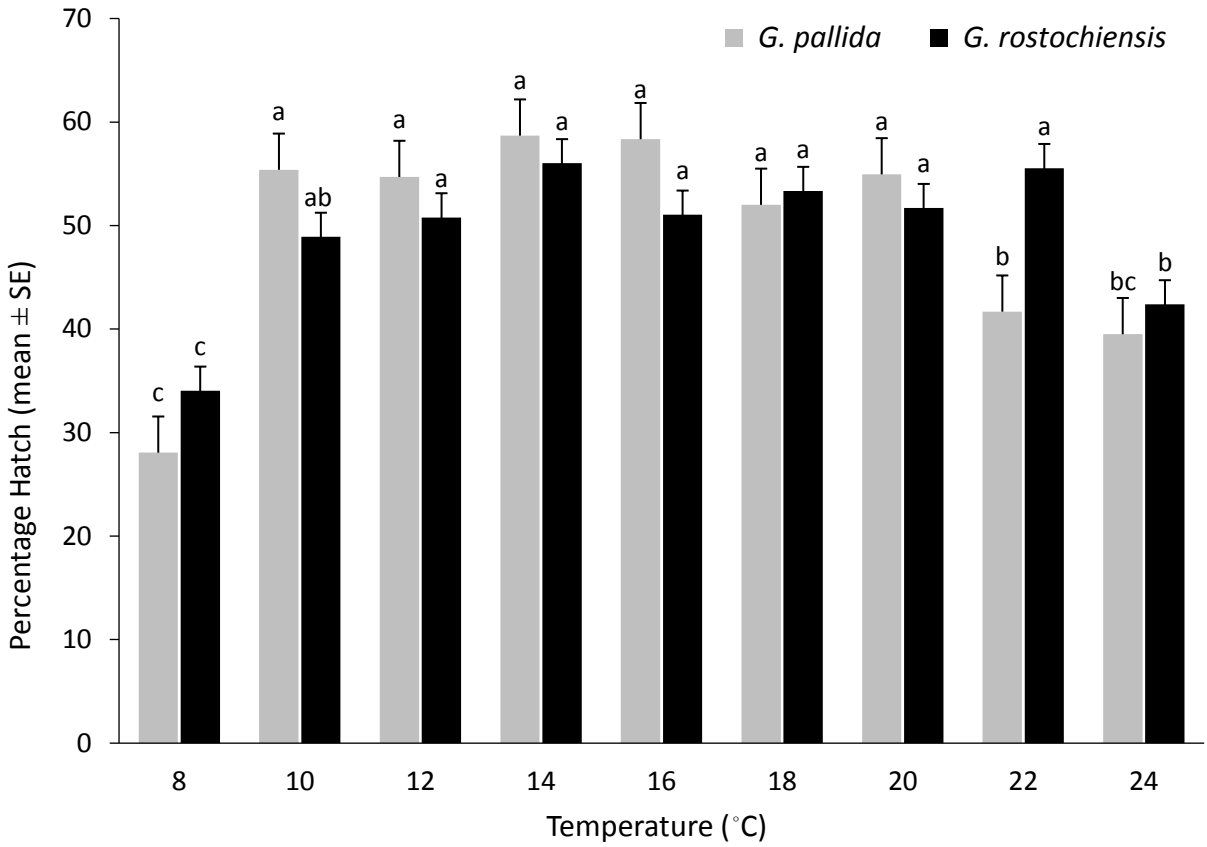


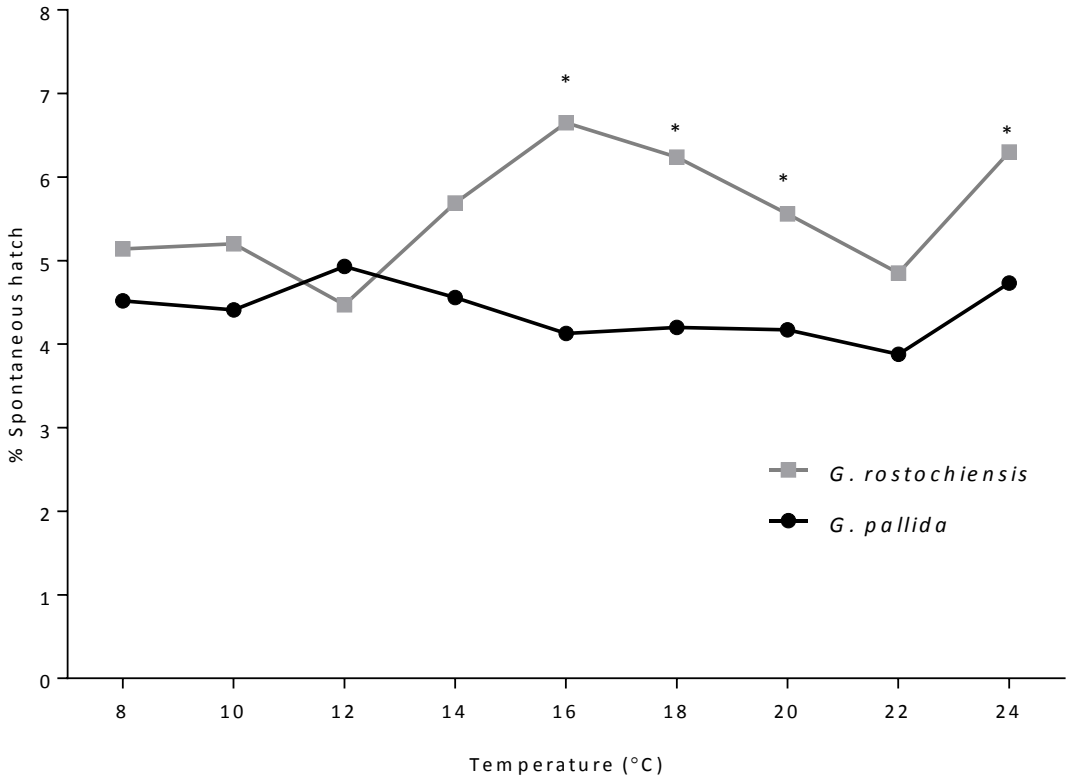
Figure 4.6 Hatching response of *G. pallida* and *G. rostochiensis* at different temperatures.



Source of Variation	DF	SS	MS	F	*P*
Temperature	8	9804.07	1225.51	13.91	<0.001
Species	1	0.09	0.09	0.001	>0.05
Interaction	8	1648.69	206.09	2.34	<0.05
Residual	144	12691.69	88.14		
Total	161	24144.54	149.97		

Figure 4.7 The effect of temperature on PCN hatch after 15 d incubation. Samples with a common letter are not significantly different ($P > 0.05$) using the Tukey test.

There was a significant difference ($P < 0.01$) in spontaneous hatch (Fig. 4.8) between species but not between different temperatures ($P > 0.05$). *G. rostochiensis* exhibited greater spontaneous hatch at higher temperatures and peaked at 16 ºC and 24 ºC. Conversely, temperature had no significant ($P > 0.05$) effect on *G. pallida* spontaneous hatch.



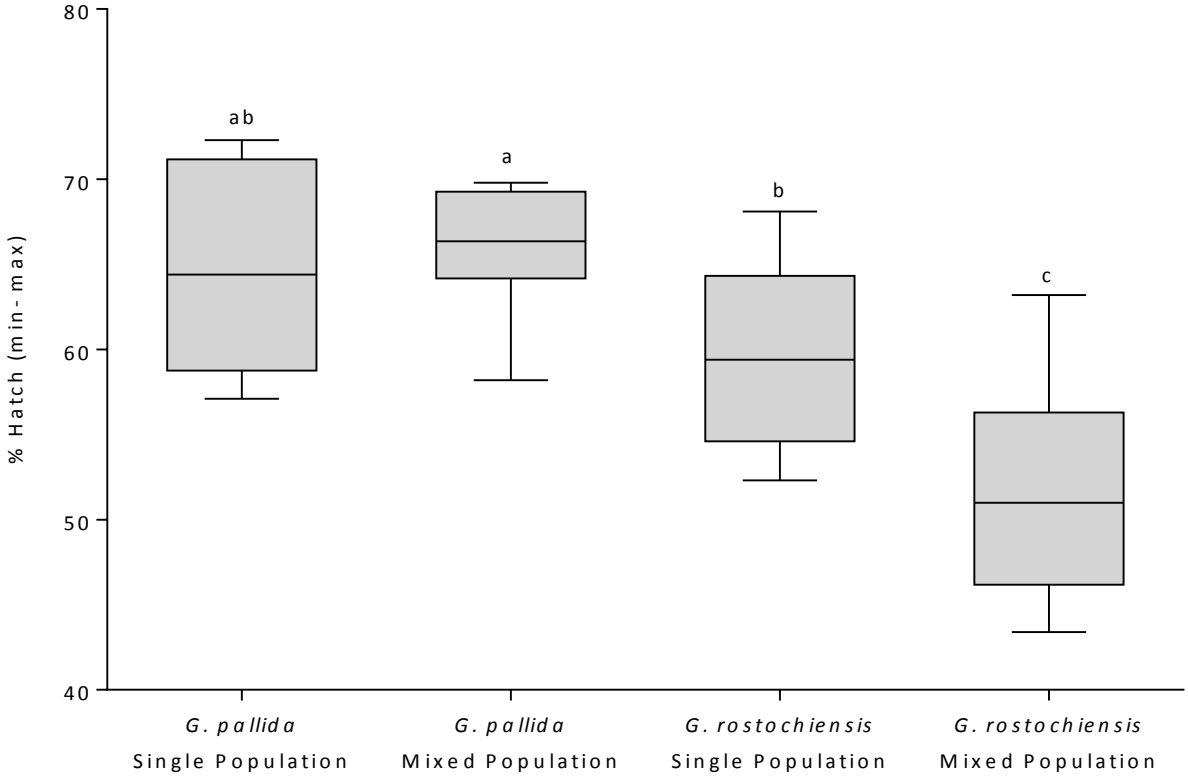
Source of Variation	DF	SS	MS	F	*P*
Species	1	57.58	57.58	7.91	<0.01
Temperature	8	41.27	5.16	0.71	>0.05
Interaction	8	14.05	1.76	0.24	>0.05
Residual	144	1048.33	7.28		
Total	161	1161.23	7.21		

Figure 4.8 The effect of temperature on the *in vitro* spontaneous hatch of *G. pallida* and *G. rostochiensis*.

4.4.4. Mixed Species

A significant difference in hatching activity was observed between species ($P < 0.001$; Fig. 4.9). *G. pallida* had a slightly higher percentage of hatch in mixed populations (mean ± SE = 64.86% ± 1.87) compared with single-species (59.54% ± 1.71), but the effect was not significant ($P > 0.05$). Conversely, *G. rostochiensis* exhibited a significant difference in hatch between single- and mixed-species treatments ($P < 0.01$). The *in vitro* hatch of *G. rostochiensis* was significantly lower in mixed-species populations (51.97% ± 2.13) than in single-species populations (59.54% ± 1.71). Overall there was no significant difference

between single- and mixed-populations ($F_{(1, 32)} = 2.65$; $P > 0.05$); however, a significant interaction (species x population) was observed ($F_{(1, 32)} = 4.87$; $P < 0.05$). There was no significant difference in spontaneous hatch between species ($F_{(1, 36)} = 0.389$; $P > 0.05$) or between single- and mixed-species populations ($F_{(1, 36)} = 2.529$; $P > 0.05$).



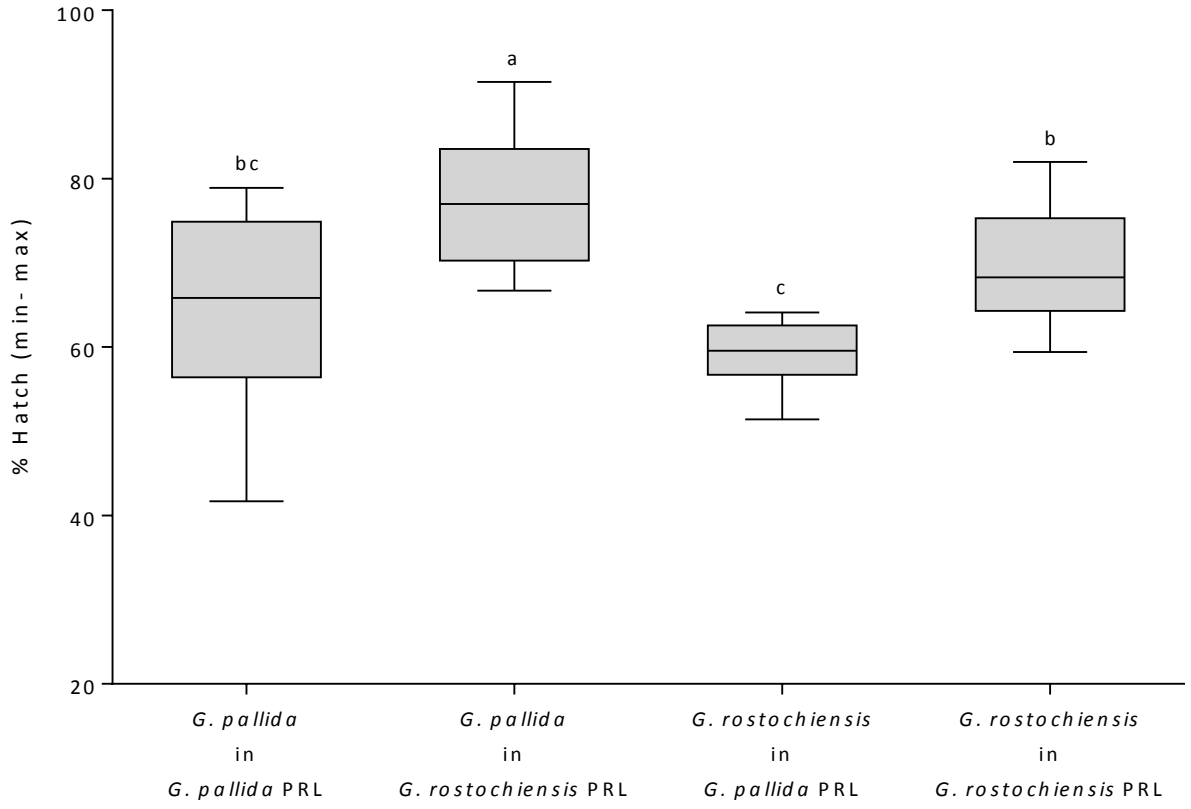
Source of Variation	DF	SS	MS	F	*P*
Species	1	842.0	842.0	23.93	<0.001
Population	1	93.1	93.1	2.65	>0.05
Interaction	1	171.2	171.2	4.87	<0.05
Residual	32	1125.9	35.2		
Total	35	2232.2	63.8		

Figure 4.9 *G. pallida* and *G. rostochiensis* hatch in single- and mixed-species *in vitro* populations. Samples with a common letter are not significantly different ($P > 0.05$), using the Tukey test.

4.4.5. Hatch responses to inter- and intra-specific PRL

Inter- and intraspecific assays revealed a significant difference between *G. pallida* and *G. rostochiensis* hatch (Fig. 4.10; $F_{(1, 36)} = 7.49$; $P < 0.01$). Both species exhibited similar hatching responses in intraspecific PRL (e.g. *G. pallida* hatch in PRL from *G. rostochiensis*), with

hatching activities (mean ± SE) of 64.59% ± 11.12 for *G. pallida* and 69.26% ± 7.07 for *G. rostochiensis*. PCN hatch in interspecific PRL revealed contrasting results. *G. pallida* hatch increased significantly ($P < 0.001$) in *G. rostochiensis* PRL (77.7% ± 2.68) and *G. rostochiensis* had a significantly ($P < 0.01$) lower hatch in *G. pallida* PRL (59.14% ± 1.22). A significant difference in hatch was observed between PRL treatments ($F_{(1, 36)} = 20.85$; $P < 0.001$). However, there was no significant interaction between species and PRL treatment ($F_{(1, 36)} = 0.36$; $P > 0.05$). Similarly, there was no significant difference between species spontaneous hatch ($F_{(1, 32)} = 2.098$; $P > 0.05$) or treatment ($F_{(1, 32)} = 0.150$; $P > 0.05$).



Source of Variation	DF	SS	MS	F	*P*
Species	1	487.34	487.34	7.49	<0.01
PRL	1	1356.76	1356.76	20.85	<0.001
Species x PRL	1	23.53	23.53	0.36	>0.05
Residual	36	2342.61	65.07		
Total	39	4210.24	107.96		

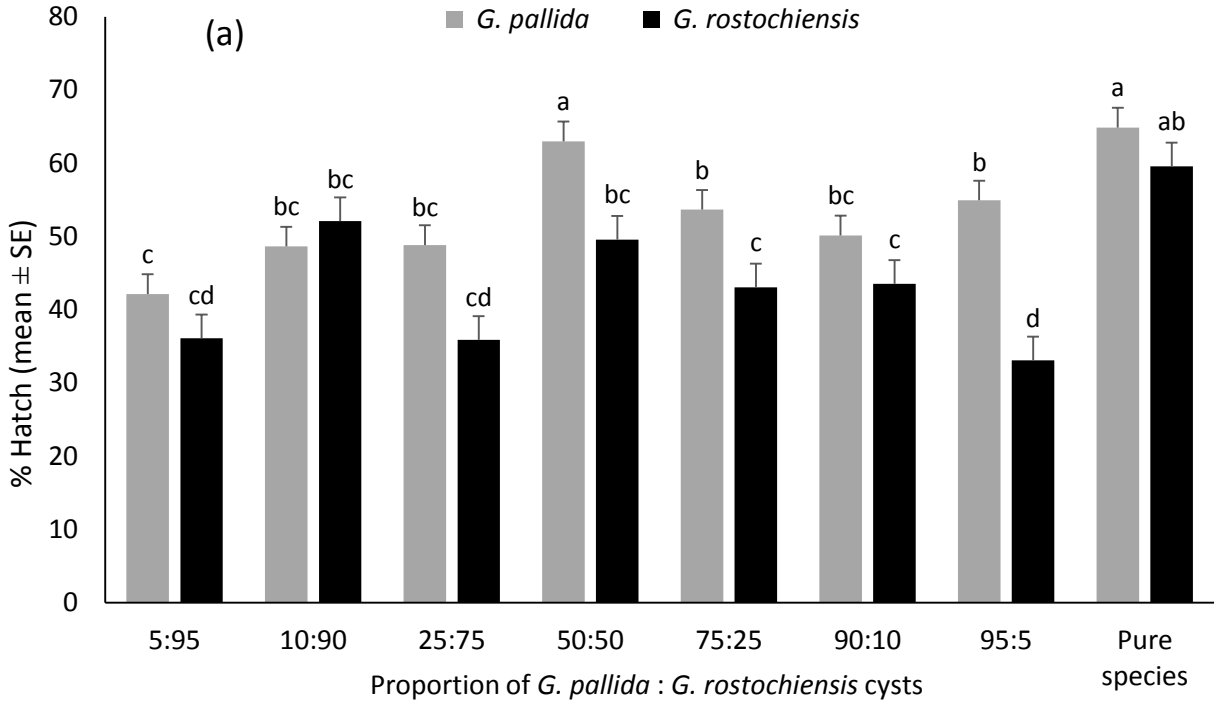
Figure 4.10 The *in vitro* hatching activity of *G. pallida* and *G. rostochiensis* in response to hetero- or homo specific PRL. Samples with a common letter are not significantly different ($P > 0.05$), using the Tukey test.

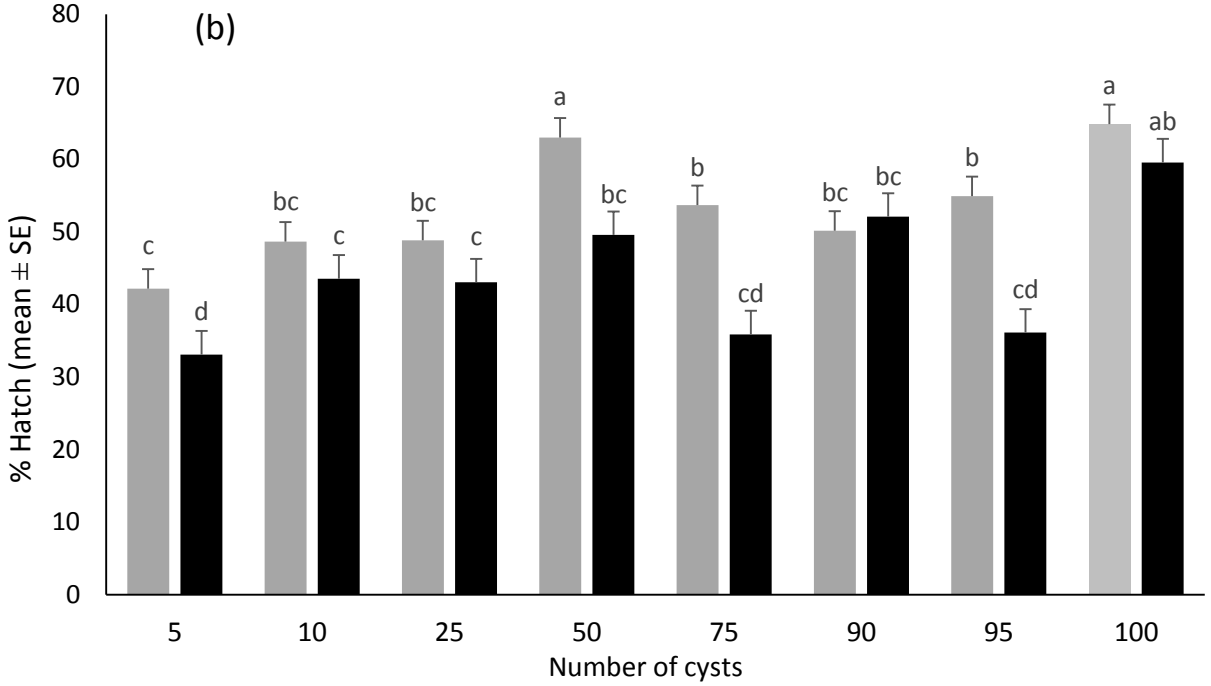
4.4.6. Species proportions within large and small population densities

A significant difference in PRL-induced hatch was evident at different population proportions in both small ($F_{(7, 212)} = 14.15$; $P < 0.001$) and large ($F_{(5, 96)} = 2.69$; $P < 0.05$) populations. A highly significant difference was evident between *G. pallida* and *G. rostochiensis* hatch in small populations ($F_{(1, 212)} = 49.67$; $P < 0.001$), with *G. pallida* exhibiting greater hatch than *G. rostochiensis* in all population proportions bar 10:90 and significant increases ($P < 0.001$) were evident when *G. pallida* represented 25%, 50%, 75% and 95% of a population (Fig. 4.11a). Furthermore, a significant interaction between species proportion and species was also observed ($F_{(7, 212)} = 4.95$; $P < 0.001$). In large populations, no significant difference was evidenced between *G. pallida* and *G. rostochiensis* ($F_{(1, 96)} = 0.55$; $P > 0.05$); however, there was a significant interaction between species and population proportion ($F_{(5, 96)} = 24.39$; $P > 0.001$).

In comparison to *G. rostochiensis*, *G. pallida* had significantly higher hatch when populations were in equilibrium (50:50) in small populations (Fig. 4.11), but not in large populations (Fig. 4.12). *G. pallida* also appeared to have the higher hatching activity (74.0%) when it represented 95% of a small population (95:5 cysts). *G rostochiensis* hatch peaked at 82.8% when it was present as 25% of a large population size (750:250 cysts). There was no significant difference in hatch between species in single-species populations in either small (Fig. 4.11) or large (Fig. 4.12) populations. The results in single-species populations were highly comparable between small and large populations, although multiplication was higher in small populations the effect was not significant ($P > 0.05$). *G. pallida* multiplication averaged 64.9% and 58.6% in small and large populations, respectively; while *G rostochiensis* averaged 59.7% in small populations and 52.9% in large populations.

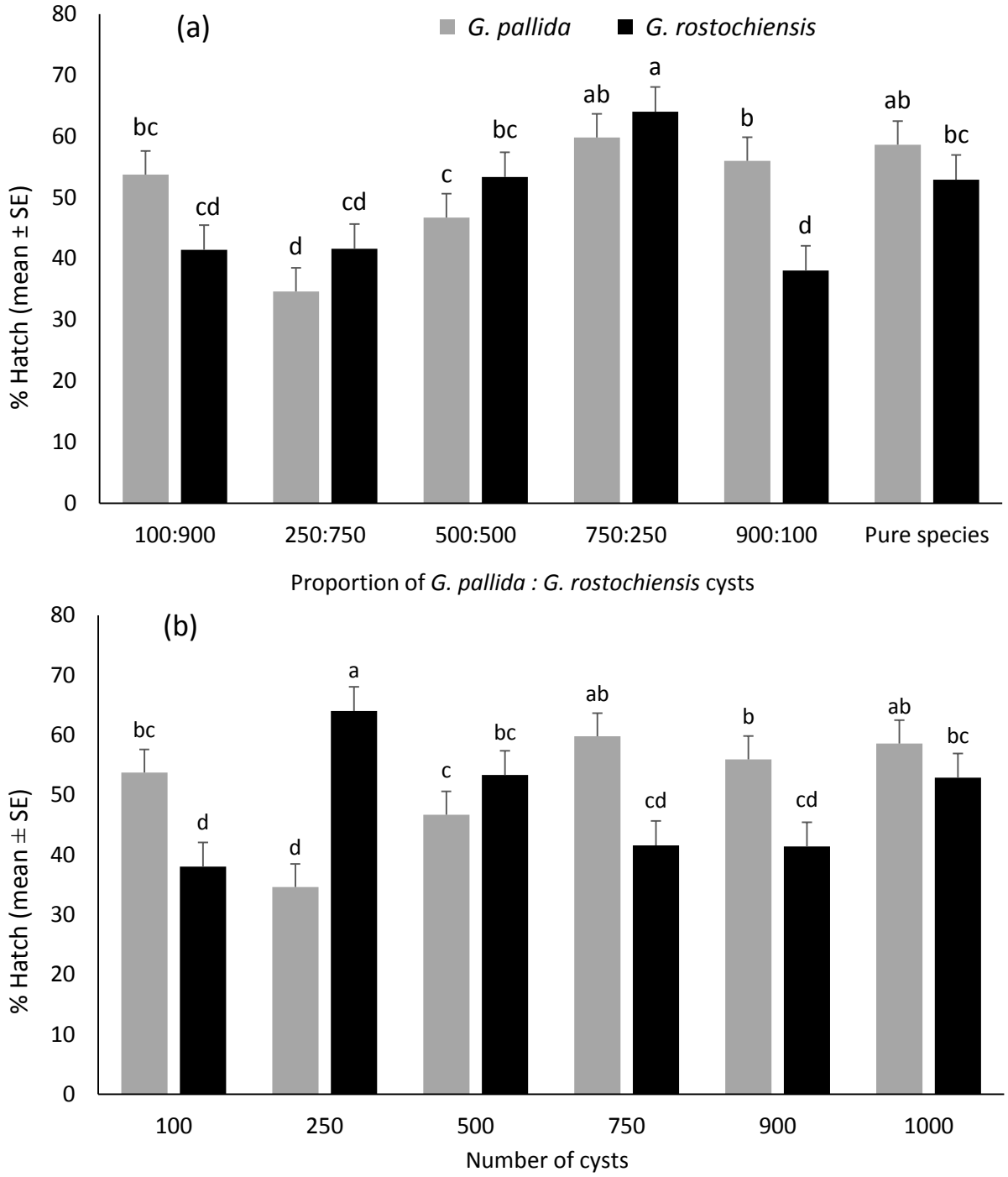
Control assays revealed a significant difference between species spontaneous hatch ($P < 0.001$), but no significant difference ($P > 0.05$) between species proportions (Table 4.2). *G. pallida* had significantly lower spontaneous hatch than *G. rostochiensis* in both small ($F_{(1, 128)} = 12.89$; $P < 0.001$) and large populations ($F_{(1, 96)} = 20.02$; $P < 0.001$).





Source of Variation	DF	SS	MS	F	*P*
Species proportion	7	8456.39	1208.06	14.15	<0.001
Species	1	4239.64	4239.64	49.67	<0.001
Interaction	7	2957.54	422.51	4.95	<0.001
Residual	212	18096.50	85.36		
Total	227	34355.14	151.34		

Figure 4.11 (A) The *in vitro* hatching response of *G. pallida* and *G. rostochiensis* at different species proportion in small populations. (B) A comparison of species hatching responses at each population density. Samples with a common letter are not significantly different ($P > 0.05$) using the Tukey test.



Source of Variation	DF	SS	MS	F	*P*
Species proportion	5	1195.62	239.12	2.69	<0.05
Species	1	48.88	48.88	0.55	>0.05
Interaction	5	10834.48	2166.90	24.39	<0.001
Residual	96	8528.64	88.84		
Total	107	20607.61	192.60		

Figure 4.12 (A) The *in vitro* hatching response of *G. pallida* and *G. rostochiensis* at different species proportions in large populations. (B) A comparison of species hatching responses at each population density. Samples with a common letter are not significantly different ($P > 0.05$), using the Tukey test.

Table 4.2 ANOVA summary table of the *in vitro* spontaneous hatch of *G. pallida* and *G. rostochiensis* at different species proportions in (a) small and (b) large populations.

(a)

Source of Variation	DF	SS	MS	F	*P*
Species proportion	7	22.17	3.17	1.05	>0.05
Species	1	38.95	38.95	12.89	<0.001
Interaction	7	8.83	1.26	0.42	>0.05
Residual	128	386.87	3.02		
Total	143	457.18	3.20		

(b)

Source of Variation	DF	SS	MS	F	*P*
Species proportion	5	13.47	2.69	1.06	>0.05
Species	1	50.98	50.98	20.02	<0.001
Interaction	5	8.71	1.74	0.68	>0.05
Residual	96	244.51	2.55		
Total	107	317.65	2.97		

4.4.1. Population size

There was no significant difference in PCN hatching activity between population sizes (Table 4.3a; $F_{(1, 264)} = 0.51$; $P > 0.05$). A significant difference was observed between species ($F_{(1, 264)} = 7.69$: $P < 0.001$) and a highly significant interaction between species and population size was evident ($F_{(1, 264)} = 33.40$; $P < 0.001$), which suggested that the two species responded differently to population size. *Post hoc* multiple comparisons with the Tukey test revealed a significant difference in hatch of the two species within small populations ($P < 0.001$), but not within large populations ($P > 0.05$). *G. rostochiensis* exhibited a significant difference in hatch between small and large populations ($P > 0.05$). *G. pallida,* however, did not exhibit a significant difference in hatch due to population size ($P > 0.05$). Population size did not have a significant effect on spontaneous hatch (Table 4.3b; $F_{(1, 215)} = 2.60$: $P > 0.05$). There was a significant difference in spontaneous hatch between the two species ($F_{(1, 215)} = 27.97$: $P < 0.001$), with *G. rostochiensis* spontaneous hatch being significantly greater than that of *G. pallida*.

Table 4.3 ANOVA summary table of the effect of population size on (a) PRL-induced and (b) spontaneous hatch of *G. pallida* and *G. rostochiensis*.

(a)

Source of Variation	DF	SS	MS	F	*P*
Population size	1	44.81	44.81	0.51	>0.05
Proportion	5	3376.29	675.26	7.69	<0.001
Species	1	2932.50	2932.50	33.40	<0.001
Population size x Proportion	5	1462.19	292.44	3.33	<0.01
Population size x Species	1	269.89	269.89	3.07	>0.05
Proportion x Species	5	4899.87	979.97	11.16	<0.001
Species x Size x Proportion	5	5031.99	1006.40	11.46	<0.001
Residual	264	23181.30	87.81		
Total	287	41198.853	143.55		

(b)

Source of Variation	DF	SS	MS	F	*P*
Population Size	1	7.31	7.31	2.60	> 0.05
Proportion	5	24.31	4.86	1.73	> 0.05
Species	1	78.46	78.46	27.97	<0.001
Population Size x Proportion	5	9.98	2.00	0.71	> 0.05
Population Size x Species	1	1.51	1.51	0.54	> 0.05
Proportion x Species	5	2.84	0.57	0.20	> 0.05
Species x Size x Proportion	5	11.33	2.27	0.81	> 0.05
Residual	192	538.59	2.81		
Total	215	674.63	3.14		

4.5. Discussion

Hatching is a critical aspect of the nematode lifecycle. The experiments presented in this chapter investigated the differential hatching responses of *G. pallida* and *G. rostochiensis* under variable abiotic and biotic parameters. The studies aimed to elucidate the mechanisms of competition between *G. pallida* and *G. rostochiensis* in mixed-species populations, specifically during the hatching stage of the PCN life cycle. Female fecundity and egg viability are primary determinants of population success. As such, comparative analysis of the two populations would suggest *G. pallida* to be a more successful species as it exhibited greater hatch, egg viability and a higher egg count relative to *G. rostochiensis*.

The greater *G. pallida* hatch supports the findings of Kaczmarek (2014), and greater proportion of viable eggs has also been reported (Marshall, 1989; Devine & Jones, 2001a).

The earlier hatch and faster hatching rate of *G. rostochiensis* hatch has been widely reported in different potato growing regions, namely Australia (Stanton and Sartori, 1990); Spain (Salazar and Ritter, 1993) and the UK (Robinson *et al.*, 1987; Kaczmarek, 2014). The data presented in these studies revealed greater *G. rostochiensis in vitro* hatch in the first 4 days; however, *G. pallida* hatch increased rapidly between 4 and 8 days and a peak in hatching activity was observed at day 8. Furthermore, the higher level of hatch exhibited by *G. pallida* appeared to be more consistent, with minimal hatch variation between days 12 and 22. Conversely, *G. rostochiensis* hatch appeared more erratic and declined more rapidly. The hatching assays measured overall hatching activity as the proportion of hatched eggs and consequently showed marked variation between sampling days. The measurement of hatch in these assays was independent of values at other days; however, this may have contributed to greater variation in hatch between days relative to cumulative hatch analysis.

A delay in PCN hatch may be observed between *in vitro* and in soil bioassays (Kaczmarek, 2014). Soil imposes both a physical and chemical barrier to migrating juveniles and to PRL by perturbing diffusion of host exudates. These factors inflict a chemotactic challenge in soil environments relative to liquid media bioassays (Dalzell *et al.*, 2011). Consequently, hatching assays should be referenced to parallel *in vitro* and *in vivo* or in-soil assays to assess differential hatching responses under different environmental parameters.

The higher hatching optimum temperature of *G. rostochiensis* compared to *G. pallida* described here has been widely reported (Franco, 1979; Robinson *et al.*, 1987; Stanton & Sartori, 1990). *G. pallida* had a hatching maximum between 14 and 16 °C compared to 22 °C for *G. rostochiensis*, a result supported by Franco (1979). The adaptation of PCN species to different hatching temperature optima may facilitate species-selection in certain

soil temperature profiles. Soil environments are subject to variable environmental regimes and seasonal and daily temperature fluctuations. Thus, the ability to adapt to such conditions could confer a competitive advantage. Temperatures decline with increasing soil depth; consequently, the lower *G. pallida* hatching temperature optima may enable this species to adaptively hatch at different soil depths and to thereby enhance its competitiveness (Barker & Koenning, 1998). The concept of niche partitioning was briefly addressed in Chapter 3 and is proposed as the main factor facilitating the coexistence of *G. pallida* and *G. rostochiensis* within a soil community.

PCN in temperate regions generally complete one life cycle within the host growing season. However, a second-generation infestation may occur if diapause is not induced because of favourable environmental conditions. Second generations of *G. rostochiensis* have previously been recorded in Italy (Greco *et al.*, 1988) and Venezuela (Jimenez-Perez *et al.*, 2009). Recent studies concluded that a second generation of *G. pallida* and *G. rostochiensis* can occur within a single growing season at soil temperatures above 17 °C (Jimenez-Perez *et al.*, 2009; Kaczmarek *et al.*, 2012). Therefore, increasing soil temperatures could potentially increase PCN multiplication and increase the risk of a second PCN generation within one season.

Furthermore, the rise in soil temperatures would significantly speed up the PCN life cycle and enhance female development and fecundity, having significant negative consequences for crop production. *G. pallida* females undergo faster embryogenesis and development than *G. rostochiensis* (Perry *et al.,* 2002). This, coupled with the lower hatching and development temperature optima of this species, infers that *G. pallida* has a distinct advantage during the early spring cultivation period (Webley & Jones, 1981).

Nematode populations used in research are typically multiplied under regulated greenhouse conditions and are subjected to warmer, less variable temperature regimes than in the field. Consequently, cultured nematode populations may have different

hatching adaptations in comparison to natural field populations. A comparative analysis of *in vitro* and *in vivo* hatching responses in cultured and naturally derived populations would be valuable to assess hatching variation within PCN populations. Storage conditions of the nematode cultures, including temperature, moisture and aeration, could significantly affect population viability and invariably affect hatching responses (Salazar & Ritter, 1993; Ingham *et al.*, 2015). The nematode populations used in this study had been maintained at 18˚C, which is conducive to the hatch of both species. The same population of *G. pallida* and *G. rostochiensis* was used for both competition and hatching studies.

Photoperiod and diurnal temperature regimes are modulators of the rate of nematode hatch (Hominick, 1986). Salazar and Ritter (1993), showed that nematodes cultured on potato plants grown at long photoperiods showed greater hatch (61 - 97 %) but had lower hatch than those cultured on plants grown under shorter photoperiods (12 - 45 %). Both parameters should be factored in to temperature-dependent hatch analyses accordingly. Hatching assays are performed at a regulated temperature that are not reflective of natural diurnal temperature fluctuations or temperature gradients present in soil profiles. However, Kaczmarek *et al.* (2014) concluded that hatch did not vary significantly in response to fluctuating or constant temperatures.

Exogenous hatching stimuli have been extensively researched and characterised (Devine & Jones, 2000a; Devine & Jones, 2003; Dalzell *et al.*, 2011). Endogenous signalling (from the host plant) regulates dormancy; however, the putative involvement of these factors on PCN hatch remains elusive. In light of this, a series of biotic hatching assays was set up to investigate the effect of endogenous factors on hatch. The mixed-species, density-dependent and interspecific PRL hatching assays investigated both intra- and interspecific nematode interactions during hatch. The assays aimed to determine whether competition between *G. pallida* and *G. rostochiensis* is operative during the hatching stage of the PCN lifecycle and, if so, to assess the underlying mechanisms of competition.

The findings suggest that signalling and chemoreception are critical elements in PCN population dynamics, which is proposed by the significant differences between single- and mixed-species population bioassays. Interestingly, the results appear to reflect the results from the *in vivo* competition assays (Chapter 3) and infer that *G. pallida* is the more successful species in mixed-species populations. Similarly, *G. rostochiensis* hatch appears to be suppressed by the presence of *G. pallida* with significantly lower hatching activity in mixed-species assays relative to single-species assays. In order for *G. pallida* to inflict an inhibitory effect on *G. rostochiensis,* interspecific signalling during hatch is the most plausible mechanism to explain this effect. Alternatively, nematode metabolites and/or semiochemicals may confer an allelopathic effect.

The PRL exchange assays were designed to investigate chemoreception and perception to species-specific compounds during hatch. The results indicated that exposure to heterospecific PRL had a significant effect on PCN hatch. *G. pallida* and *G. rostochiensis* hatch was induced in PRL and populations were maintained to facilitate the accumulation of nematode semiochemicals and the putative production of novel hatching chemicals. The data suggest that nematodes are sensitive and chemoreceptive to heterospecific compounds. *G. pallida* hatch was significantly greater in *G. rostochiensis*-sensitised PRL, while *G. rostochiensis* hatch was significantly inhibited in *G. pallida*-sensitised PRL, which is similar to the results from the *in vivo* competition studies (Chapter 3).

It could be speculated that species-specific hatching signals elicit these responses. In this case, *G. rostochiensis* compounds potentially act as kairomones to *G. pallida*, as *G. rostochiensis* perceives *G. pallida* compounds as allomones. Characterisation of the nematode-secreted chemicals is essential to substantiate the findings presented. Further investigations should involve concentration-dependent analyses and chemical profiling to identify putative signalling compounds that may influence nematode interactions and interspecific communication. The isolation of such compounds would be invaluable in future

bioassays, entailing nematode behaviour studies. Similarly, there is significant potential for such compounds in the development of novel PCN control agents.

Plant-parasitic nematodes are known to emit sex and aggregation pheromones, and interspecific responses have been reported. Riga *et al.* (1997) reported that males of *G. pallida* are attracted to *G. rostochiensis* female sex pheromones but *G. rostochiensis* males are not attracted to the *G. pallida* female pheromones in *in vitro* bioassays. If *G. pallida* respond to female sex pheromones of *G. rostochiensis*, it is quite possible that they respond to heterospecific aggregation pheromones; the interaction of such populations with endogenous semiochemicals from a different species merits investigation (Wang *et al.*, 2011).

The effect of population density on PCN multiplication has been extensively researched (Seinhorst, 1970; La Mondia & Brodie, 1986). Competition for host-based resources is the main limiting factor governing PCN multiplication. The assays revealed significant differences in hatching activity between different species proportions. The data appeared similar to the results from the *in vivo* competition assays (§ 3.5.3), with *G. pallida* displaying the greater overall hatch. The *in vitro* species proportion hatching assays were not resource dependent; the hatching stimulus was standardised to elicit large-scale hatch in both species. In effect, the density-dependent species proportion hatching assays suggest the involvement of potential hatching signals that may regulate hatch. This would result in differential hatching responses to inter- and intraspecific signals and that would modulate competition between species. This phenomenon is evident in many invertebrate parasites where ecdysones (hatching hormones) and epideictic pheromones play crucial roles in population regulation (Barker & Rees, 1990; Lee, 2002).

The genes responsible for eclosion and ecdysis are highly conserved between nematodes and insects (Gáliková *et al.*, 2011; Niwa & Niwa, 2014). Therefore, the potential role of signalling compounds in PCN hatch warrants further investigation. Signalling cues,

namely ascarosides (dauer pheromones), have been extensively studied in *Caenorhabditis elegans* and *Pristionchus pacificus* (Sommer & Ogawa, 2011). Dauer pheromones have a role in regulating population density (Bento *et al.*, 2010). As such, it is highly plausible that the effects evidenced in the PCN density-dependent assays may be attributable to endogenous signalling.

In a given population approximately 20 – 30% of nematodes eggs do not hatch until later years (Hockland, 2002). This has two distinct advantages, namely the preservation of genetic diversity and assurance of inoculum for successive populations. It may also control the number of infective nematodes that are released upon stimulation by host root exudates as a means of ecological facilitation to reduce intraspecific competition. It may be assumed that this proportion of eggs is genetically predetermined. More than 90% PCN hatch can be elicited by exogenous stimuli (Fig 4.3), and concentration-dependent hatching responses to external stimuli have been reported (Devine & Jones, 2000b; Byrne *et al.*, 2001; Dalzell *et al.*, 2011). Therefore, it is arguable that the proportion of eggs that remain in quiescence is variable and regulated by endogenous as well as exogenous stimuli.

4.5.1. Future recommendations and critical analysis.

Future research would benefit from behavioural assays involving an array of species-specific pheromones to assess differential responses to exogenous chemicals. Assay consistency is an important factor in hatching analyses. PRL is subject to degradation over time and *in vitro* hatching assays are subject to microbial contamination. As such, the accumulation of both nematode and microbial metabolites can significantly affect PCN hatch. Assay consistency could be improved by removing PRL on a regular basis and counting the number of hatched juveniles rather than egg counting.

Although the PRL was sterilised prior to use in experiments, the assays are not maintained in an aseptic environment and the carbon-rich PRL medium is a prime source for microbial colonisation. The application of fresh PRL would mitigate medium degradation

and would possibly be more reflective of a natural soil environment where PRL is continuously leached into the rhizosphere. Furthermore, cyst storage condition can significantly affect hatch, egg viability and influence dormancy. The timing of hatching assays is critical to ensure that populations are in quiescence and amenable to hatch. In effect, diapause may be artificially disrupted by storing the cysts at 4°C for 3 months (Muhammad, 1994; Devine, 2010), or avoided by storing cysts in hydrated form (Ingham *et al.*, 2015).

In vitro hatching assays have provided substantial data on PCN hatching mechanisms, but the study of PCN hatch should also entail *in vivo* and in-soil hatching assays integrating quantitative analysis of hatched eggs, juveniles and nematode viability. The rate of hatch in *in vitro* hatching assays lacks the complexity and variation evident in the field. Physicochemical properties of soil have a significant impact on PRL diffusion and associated concentration gradients. Thus, the rate of hatch and juvenile emergence in soil and *in vitro* can vary significantly and species-specific responses to different HF concentrations and elutions may vary accordingly. Natural PCN populations are likely to exhibit greater hatching variability due to the different PCN generations present in soil, relative to the single-generation PCN populations used in the research described in this chapter.

Manual egg counting is laborious, and operators are often susceptible to fatigue and errors: thus repeatability and reproducibility can be compromised. The use of a counting chamber with standardised surface area, volume and reference gridlines to facilitate counting would greatly minimize errors and over/under counting. Image-assisted analyses may provide more conclusive data, and are less subject to error as image outputs may be saved for future reference. Furthermore, automated image analysis and cell counting applications can simultaneously process a large volume of samples efficiently and accurately.

The experiments in this chapter signify the importance of abiotic and biotic factors on the differential hatching responses of *G. pallida* and *G. rostochiensis,* and the resultant effect on PCN population dynamics. *G. pallida* proved to have the highest rate of hatch in all *in vitro* hatching assays and appeared to "out-hatch" *G. rostochiensis* under numerous hatching parameters. The greater hatch efficiency and lower spontaneous hatch of *G. pallida* may be the principal determinants of its greater hatch in these studies.

PCN hatch is a critical survival mechanism in the pest life cycle. Therefore, disruption of the hatching process is a primary target in nematode control; hatching inhibitors in particular have vast potential in the development of novel control agents (González *et al.*, 1994; Perry, 1997; Byrne *et al.*, 1998).

4.6. Bibliography

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Chapter 5 Host root responses to PCN attack

Abstract

PCN infection may elicit host defences, namely induced systemic resistance (ISR). PCN, however, downregulate host defences and modulate phytohormones to facilitate a biotrophic interaction with the host. Furthermore, PCN attack may instigate structural changes in host roots due to damage incurred during initial invasion and syncytium development. Split-root trials were conducted to assess the effect of ISR on PCN multiplication at different stages of inoculation after the initial PCN attack. The results indicated a significant *G. pallida*-induced reduction in *G. rostochiensis* multiplication at both 7 and 14 dpi. Conversely, *G. pallida* was not affected by *G. rostochiensis* infection and there was no significant difference in *G pallida* multiplication at 7 or 14 dpi. In the single-species split-root trials, *G. rostochiensis* multiplication was significantly reduced at 14 dpi; whereas *G. pallida* did not exhibit a significant difference in multiplication. Root observation assays using rhizotrons were conducted to evaluate changes in root architecture in PCN-infected and uninfected plants. The results indicated an increase in root tip density upon PCN infection; albeit not significantly. *G. pallida* multiplication was significantly greater in mixed-species rhizotrons relative to single-species rhizotrons. Split-root rhizotrons were established to assess systemic changes in root morphology: one-half of the root system was infected with PCN and the other half remained uninfected. Significant correlations ($P < 0.05$) between root area and PCN multiplication were observed in both infected and uninfected host roots. *G. rostochiensis* multiplication was significantly lower in the mixed-species split-root rhizotrons than in single-species split-root rhizotrons. Conversely, *G. pallida* multiplication was greater in mixed-species split-root rhizotrons, but not significantly.

5.1. Introduction

The rhizosphere of PCN-infected soil is a region of complex plant-nematode-microbial interactions. Up to 40% of a plant's photosynthate is secreted into the rhizosphere, which is exploited by specific beneficial microorganisms including rhizoplane and ecto- and endorhizospheric bacteria and mycorrhizal fungi (Jones *et al.*, 2009; Baetz & Martinoia, 2014). Root exudates also attract an array of pest and pathogenic species (Badri & Vivanco, 2009), so that the roots are continuously subjected to biotic stress. Plants possess a vast array of physical and chemical-based defences to detect pest and pathogen invasion and to prevent progressive damage and disease. The mechanisms of defence are highly variable from constitutively expressed defences to inducible defences that are expressed temporally and spatially in response to pest attack.

5.1.1. Plant immunity

Plant immunity comprises innate constitutive physical and chemical defences, including structural barriers, e.g. cutin and suberin, and chemical defences, such as secondary metabolites (Serrano *et al.*, 2014; Doughari, 2015). Inducible responses augment preformed defences and typically include localized cell wall fortifications, *de novo* synthesis or activation of secondary metabolites including phytoalexins and the hypersensitive response (HR), including localized apoptosis to impede the spread of biotrophic pests and pathogens.

The initial stage of induced plant defence is the recognition the pest or pathogen. Host recognition receptor protein include an array of glycoproteins, oligosaccharides and polysaccharides of conserved elicitor molecules, known as pathogen- or damage-associated molecular patterns (PAMPs or DAMPs; Jones and Dangl, 2006). Localized trans-membrane pattern recognition receptors (PRRs), on the surface of plant cells, recognize PAMPs and immediately initiate PAMP-triggered immunity (PTI; Wiesel *et al.*, 2014). Effector-triggered immunity (ETI) entails the recognition of a specific pathogen-derived effector (i.e. *avr*-gene products) by plant *R*-proteins (§ 1.7.2.3). ETI is an

accelerated and amplified version of the PTI response that exceeds the threshold for HR induction to confer effective localized resistance against biotrophic pests and pathogens (Jones & Dangl, 2006; Newman *et al.*, 2013).

5.1.1.1. Localized induced defences

Invading pests and pathogens physically disrupt the cell structure; the resultant membrane depolarization and Ca^{2+}-mediated ion flux instigates an imbalance in cytosolic redox potential (Sheridan *et al.*, 2004). In host-PPN interactions, this results in HR to immobilize invading nematodes and inhibit subsequent attack (Reitz *et al.*, 2002). HR is correlated with *R*-gene-mediated resistance (§ 1.7.2.3); nematode elicitor molecules activate an ion flux, involving hydrogen peroxide (H_2O_2) and K^+ efflux, as well as Ca^{2+} and H^+ influx. This is followed by an immediate influx of toxic, necrotizing reactive oxygen species (ROS), including H_2O_2, nitric oxide (NO), the superoxide anion (O_2^-), and the hydroxyl radical (OH^-). This oxidative burst initiates physical defences including lignin and callose deposition, membrane phosphorylation and hydroxylation, and the synthesis of hydroxyproline-rich glycoproteins (HRGP) involved in cell wall fortification (Huang, 1998). In host-PPN interactions, this results in the formation of a necrotic layer of cells around a syncytium to impede development (Sobczak *et al.*, 2011).

5.1.1.2. Defence signalling

HR is a necessary precursor of systemic defence responses. It essentially initiates signal transduction pathways to upregulate the production of the defence-signalling chemicals salicylic acid (SA), ethylene (ET) and jasmonic acid (JA). SA elicits systemic acquired resistance (SAR), that confers durable local and systemic resistance by upregulating the expression of defence genes coding for phytoalexins and pathogenesis-related proteins (PR; Loon *et al.*, 2006). HR also activates lipoxygenases (LOX), essential catalysts of the octadecanoid biosynthetic pathway, which culminates in JA synthesis (Yang *et al.*, 2009).

PCN infection elicits a wound response due to stylet perforation of the root cortex. Additionally, elicitors in nematode secretions, namely cellulases (β-1,4-endoglucanases) and pectinases, including pectate lyases and polygalacturonase, elicit JA-mediated resistance (Abad *et al.*, 2003). Induced responses involve the upregulation of defence-related proteins and defence chemicals i.e. peroxidases, chitinases, LOX, extensins and proteinase inhibitors (PIs; van Loon *et al.*, 2006). Host resistance is downregulated by PCN avirulence factors. The *G. pallida*-specific *avr* gene product rGp-FAR-1 hinders the JA pathway by binding to oxylipins, i.e. linolenic and linoleic acids, thereby preventing lipoxygenase-mediated peroxidation of linolenic acid, a crucial precursor of JA biosynthesis (Prior *et al.*, 2001). The *G. rostochiensis*-specific effector Gr-VAP1 also suppresses host defence responses (Lozano-Torres *et al.*, 2014). Thus, nematode effectors suppress PTI, resulting in effector-triggered susceptibility (ETS; Jones and Dangl, 2006).

5.1.1.3. Systemic Responses

Host plants exhibit a differential response to PCN infection. SAR is generally initiated in response to biotrophic pathogen attack, whereas JA-mediated defences are elicited in response to pests, wounding or necrotrophic attack (Pieterse & van Loon, 1999; Wasternack, 2014). Endoparasitic nematodes elicit SA-mediated SAR particularly during the sedentary biotrophic stage of parasitism (Uehara *et al.*, 2010). PPN avirulence factors are highly conserved among species; the nematode secretions involved in the infection process are closely related to bacterial factors (Davis *et al.*, 2000; Barker, 2003). In addition, proteins and oesophageal secretions (i.e. endoglucanases and the products of nematode avirulence genes) are analogous to those of plant pathogenic bacteria. Such products are highly conserved among species, suggesting a horizontal gene transfer from bacteria. Thus, PCN elicit a SAR response: indeed, many of the nematode *R*-genes, including *Hero* (Ernst *et al.*, 2002), *Gpa2* (van der Vossen *et al.*, 2000) and *Gro-1* (Paal *et al.*, 2004), upregulate SA-mediated defences (Poch *et al.*, 2006).

In general terms, JA coordinates defence responses effective against necrotrophic pathogens and chewing insects, while SA targets mainly biotrophic pathogens, such as viruses and biotrophic fungal pathogens. However, synergistic cross-talk between signalling pathways enables the plant to tailor immune responses that are triggered against specific invaders encountered (Pieterse & van Loon, 1999; Pieterse *et al.*, 2009).

Induced resistance (IR) is a JA-mediated defence mechanism initiated by challenged plants to potentiate a vast array of physical and chemical host defences. Induced Systemic Resistance (ISR) prevents further infection and reduces host susceptibility to subsequent pest attack (Manosalva *et al.*, 2015). IR is a highly specific defence response and is meditated by host *R*-proteins, of the nucleotide-binding leucine-rich repeat (NB-LRR) superfamily, which recognize specific pest *avr* proteins. An incompatible *avr-R* interaction results in the immediate upregulation of extensive physical and chemical defence responses at the site of injury and proximal tissue to curtail further infection. The response may subsequently elicit systemic defences via activation of signal transduction cascades.

5.1.1.4. PCN-effector triggered susceptibility

PCN have evolved several physical and biochemical adaptations to contest induced host defence responses by avoiding detection or by suppressing host defence mechanisms (Gheysen & Jones, 2006). Successful parasitism is often contingent upon overcoming the host's initial local defence responses, particularly HR, which can prevent syncytium formation or attenuate its development (Robertson *et al.*, 2000).

The nematode cuticle and epidermis are the primary lines of defence to host resistance and function as both physical and chemical barriers against biotic and abiotic elements. Additional protection is afforded by the presence of a surface coat, a layer composed of glycoproteins and glycolipids (Decraemer & Hunt, 2013). Surface coat effectors are produced in the hypodermis and constitute antioxidant quenching enzymes, such as catalase, superoxide dismutase and ascorbate peroxidase. Such compounds metabolize

host ROS and thereby provide immunity to the oxidative burst and HR (Gheysen & Fenoll, 2002). Extensive research has been focused on PCN effector molecules (Jones *et al.*, 2009; Cotton *et al.*, 2014; Thorpe *et al.*, 2014; Ali *et al.*, 2015). Several effectors have been characterised, including the conserved SPRYSEC proteins in *G. rostochiensis,* which suppress *R*-mediated defence responses (Rehman *et al.*, 2009; Sacco *et al.*, 2009; Postma *et al.*, 2012a; Moffett *et al.*, 2015).

Avoiding detection at the host-parasite interface is the primary adaptation deployed by PCN to negate host defences. PCN-derived host defence inhibiting compounds are typically expressed in oesophageal secretions to facilitate J2 mobilization and synycitium induction. Nematode oesophageal glands express chorismate mutase enzymes, involved in the early stages of the shikimic pathway, to modulate plant biosynthesis of aromatics and hormones, such as auxins and defence signals including SA (Jones *et al.*, 2003; Chronis *et al.*, 2013). Furthermore, host recognition of nematode antigens is minimized by the continuous shedding and replenishment of the surface coat (Lozano-Torres *et al.*, 2014). Host mimicry is also implicated in PCN, whereby the surface coat produces proteins analogous to host cellular proteins as a disguise to negate detection by the host. PCN secrete effectors, which mimic plant CLAVATA3/ESR-related (CLE) ligand proteins (Eves-van den Akker *et al.*, 2014). Plant CLE peptides are involved in stem cell differentiation from the vascular bundle and apical meristems. PCN secrete CLE-like effectors and exploit CLE ligand mimicry in the sedentary nematode-host interaction (Lu *et al.*, 2009; Chen *et al.*, 2013).

5.1.2. PCN-induced root morphogenesis and PRL

It is proposed that PCN-induced damage at the root tips may increase lateral root formation, resulting in an increased number of feeding sites for the later-emerging *G. pallida* juveniles. Similarly, PCN inflicted damage on host roots during invasion and subsequent syncytium development may result in quantitative and/or qualitative changes in the host root exudates (PRL). This may subsequently influence PCN hatch and

multiplication (Back *et al.*, 2010). Furthermore, the temporal expression of species-selective HF can have a significant effect on PCN hatch (Devine *et al.*, 2001).

Larger root systems produce more PRL and thus produce greater quantities of HF. Therefore, quantitative changes in PRL due to PCN infection may fulfil the elevated HF threshold requirements for *G. pallida* hatch (Byrne *et al.*, 2001). Root branching pattern, as opposed to root system size *per se*, is inferred in the greater quantitative production of HF. Furthermore, there appears to be a positive correlation between lateral root density and both root exudate production and PCN hatch (Rawsthorne & Brodie, 1986). As such, one of the main objectives of this research is to investigate whether the early-hatching *G. rostochiensis* juveniles induce changes in host root development, which would favour the later-hatching *G. pallida*.

5.2. Aims

The research described in this chapter investigated whether induced root changes associated with PCN challenge were associated with the greater competitiveness of *G. pallida* in mixed-species populations. The main aims included:

- To determine whether ISR is elicited and to evaluate PCN multiplication in unprimed and primed-root systems.
- To evaluate PCN multiplication in primed-roots at different time intervals.
- To analyse changes in root architecture due to PCN infection.
- To determine if PCN infection induces structural changes in uninfected roots via rhizotron and split-root rhizotron experiments.
- To evaluate differential host responses to *G. pallida* and *G. rostochiensis* infection in single- and mixed-species populations using rhizotrons.

5.3. Materials and Methods

5.3.1. Plant Material

Chitted seed tubers (§ 3.3.4.1) of late maincrop potato variety 'Rooster' were planted in all rhizotrons and split-root assays.

5.3.2. Soil

A silty loam topsoil was used throughout all experiments (§ 3.3.3).

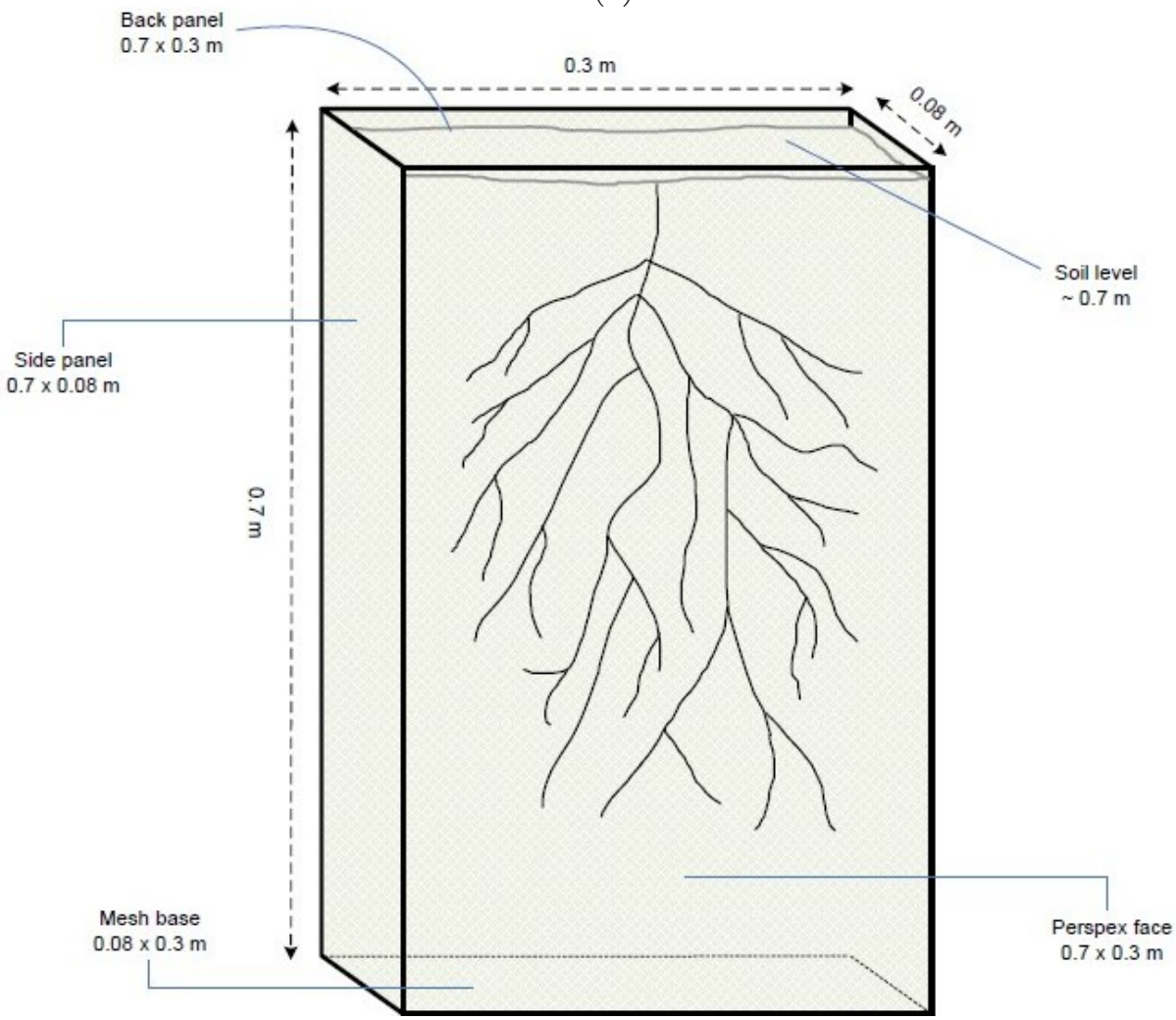
5.3.3. Nematode populations

Single generation populations of *G. pallida* Pa 2/3 and *G. rostochiensis* Ro 1 cysts, generously supplied by Dr. Colin Fleming, AFBI, Belfast, UK, were used throughout the experiment. Hatching assays were conducted before each trial to ascertain egg viability and hatching efficiency (§ 3.3.2). PCN cysts were pre-soaked for 1 week (§ 3.3.1). Thereafter, replicate samples, each of five cysts, were soaked for 5 d in 1.5 ml microtubes containing a 250 µl solution of diluted PRL (§ 3.3.6) to induce juvenile hatch. The initial population inoculum was equivalent to 100 cysts (26500 eggs or 5 eggs g soil^{-1}). Mixed populations comprised a 1:1 ratio of *G. pallida* and *G. rostochiensis*. All rhizotron and split-root pot assays were inoculated after 3 weeks with an egg suspension and administered directly to the root area as described in § 3.3.5.3.

5.3.4. Rhizotron assay

A rhizotron unit was constructed from marine plywood and Perspex (Fig. 5.1). Soil used in rhizotrons was sieved through a 1000 µm sieve prior to use to assure an even soil composition to facilitate image analysis. The soil was applied to the rhizotron in even strata and gently compacted at regular intervals to ensure no air spaces were visible on the Perspex face.

(A)



(B)

Figure 5.1 (A) Schematic rhizotron design and (B) a rhizotron unit positioned at a 45º angle to facilitate root growth along the transparent Perspex plate.

Each unit was filled with soil and the seed tuber (one per rhizotron) was planted 5 cm from the soil surface. The rhizotron was placed at a 30° angle with the Perspex being the lower face to encourage root growth along the transparent plate. An opaque black plastic material was used to shelter the transparent face from light and thereby to avoid UV damage. Four treatments were established that included:

- Control (0 eggs g soil^{-1})
- *G. pallida* (5 eggs g soil^{-1})
- *G. rostochiensis* (5 eggs g soil^{-1})
- *G. pallida* (2.5 eggs g soil^{-1}) and *G. rostochiensis* (2.5 eggs g soil^{-1})

Each treatment consisted of ten replicates which were arranged in a replicated randomized block design. Two soil-filled rhizotrons were placed at the beginning and end of each row to standardise compaction pressure throughout each row. Rhizotrons were maintained in a glasshouse between May and October and were watered on alternate days.

5.3.4.1. Nematode inoculation

Plants were inoculated 14 d after shoot emergence. Nematodes were applied directly to the rhizosphere as an egg suspension to eliminate, where appropriate, any effect of the delayed *G. pallid*a hatch (§ 3.3.5.3).

5.3.5. Image acquisition

All images were acquired 21 d after J2 inoculation. Each unit was scanned at a 300 dpi resolution using a modified flatbed scanner (CanoScan N650U). The scanner was aligned parallel to and secured to the Perspex face and an image was acquired for the upper and lower sections of the root area.

5.3.6. Image analysis

Digital image outputs were merged and analysed in JPEG format using SigmaScan™ Pro 5.0 (SPSS, Inc.) image analysis software. Root density and root counting were performed

using Adobe Photoshop V.10.1 (Adobe Systems Incorporated). Roots were traced using a digitising tablet (Manhattan PF-1209). A pseudo-colour line overlay was applied to delineate and vectorise the root system for digital measurement. Root length was represented in pixels, and equated to (mean ± SD) 117.95 ± 0.86 pixel cm^{-1}, which was recalibrated in subsequent trials to ensure area accuracy and reproducibility.

5.3.6.1. Root area

The root area was calculated by selecting triplicate 1000 x 1500-pixel sections from each replicate. The selected area was standardised by defining sampling coordinates. Images were converted to 8-bit format and minimum and maximum threshold limits were established. Total root area was quantified as the number of pixels within the defined threshold limits. The pixelated root area was calculated as a percentage of the total pixel area. Data are presented as the average of triplicate values for each replicate.

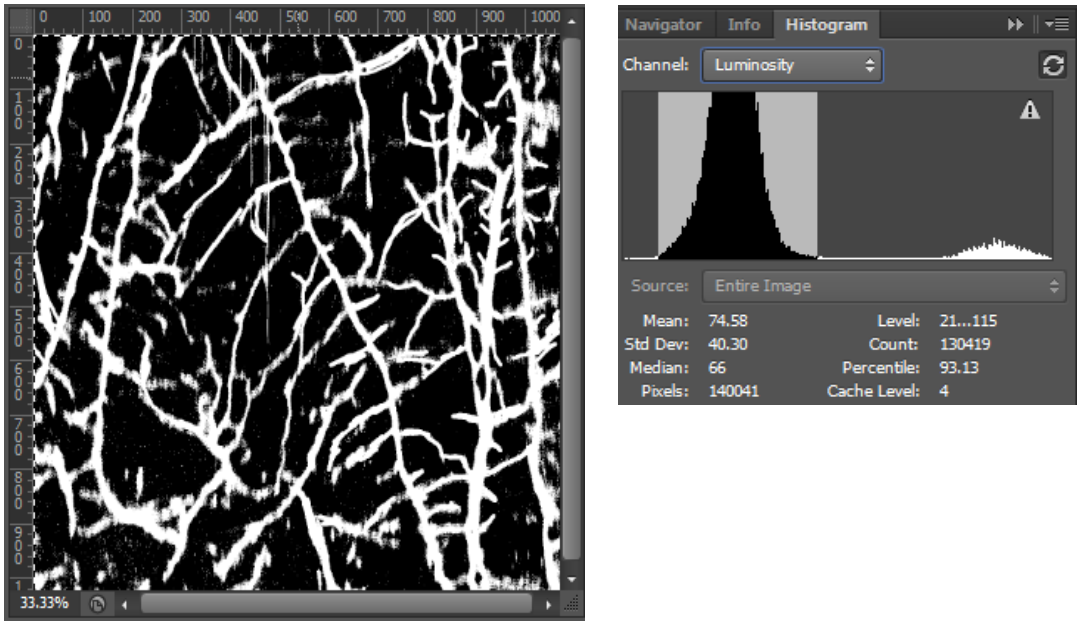


Figure 5.2 Threshold analysis of a PCN-infected root system. Root area is represented as percentage of the total number of compliant pixels within the defined threshold limits.

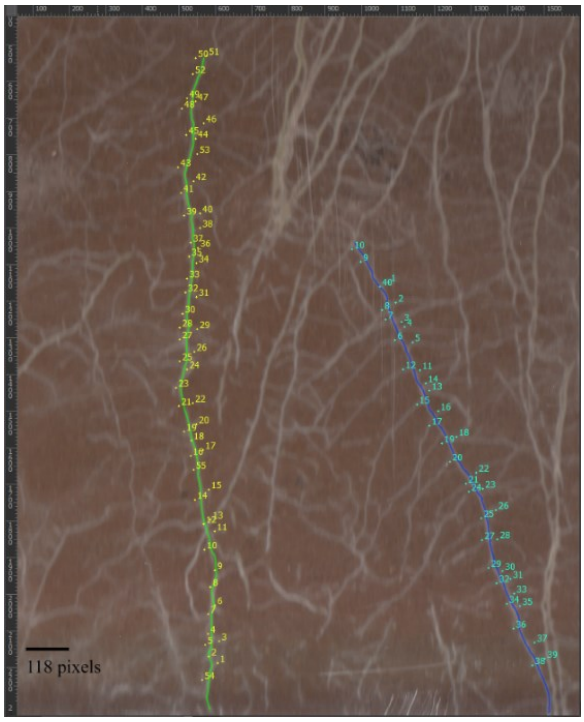


Figure 5.3 Image output of PCN infected root system. The length of the root section was digitally mapped and pixel length was measured. The number of root tips were counted using a count tool.

5.3.6.2. Root tip density

The average number of root tips was measured by selecting eight tertiary roots that intersected a transect line at defined coordinates. The length of the selected roots and the number of root tips per section were counted: each section was a minimum 6,000 pixels (50.85 cm) in length. Root tip density was calculated as the number of root tips cm^{-1}.

5.3.6.3. Biomass

Plants were cut at soil level 42 d after planting. Roots were subsequently removed from the rhizotron and shaken over a 250 µm sieve to collect any adhering cysts. Thereafter, roots were thoroughly washed by immersion in water to remove soil particles. Roots were further rinsed in a 250 µm sieve to collect fine roots. Individual samples were placed in paper bags and dried in an oven at 60 °C for 48 h and weighed after drying.

5.3.7. Split-root rhizotron assay

Rhizotrons (§ 5.3.1) were modified to facilitate a split-root system. A physical barrier was incorporated into each unit to create an impermeable vertical partition to separate the soil

systems (Fig. 5.4). The barrier consisted of duct tape, which was sealed to the back panel and the Perspex front panel of the rhizotron. The unit was filled with soil (§ 5.3.4) with a 10 cm headspace to accommodate the seed tuber and an impermeable plastic membrane (cling film) was placed on the top layer of soil. A seed tuber was placed directly on the membrane directly over the soil partition. The membrane was perforated with two holes (approx. 2 cm diameter) on either side of the partition to facilitate root division and growth in either of the divided sections. The membrane was positioned to create a bund or impermeable barrier around the seed tuber and thereby to permit watering to both soil divisions.

The efficacy of the split-root system to impede J2 migration between soils was validated by testing nematode populations (§ 3.3.8 - 3.3.11) for foreign *G. pallida* or *G. rostochiensis* that may have migrated between the partitioned soils. Only one-half of the split rhizotron (selected at random) was inoculated with PCN to facilitate the direct comparison of PCN-infected and uninfected roots within a single host. Inoculum was applied as an egg suspension (§ 5.3.3). Four treatments were established on the split-roots, which involved:

- *G. pallida* (5 eggs g soil^{-1}) vs. no PCN
- *G. rostochiensis* (5 eggs g soil^{-1}) vs. no PCN
- *G. pallida* (2.5 egg g soil^{-1}) + *G. rostochiensis* (2.5 egg g soil^{-1}) vs. no PCN
- Control (0 eggs g soil^{-1}) vs. no PCN.

The split-root rhizotrons were incubated outdoors from April to September and root area was analysed after 21 d after J2 inoculation as described in § 5.3.1.

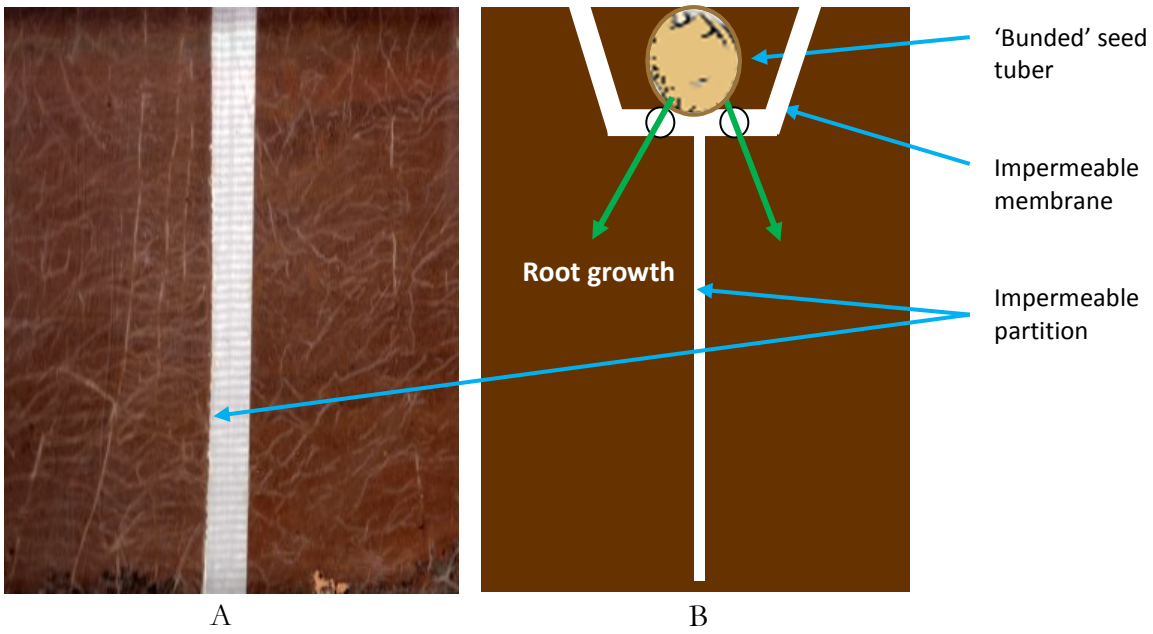


Figure 5.4 Split-root rhizotrons system (A) with an impermeable barrier. (B) split-root rhizotron design incorporating an impermeable membrane and partition to impede J2 mobility between partitioned soil.

5.3.8. Split-root pot assay

Each replicate of the split-root assay comprised three pots in a two level pyramid design (Fig. 5.5). Each upper pot (10 cm diameter) was nested above two base pots (15 cm diameter), which were filled with soil (§ 3.3.3) to the surface level to support the upper pot. One chitted seed tuber cv. 'Rooster' (§ 5.3.1) was placed at the bottom of the upper pot and was filled to the surface level with soil after planting. The "tuber pot" was modified to contain two holes (3 cm in diameter), one in each hemisphere of the pot to facilitate root development in two separate rhizospheres. The efficacy of the split-pot system was validated by species detection assays (§ 3.4.8 - 3.4.11).

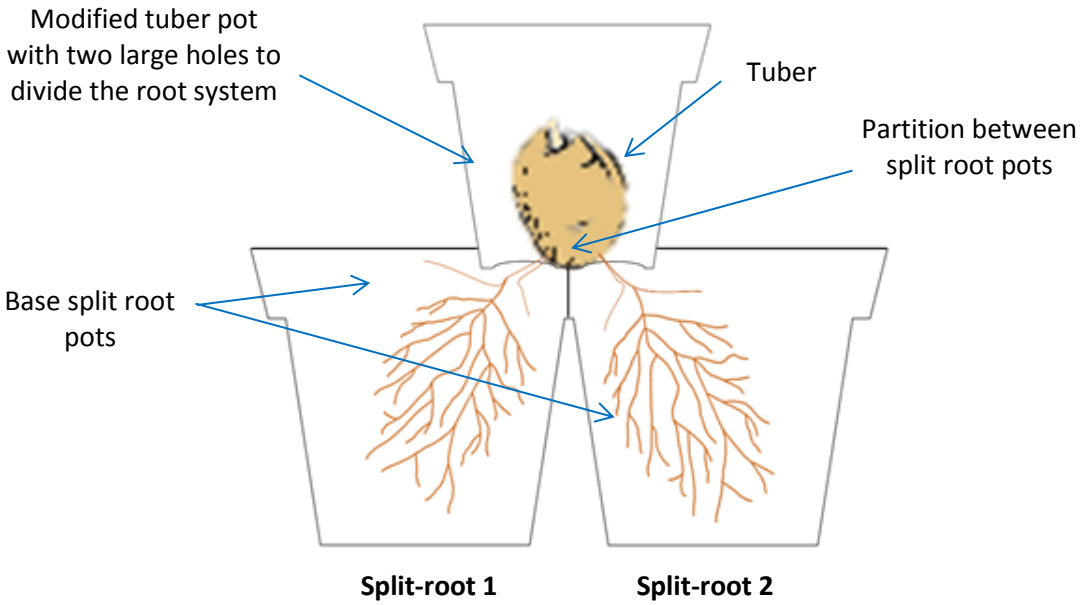


Figure 5.5 Split-root system pot assay, consisting of a pyramid design with the tuber pot nested over two base pots, each accommodating one half of the split-root system.

The first split-root pot (split-root 1) was inoculated two weeks after shoot emergence (0 d). The second half of the root system (split-root 2) was inoculated at 0, 7 or 14 dpi (days post split-root 1 inoculation). Each pot was inoculated with an egg suspension of 5 eggs g soil^{-1} (§ 3.3.5.3). Eleven treatments were established, with 8 replicates per treatment (Table 5.1).

Table 5.1 Split-root pot treatments.

Split-root 1		Split-root 2	
Species	**Time**	**Species**	**Time**
G. pallida	0 d	*G. rostochiensis*	0 dpi
G. pallida	0 d	*G. rostochiensis*	7 dpi
G. pallida	0 d	*G. rostochiensis*	14 dpi
G. rostochiensis	0 d	*G. pallida*	7 dpi
G. rostochiensis	0 d	*G. pallida*	14 dpi
G. pallida	0 d	*G. pallida*	0 dpi
G. pallida	0 d	*G. pallida*	7 dpi
G. pallida	0 d	*G. pallida*	14 dpi
G. rostochiensis	0 d	*G. rostochiensis*	0 dpi
G. rostochiensis	0 d	*G. rostochiensis*	7 dpi
G. rostochiensis	0 d	*G. rostochiensis*	14 dpi

5.3.8.1. Plant material

The plants were grown outdoors from early May to late September and were maintained in a temperature-stabilising plunge pit throughout the experiment (§ 3.3.4.1). Soil temperature ranged from 15 to 18 °C. The basal pots were contained in plastic bags to prevent cross contamination between samples due to nematode migration.

5.3.9. Cyst extraction and purification

Cysts from one soil subsample (400 cm^3) from each replicate were extracted by elutriation and subsequent purification (§ 3.3.4.3).

5.3.10. Nematode quantification

See section 3.3.8 – 3.3.11

5.3.11. Statistical analysis

All data were analysed using SigmaPlot V12.5 (Systat Software, Inc. Erkrath, Germany). All data were checked for normality and homogeneity of variance; in certain cases, variables were normalized by data transformation. Data were analysed for significance by ANOVA. Data from these analyses were presented as the means of the untransformed data. Split-root pot assays were analysed by three-way ANOVA. The rhizotron and split rhizotron assays were analysed by two-way ANOVA. Haulm and root biomass data were analysed by one-way ANOVA. *Post hoc* multiple comparison analyses were performed using the Tukey test. Linear parametric correlations between root data and nematode multiplication were analysed using Pearson's test and plotted by linear regression. Root tip density data were non-parametric and analysed by the Kruskal-Wallis test.

5.4. Results

5.4.1. Split-root assay

Three-way interaction ANOVA revealed a significant difference in PCN multiplication between species ($F_{(1, 175)} = 4.0$; $P < 0.05$), but there was no significant difference between inoculation time ($F_{(2, 175)} = 0.18$; $P > 0.05$) or between mixed- and single-species populations (Table 5.2a; $F_{(1, 175)} = 0.11$; $P > 0.05$). No significant interactions were observed ($P > 0.05$). The effect of time interval between inoculations revealed similar results, with a highly significant difference between species (Table 5.2 b; $F_{(1, 175} = 8.30$; $P < 0.001$).

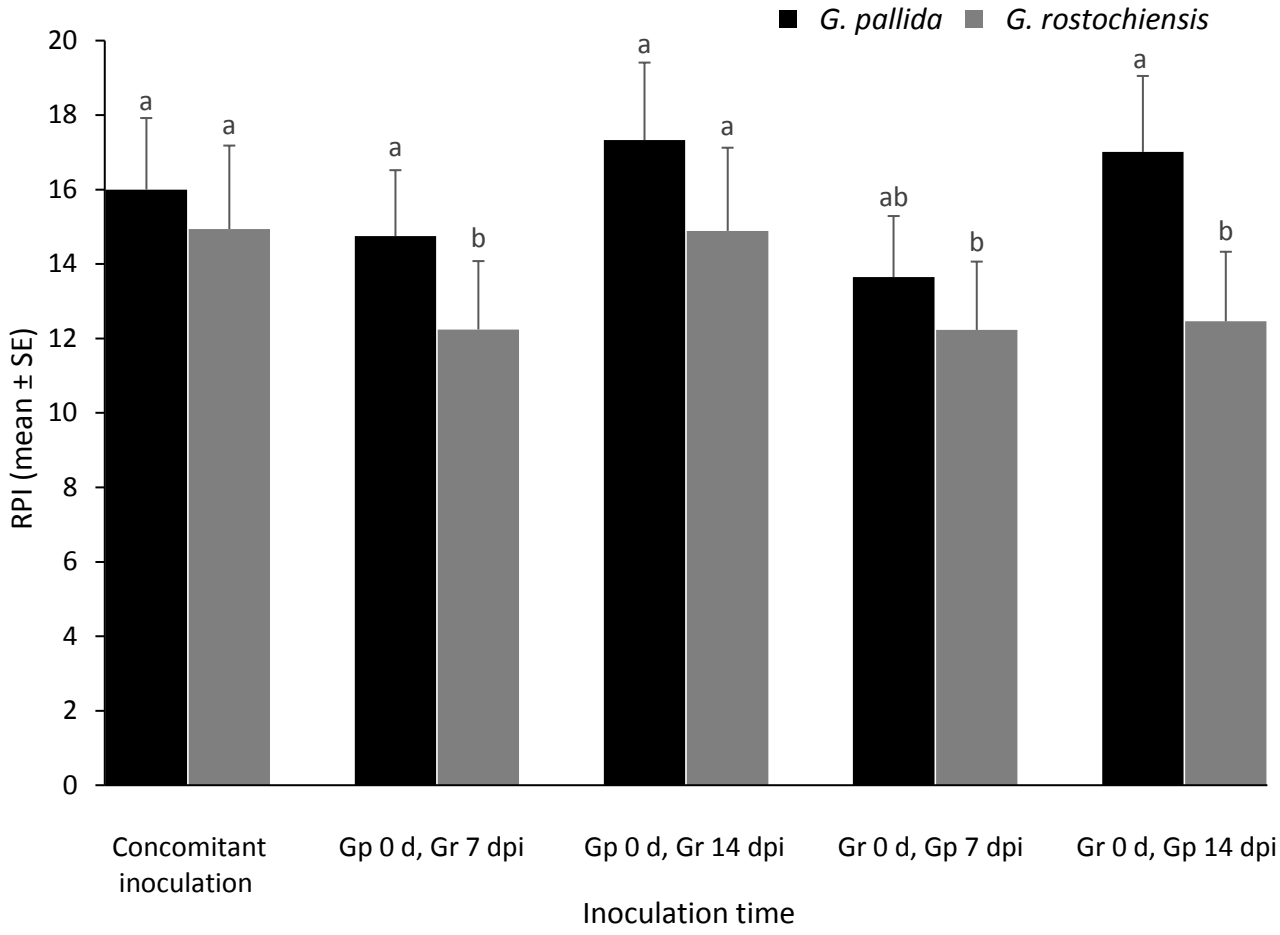
When mixed-species studies were investigated, the split-root pot assays revealed significant differences between species multiplication rates at different inoculation times. There was minimal difference between species multiplication upon simultaneous inoculation of the split-root system (Fig. 5.6), and there was no significant difference in PCN multiplication between inoculation times ($F_{(2, 79)} = 0.43$; $P > 0.05$) or between species ($F_{(1, 79)} = 0.83$; $P > 0.05$). When the split-root system was first inoculated with *G. pallida*, there was no effect of *G. rostochiensis* on *G. pallida* multiplication ($P > 0.05$) However, *G. rostochiensis* multiplication on split-root 2 was significantly reduced when split-root 1 had been inoculated with *G. pallida* 7 d earlier and also at 14 d earlier, albeit not significantly (Fig. 5.6).

When the split-root system was first inoculated with *G. rostochiensis*, *G. pallida* multiplication was not significantly affected ($P > 0.05$), regardless of when *G. pallida* was inoculated onto split-root 2 at either 7 or 14 d. When single-species split-root inoculations were carried out at different intervals, there were no significant differences for either species, although there was a significant species main effect (Fig. 5.7; $F_{(1, 90)} = 0.59$; $P < 0.01$).

Table 5.2 ANOVA summary of the effect of (A) inoculation time and (B) interval between inoculation on *G. pallida* and *G. rostochiensis* multiplication in split-root pot systems.

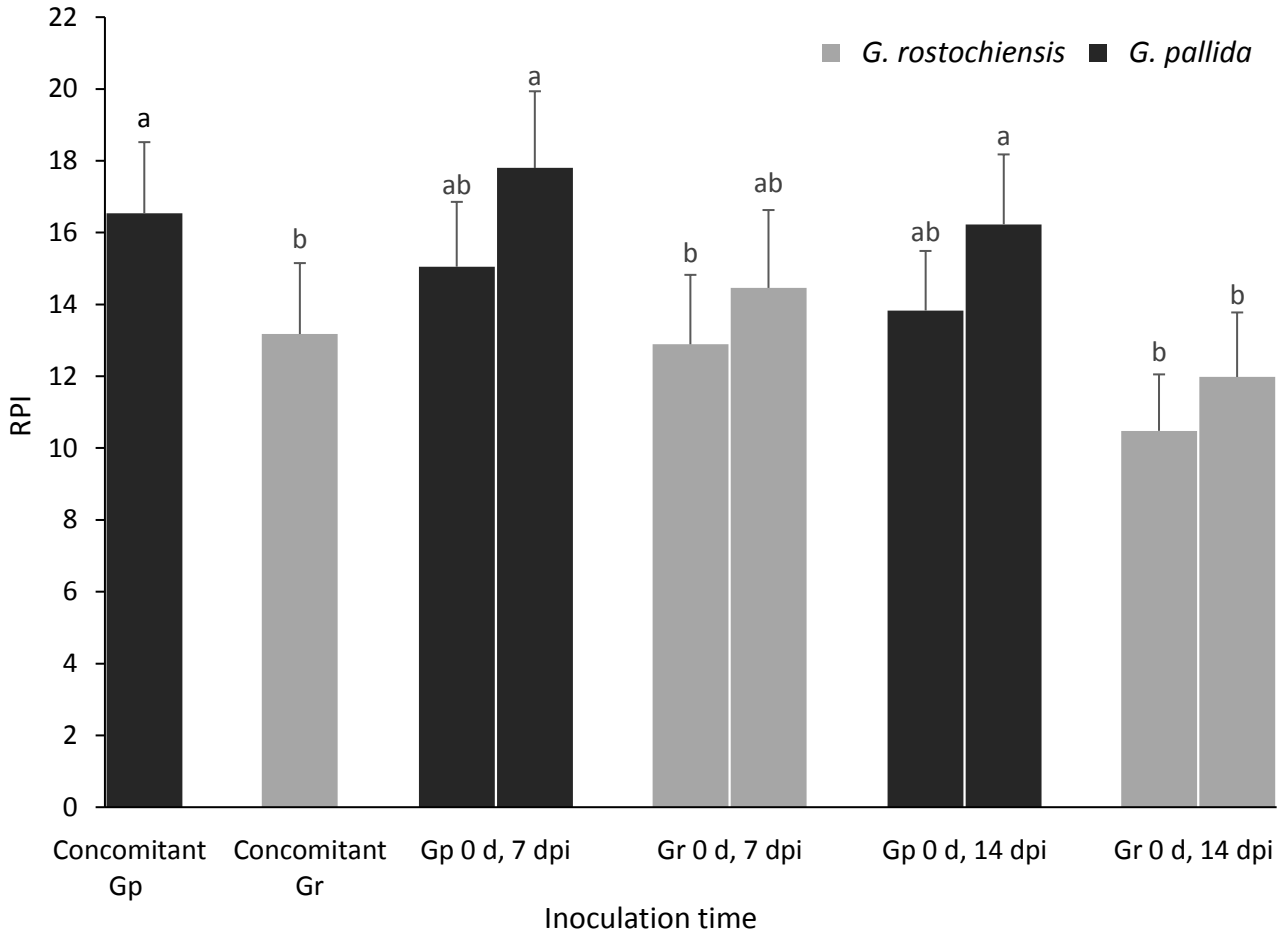
(A) Variation in inoculation time	**DF**	**SS**	**MS**	**F**	***P***
Population	1	8.0	8.00	0.11	>0.05
Inoculation	2	25.6	12.79	0.18	>0.05
Species	1	289.7	289.67	4.00	<0.05
Population x Inoculation	2	120.7	60.33	0.83	>0.05
Population x Species	1	17.2	17.22	0.24	>0.05
Inoculation x Species	2	22.6	11.28	0.16	>0.05
Population x Inoculation x Species	2	6.3	3.17	0.04	>0.05
Residual	164	11865.3	72.35		
Total	175	12400.5	70.86		

(B) Variation in interval	**DF**	**SS**	**MS**	**F**	***P***
Population	1	2.71	2.71	0.04	>0.05
Interval	2	129.31	64.65	1.03	>0.05
Species	1	520.50	520.50	8.30	<0.001
Population x Interval	2	1.46	0.73	0.01	>0.05
Population x Species	1	2.71	2.71	0.04	>0.05
Interval x Species	2	7.81	3.91	0.06	>0.05
Population x Interval x Species	2	1.46	0.73	0.01	>0.05
Residual	164	10282.69	62.70		
Total	175	10929.39	62.45		



Source of Variation	DF	SS	MS	F	*P*
Inoculation Time	2	74.66	37.33	0.43	>0.05
Species	1	72.47	72.47	0.83	>0.05
Interaction	2	22.84	11.42	0.13	>0.05
Residual	74	6428.78	86.88		
Total	79	6622.65	83.83		

Figure 5.6 The effect of inoculation time of *G. pallida* (Gp) and *G. rostochiensis* (Gr) at different intervals (0 , 7 and 14 dpi) in mixed-species split-root systems, where one split-root was inoculated with *G. pallida* and the other with *G. rostochiensis*. Samples that share a common letter are not significantly different ($P > 0.05$), using the Tukey test.

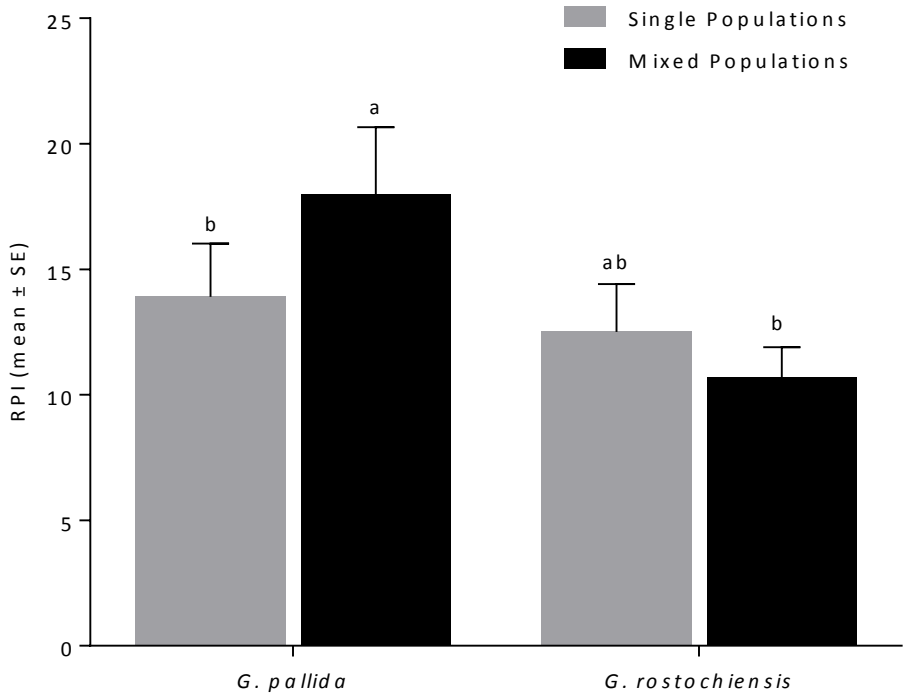


Source of Variation	DF	SS	MS	F	*P*
Inoculation Time	2	71.28	35.64	0.59	>0.05
Species	1	261.40	261.40	4.33	<0.01
Interaction	2	4.48	2.24	0.04	>0.05
Residual	90	5436.39	60.40		
Total	95	5773.55	60.77		

Figure 5.7 The effect of inoculation time of *G. pallida* (Gp) and *G. rostochiensis* (Gr) at different intervals (0 d, 7 d and 14 d) in single-species split-root systems, where split-root pots 1 and 2 were inoculated with the same species. Samples with a common letter are not significantly different ($P > 0.05$), using the Tukey test.

5.4.2. Rhizotron assay

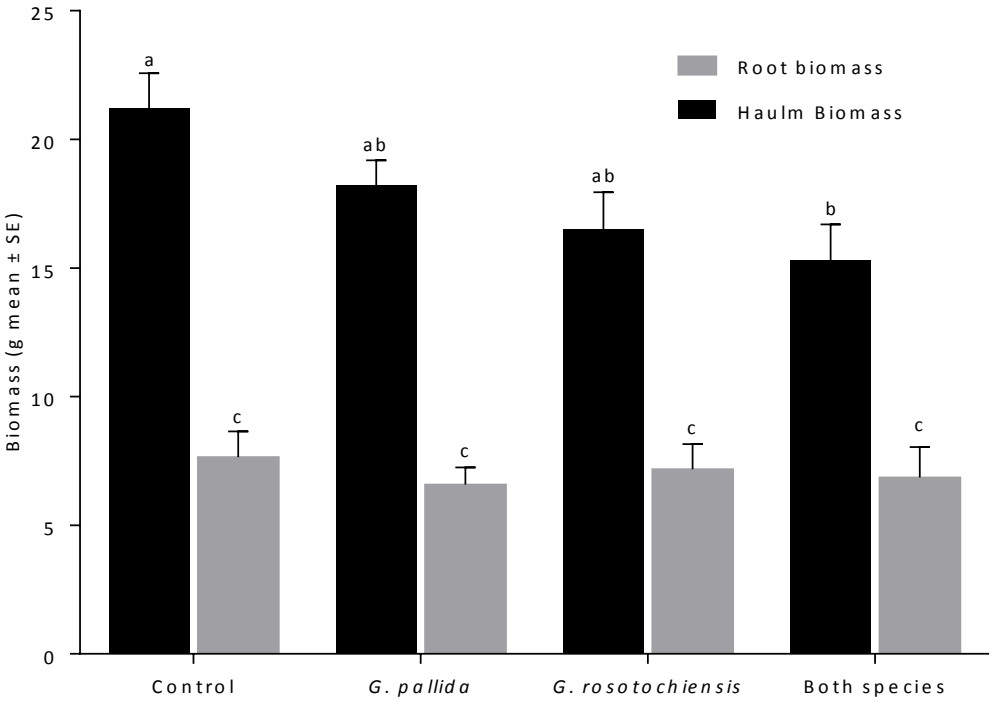
PCN multiplication within the rhizotron assays revealed a significant difference in multiplication (RPI) values between species ($F_{(1, 39)} = 4.54$; $P < 0.01$). *G. pallida* significantly outcompeted *G. rostochiensis* in the mixed-species population rhizotrons (Fig. 5.8), with a multiplication rate (mean ± SE) of 18.0 ± 0.260, compared to 10.70 ± 0.260 for *G. rostochiensis*. Indeed, *G. pallida* exhibited higher multiplication in mixed- than in single-species populations (Fig. 5.8). However, there was no significant difference in multiplication between the two species when studied as a single-species population (Fig. 5.8). There was no significant difference between populations ($F_{(1, 39)} = 0.305$; $P > 0.05$) and there was no significant interaction between population and species ($F_{(1, 36)} = 2.08$; $P > 0.05$).



Source of Variation	DF	SS	MS	F	*P*
Population	1	12.77	12.77	0.305	>0.05
Species	1	190.10	190.10	4.545	<0.01
Interaction	1	87.03	87.03	2.080	>0.05
Residual	36	1505.85	41.83		
Total	39	1795.74	46.05		

Figure 5.8 PCN multiplication in single- and mixed species rhizotrons. Any two samples with a common letter are not significantly different ($P > 0.05$) using the Tukey test.

PCN infection had a significant effect on haulm biomass ($F_{(3, 39)} = 3.87$; $P < 0.01$), with only mixed-species populations having a significant effect on haulm biomass. Root biomass was not significantly affected in any treatment ($F_{(3, 39)} = 0.23$; $P > 0.05$). The mixed-species rhizotron conveyed the greatest RPI values and the lowest haulm biomass (Fig. 5.9). The uninfected control plants had the greatest haulm (mean ± SE) and root dry biomass yields of 21.22 g ± 0.214 and 7.65 g ± 0.176, respectively (Fig. 5.9). There was no significant correlation (Fig. 5.10) between RPI and haulm biomass ($r = -0.157$; $P > 0.05$) and root biomass ($r = -0.022$; $P > 0.05$).



Variation in haulm biomass	DF	SS	MS	F	*P*
Between Groups	3	197.14	65.71	3.87	<0.01
Residual	36	612.05	17.00		
Total	39	809.19			

Variation in root biomass	DF	SS	MS	F	*P*
Between Groups	3	6.58	2.19	0.23	>0.05
Residual	36	341.10	9.48		
Total	39	347.68			

Figure 5.9 The effect of PCN infection on haulm and root dry biomass. Samples with a common letter are not significantly different ($P > 0.05$) according to the Tukey test.

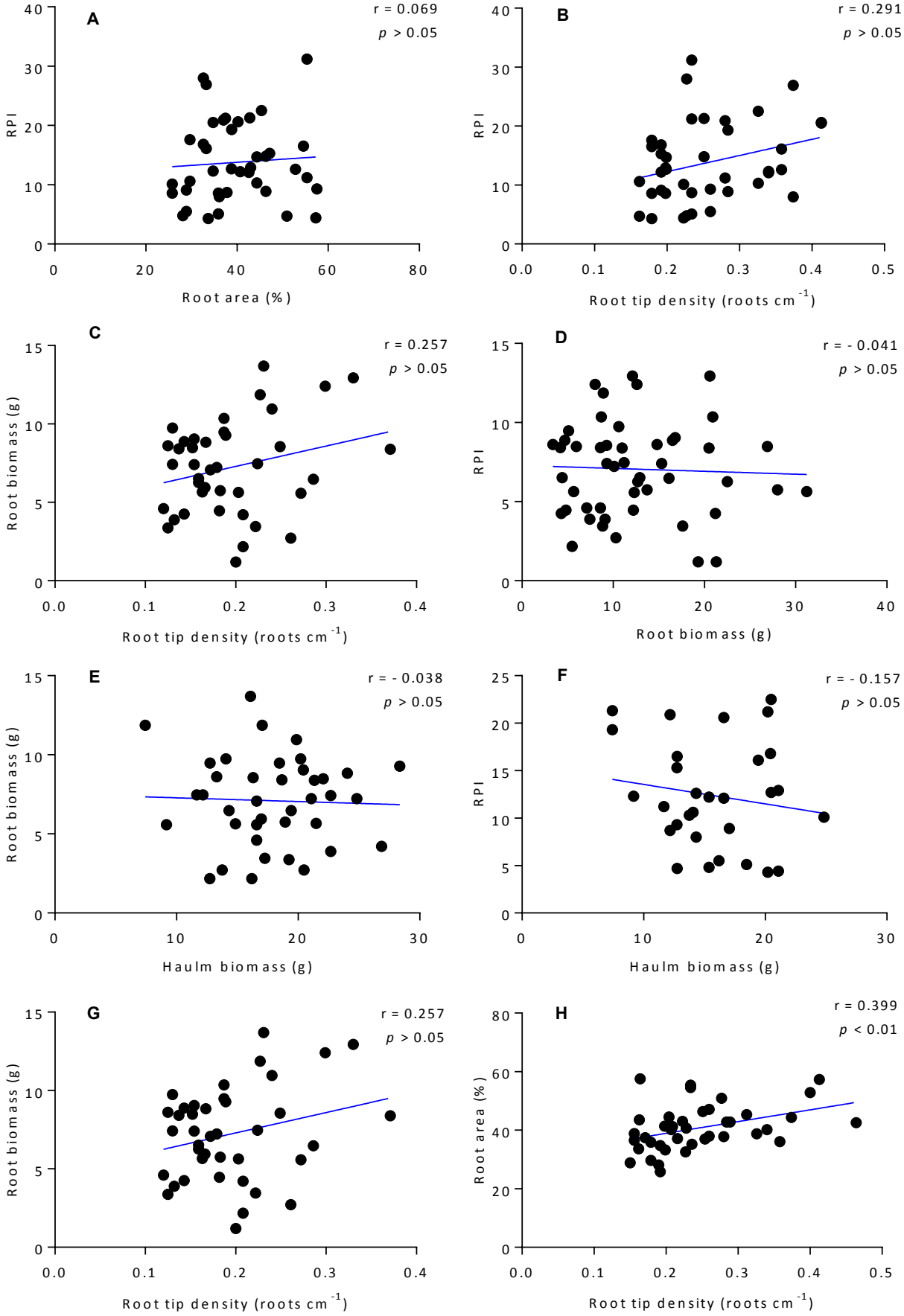


Figure 5.10 The effect of PCN multiplication on plant morphology.

Root system mapping revealed an increase in root tip density (Fig. 5.11) when roots were infected with *G. pallida* (5.44%) and *G. rostochiensis* (15.84%) and both species (8.44%); however, the effect was not significant (r = 0.291; $P > 0.05$). Furthermore, there was no significant difference in root tip density between treatments or controls (H = 2.547; $P > 0.05$).

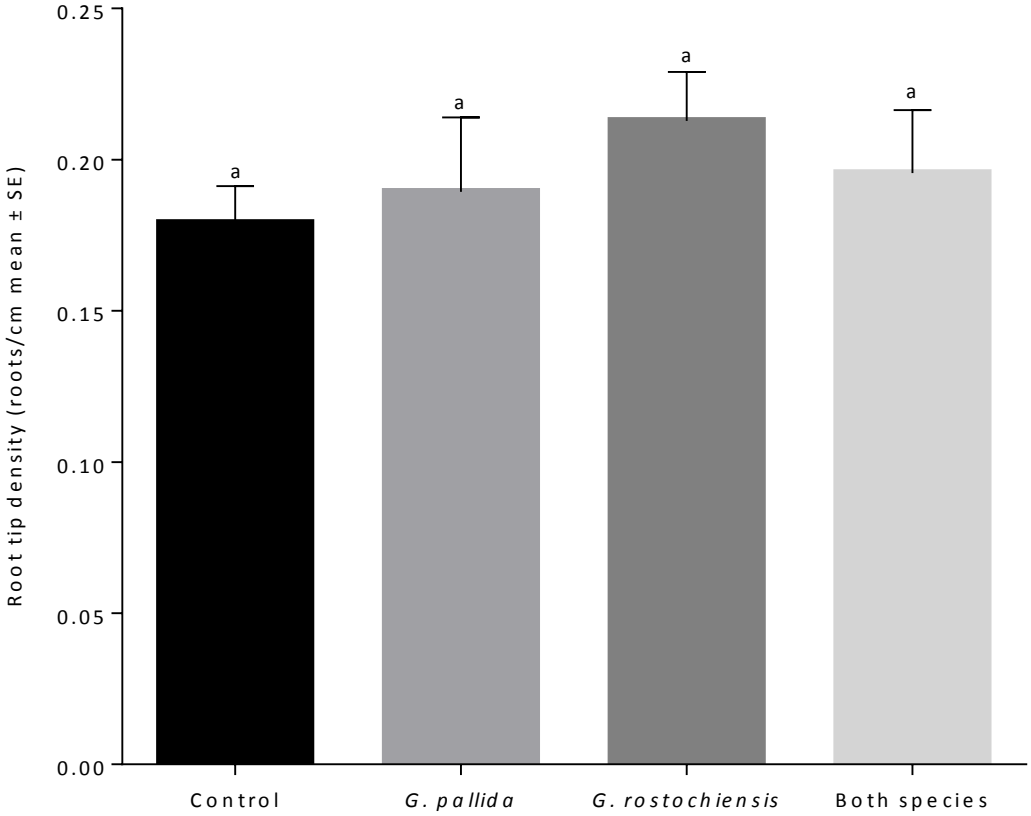
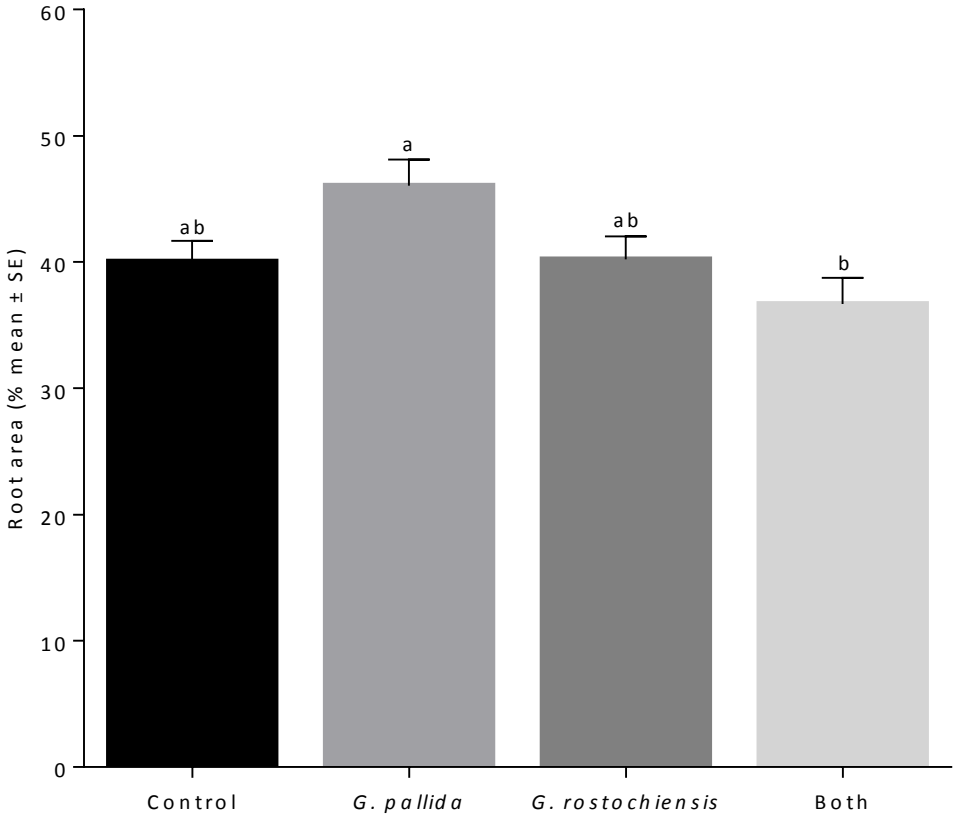


Figure 5.11 Root tip density of PCN-infected roots and uninfected roots as controls.

There was a significant correlation between root area and root tip density (Fig. 5.10; r = 0.399; $P < 0.01$). Furthermore, a significant difference in root area was observed between treatments (Fig. 5.12; $F_{(3, 119)} = 4.13$; $P < 0.01$). *G. pallida*-infected root systems exhibited the highest (mean ± SE) total root area percentage (46.05 % ± 11.39). Roots infected with both *G. pallida* and *G. rostochiensis* had the lowest root area (36.68 % ± 11.38).



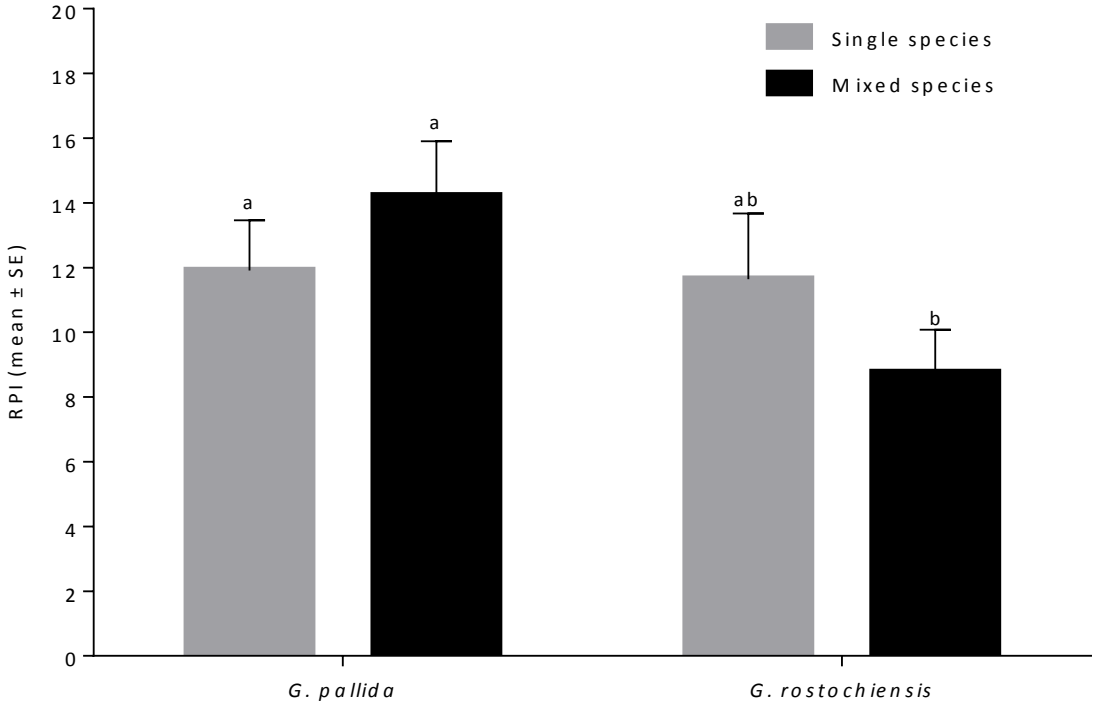
Source of Variation	DF	SS	MS	F	*P*
Between Groups	3	1363.48	454.49	4.13	<0.01
Residual	116	12765.12	110.04		
Total	119	14128.59			

Figure 5.12 The effect of PCN infection on root. Samples with a common letter are not significantly different ($P < 0.05$) using the Tukey test.

5.4.3. Split-root rhizotron assay

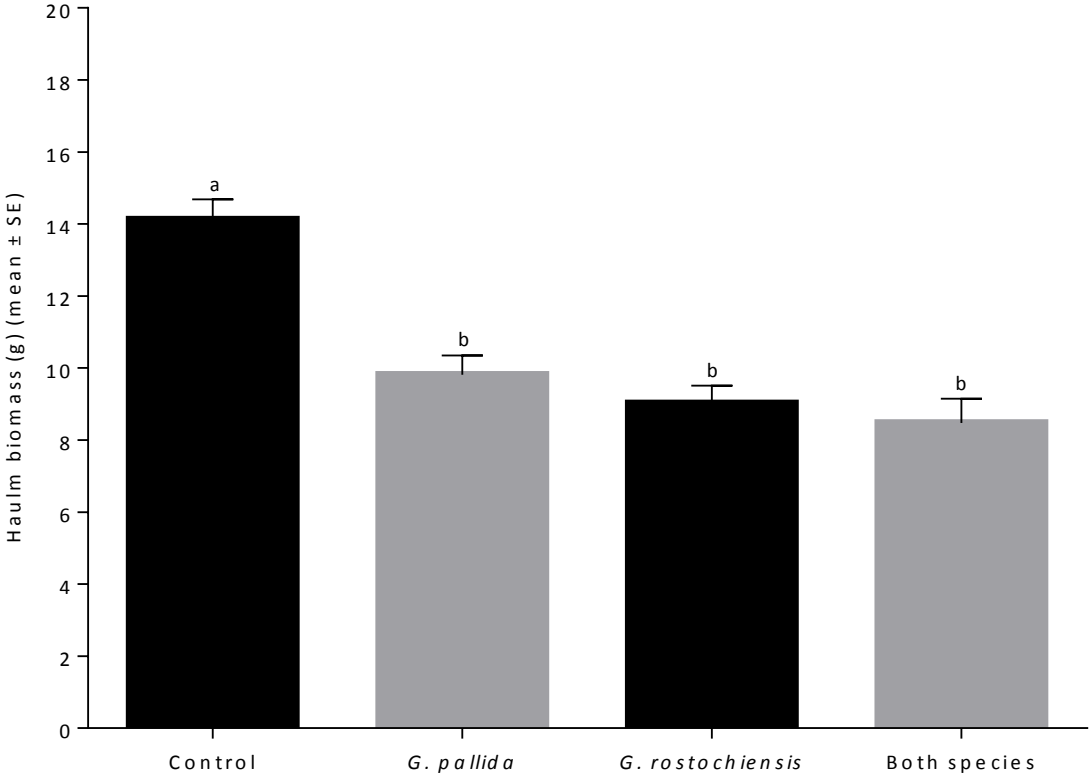
A significant difference in multiplication rates between single- and mixed-species populations was observed in split-root rhizotrons ($F_{(1, 39)} = 4.57$; $P < 0.01$). The Tukey test revealed a significant difference between *G. pallida* multiplication in mixed-species and *G. rostochiensis* multiplication in single-species populations (Fig. 5.13). Despite this, there was no significant difference in multiplication between species ($F_{(1, 39)} = 3.29$; $P > 0.05$). Similarly, there was no significant interaction between population and species ($F_{(1, 36)} = 1.71$; $P > 0.05$). Both *G. pallida* and *G. rostochiensis* had similar multiplication rates when

present in single-species populations, with RPI (mean ± SE) of 11.91 ± 0.221 and 11.61 ± 0.233, respectively. A slight difference between single- and mixed-species populations was evident within individual species multiplication rates. *G. pallida* had a greater RPI in mixed-species populations (14.22 ± 0.231); conversely, *G. rostochiensis* RPI was reduced in mixed-species populations (8.763 ± 0.204), compared to species-species populations, although the difference was not significant ($P > 0.05$). There was a significant difference in haulm biomass between treatments ($F_{(3, 39)} = 17.27$; $P < 0.001$). The PCN-infected samples had significantly lower yields relative to control samples (Fig. 5.14).



Source of Variation	DF	SS	MS	F	*P*
Population	1	133.23	133.23	4.57	<0.01
Species	1	96.04	96.04	3.29	>0.05
Interaction	1	49.91	49.91	1.71	>0.05
Residual	36	1049.60	29.16		
Total	39	1328.77	34.07		

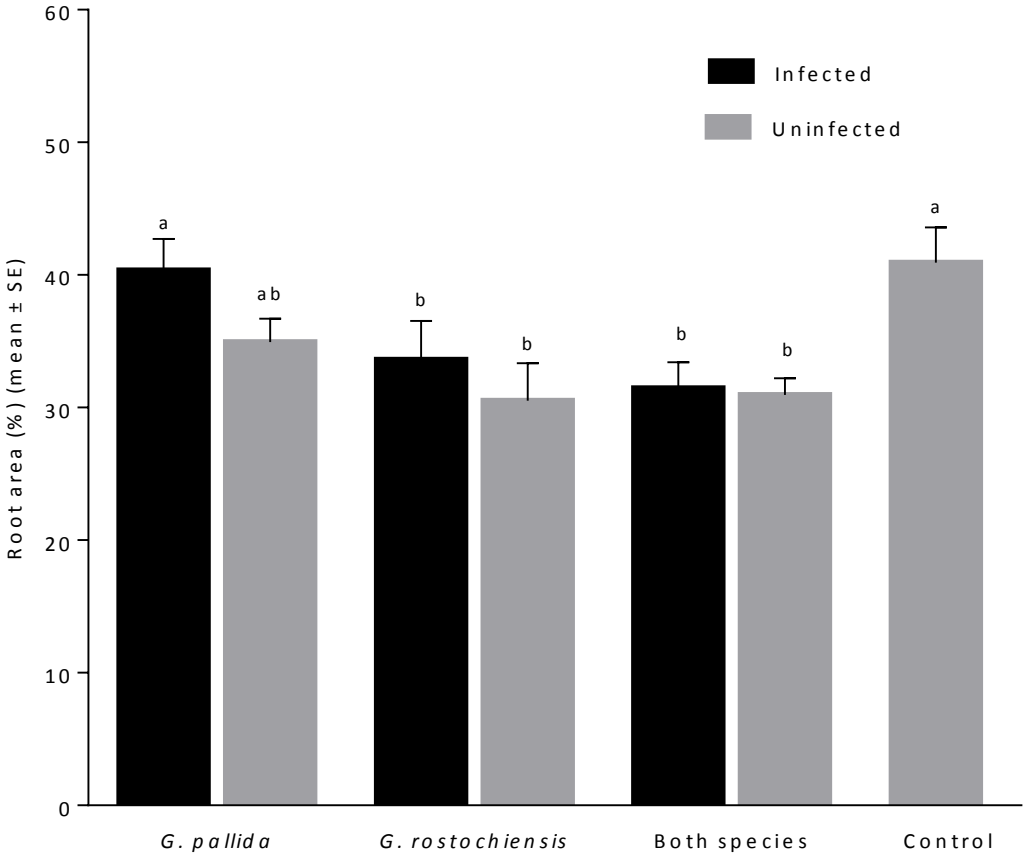
Figure 5.13 The effect of PCN multiplication in single- and mixed-species populations in split-root rhizotrons. Samples with a common letter are not significantly different ($P < 0.05$), using the Tukey test.



Source of Variation	DF	SS	MS	F	*P*
Between Groups	3	218.57	72.86	17.27	<0.001
Residual	36	151.86	4.22		
Total	39	370.42			

Figure 5.14 The effect of PCN multiplication (in single- and mixed-species populations) on haulm biomass in split-root rhizotrons. Samples with a common letter are not significantly different ($P > 0.05$), using the Tukey test.

A significant difference in root area between root systems infected with different PCN species was observed (Fig. 5.15; $F_{(3, 239)} = 23.06$; $P < 0.001$), with *G. pallida*-infected roots having the greater root area compared to *G. rostochiensis*-infected roots (Fig. 5.15). No significant difference between infected and uninfected root systems was observed ($F_{(1, 239)} = 1.00$; $P > 0.05$). However, there appeared to be a trend towards greater root area in PCN-infected roots relative to uninfected roots. This effect was evident, particularly in *G. pallida*-infected root systems and to a lesser extent in *G. rostochiensis*-infected root systems. The control treatments exhibited the highest (mean ± SE) percentage root area (40.67% ± 0.29), which was comparable to infected *G. pallida* treatments (40.22% ± 0.274).



Source of Variation	DF	SS	MS	F	*P*
Species	3	6279.99	2093.33	23.06	<0.001
Treatment	1	90.44	90.44	1.00	>0.05
Interaction	3	283.65	94.55	1.04	>0.05
Residual	232	21064.74	90.80		
Total	239	27718.83	115.98		

Figure 5.15 The effect of PCN multiplication (single- and mixed-species populations) on root area. Samples with a common letter are not significantly different ($P > 0.05$), using the Tukey test.

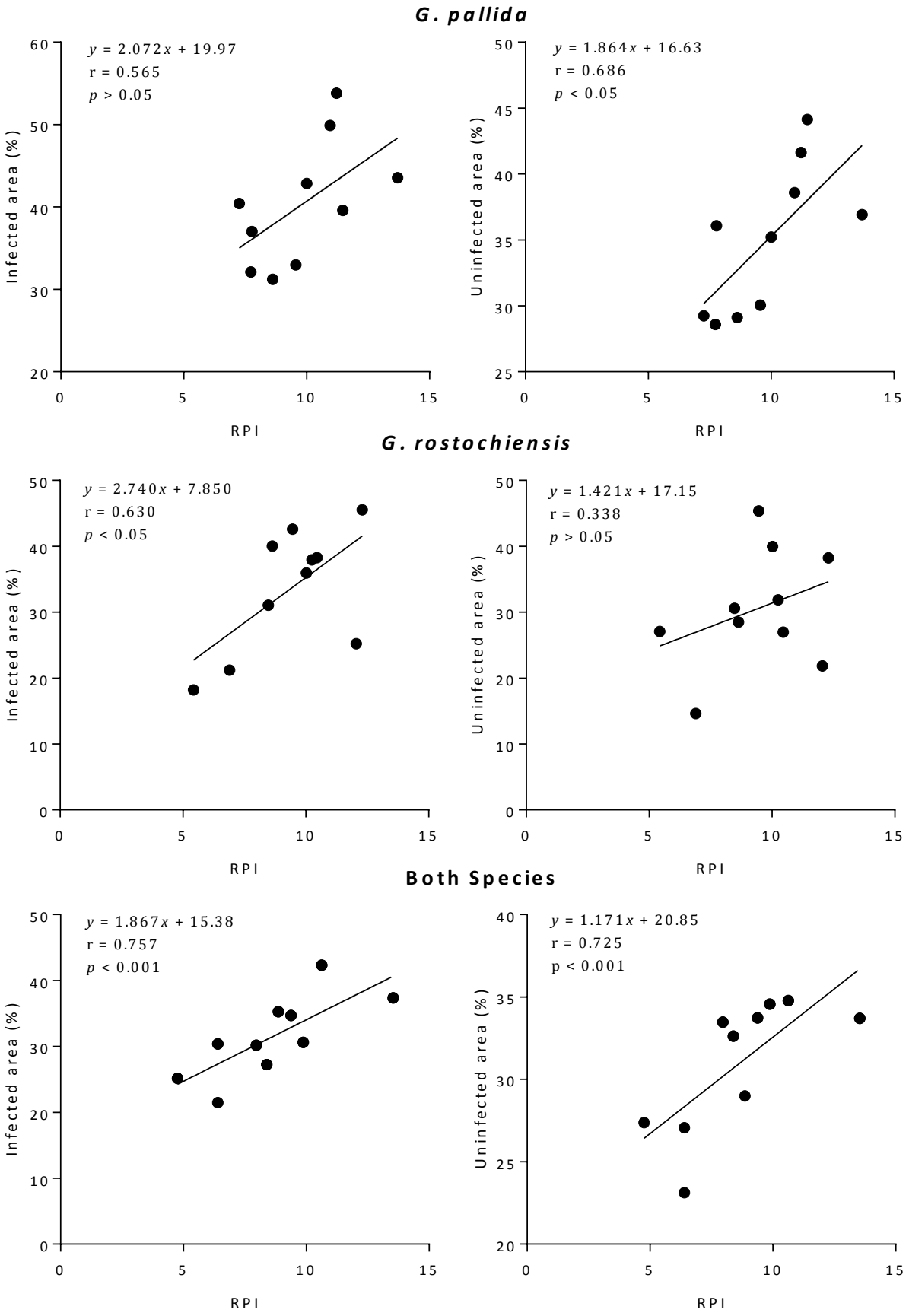


Figure 5.16 Relationships between PCN multiplication and root area in split-root rhizotrons. Data points are expressed as the mean of triplicate values.

Significant positive correlations were observed between PCN multiplication and root area in both infected and uninfected root systems (Fig. 5.16). A significant correlation was evident between *G. pallida* multiplication and root area in *G. pallida*-uninfected roots systems (r = 0.686; $P < 0.05$). A similar trend was observed in *G. pallida*-infected roots; although the effect was not significant (r = 0.565; $P = 0.06$). Conversely, there was a significant correlation between multiplication and *G. rostochiensis*-infected root area (r = 0.630; $P < 0.05$). However, the effect was not observed in uninfected roots (r = 0.338; $P > 0.05$). PCN multiplication and root area were highly correlated in mixed-species populations in both PCN-infected (r = 0.757; $P < 0.001$) and uninfected (r = 0.725 $P < 0.001$) roots.

5.5. Discussion

Consequences of PCN-host manipulation include nematode-induced phenotypic responses such as hypertrophied cells (Fudali *et al.*, 2008), syncytium induction and lateral root branching (Goverse *et al.*, 2000; Mathesius, 2003). Several studies have reported host-induced defences in response to PCN infection, namely ROS accumulation, HR and SA-mediated defences (Postma *et al.*, 2012b; Ali *et al.*, 2015). However, relatively few studies have analysed ISR against PCN. Wondafrash *et al.* (2013) reported ISR against RKN in tomato roots that was instigated by foliar herbivory, while microbial-mediated ISR against *G. pallida* has been reported (Hasky-Günther *et al.*, 1998; Reitz *et al.*, 2002).

5.5.1. Split-root pot assay

The split-root experiments in this chapter investigated whether ISR is potentiated in distal uninfected roots in response to PCN infection. The split-root rhizotron further investigated this effect by analysing multiplication and PCN-induced root morphogenesis. In Chapter 3, *G. pallida* proved to be the more competitive species, particularly in mixed-species populations, at the expense of *G. rostochiensis*. The split-root systems physically segregate *G. pallida* and *G. rostochiensis* populations by containment of each population in separate pots. Therefore, direct interspecific competition is negated, facilitating the

evaluation of species-specific responses to host ISR. In effect, the *G. rostochiensis* multiplication rate appeared relatively consistent in both the mixed- and single-species split-root systems. Nevertheless, the data presented indicated that *G. pallida* was the more successful species in both single- and mixed-species split-root systems, achieving higher multiplication than *G. rostochiensis* at all inoculation times. Furthermore, the results suggest that inoculation of part of the root system with *G. pallida* (before or after *G. rostochiensis* infection) inhibited *G. rostochiensis* multiplication in the other half of the root system. This effect was observed even if *G. rostochiensis* was inoculated before *G. pallida*. As such, it would appear that *G. pallida* infection had a negative systemic effect on *G. rostochiensis* multiplication.

The observed decrease in *G. rostochiensis* multiplication would propose the upregulation of systemic root defences, due to the *G. pallida* infection in one half of the rhizosphere. Furthermore, an increase in PCN multiplication could also infer increased host susceptibility due to PCN-mediated downregulation of host resistance via suppression of defence signalling pathways (Quentin *et al.*, 2013). Suppression of SA-mediated defences is a distinct advantage that may prevent SAR. Similarly, nematode effectors involved in the downregulation of JA-mediated responses may suppress ISR responses elicited due to wounding. Indeed, suppression of JA-mediated host resistance by both *G. pallida* and *G. rostochiensis* has been reported (Goverse & Smant, 2014; Moffett *et al.*, 2015).

Alternatively, the greater PCN multiplication evidenced at later inoculation (7 and 14 dpi) may be attributed to PCN-induced changes in root architecture and root proliferation. This would enhance multiplication by increasing root area for later-colonising J2 and thereby alleviate intraspecific competition. However, an increase in root area may also be attributed to plant growth as the root system is likely to be more developed at 14 d, relative to earlier treatments. However, the results from the rhizotron study would suggest an association between PCN-induced root changes and increased multiplication.

5.5.2. Rhizotron studies

Both the mixed-species and split-root rhizotron studies revealed similar PCN multiplication patterns as those evidenced in the competition trials (Chapter 3). *G. pallida* was the more successful species, with significantly higher multiplication in mixed-species populations relative to single-species populations. Similarly, *G. rostochiensis* multiplication was significantly lower in mixed- than in single-species populations. It would appear that the host root system exhibits species-specific responses to PCN attack. *G. pallida* single-species treatments recorded the greatest root area, yet it had a similar root tip density to the control and mixed-species treatment.

G. rostochiensis treatments yielded the highest root tip density but this did not correlate with increased root area. In the field, it is possible that this contributed to the greater *G. pallida* multiplication evidenced in mixed-species populations. The *G. rostochiensis*-induced increase in root tip density could be exploited by the later hatching *G. pallida*. In essence, treatments infected with both *G. pallida* and *G. rostochiensis* had the lowest root area and yielded the lowest haulm and root biomass. Thus, it may be proposed that dual *G. pallida* and *G. rostochiensis* infection has a particularly deleterious effect on the host. *G. pallida* appears to outcompete *G. rostochiensis* and be a more aggressive pest in mixed-species populations; therefore, the host responses evident in mixed-species infection may be partially due to *G. pallida* competitiveness.

In the split-root rhizotrons a trend towards increased root area in PCN-infected roots was evident. Indeed, PCN multiplication and root area were also significantly positively correlated. The results indicated that PCN-induced changes in root architecture invariably facilitate colonization by later emerging J2. Upon host invasion, J2 are attracted to and aggregate in the region of cell elongation and primordia of secondary roots (Prot, 1980). These regions constitute the main areas of new rootlet development. Widdowson *et al.* (1958) observed that new lateral rootlets generally formed at the point of J2 aggregation on the root, which would correlate with the findings from this research. Furthermore, the

PCN-induced root changes are also consistent with the studies of Fatemy and Evans (1986). They reported an increase in root growth upon *G. rostochiensis* infection root growth coupled with a reduced shoot-to-root ratio and a decline in haulm biomass. Indeed, the positive correlations between root area, root tip density and PCN multiplication are more discernible in roots directly infected with juveniles relative to uninfected roots.

5.5.3. Critical evaluation and future research recommendations:

It is difficult to determine whether ISR is elicited based on analysis of PCN multiplication and root studies alone. As such, the split-root experiments would benefit from supplemental temporal and spatial gene expression analysis to evaluate if ISR is instigated in uninfected roots. Moreover, biochemical analysis of root leachates in uninfected roots merits investigation to evaluate qualitative and/or quantitative changes in PRL due to PCN attack. Phytohormones, particularly auxins, are modulated by PCN parasitism; therefore, it is critical to investigate the impact of PCN-induced host responses on phytohormone accumulation and distribution and the consequential impacts on root proliferation. Thus, future research would benefit from studies combining gene expression and phytohormone analysis in concert with root biometric analyses.

The results indicated different host root responses to single- and mixed-species populations. Therefore, it is important to evaluate if an effect conferred by single-species populations is similar to that of mixed-species populations. As such, the effect of simultaneous PCN infection versus single *G. pallida* or *G. rostochiensis* infections would be important elements of future research. It would also be beneficial to include an additional treatment in the split-root pot assays, whereby one pot is inoculated with both species and the other pot contains inoculum from one species alone. This would also help assess differential species responses to priming incurred by pure *G. pallida* or pure *G. rostochiensis* populations and by mixed-species populations.

Soil biology, particularly, plant-microbe interactions, indirectly influence PCN multiplication by modulating the synthesis, composition and mobility of host root leachates (Ryan *et al.*, 2000), and eliciting microbe-mediated ISR (Reitz *et al.*, 2002). Future split-root pot and rhizotron research integrating PGPR and AMF isolates would permit analysis of the role of microbes in the PCN-host interaction. Mycorrhized root systems are typically larger and have a more developed architecture than uninoculated roots (Atkinson *et al.*, 1994). Therefore, the role of microbes in modulating root structure and the elicitation of ISR effective against PCN merits investigation.

Biometric root analyses using various different image acquisition and analysis techniques can provide considerable valuable data for root studies. The rhizotron was an efficient system for the non-destructive analysis of PCN infected root systems and it worked very well for root tip density and area quantification. However, there were limitations to this method, namely image resolution and sensitivity. This was largely due to the soil medium and problems with condensation. The selection and preparation of an appropriate soil medium is critical to assay sensitivity. The soil is compacted to reduce and refine the soil root interface to optimize the definition of the hosts' roots. Compaction may however, affect root development and potentially affect PRL diffusion and impact nematode migration.

Higher image resolution would also permit analyses that are more accurate. However, the greater sensitivity of higher resolution image capture would require refining the transparent face to a thinner, yet robust material with excellent clarity for image acquisition. Higher resolution imaging would require a scanning or camera device with high specifications, but also compatibility with the transparent face to assure minimal diffraction or reflection. Studies using mini-rhizotrons would overcome such limitations due to the advanced imaging capture devices and high optical specifications. Mini-rhizotrons are essentially cylindrical transparent probes containing an imaging device (Taylor *et al.*, 2014). The probes are inserted into the rhizosphere and roots grow adjacent

to and surrounding the probe. Numerous commercial rhizotrons are available which offer high resolution and magnification. The 3D imaging component of new technologies would be an invaluable asset to studies entailing syncytium and root tip development.

It would be worthwhile comparing different inoculation methods; J2 inoculum results in inundative infection, whereas hatching from cysts occurs over a longer period and more closely resembles natural inoculation. Thus, future rhizotron studies should evaluate temporal PCN-induced changes in root architecture, supplemented by gene expression analyses and biochemical PRL analyses. Additionally, future research should assess host root responses to different PCN infection densities.

Host induced resistance is critical in the battle against PCN. Therefore, a comprehensive understanding of PCN-induced structural changes in root architecture and biochemistry for host defence is imperative to optimise current PCN control strategies and aid the development of novel control methods.

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Chapter 6 General Discussion

PCN have a highly specialised interaction with their host, as a result of which they are among the most damaging pests of potato. PCN infestation incurs an average economic impact of €600 million in the EU and an annual 12.2% deficit in global potato crop yields (Urwin *et al.*, 2001; Nicol *et al.*, 2011). It is unequivocally the most important nematode threat to potato production globally (Baker *et al.*, 2012). In light of the importance of this pest, this research primarily focused on what is probably the most significant development in PCN management, the increasing importance of *G. pallida*.

6.1. PCN detection and population surveillance

The technical difficulties encountered in nematode detection, identification and the imposed constraints on population assessments were addressed in Chapter 2. Species discrimination and quantification are critical elements of population monitoring. An enhanced knowledge of population density and species composition of the PCN population is critical to the application of appropriate, effective PCN control methods. Traditional morphometric methods of cyst and juvenile identification are impractical for population analyses and cPCR methods require time-consuming post-reaction processing. Although qPCR has facilitated automated high-throughput screening (HTS), the detection process is still constrained at the cyst and juvenile extraction stage. Nematode enrichment from soil samples is a prerequisite for all assays.

HTS integrating molecular-based diagnostics has proved successful in the analysis of soil nematode populations (Porazinska *et al.*, 2009). Such analyses provide qualitative and quantitative population data and have much potential as a method for PPN detection and populations analysis (Porazinska *et al.*, 2012; Darby *et al.*, 2013). Furthermore, Sapkota and Nicolaisen (2015) demonstrated the successful isolation and amplification of nematode DNA for HTS nematode community analysis via direct DNA extraction from soil samples. This technique does not require a nematode enrichment step and therefore, has immense potential for nematode population and infestation density analyses. In certain cases, speciation is not sufficient and pathotyping may be necessary, particularly in the

event of a PCN species overcoming varietal resistance (2007/33/EC; Hockland *et al.*, 2012). Indeed, variations in hatch and virulence among pathotypes and ecotypes have implications for control, which further signifies the importance of accurate species and pathotype identification in PCN management (Turner *et al.*, 2009).

6.2. PCN incidence in Ireland

This project aimed to assess the status of PCN in Ireland, investigate how *G. pallida* out-competes *G. rostochiensis* in mixed-species populations and deduce whether this greater *G. pallida* competitiveness is related to its delayed hatch. The transition in PCN populations from *G. rostochiensis*- to *G. pallida*-dominant is well documented in the UK and the Netherlands (Minnis *et al.*, 2002; Taylor & Hockland, 2010; FVO, 2013). This trend is largely attributed to the effective control of *G. rostochiensis* relative to *G. pallida,* although data presented in Chapter 2 suggest that this effect may also occur in the absence of conventional control measures, as in Ireland. Indeed, the competition trials described in Chapter 3 indicated that the greater *G. pallida* competiveness in mixed-species populations is a critical factor for the increased incidence of this species. All competition experiments unanimously produced results signifying that *G. pallida* is an antagonist of *G. rostochiensis*, supporting the findings of Den Nijs (1992b) as well as those of Ryan *et al.* (2005) and Lettice (2014) from this laboratory. The success of *G. pallida* was not strictly limited to mixed-species competition assays; it was also reflected in single-species assays and also in the hatching bioassays. Essentially *G. pallida* was the more successful species regardless of situation.

Kaczmarek (2014) investigated PCN population dynamics with regards to initial population density and species composition in the field. Kaczmarek reported a reduction in *G. rostochiensis* multiplication in mixed-species populations when *G. pallida* was present at equal or greater P_i on the PCN susceptible cultivar 'Desirée' and *G. pallida*-partially resistant 'Vales Everest'. Conversely, when *G. rostochiensis* was present at a greater P_i, *G. pallida* multiplication was significantly reduced on 'Vales Everest'. It is difficult to draw

any substantial comparisons from these studies as they were field trials conducted under colder soil conditions and on different varieties. However, it is worthwhile noting that *G. pallida* multiplication was only reduced in the presence of *G. rostochiensis* on partially *G. pallida*-resistant 'Vales Everest', which was, in part, due to host resistance, as well as greater *G. rostochiensis* infestation densities.

6.3. Population dynamics

Numerous population models have been developed integrating root area as the main limiting factor contributing to competition (Elston *et al.*, 1991; Phillips *et al.*, 1991; Seinhorst, 1993). Few models, however, describe the effect of interspecific competition (van den Berg *et al.*, 2006; Maneva & Trifonova, 2015). The data presented in Chapter 3 would suggest that interspecific competition can have a detrimental impact on populations, possibly more so than intraspecific competition. This has important implications for PCN population modelling and the application of models in the establishment of appropriate PCN control programmes. As such, both inter- and intraspecific competition, in conjunction with species virulence on specific varieties, should be factored into PCN population and yield loss simulation models.

6.4. Factors influencing competition

Several plausible explanations for the greater competitiveness of *G. pallida* have been proposed. Intrinsic pest characteristics may significantly affect competition between species, particularly virulence (Phillips *et al.*, 2006), infectivity and persistence (Robinson *et al.*, 1987), differential development and hatching mechanisms (Stanton & Sartori, 1990; Ebrahimi *et al.*, 2014) and species-specific responses to host chemical cues (Devine & Jones, 2003). In Chapter 4, the differential hatching responses of the two species were investigated. *G pallida* had greater viability and achieved higher hatching rates, relative to *G. rostochiensis*. The greater hatch efficiency of *G. pallida* supports the findings of both Den Nijs (1992a) and Kaczmarek (2014) and it is proposed that the more efficient hatch of *G.*

pallida may allow it to colonise roots more effectively and thereby reduce available sites for *G. rostochiensis*.

G. pallida exhibits greater selectiveness and dependence on host chemical cues and consequently, displays a more conservative and specific hatching behaviour requiring a higher minimum HF threshold concentration to stimulate hatch (Byrne *et al.*, 2001). Indeed, the greater quantities of *G. pallida*-specific HF in PRL and the associated lower level of spontaneous hatch is well documented (Byrne *et al.*, 2001; Devine *et al.*, 2001; Devine & Jones, 2001). This research also suggests the greater chemical dependence of *G. pallida* compared with *G. rostochiensi*s. *G. pallida* responses to interspecific sex pheromones have also been reported (Riga *et al.*, 1997), while the hatching data from this research suggest interspecific hatching responses to *G. rostochiensis*-derived PRL.

The greater *G. pallida*-HF selectiveness is proposed as one of the main reasons for its delayed hatch and increased persistence in the field (Ryan *et al.*, 1999; Byrne *et al.*, 2001). *G. rostochiensis* emerges earlier and faster than *G. pallida* and therefore colonises the root system before *G. pallida* emergence. Despite this advantage, it achieves lower multiplication relative to *G. pallida* in mixed-species populations. Therefore, it was proposed that the earlier-*G. rostochiensis* infestation could potentially instigate root proliferation and elicit ISR, a hypothesis which was addressed in Chapter 5.

In Chapters 3 and 5, time-dependent competition assays assessed whether the later *G. pallida* hatch is its main competitive advantage and if PCN-induced changes in root architecture due to the challenge of earlier *G. rostochiensis*-hatch are implicated in the greater *G. pallida* multiplication. The effect of inoculation timing was evident in the mixed-species competition assays (Chapter 3) even though *G. pallida* consistently outcompeted *G. rostochiensis* despite differences in inoculation time. However, concomitant inoculation significantly reduced *G. pallida* multiplication.

Lettice (2014) similarly investigated the manipulation of hatching time and inoculation to determine whether early hatching *G. pallida* would exhibit the same degree of competitiveness as late hatching *G. pallida*. The author concluded that *G. pallida* multiplication was reduced when it colonised 'Golden Wonder' roots before *G. rostochiensis,* but the effect was not significant. However, *G. rostochiensis* multiplication was significantly reduced when juveniles were applied after *G. pallida*. Results from the research in this thesis reveal similar findings on 'Golden Wonder'; however, there was a significant difference in species multiplication when both were applied concomitantly. The disparity between the two studies may be due to the different inoculation techniques employed in Lettice's experiments, in which the initial PCN inocula comprised cysts and subsequent inocula were juveniles. Conversely all time-dependent trials in this research utilised juvenile inoculum to negate the effect of delayed hatch.

The split-root assays facilitated analysis of the effect of inoculation timing in the absence of interspecific competition. The results suggest that later-hatching juveniles would have a competitive advantage. Indeed, both species exhibited a trend toward greater multiplication when applied at later inoculation times, but *G. pallida* was the only species to exhibit a significant increase in multiplication.

6.5. ISR

It was proposed that the earlier *G. rostochiensis* challenge could elicit ISR, resulting in the upregulation of host defences in response to infestation. The split-root mixed-species trials revealed a reduction in *G. rostochiensis* multiplication when *G. pallida* was inoculated 7 d before and at 14 d after *G. rostochiensis* inoculation. As such, it appeared that *G. pallida* infection had a negative systemic effect on *G. rostochiensis* multiplication. In the single-species split-root assay there appeared to be a trend towards increased multiplication when juveniles were inoculated at 7 and 14 dpi. It is also possible that the later-infecting juveniles elicited ISR, thereby affecting the sedentary feeding nematodes in split-root pot 1. This would in turn affect female fecundity which may account for the lower

multiplication evident in the first inoculated treatments. Alternatively, the initial inoculation may have suppressed host defences; however, gene expression assays targeting marker genes of the defence signalling pathways, JA/ET and SA, would be necessary to substantiate these speculations.

Few ISR studies have been conducted on PCN; as a result, it is difficult to compare the findings from Chapter 5 with other published work. Furthermore, most PPN gene expression studies pertain to the local induced responses at the feeding site and few have investigated systemic responses in roots (Wondafrash *et al.*, 2013). Puthoff *et al.* (2003) found that *Heterodera schachtii* elicited JA-mediated systemic resistance, which was potentiated 3 d after infestation. Similar evidence was found in roots challenged with *Heterodera glycines* (Alkharouf *et al.*, 2006), in which ISR was potentiated up to 10 d after infestation (Ithal *et al.*, 2007).

Kyndt *et al.* (2012) investigated ISR elicitation following migratory and sedentary nematode attack in rice and reported that induced defences had declined after 7 d. As such, the effect of PPN-elicited ISR and subsequent dissipation or nematode suppression of host defences during syncytium formation is quite plausible. Further research into *Globodera*-specific elicitation of defence signalling pathways and ISR coupled with nematode-induced changes in root structural architecture are highly recommended. Similarly, PCN-manipulation of host hormones (Gheysen & Mitchum, 2011) and the differential effect of either species infection on root structure merits investigation.

6.6. Changes in root development

Nematodes are known to modulate host root development and cause excessive branching due to root infestation (Gheysen & Mitchum, 2011; Ravichandra, 2014). It was therefore postulated that the early-colonizing *G. rostochiensis* juveniles induced changes in root development and architecture, due to damage incurred during host infestation. PCN infestation may stimulate compensatory lateral root growth and consequently increase the

root surface area for the later-hatching *G. pallida*. In the split-root trials a distinct correlation between nematode infection and root area was observed. Surprisingly, the rhizotron and split rhizotron assays did not exhibit similar trends in root area. This disparity may be due to growth conditions; the mixed-species rhizotrons trials were grown in the glasshouse and the split-root rhizotrons were grown outdoors. Consequently, the latter trial may have been subject to abiotic and biotic stress in concert with PCN infection, which may account for the lower recorded root area values. Nevertheless, both trials had very similar patterns of PCN multiplication, although the glasshouse trial resulted in higher multiplication.

6.7. PCN multiplication on different varieties

Resistant varieties are a valuable component of IPM; however, the absence of major gene resistance to *G. pallida* is a major limitation in management programmes against this species, although partially resistant varieties confer a certain degree of resistance. As indicated in Chapter 2, the types of varieties grown in Ireland are generally determined by consumers, who generally dictate the growth of older, more traditional varieties. Consequently, few resistant or partially resistant varieties are grown in Ireland. Variety is an important consideration in nematode management, although maturity class should also be considered. Early varieties have shorter life cycles, faster maturation and are harvested earlier. In theory, they should be less susceptible to nematode attack, relative to maincrop varieties, as they are harvested before PCN completes its lifecycle, so that PCN populations should fail under early varieties. On the other hand, maincrop varieties are planted later and are grown for a longer duration, which facilitates nematode feeding damage, multiplication and potentially a second generation (Greco *et al.*, 1988; Kaczmarek *et al.*, 2014).

Both PCN species exhibit differential responses to hatching factors in PRL, which are qualitative and quantitatively variable according to variety, maturity class, host physiology and plant age (Chapter 4). Indeed, the effect of variety on PCN multiplication was highly

variable between experiments. The experiments in this project investigated PCN multiplication on four different varieties that reflected different maturity classes (Chapter 3). 'Golden Wonder' seed tubers were only available in the first year of the pot trials due to a poor harvest that hindered supply and were therefore omitted from subsequent years' trials. Lettice (2014) observed greater *G. pallida* multiplication on early varieties; namely, 'Home Guard' and 'British Queen' (second early). Conversely *G. rostochiensis* performed better on the later-maturing varieties, which is consistent with the hatching preferences of this species for later-maturing varieties (Byrne *et al.*, 2001). Similar species-specific varietal preferences were evident in this research (Figure 3.4).

As noted in Chapter 3, one of the main defects of the competition assays was the omission of plant attributes, such as yield and biomass analyses. It would be worth comparing PCN multiplication to yield loss in order to assess the relationship between multiplication and decline in yield and to identify levels of susceptibility and/or tolerance. Research of this nature would contribute significantly to PCN population modelling and loss prediction (Trudgill *et al.*, 2014). Furthermore, it would be beneficial to comparatively analyse root systems of different varieties with rhizotron experiments.

6.8. Conclusions

G. rostochiensis is the dominant species in Ireland; however, it poses less of a threat than *G. pallid*a as it can be managed effectively with resistant varieties and nematicides, although these strategies are not widely used in this country. It is evident that *G. pallida* could become a significant threat to potato production in Ireland (Chapter 2) and efforts to curtail further PCN infestation are required. *G. pallida* populations tend to be more genetically diverse than *G. rostochiensis*, with a considerable degree of variance in virulence among pathotypes (Plantard *et al.*, 2008; Hoolahan *et al.*, 2012). This research has demonstrated the greater *G. pallida* multiplication when in competition with *G. rostochiensis*. Furthermore, mixed-species infection exhibited a more deleterious effect on the host than single-species infection (Chapter 5). Greater *G. pallida* virulence and increased

aggressiveness in mixed-species populations could have devastating consequences for potato growers if adequate PCN management efforts are not implemented (Phillips *et al.*, 2006; Hockland *et al.*, 2012). Further research in the area of novel *G. pallida*-management is essential. An informed knowledge of species composition and infestation level is critical in order to implement efficient PCN control methods.

6.9. Main findings from this research

- qPCR proved to be the more efficient method for PCN detection and speciation.
- A significant increase in the incidence of *G. pallida* in Irish PCN populations was reported from 2002-2011.
- *G. pallida* multiplication is stimulated in the presence of *G. rostochiensis,* while *G. rostochiensis* multiplication is significantly inhibited by *G. pallida* in mixed-species populations.
- *G. pallida* multiplication was greatest when it was inoculated after *G. rostochiensis* and it was reduced in concomitant inoculation with *G. rostochiensis.*
- PCN species exhibited different levels of hatch in mixed- and single-species populations *in vitro*; *G. pallida*-sensitised PRL inhibited *G. rostochiensis* hatch, *G. rostochiensis*-sensitised PRL stimulated *G. pallida* hatch.
- A positive correlation between multiplication and root area was demonstrated.
- *G. pallida* infestations had a significant negative systemic effect on *G. rostochiensis* in split-root rhizotrons.

6.10. Future research recommendations

- Extrapolation of *in vitro* studies to the field should be conducted.
- Time- and density-dependent inter- and intraspecific competition assays to be carried out with higher infestation densities and different inoculation regimes.

- Studies with both cyst and juvenile inocula to be carried out for comparative analysis. Cysts should be retrievable to assess hatching efficiency and to determine reproductive success.
- The development of inter- and intraspecific competition coefficients to be carried out for applications in PCN population models.
- The rhizotron system should be further exploited to carry out *in situ* studies investigating PCN competition e.g. the time course of PCN multiplication in single- and mixed-species populations (using immature cyst colour to distinguish between species).
- Split-root and rhizotron assays to be conducted with different varieties and different inoculation times, coupled with analysis of phytohormone levels in locally infected and distal roots, and ISR gene expression analysis to determine PCN-induced temporal and spatial ISR elicitation.
- Further split-root rhizotron assays to assess PCN-induced root changes on different varieties and at different infestation densities.
- Split-root rhizotron studies to be carried out to assess relationships between PCN multiplication, gene expression, phytohormone levels and changes in root architecture.
- Further *in vitro* bioassays to be carried out to investigate intra- and interspecific communication and field trials using cysts exposed to homo- and heterospecific PRL as inoculum.

6.11. Bibliography

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Appendix I: Distribution of *G. pallida* (EPPO, 2014)

Algeria
Argentina
Austria
Belgium
Bolivia
Bulgaria
Canada
Canada
Chile
Colombia
Costa Rica
Croatia
Cyprus
Czech Republic
Denmark
Ecuador
Falkland Islands
Faroe Islands
Finland
France
Germany
Greece
Hungary
Iceland
India
Ireland
Italy
Luxembourg
Malta
Netherlands
New Zealand
Norway
Pakistan
Panama
Peru
Poland
Portugal
Romania
Slovenia
Spain
Sweden
Switzerland
Tunisia
Turkey
United Kingdom
United States of America
Venezuela

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Appendix II: Distribution of *G. rostochiensis* (EPPO, 2014)

Albania
Algeria
Armenia
Australia
Australia
Austria
Belarus
Belgium
Bolivia
Bosnia and Herzegovina
Bulgaria
Canada
Chile
Colombia
Croatia
Cyprus
Czech Republic
Denmark
Ecuador
Estonia
Faroe Islands
Finland
France
Germany
Greece
Hungary
Iceland
India
Indonesia
Iran
Ireland
Italy
Japan
Latvia
Lebanon
Libya
Liechtenstein
Lithuania
Luxembourg
Malta
Mexico
Netherlands
New Zealand
Norway
Oman
Pakistan
Panama
Peru
Philippines
Poland
Portugal
Romania
Russia
Serbia
Sierra Leone
Slovakia
Slovenia
South Africa
Spain
Sri Lanka
Sweden
Switzerland
Tajikistan
Tunisia
Turkey
Ukraine
United Kingdom
United States of America
Venezuela

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