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Characterisation of potato cyst nematode populations in Great Britain for sustainable crop management

A thesis submitted to Harper Adams University in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Abstract

Potato cyst nematodes (PCN), comprised of two related species (*Globodera rostochiensis* and *G. pallida*), are the most destructive pests of potato crops in Great Britain (GB) and other temperate regions. It is estimated that PCN are the second, after late blight, greatest cause of losses in potential yield and quality worth nearly £26 million per year.

Development of potato genotypes with the H_1 resistance gene proved to be highly effective in controlling *G. rostochiensis* but at the same time increased the risk of selection of *G. pallida*. The management of the latter is expensive and more challenging due to the pressure to replace chemical control products and the lack of potato genotypes fully resistant. In the most recent survey *G. pallida* was identified as being the dominant species and present in approximately 92% of the infested potato fields, which indicated that the species distribution of PCN has been changing.

To fully understand a possible infestation level, the assessment of PCN population densities should include a measurement of the viability of the eggs. The absence of a precise viability assessment might lead to inaccurate determination of field populations and inappropriate selection of management strategies. At the moment viability tests are not often commercially performed as they are still too expensive and time consuming to be widely accessible for the growers.

Although, the complexity of PCN virulence is not well defined by the methodology used in the pathotype scheme from 1977, which includes a limited selection of resistance genes and lacks an important genotype currently used in potato breeding programs, the ideology used to develop this scheme is still in use. This makes the search for markers that can be used to monitor populations even more important. Recently, certain populations of *G. pallida* were found to contain mixtures of genotypes, which are likely to represent three distinct geographical introductions from the Andes. These haplotypes were renamed as mitotypes. This novel method provides an efficient way to assess the complexity of field populations by investigating occurrence of partial sequence of cytochrome *b* gene descriptive of these introductions.

Understanding PCN population's characteristics is of paramount importance to the selection of appropriate management methods and to indicate the direction of the future research. This study aimed to characterise current PCN populations by conducting a PCN survey of ware potato growing land in GB. Collected survey

samples were further investigated to provide up-to-date information about the regional prevalence of PCN, the ratio between PCN species, the virulence and mitotype composition of selected populations and the viability determined using a test validated as suitable for field samples of *G. pallida* populations.

The results identified a decrease in the incidence of PCN (48%) compared with the previous survey and confirmed the continuous shift towards G*. pallida* as the predominant species. Of populations found to be PCN infested, 89% were *G. pallida*, 5% were *G. rostochiensis* and 6% contained both species. Viability testing by hatching in potato root diffusate assay did not take into account the viability of the unhatched, possibly dormant, eggs and should not be used as the sole indication of field population density. Assessment of *G. pallida* populations using techniques based on staining with Meldola's blue overestimated the number of viable eggs in heat-treated samples. The trehalose assay was proven to be a good alternative to the other methods. *Globodera pallida* field population's virulence assessment showed that potato genotypes with resistance derived from *S. vernei* controlled reproduction better than when derived from *S. tuberosum* spp. *andigena* CPC2802. Genotype with resistance derived from *S. multidissectum* PH1366 showed partial resistance to all of the field populations. This indicates that using these three genotypes in the field has potential to effectively reduce *G. pallida* infestations. Finally, next generation sequencing revealed that mitotype 2 was not present in any of the *G. pallida* field populations from England. All tested populations were found to be mitotype 1, mitotype 3 and a mixture of mitotypes 1 and 3.

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List of abbreviations, units and symbols

A	absorbance
ADAS	Agricultural Development and Advisory Service
AHDB	Agriculture and Horticulture Development Board
ANOVA	analysis of variance
APHA	Animal and Plant Health Agency
b.p.	base pair
c.	*circa*
CABI	Centre for Agriculture and Biosciences International
cyt-*b*	cytochrome b
DAP	days after planting
DEFRA	Department for Environment, Food and Rural Affairs
DNA	deoxyribonucleic acid
e.g.	*exempli gratia* (for example)
EF1-α	elongation factor 1-α gene
ELISA	enzyme-linked immunosorbent assay
EPPO	European and Mediterranean Plant Protection Organization
et al.	*et alia* (and others)
FAO	Food and Agriculture Organization of the United Nations
Fera	Fera Science Limited
FW	fresh weight
g	unit related to gravitational force
g	gram
GB	Great Britain
GLM	general linear model (statistics)
gpd-1	glycerol-3-phosphate dehydrogenase gene
ha	hectare
HAU	Harper Adams University
HFs	hatching factors
i.e.	*id est* (that is)
IEF	isoelectric focusing analysis
ITS	ribosomal internal transcribed spacer
J1-5	*Globodera pallida* life cycle stages
JHI	James Hutton Institute
JIC	John Innes Centre

kg	kilogram
l	litre
M	molar
MAbs	monoclonal antibodies
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
mRNA	messenger ribonucleic acid
Mt	metric tonne (a unit of weight equal to 1,000 kilograms)
mtDNA	mitochondrial DNA
n	number of observations
$NADPH^+$	nicotinamide adenine dinucleotide phosphate
nm	nanometer
OJEU	Official Journal of the European Union
P	probability value in statistics
Pa	pathotype nomenclature for *Globodera pallida*
PCN	potato cyst nematodes
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism
Pf	final nematode population density
Pf/Pi	multiplication ratio
Ph	power of hydrogen
Pi	initial nematode population density
PMB	Potato Marketing Board
pmol	picomoles
PRD	potato root diffusate
qPCR	quantitative real-time polymerase chain reaction
R	correlation coefficient
R^2	coefficient of determination
RAPD	random amplified polymorphic DNA
rDNA	ribosomal deoxyribonucleic acid
real-time PCR	real-time polymerase chain reaction

RNA	ribonucleic acid
Ro	pathotype nomenclature for *Globodera rostochiensis*
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
SASA	Science and Advice for Scottish Agriculture
SEM	standard error of the mean
SNP	single nucleotide polymorphism
sp.	species
spp.	multiple species
TRD	tomato root diffusate
U	enzyme unit
UK	United Kingdom
v/v	volume per volume
w/v	weight per volume

∆A	change in absorbance
ºC	degree Celsius
♀	female
♂	male
°S	latitude (degree south)
<	less than
≤	less than or equal to
>	more than
~	approximately equal
µg	microgram
µl	microliter
µm	micrometre
%	percent
£	pound sterling
$	United States of America dollar
®	registered trademark
Λ	wavelength (nm)

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Chapter 1

A review of literature relevant to potato cyst nematodes and their characterisation in Great Britain.

1.1. The potato crop

1.1.1. *Crop description*

The potato (*Solanum tuberosum* L.) is a starchy, tuber-bearing plant from an economically important family of flowering plants the Solanaceae, and shares the genus *Solanum* with other well-known species like tomato (*S*. *lycopersicum*) and eggplant (*S*. *melongena*). Potatoes are herbaceous perennials that are usually propagated using seed tubers which are the only parts of the plant suitable for consumption (Figure 1.1.). During the first phase of potato growth, sprouts emerge from the seed tuber, which grow upward to develop into shoots, and the production of roots from the base of emerging sprouts begins. Photosynthesis starts as the leaves and branch stems develop from nodes on aboveground sprouts, and roots and stolons are formed at belowground nodes. The tips of these stolons swell and develop into new tubers which are highly adapted parts of the stem organised for food storage and vegetative reproduction. The end of this phase is often associated with flowering after which, potato plants produce small green fruits that look like green cherry tomatoes containing true (sexual) botanical seed (Almekinders and Struik, 1996). Following tuber initiation, changes in the resource distribution occur when the plant begins to direct the majority of its resources into newly formed tubers which is often described as tuber bulking. After flowering, fruiting and tuber

formation, the maturation phase takes place when the aerial parts senesce and die back, the tubers skin harden, and their sugars convert to starches (Cutter, 1992).

Figure 1.1. Potato plant - *Solanum tuberosum* L. (Source: https://dranthea. wordpress.com/tag/chaconine/).

Based on the developing stem growth habit, potato varieties are classified as either determinate or indeterminate. The determinate growth pattern is presented in early-season varieties in which inflorescence forms on the tip of the main stem which naturally stop stem growth hence their short growth cycle. After flowering of the main stem, production of the new branches is limited and always occur from nodes on the main stem. The indeterminate growth pattern is characteristic of late-season varieties with a long life cycle. In these genotypes the inflorescence of the main stem

does not terminate vegetative growth. It continues by development of the second-order stems from axillary buds developed on the main stem below its inflorescences. These branches also produce leaves, inflorescences and axillary buds which may produce third-order stems which again can complete the development cycle and increase the degree of stem branching, extending the flowering period (Almekinders and Struik, 1996). Tuber initiation and bulking take place earlier in determinate when compared with indeterminate genotypes which have higher yield potential if a longer growing season is permitted. Determinate varieties produce short plants while indeterminate plants tend to grow tall and produce greater total leaf area, inflorescences and true seeds (Struik, 2007).

The potato tuber follows only rice and wheat as a food crop for human consumption worldwide (Gibson and Kurilich, 2013). Camire *et al*. (2009) summarised that potato is a nutrient dense food that is high in carbohydrates, 20.13g of 100g (fresh weight) (FW) of potato boiled in skin, and low in fat, 0.1g of 100g (FW) of potato boiled in skin. It can supply significant nutrients with only 87 kilocalories in 100g (FW) of potato boiled in skin. Additionally potatoes are a good source of vitamin C, vitamin B and potassium while the skin is rich in fibre (1.8g of 100g (FW) of potato boiled in skin). Potatoes have a low protein content (1.87g of 100g (FW) of potato boiled in skin) which is balanced by their excellent biological value (a measure of the proportion of absorbed protein from a food which becomes incorporated into the proteins of the organism's body) of 90–100. Finally, many compounds in potatoes contribute to antioxidant activity (Camire *et al*., 2009).

1.1.2. *Crop history*

The history of potato crop in Europe begins in the last quarter of the sixteenth century when the *S. tuberosum*, most likely, subspecies *andigena* was introduced

into Europe from the Andes in South America (Hawkes, 1990) (Figure 1.2.). Although there are statements in the literature which suggest an earlier introduction, the earliest reliable date claims that first potatoes were brought to Spain in 1570 (Salaman and Burton, 1949). A separate introduction into England was recorded between 1588 and 1593. It was more clearly defined by Hawkes (1990) who discussed the evidence that the actual date of English introduction was 1590. Evidence obtained from post conquest chronicles and archaeological remains indicate that the potato was established as a domesticated plant and cultivated even before the discovery of South America. Despite this, due to the adaptation to short-days, Andean subspecies at first were grown in Europe as a botanical curiosity. It was around the middle of the eighteenth century when this adaptation (to short day length) was bred out and potatoes started to play a role as a field crop (Hawkes, 1992).

Figure 1.2. Worldwide spread of potato species (Source: Turner and Evans, 1998).

1.1.3. *Production*

The Food and Agriculture Organization of the United Nations (FAO) estimated that just over two thirds of the global potato production (69.4%) in 2005 was consumed by people as food (FAO, 2008) while the rest was non-food used for starch production and as farm animal feed. This means that the annual diet of an average global citizen in this year included about 33.7kg of potatoes (FAO, 2008). World potato consumption in 2011 increased slightly and was 34.35 kg/person/year (FAO, 2018). In the same year, Great Britain's (GB) average annual potato intake was considerably higher at 91.7kg/person (AHDB, 2012).

The Food and Agriculture Organization of the United Nations further reports that the world production of potatoes in 2016 was about 377 million metric tonnes (Mt) which makes potato the 10th most important crop by quantity, after cereals (total), sugar cane, coarse grain (total), vegetables primary, maize, fruit primary, roots and tubers (total), wheat and rice (paddy) (FAO, 2018). In the same year, the United Kingdom (UK) potato crop was the 6th most produced crop by quantity (*c*. 5.4 million tonnes) after cereals (total), wheat, coarse grain (total), barley and sugar beet and 2nd, after wheat, by values (*c*. $ 846 million) (FAO, 2018).

Potato varieties are categorised based on the number of days from planting of seed tubers to maturity of the next generation. In GB they are classified into three main groups: earlies, second earlies and maincrops (subdivided into early maincrop and late maincrop). The majority of potatoes are grown for ware production (85%) which is mostly comprised of pre-pack and processing markets followed by fresh chipping, fresh bags and other ware markets. The remaining sector specialises in seeds production (15%). The national crop in 2016 was grown on 116,200 ha which were managed by 1,925 growers and yielded at 5.22 million tonnes (AHDB, 2017).

Producing a high yielding potato crop is partly achieved by providing sufficient macro- and micro-nutrients and irrigation for optimum plant growth. Potato plants are at risk from a variety of different pests and pathogens at each stage of growth. A range of plant parasitic nematodes are recognized to be a threat to potatoes in the UK including needle nematodes (*Longidorus* species), root-lesion nematodes (*Pratylenchus* species), potato tuber rot nematode (*Ditylenchus destructor*), stubby-root nematodes (*Trichodorus* and *Paratrichodorus* species) and cyst nematodes (*Globodera* species) (AHDB, 2015a) from which the latter are considered to be the most important pests of the crop (AHDB, 2015b).

1.2. Potato cyst nematodes

1.2.1. *Taxonomy*

Taxonomic classification of potato cyst nematodes (based on Turner and Subbotin, 2013) is shown below:

Kingdom: Animalia

Phylum: Nematoda

Class: Chromadorea

Subclass: Chromadoria

Order: Rhabditida

Suborder: Tylenchina

Infraorder: Tylenchomorpha

Superfamily: Tylenchoidea

Family: Hoplolaimidae

Subfamily: Heteroderinae

Genus: Globodera

Species: Globodera pallida and *G. rostochiensis*

Nematodes, with their simple body structure (Brzeski, 1993), are found in almost all habitats and some of them are parasites of plants and animals. Among the plant parasitic nematodes the family Heteroderidae, which is represented by the genus *Globodera* (Figure 1.3.), is especially important due to the high level of damage caused to crops. There are two species of PCN: *Globodera pallida* and *G. rostochiensis*. Cyst nematodes specific to potato, before being recognised as individual species and seperated by Wollenweber in 1923, were thought to be a subspecies of *Heterodera schachtii* (sugar beet cyst nematode). A population from a field in the Rostock region of Germany was isolated and, based on morphological differences, described as *Heterodera rostochiensis* Woll. Although differences between the populations led to the realisation that two species of PCN were involved (Jones *et al*., 1970) both currently known species of PCN were classified under one name for a long time. It was only in 1973 when Stone described the pathotypes of *H*. *rostochiensis* Woll. 1923 with white or cream females as a new species named *Heterodera pallida* (Stone, 1972).

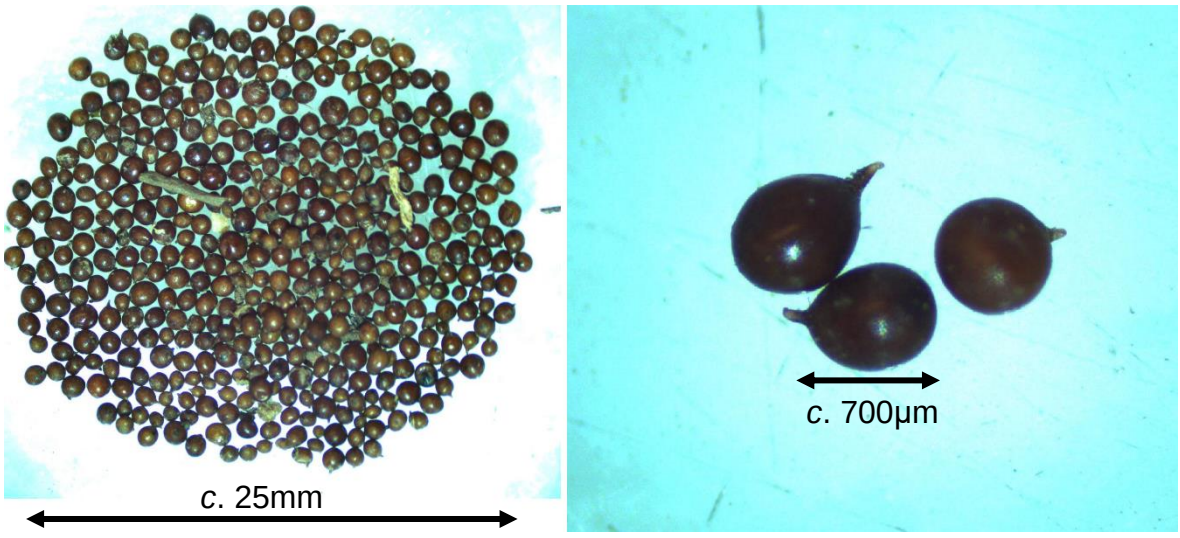


Figure 1.3. *Globodera* spp. cysts extracted from field soil sample collected as a part of PCN survey (Source: Author's own).

Some authors concluded that the subgeneric name *Globodera* established by Skarbilovich (1959) was then elevated to generic rank, to which round cyst

nematode species including PCN were assigned, by Behrens (1975). Loof and Bakker (1992) disagreed with this statement and present arguments which demonstrate that the combination of the specific name *rostochiensis* Woll. with the generic name *Globodera* is to be attributed to Skarbilovich (1959) while the authorship of the combination *G. pallida* should be given to Stone (1972).

1.2.2. *Morphology*

One of the most recognizable differences between sibling species of PCN is the external colour of the females which can be observed shortly after they rupture the root cortex. Stone (1972) observed that maturing females of *G. pallida* become cream or remain white, while in contrast *G. rostochiensis* females become golden yellow. *Globodera pallida* juveniles, adults and cysts are very similar to *G. rostochiensis* and often morphometric comparison between these two species does not provide strong differentiation tools but several important morphological characteristics for such a purpose are listed below and in Table 1.1.

Cyst:

- mean distance from anus to vulval basin is noticeably **shorter** in *G. pallida*;
- **fewer** ridges between anus and vulval basin in *G. pallida*;
- mean Granek's ratio, a distance between anus and fenestra, is **shorter** in *G. pallida* (Turner and Subbotin, 2013);

Second-stage larvae:

- average stylet, body and tail is **longer** in *G. pallida*;
- stylet basal knobs with **forward projection** on anterior face in *G. pallida* (rounded with slight backward slope in *G. rostochiensis*);
- tail terminus **more pointed** in *G. pallida* (rounded in *G. rostochiensis*);

- contours of oral disc and lips is **rectangular** in *G. pallida* (ovate in *G. rostochiensis*) (Turner and Subbotin, 2013);

Male:

- average distance between stylet knobs and junction of the dorsal gland duct is **shorter** in *G. pallida* (Stone, 1972).

Table 1.1. Morphological characteristics for differentiation between *G. pallida* and *G. rostochiensis* (measurements in µm) (Source: Turner and Subbotin, 2013 - modified; Willmott *et al*., 1973).

	Cyst			Second-stage juvenile			Male
	Distance from anus to vulval basin	Number of ridges between anus to vulval basin	Granek's ratio	Body length	Stylet length	Tail length	Distance from stylet knobs to dorsal gland duct junction
Globodera pallida	35-55	7-17	1.2-3.6	380-533	23-25	40-57	2-7
Globodera rostochiensis	50-77	16-22	2.3-7.0	366-502	19-23	37-57	4-8

1.2.3. *Intraspecific diversity*

Comparison of morphological differences between *G. pallida* and *G. rostochiensis* enabled separation of the species but also highlighted the diversity within both. Further attempts to explain the differences between populations of the same species result in more complex separation within each species into pathotypes, based on the virulence, and more recently mitotypes, based on the differences in a region of mitochondrial DNA between populations of different geographical origin.

Pathotype is a term used to describe virulence, a measure of pathogenicity for populations within the same species. Pathotypes reflect the ability of individual PCN populations to multiply on various potato genotypes with a known resistance gene (or group of genes). Before the recognition of the second PCN species (Stone,

1972) the name ‘pathotype’ was used to describe the differences in the biology between *H. rostochiensis* populations which could actually have been different *Globodera* species. British (three pathotypes designated by the letters A, B and E) and Dutch (four pathotypes designated by the letters A - D) National Schemes for pathotypes of *H. rostochiensis* were used widely in Europe and elsewhere (Kort *et al*., 1977) before Stone introduced a second species of PCN in 1973. In 1966 the dissimilarities in the life cycle between pathotypes of *H. rostochiensis* (later known as *Globodera* species) were observed by Guile (1966). Author reported that maturing females of pathotype A became golden yellow (later *G. rostochiensis*), those of pathotype B became cream and those of pathotype C remained white until they died when they changed colour to brown (later *G. pallida*).

The requirement for pathotype schemes arose because classification of PCN into two separate species did not account for all the variabilities between PCN populations. Trudgill and Parrott (1972) presented the evidence of the existence of two species and at the same time suggested a scheme of pathotype nomenclature for both, although based on the similarity between the Dutch and British pathotypes, they used different test plants from those used in Netherlands. Shortly after, in 1977, two proposals for a new system of classification were published simultaneously. A scheme based on PCN populations originating from South America categorizing PCN into races and sub-races (Table 1.2.) was published by Canto-Saenz and de Scurrah (1977) but was generally not used in Europe. Kort *et al*. (1977) criticised it for unnecessary complexity. Concurrently the scheme published by Kort *et al*. (1977) was based on European populations assigned into pathotypes (Table 1.2.): five of *G. rostochiensis* prefixed with Ro (1-5) and three of *G. pallida* prefixed with Pa (1-3). Trudgill (1985) produced a critical review of this scheme highlighting that many of their pathotypes are not naturally present and were created only as a result

of the design of the investigative procedure and that multiplication rates vary greatly due to environmental factors making them too unstable to use as a measure of nematode virulence. He also concluded that most PCN populations in Europe are mixtures of pathotypes and, when assessed for virulence genes, are heterogeneous, which emphasised the need for a system of pathotyping with greater accuracy. The flaw of the work by Kort *et al*. (1977) is also discussed by Stone (1985) who pointed that only three (out of seven) of the potato differential genotypes possessed known resistance genes, which does not fit with the proper definition of pathotypes. Notably, Stone *et al*. (1986) reported the difficulty in distinguishing between *G. pallida* pathotypes Pa2 and Pa3. They concluded that this separation is impractical and suggested that both pathotypes should be combined under one name - Pa2/3. Similarly Phillips and Trudgill (1998a) showed that separation of *G. pallida* populations into Pa2 and Pa3 poses problems due to a continuous range of variation in virulence between the two sources of resistance used. Regardless of these critical appraisals, the system for pathotype identification and classification proposed by Kort *et al*. (1977), is generally accepted and it is still in use (CABI and EPPO, 2018). Most recently, the European and Mediterranean Plant Protection Organization (EPPO) protocol for testing of potato varieties to assess resistance to *G. rostochiensis* and *G. pallida* (EPPO, 2006) was suggested as a method for testing new or unusual populations. The virulence of populations overcoming the resistance in varieties currently used by potato industry should be tested by assessment of their multiplication ability on a set of potato genotypes (EPPO, 2017). A relative susceptibility, percentage of newly formed cysts or eggs in comparison to susceptible genotype, was simplified and converted into score-based scale. The highest level of resistance is described by score 9 and the lowest by score 1 (EPPO, 2006).

Table 1.2. Pathotype groups of potato cyst nematodes: *G. rostochiensis* and *G. pallida* (Source: Turner and Subbotin, 2013).

	Species	*G. rostochiensis*					*G. pallida*						
	Globodera species virulence groups [a]	*Ro1*		*Ro3*		*Ro5*	*Pa1*				*Pa2/3*		
	European pathotypes [b]	*Ro1*	*Ro4*	*Ro2*	*Ro3*	*Ro5*	*Pa1*				*Pa2*	*Pa3*	
	South American pathotypes [c]	*R1A*	*R1B*	*R2A*	*R3A*	−	*P1A*	*P1B*	*P2A*	*P3A*	*P4A*	*P5A*	*P6A*
Species and accession	Ploidy, resistance gene												
Solanum tuberosum ssp. *tuberosum*	4x, minor	+	+	+	+	+	+	+	+	+	+	+	+
S. tuberosum spp. *andigena* CPC 1673	4x, H1 on chromosom 5	−	−	+	+	+	+	“	“	“	+	+	“
S. kurtzianum KTT 60.21.19	2x, K1 K2 A and B	−	(+)	−	(+)	(+)	+	+	+	+	+	+	+
S. vernei GLKS 58.1642.4	2x, quantitative	−	+	−	−	+	+	+	+	−	+	+	+
S. vernei Vtn 62.33.3	2x, quantitative		−	−	−	+	−	+	−	−	−	+	+
ex. *S. multidissectum* hybrid P55/7	2x, 1 + polygenes H2	+	+	+	+	+	−	−/+	−	+	+	+	+
S. tuberosum ssp. *andigena*	H3 + polygenes	+	“	“	“	“	(−)	“	“	“	(−)	(−)	“
CIP 280090.10	Quantitative	“	“	“	“	“	“	“	“	“	−	−	+
S. vernei hybrid 69.1377/94	2x, polygenes	−	−	−	−	−	−	“	“	“	−	−	“
S. vernei hybrid 63.346/19	2x, polygenes	−	−	−	−	−	+	“	“	“	+	+	“
S. spegazzinii	2x, Fa = H1	−	−	+	+	+	“	“	“	“	“	“	“
S. spegazzinii	2x, Fb +2 minor	+	−	+	−	−	“	“	“	“	“	“	“
	Glo1 on chromosom 7	(−)	+	+	+	+	“	“	“	“	“	“	“

[a]Trudgill (1985); [b]Kort *et al*. (1977);[c]Canto-Saenz and de Scurrah (1977).

Note : + = compatible interaction: nematode multiplication, potato susceptible; − = incompatible interaction: nematode no multiplication, potato resistant; ( ) = partial or uncertain interaction; “ = no information.

Nevertheless, it is well known that the classification schemes described do not accurately reflect the complexity of virulence found among field populations of PCN and continuous attempts are being made to find markers which readily distinguish and group populations. Recently various studies using biochemical and molecular methods investigated the genetic diversity between *G. pallida* populations in relation to the possible number of introductions into Europe. A distinct geographical origin might be reflected in the population's different virulence characteristics (Phillips and Trudgill, 1998a) or reveal the differences which will allow the separation of populations by other factors. In one approach Peruvian PCN populations were genotyped with partial sequence of the mitochondrial gene, cytochrome *b* (cyt-*b*), as a molecular marker and showed five biogeographically distinct clades (groups of populations that have a common ancestor) (Picard *et al*., 2007). Subsequently, European PCN populations were tested using the same molecular marker and, by comparison with previously described Peruvian clades, resulted in identification of their origin with high accuracy (Plantard *et al*., 2008). The phylogenetic analysis of DNA sequences comprising 872 base pair (bp) of cyt-*b* gene from *G. pallida* populations were presented by Hockland *et al*. (2012) and later reviewed by Eves-van den Akker *et al*. (2015). The authors observed that three discrete sub-clades contained the European populations with phenotypes of pathotype 1, 2 or 3 and also contained Peruvian populations assigned by Plantard *et al*. (2008). These three Peruvian cyt-*b* gene haplotype groups were combined with Western European populations in clade I. Based on these findings of *G. pallida* mitotypes, it was suggested that these three 'mitotypes' represent a promising opportunity to study the diversity of *G. pallida* (Eves-van den Akker *et al*., 2015).

1.2.4. *Origin and introduction to Europe*

It is generally accepted that *Globodera* species are native to South America where they evolved into individual species (Figure 1. 4.), through their interaction with their Solanaceous hosts, and by adapting to different climatic regions, and day lengths at different latitudes (Evans *et al*., 1975). The same authors further reported the geographic separation between species, with *G. pallida* occurring to the north of Lake Titicaca (15.6°S) in Peru, Ecuador and Colombia, while its sibling species, *G. rostochiensis*, is found to the south of this latitude in Peru and Bolivia.

The introduction of PCN into Europe is thought to have originated from soil adhering to seed tubers which, after the catastrophic outbreak of potato blight (*Phytophthora infestans*) in 1845 and 1846 in Ireland, were imported from South America for breeding resistance against this pathogen (Evans *et al*., 1975). The first record of cyst-forming nematodes in Europe was by Kühn (1881) who observed PCN on potatoes growing in Germany but attributed the damage to *H. schachtii*. Inagaki and Kegasawa (1973) detected viable *G. rostochiensis* cysts in Peruvian guano (the excrement of seabirds and bats) used as fertiliser in Japan and discussed this as a possible route for PCN distribution into Europe. Franco *et al*. (1998) agreed with this theory, although they argued that it was guano shipped to England and Germany from the 1840s onwards, in PCN-contaminated potato bags rather than contamination of guano, after birds fed on Peruvian PCN infested lands. Further distribution of PCN could have been associated with infested soil transported by military vehicles, like in the USA where the introduction of PCN is attributed to contaminated military equipment returning from Europe after the First World War (Brodie and Mai, 1989). It could also, especially in Europe, be the result of national and international trade of potato tubers for consumption or as the seeds of the improved varieties (Franco *et al*., 1998).

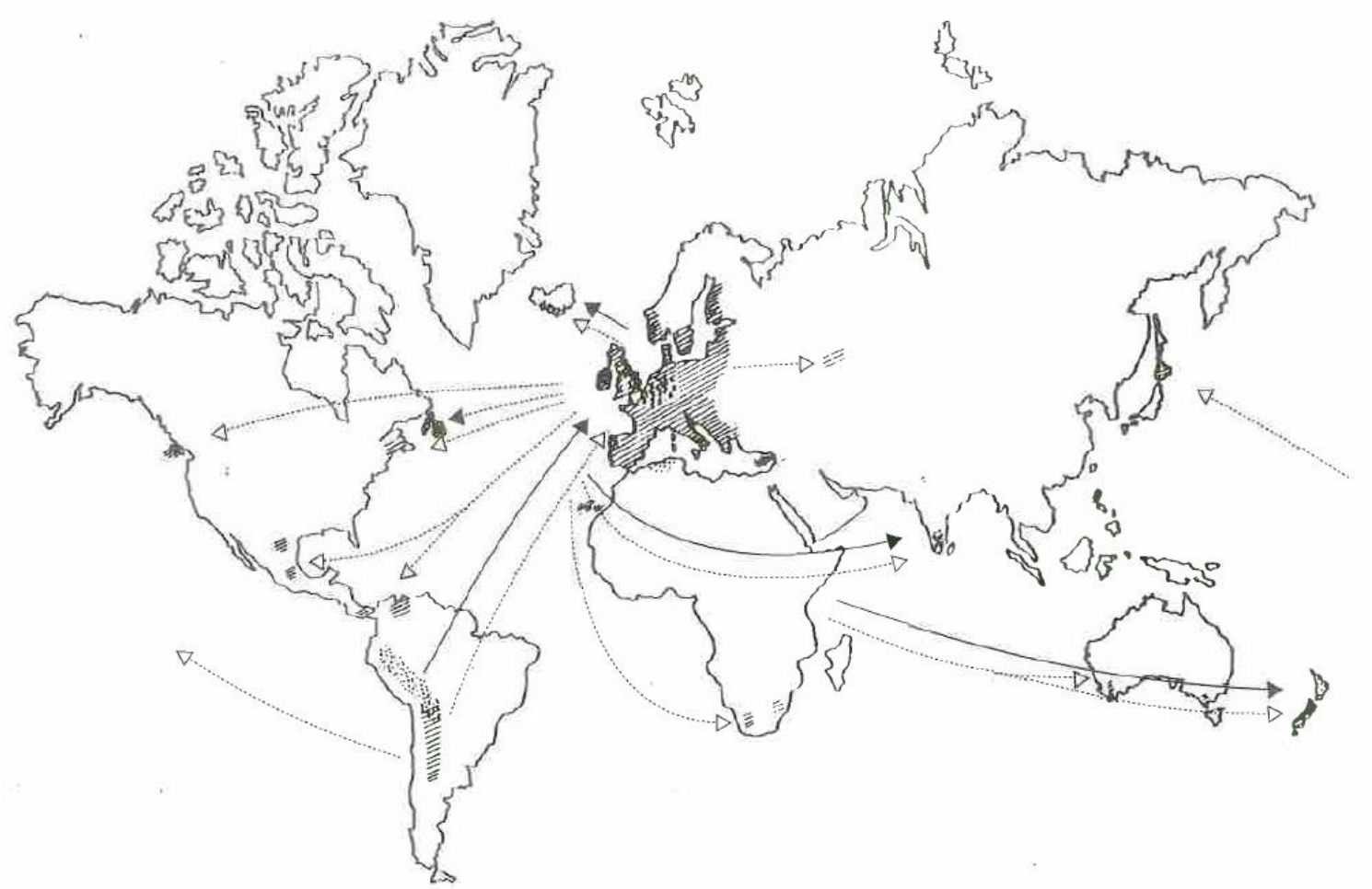


Figure 1.4. Worldwide spread of PCN species from South America (Andes): solid line-*G. rostochiensis*, dotted line-*G. pallida* (Source: Turner and Evans, 1998).

1.2.5. *Occurrence and distribution of potato cyst nematodes in Great Britain*

The symptom of PCN infestation, a poorly growing patch of the crop grown in the field used for storage of potatoes the previous year, was noticed for the first time in the East Riding of Yorkshire in 1904. Frequent potato cropping in this field resulted in complete infestation by 1917 and spread, over a period of 21 years, to three other fields on the same farm (Strachan and Taylor, 1926). Authors described the pathogen causing damage to the crop as a potato eelworm due to symptoms similarity to those caused by beet eelworm. Spears (1968) confirmed that the golden nematode in England and Wales was confined to Yorkshire in 1917 but he also further suggested that it was most likely present even 10 to 15 years earlier. The first information about cyst–forming nematodes on potatoes in Scotland was by Massee (1913) who described crop damage due to the infestation of *H. schachtii* based on the similarity between symptoms observed on sugar beet. Several years later, Warburton (1919) reported a few additional cases of *H. schachtii* affecting potatoes in the UK. Eelworm in potatoes were next reported in Lincolnshire in 1924 where severe losses resulted from the failure of the potato crop (Morgan, 1925). Soon after, in 1927, *H. schachtii* was identified in many parts of Lancashire and Cheshire where infestation also caused the potato crop to fail (Smith and Prentice, 1929). When *H. schachtii* was reported in these early publications it can be assumed that the infestations were caused by *Globodera* species because potato plants are not considered to be a host for *H. schachtii*. The rapid spread and extent of the problem was highlighted in a survey by Winfield in 1965 who sampled one field randomly from each of 100 selected farms in Lincolnshire and showed that 84% of the soil samples contained cysts (Winfield, 1965).

The occurrence and distribution of both *Globodera* species in the UK were unwittingly recorded even before the separation into two species. In these studies

differences between populations were attributed to variation in virulence often described as ‘pathotypes’. Jones and Pawelska (1963) tested the ability of 47 populations to multiply on resistant potato genotypes, derived from *S. tuberosum* ssp. *andigena*, *S. multidissectum*, *S. vernei*, S*. sanctae-rosae*, *S. famatinae* and *S.* × *juzepczukii*, and on *S. nigrum*, *S. sarachoides* and *Nicotiana* spp which showed the differences in virulence and also geographic separation. Populations which were unable to produce cysts on resistant varieties (later *G. rostochiensis*) were mostly found in East Anglia, south-east England and Northern Ireland and those which were able to complete life cycles (later *G. pallida*) were common in Yorkshire, the East Midlands and the Channel Islands. Other regions could not be simply classified as they were represented by a mixture of the populations. The distribution of the pathotypes A (later *G. rostochiensis*), B and C (later *G. pallida*) in the East Midlands was studied in pot tests and field trials (Guile, 1967). The author reported that 82% of samples tested had only white and creamy-white females (pathotypes B and C), 7% had only golden females (pathotypes A) and 11% were of mix pathotypes. In another pathotype survey in south-west Lancashire, 37 populations were tested from which 70% were able to overcome the resistance (later *G. pallida*) and 30% were reported as pathotype A (later *G. rostochiensis*) (Dixon *et al*., 1968). Brown (1970) studied populations in England and Wales also by testing their ability to produce cysts on resistant potato varieties. In his survey of Bedfordshire, Essex, Kent and regions of East Anglia with peaty soils, he found them to be infested with non-resistance breaking populations (later *G. rostochiensis*), while East Midlands and Yorkshire had populations which were able to produce cysts (later *G. pallida*) while the rest of the English and Welsh lands tested had populations of mixed ability to overcome the resistance. Potato cyst nematodes were found in most of the UK

potato growing area by the early 1970s, with *G. rostochiensis* dominating East Anglia and *G. pallida* being predominant in Northern England (Trudgill *et al*., 2003).

Publications on the occurrence and distribution following the recognition of two species of PCN in 1973 distinguish *G. pallida* (sometimes referred to as white PCN) and *G. rostochiensis* (sometimes referred to as yellow/gold PCN). In 1978 *G. rostochiensis* was reported to be predominant in East Anglia, the South East, the West Midlands and Scotland (Jones and Kempton, 1978). Stone *et al*. (1986) tested 144 fields from England, Wales and Scotland and showed that *G. rostochiensis*, although detected in all tested regions, was more common in south-western counties while *G. pallida* was the predominant species in the north and east. However the authors themselves concluded that the results do not correctly describe the distribution as the selection of the fields in this survey was biased towards *G. pallida*. Between 1982 and 1986 the Agricultural Development and Advisory Service (ADAS) tested sets of samples submitted for advisory purposes from Cambridge, Kirton, Leeds and Shardlow Laboratories and reported that 62% of the samples contained PCN (Hancock, 1988). The counties with historically the worst PCN infestation problems (Lincolnshire, Cambridgeshire, Norfolk and North Yorkshire) (Hancock, 1988) also represented the regions of the country with the most intensive ware potato cropping. Whereas Devon, Cornwall, South Wales, Suffolk and Essex were mainly associated with early potato production and faced PCN infestation as a new problem.

Taxonomic separation into *G. pallida* and *G. rostochiensis* allowed researchers to use consistent nomenclature and to focus more clearly on pathotypes by investigating the differences between populations within each of these two species. In the UK just a few of the pathotypes proposed by Kort *et al*. (1977) are known: Ro1 for *G. rostochiensis*, which corresponds to pathotypes A,

and Pa1, Pa2 and Pa3 for *G. pallida* which correspond to pathotypes B and elements of pathotype E in the former British scheme (Stone *et al*., 1986). *Globodera pallida* pathotype Pa2 was found to be predominant in the UK after seven populations from the major potato growing areas (the East Midlands, north-west England and north-east England) were tested (Stone *et al*., 1979). Examination of seven *G. rostochiensis* populations from England, Northern Ireland and Wales showed that they were all pathotype Ro1 (Stone and Valenzuela, 1979). The authors concluded that other existing pathotypes of *G. rostochiensis* are apparently absent or rare in the UK.

A survey by the Potato Marketing Board (PMB) in 1992 estimated that 42% of potato growing land in England, Wales and Scotland was infested with PCN with the highest proportion of infested land in the eastern counties. Analysis of the soil samples, from the counties most affected (Bedfordshire, Cambridgeshire, Norfolk and Northamptonshire), showed that 78% of growers submitted samples with viable cysts (Hancock, 1996). Samples sent to ADAS between 1993 and 1995, and tested for statutory and advisory reasons, showed that viable PCN were present in approximately 36% of sites sampled, and of the populations found, 54% were predominantly *G. pallida*, 5% were predominantly *G. rostochiensis* and 41% contained both species. It is however, likely that soil samples submitted for statutory reason would be from land believed to be PCN free. Testing samples sent to ADAS from land cropped with potatoes for advisory purpose only in 1994 and 1995 showed a higher PCN infestation level of 67% which confirmed the above assumption (Hancock, 1996). Potato cyst nematodes were found in 88% of samples when a total of 508 fields from Cambridgeshire, Hereford and Worcester (now Herefordshire and Worcestershire), Lincolnshire, Norfolk and North Yorkshire, which included potatoes as part of the rotation, were tested by ADAS in 1997 and 1998. Due to the

low viability of PCN within the samples, only 55% of fields where PCN were found could be further tested to a species level.

Globodera pallida was found to be the predominant species in Cambridgeshire, Lincolnshire, Norfolk and North Yorkshire where over 90% of samples with a positive result from the speciation test was detected as pure *G. pallida* populations. Hereford and Worcester, defined by the author as one county, was the only county where pure *G. rostochiensis* was accounted for 56% of samples with a positive result for species detection. Mixed populations, across all counties, were found in 10% of all positive species tests (Parker, 1998). These results confirmed earlier findings by Stone *et al*. (1986) which suggested different geographical distributions of the sibling species. Parker (1998) concluded, after analysis of cropping history in tested counties, that intensive use of varieties with resistance to *G. rostochiensis*, in some cases, was a major factor contributing to the high occurrence of *G. pallida*. A significant relationship between the species of PCN present and the use of resistant and non-resistant varieties was also reported by Minnis *et al*. (2002). The survey, by the same authors (Figure 1.5.), of ware potato growing land sampled in 1998-1999 in England and Wales showed that PCN were present in 64% of sites sampled and that the populations were distributed in the proportions 67% *G. pallida*, 8% *G. rostochiensis* and 25% both species (Minnis *et al*., 2002).

Figure 1.5. Sites where PCN were found in the most recent PCN survey conducted in 1998-1999 (Source: Minnis *et al*., 2002).

Haydock (2003) quoted a personal communication which said that in Scotland soil testing by the Scottish Agricultural College in 1996-1997 found that approximately 23% of potato ware land was infested, with these populations consisting of 24% *G. pallida*, 53% *G. rostochiensis* and 23% mixed species. Samples for export crops (42%) and from seed potato land (58%) from England were tested in 2008-2009 by the Nematology team at the Food and Environment Research Agency (FERA). Notably, only 1.41% of the samples were found to be infested with PCN, with 63% being *G. pallida*, 29% *G. rostochiensis* and 8% containing both species. Interestingly of all PCN detected samples, 91.5% originated in ware land (Taylor and Hockland, 2010).

Recently, high-throughput species identification using next-generation sequencing technology was used to describe the distribution of *G. pallida* mitotypes across Scotland. *Globodera pallida* was detected in ~76% of soil samples from potato seed and ware land collected by Science and Advice for Scottish Agriculture (SASA) as a part of annual surveys between 2011 and 2014. Positive samples were further tested and categorised as: a single mitotype (~79%), a mix of two mitotypes (~18%) and a mixture of all three mitotypes (~2.3%) (Eves-van den Akker *et al*., 2015).

In his thesis Minnis (2000) discussed soil sampling strategies highlighting that a protocol and equipment used in the survey for soil collection can have a strong impact on the results. Turner (1993) investigated the efficiency of several soil sampling methods and concluded that in agricultural land, regardless of the technique used, there was always some level of inaccuracy. The most recent directive 2007/33/EC on the control of PCN by the Official Journal of the European Union (OJEU) took effect from 1 July 2010, and standardised soil sampling for PCN detection (OJEU, 2007).

1.2.6. *Biology*

Potato cyst nematodes are unsegmented roundworms belonging to the genus *Globodera*. They are soil living obligate plant parasites found primarily in temperate climates. Their sedentary endoparasitic lifestyle leads to extensive plant damage due to prolonged activity. Their success can be attributed to their strong host-specific relationship (see section 1.2.6.2.), survival strategies and high reproduction ratio. The host range for these nematodes is limited to members of the Solanaceae family (Willmott *et al*., 1973).

1.2.6.1. *Life cycle*

The life cycle of PCN, consists of an egg stage, three juvenile stages (J2-J4) and the adult male or female (Figure 1.6.) and requires a period of 6-10 weeks to be completed which may vary depending on environmental conditions *e.g*. soil temperature (Turner and Subbotin, 2013). The life cycle can be divided into two phases: active and non-active (Turner and Evans, 1998). Transition from a non-active into an active phase marks the beginning of the life cycle and is initiated by hatching of a second-stage juvenile (J2), which is the parasitic stage of *Globodera* species that invades the roots. This process occurs, mainly, in suitable environmental conditions and in presence of a solanaceous host crop (Fenwick, 1949). The changes in egg shell integrity which lead to hatching are triggered by chemicals (described in the section 1.3.3.1.) released from the roots of the host plants (Robertson and Forrest, 1989). The J2 uses its stylet to free itself by cutting a slit in the eggshell and then leaves the cyst through the natural openings which are in the fenestral region or the neck of the cyst (Turner and Subbotin, 2013). Once released into the soil, the J2 is deprived of cyst wall-eggshell protection and further development is possible only after induction of the specific feeding site, the syncytium, inside the root. In the search for the host, the J2 follows gradients of chemicals, released by the root system, possibly using sense organs (amphids) positioned on the nematode's head, for the location of the root tip (Perry, 1998). Usually penetration of the root occurs directly behind the growing root tip (in elongation and maturation regions) or near a lateral root (Evans and Stone, 1977). To overcome the plant's first line of defence (epidermis) and enter the root, the J2 cuts through the epidermal cell walls with its stylet (Turner and Evans, 1998). After entering the first epidermal cell, the larvae migrate intracellularly perforating the wall of cortical and endodermal cells. The migration from cell to cell is possible not only

due to mechanical breakage of the walls caused by the robust stylet but also as a result of enzymes secreted by the nematode from the esophageal glands called pathogenesis factors (Golinowski *et al*., 2003). These include β-1,4-endoglucanases that degrades cellulose, a main component of the plant cell walls, which predominantly occurs in long rigid microfibrils (Smant *et al*., 1998) and polygalacturonases that degrade pectins, another major polysaccharide of the cell wall which also glue neighbouring cells together (Mahalingam *et al*., 1999). The destructive movement terminates when the J2 enters the vascular cylinder and the process of initial syncytial cell selection begins. The syncytium is usually initiated in the cortex or in the pericycle and procambium (vascular cylinder) (Sobczak *et al*., 2005). At this stage, the juvenile which has successfully established a syncytium loses mobility and, while actively extracting nutrients from the feeding site, undergoes three moults: to the third-stage (J3), to the forth-stage (J4) and finally to the adult (sometimes referred to as J5). The juvenile in second-stage cannot yet be determined as male or female. The sexes can be distinguished just at the beginning of the third stage when the first anatomical and morphological differences appear (Evans and Stone, 1977). The male juvenile (J4) permanently ceases nutrient uptake and moults to an adult vermiform shape and is mobile. The free-living male leaves the root in search of the adult female and is attracted by sex pheromones (Riga *et al*., 1996). Nematodes reproduce sexually and an adult male may mate with several females over the period of sexual activity which starts immediately after moulting to J4 and, under temperate conditions, continues for 9-10 days. The female on the other hand remains fertile for many weeks (Evans, 1970) and relies on the nutrients extracted from syncytium, while remaining immobilised for the whole period. After moulting from stage three to four, extensive growth of female body, now spherical in shape, causes the rupture of the root cortex. The nematode

remains attached to the root by the head and neck while the rest of the body, including the reproductive system, is exposed to the soil. After fertilization of J1 eggs by the male, the embryo development takes place within the female's body and when J2 are formed, the female dies and her body transforms into a thickened cyst to protect the eggs against unfavourable environmental conditions and antagonists. Transition from an active into a non-active stage marks the completion of the life cycle and juveniles will remain in it until favourable environmental conditions return.

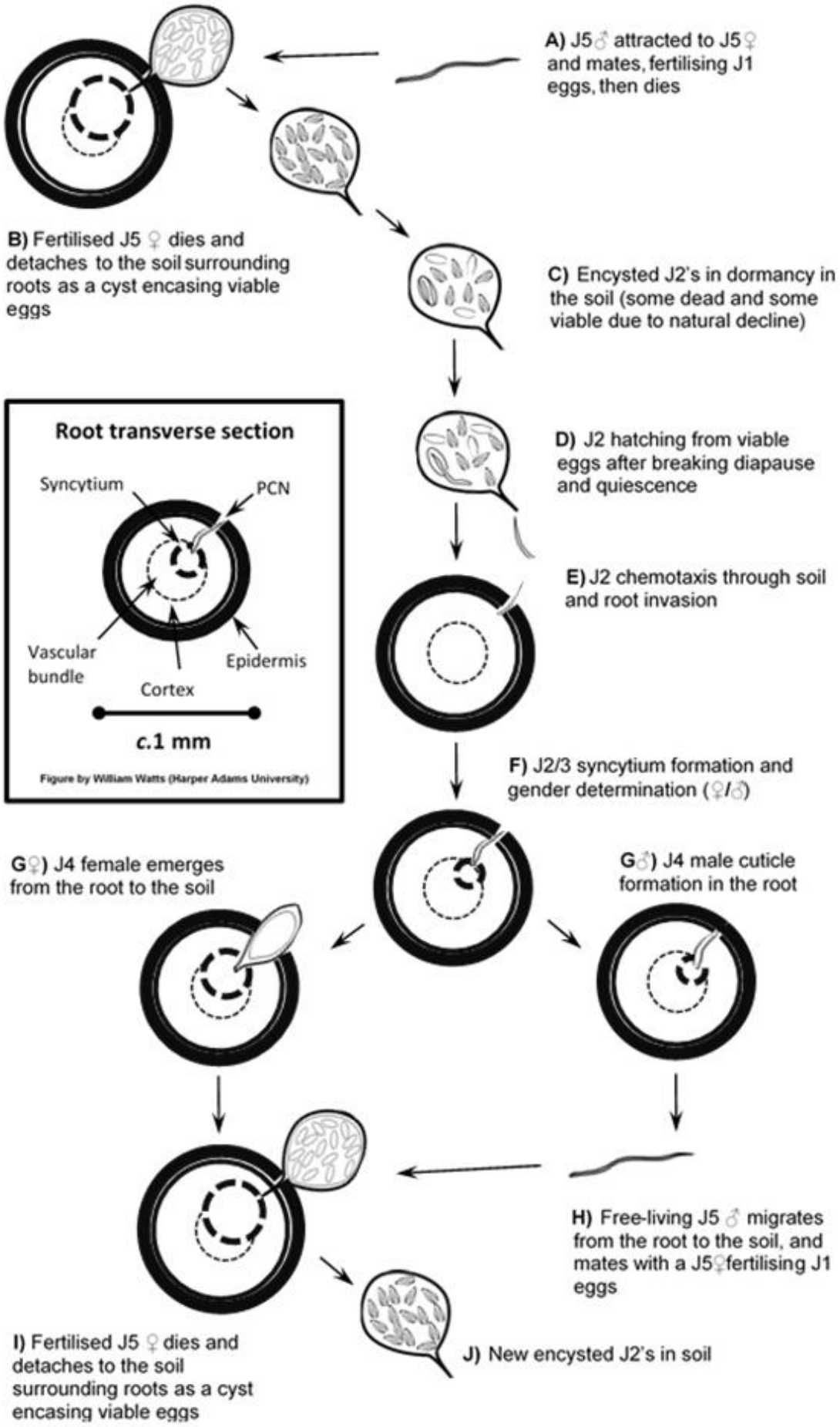


Figure 1.6. Potato cyst nematodes life cycle (Source: Watts, 2018).

1.2.6.2. *Natural decline*

The natural decline of PCN populations in field occurs as a result of spontaneous hatch of juveniles in the absence of a host crop (den Ouden, 1960) and in-egg mortality (Devine *et al*., 1999). The eggs are stimulated to hatch by the root diffusate produced by growing potato plants while remaining unhatched or hatching at very low rate, *c*. 3%, in water under laboratory condition (Triffitt, 1930). This unique host-plant interaction is as an adaptation to provoke the greatest hatch of the juveniles in the presence of a host plant and increases the chance of infection. Even with a strong synchronisation of life cycles between plant and pathogen, some of the eggs hatch in the years when no host plants are cultivated thus causing a reduction in the number of eggs able to infect the roots when the host plant becomes available. Jones (1970) demonstrated that the hatch of PCN eggs in the presence of potato root diffusate (PRD) might reach over 80% while spontaneous hatch with no host plant would be on average one-third every year (Cooper, 1953). Cole and Howard (1962) showed a *c.* 80% annual reduction in the egg population in microplots of a sandy loam as a result of growing resistant potatoes and a 60% reduction in the first year of growing a non-host crop. In absence of the host plants *G. pallida* population's density declined at an annual average rate of 20% while *G. rostochiensis* populations declined at an annual average rate of 22.3% which showed no significant difference between the species (Whitehead, 1995). Much slower rate of decline of *G. pallida* population in the absence of the host plant, about 50% during nearly five years, was reported by Radivojevic and Grujic (2010).

The decline of a population as a result of in-egg mortality can be due to incomplete hatch which increases susceptibility of partially hatched juveniles to negative environmental conditions and/or parasitism (Forrest, 1989). In nature nematodes have many antagonists which can act as parasites, including fungi

(Kerry and Crump, 1977) and bacteria (Stirling, 1988; Crump, 1989), which have been widely investigated as a method for biological control, or as predators (Kerry, 1986). The group of PCN natural enemies acting as predators is composed by other soil dwelling nematodes of the orders *Mononchida*, *Diplogasterida*, *Dorylaimida* and *Aphelenchida* (Khan and Kim, 2007).

The rate of decline is not constant between studies and the differences in the results might be due to many variables such as the density of soil infestation (Whitehead, 1995), soil type (Cole and Howard, 1962; Jones and Perry, 1978), temperature (Clarke and Perry, 1977) and the potato variety used (Evans, 1983). In addition the methodology to assess population densities varies between studies (Whitehead, 1995).

1.2.6.3. *Diapause and quiescence*

To increase the chance of successful invasion, plant parasitic nematodes synchronize their life cycle with the life cycle of the host plant by entering into a survival stage and staying inactive until conducive conditions occur (Perry, 1989a). This part of nematode life cycle is complex with different stages of dormancy being recognised including quiescence and diapause which is further subdivided as being obligate and facultative (Evans and Perry, 1976). The same authors proposed to use the cause of arrest in development as a standard for the distinction between the states and describe diapause as a type of dormancy which cannot be reversed, even under favourable conditions, until specific requirements have been satisfied, and quiescence as the nematode's response to unfavourable conditions which can be reversed when conducive conditions return. Obligate diapause is observed during the first season of development, but from the second season onwards facultative diapause can be initiated by external factors (Turner and Subbotin,

2013). In field conditions, PCN populations can consist of a mixture of diapaused and quiescent nematodes and as the majority of diapaused nematodes are insensitive to root diffusate, not all eggs will hatch. Palomares-Rius *et al*. (2013) showed that hatching of both populations, in quiescence and diapause, was significantly influenced by PRD treatment (variety 'Desiree') but the number of hatched quiescent nematodes was much greater than diapaused. The importance of diapause was highlighted by Kroese *et al*. (2011). They showed that the viability of older eggs, which have passed through diapause, was similar when assessed with a hatching assay and Meldola's blue stain while viability of newly produced eggs, which were still in diapause, differ greatly between these two assessment methods. Possible factors that could stimulate a break in diapause include exposure to a minimum environmental temperature for a defined length of time, photoperiod, light intensity or chemical hatching factors (Salazar and Ritter, 1993).

1.2.6.4. *Hatching temperature*

Differences in the hatch of PCN populations at different temperatures were reported by researchers even before the separation into the two species took place. McKenna and Winslow (1972) reported variation in the hatchability between 'pathotypes' of *H. rostochiensis* which could in fact represent species currently recognised as *G. pallida* and *G. rostochiensis*. In the publications since 1972, the highest hatch for most of *G. pallida* and *G. rostochiensis* populations tested (12 out of 14 and 7 out of 12, respectively) occurred at 20°C, remaining populations emerged best at 15°C for *G. pallida* and at 25°C for *G. rostochiensis*. These results led the researchers to the conclusion that *G. pallida* is better adapted to lower hatching temperatures (Parrot and Berry, 1976). Also Franco (1979) investigated the optimum temperature for hatching of PCN species and found that number of

hatched juveniles for *G. pallida* was highest in a range of temperatures between 10-20°C while for *G. rostochiensis* in a range of temperatures between 15-20°C. Similarly the hatching assays conducted by Robinson *et al*. (1987) in PRD under different temperature regimes showed the highest number of juveniles emerging from the eggs for *G. rostochiensis* occurred at 20°C while most efficient hatch for *G. pallida* occurred at 15°C. More recent research by Kaczmarek *et al*. (2012) confirmed earlier findings by showing that *G. rostochiensis* has a preference for a higher temperature range (15-21°C) for hatching than *G. pallida*. To investigate the effect of climate change on PCN populations Jones *et al*. (2017) tested the growth of PCN females at temperatures between 15 and 25°C. When *G. pallida* was tested on the potato variety 'Desiree' they observed a progressive decline in the number of females at temperatures above 17.5°C. Under the same conditions, *G. rostochiensis* achieved its optimum between 17.5 and 22.5°C. Reproductivity at 15°C was more successful in the case of *G. pallida* than *G. rostochiensis.*

Evans (1983) who reported faster emergence of *G. rostochiensis* juveniles when tests were conducted at the temperature (20°C) for both species. His results agreed with Whitehead (1992) observation that *G. pallida* hatched less vigorously than *G. rostochiensis* even when both populations were hatched at their optimum temperature, 15°C and 20°C, respectively. Kaczmarek *et al*. (2014) investigated the hatching pattern of both PCN species over a range of temperatures, graded from 5 to 29ºC, and noticed that overall *G. rostochiensis* hatched more quickly. The greatest cumulative percentage hatch of J2 occurred between 15 and 27°C for *G. rostochiensis* and 13-25°C for *G. pallida*.

1.2.7. *Crop damage*

Cyst nematodes (*Globodera* and *Heterodera*) and root-knot nematodes (*Meloidogyne* spp.) are considered to be most economically damaging of all plant-parasitic nematodes (Jones *et al*., 2013). *Globodera pallida* and *G. rostochiensis* cause serious damage to potato crops world-wide by restricting root growth and uptake of nutrients, reducing foliar growth and eventually leading to a loss in tuber yield (Phillips *et al*., 1998b).

Apart from potato, tomato (*S*. *lycopersicum* L.), aubergine (*S*. *melongena* L.) and some other Solanaceae are recorded as hosts for PCN including the European weeds *S*. *sarrachoides* Sendt. (Hairy nightshade) and *S*. *dulcamara* L. (Bittersweet) (Goodey *et al*., 1965).

Annually, PCN are thought to account for losses of more than 12%, on average, of the world potato crop yield (Urwin *et al*., 2000) with associated annual costs of up to £300 million in the European Union (Ryan *et al*., 2000). Yield losses resulting from damage caused by PCN in the UK alone has been estimated to cost approximately £43 million annually, based on the mean value of the crop from 1990–1995 (Haydock and Evans, 1998). According to more recent assessment, PCN are the second, after late blight, greatest, cause of losses in potential yield and quality worth nearly £26 million per year, mainly affecting the processing and fresh market sectors (Twining *et al*., 2009).

The effects of PCN parasitism are visible on both the foliage and the roots of potatoes. The mechanical damage to the root system, caused by intracellular migration of PCN juveniles cutting through cell walls, allows parasitic fungi and bacteria to invade wounded roots, the process which normally could be prevented by intact plant tissue (Turner and Evans, 1998). Back *et al*. (2004) reported an indirect interaction between *G. rostochiensis* and the fungus *Rhizoctonia solani*. A

positive relationship found between nematode densities and fungi infection may be linked to root damage caused by migrating juveniles and the release of root metabolites attractive to *R. solani*. Further phases of PCN infection cause a number of cytological and physiological changes in the cells which ultimately lead to the formation of the parasite nourishing structure – the syncytium (Dropkin, 1969). This multicellular and multinucleate structure, the only source of food for developing PCN juvenile, occurs as a result of hypertrophy, followed by fusion of the neighbouring protoplast. This fusion takes place through openings resulting from the dissolution of the cell wall (Jones and Northcote, 1972). The infestation, and consequently the production of syncytium, leads to reduction of the size and damage to the roots which penetrate into a smaller volume of soil that normally results in a reduced intake of water and minerals whose deficiency represents a significant reduction in yield (Turner and Evans, 1998). The aboveground symptoms can be visible as patches of wilted or/and plants with fewer stems and newly formed leaves while developed leaves are smaller, thicker and senesce earlier (Turner and Evans, 1998). Overall the infected plants are stunted and become water stressed, wilt easily, suffer nutrient shortage, become chlorotic (Figure 1.7.), and eventually die prematurely as a result of poor and inefficient development of root system (Haydock and Evans, 1998).

Figure 1.7. Comparison of a healthy potato plant (left) with a plant infected with potato cyst nematodes (right) (Source: Ulrich Zunke, University of Hamburg, Bugwood.org).

The economic threshold for crop loss due to PCN damage varies with environmental conditions, variety tolerance and soil type but it is usually accepted that in GB potato crops can be grown with modest yield loss at population densities below 20 eggs g^{-1} of air dried soil (Evans and Stone, 1977). Damage can be prevented when the soil population is reduced to the damage-threshold which for an intolerant variety is around 2 eggs g^{-1} soil (Whitehead and Turner, 1998). The reduction of the PCN population density to the damage-threshold can be achieved by using cultural, biological and chemical management strategies independently or combined in an integrated control program.

1.2.8. *Management*

Options for PCN management include: the prevention of infestation, the monitoring of population density and species composition of infested land, and the

implementation of field control methods (Haydock and Evans, 1998) which can be separated into cultural, biological and chemical. The cultural management methods are, most of the time, used as a preventative measure. It includes the rotational growing of non-hosts or trap crops, use of resistant or tolerant host varieties, soil management, and control of weed potatoes or other hosts and fallowing (Trudgill *et al*., 1992). Biological management intends to control nematode populations via the introduction of a natural enemy or predator (Stirling, 1991). Chemical management involves a treatment applied either before planting to reduce pre-planting populations or a treatment applied at or after planting which aims to decrease the level of attack from the pathogen (Trudgill *et al*., 1992).

1.2.8.1. *Prevention*

To prevent contamination of non-infested land, sanitation, legislative control and waste disposal should be adopted. The spread of PCN from an infested field can result from air borne soil aggregates, flood water run-off and soil adhering to machinery or passing animals. To minimise the risk of cysts being distributed by these methods, prevention measures, like constructing natural wind breaks or soil movement restrictions, and hygiene practices should be applied (Turner and Subbotin, 2013). Introducing legislative measures might also reduce this risk by regulating crucial for the industry areas like: the production of certified seed, rotation, use of resistant varieties and soil sampling systems for determining PCN presence in ware and seed lands (OJEU, 2007). Contaminated waste soil *e.g*. from machinery used in the infested field, can be disposed on non-agricultural lands or disinfested by steaming, chemicals and inundation (van Loenen *et al*., 2003; van Overbeek *et al*., 2014). To assist with reduction of PCN infestation occurring on clean fields for the potato seed production and prevent further spread an updated directive

2007/33/EC on the control of PCN took effect from 1 July 2010. Implemented changes increased the intensity of sampling and imposed an official pre-planting soil testing on land used for potato seed production (OJEU, 2007).

1.2.8.2. *Resistant and tolerant varieties*

The definition of resistance by EPPO states that a potato variety should be considered resistant when it significantly inhibits the development (hinders or reduces the growth, development or multiplication) of a particular PCN population (EPPO. 2006). Williamson and Hussey (1996) summarised that in some cases resistance comes from dominant or semi-dominant genes (monogenic or major gene resistance) and in others its inheritance is polygenic (explained genetically by a composition of dominant major genes and minor genes which may modulate the response). They further describe that the resistance can be specific to one nematode species or even a selected pathotype, but also could be effective against several nematode species.

The H_1 dominant resistance gene has, since the discovery in 1952 (Ellenby, 1952), been widely and successfully used in potato varieties. This single major resistance gene, derived from *S. tuberosum* ssp. *andigena* CPC1673, provides almost total resistance to *G. rostochiensis* pathotype Ro1 populations but has no effect on *G. pallida* populations. All populations of *G. rostochiensis* in the UK are exclusively the avirulent pathotype Ro1 and are lacking the gene that confers virulence against the H_1 gene (Trudgill *et al*., 1987; Turner *et al*., 2006). The first commercial variety to be grown in the UK with the H_1 gene was Maris Piper released in 1966. At this time *G. rostochiensis* infestation posed a greater threat to potato crop than *G. pallida*. Maris Piper has been grown frequently ever since alongside other varieties containing the H_1 gene (Turner *et al*., 2006). Only partial (polygenic)

resistance is available in *G. pallida* resistant varieties which, when compared with susceptible varieties, reduce PCN population multiplication but do not completely prevent it (Haydock and Evans, 1998).

The H_2 gene, derived from *S*. *multidissectum* PH1366, was the first major gene found to confer nearly complete resistance to pathotype Pa1 (Dunnett, 1961). Howewer, this pathotype rarely occurs in the UK (Kort *et al*., 1977). Another major gene against *G. pallida* was derived from *S. tuberosum* ssp. *andigena* CPC1673 (Arntzen *et al*., 1993a). This gene (*Gpa2*) confers resistance to a Dutch population of pathotype Pa2 (Bryan *et al*., 2002). Other more complex sources of resistance to *G. pallida* derived from *S. tuberosum* ssp. *andigena* CPC2802 and *S. vernei* confer a high level of resistance to pathotype Pa2 and Pa3 which are common in the UK and occur mainly as a mixture (Bryan *et al*., 2002). The invasion of PCN juveniles into resistant and susceptible potato varieties is similar but in resistant varieties a degradation of the syncytium and a lack of nutrients prevents female development and no reproduction takes place (Haydock and Evans, 1998). Trudgill *et al*. (1987) reported that growing resistant varieties can decrease the density of Ro1 *G. rostochiensis* populations, depending upon the proportion of eggs which remain unhatched, by up to 70-80%. Jones and Parrott (1968) observed faster density decline of some field populations under resistant varieties than it would occur under non-host crop. These authors concluded that nearly all juveniles developed into the males, and with only few available females, the reproduction was slight.

The difficulty in effective control of *G. pallida* populations with resistant varieties can be explained by the greater genetic diversity of this species introduced into Europe than of *G. rostochiensis* (Blok and Phillips, 1995). In the UK, continuous growth of *G. rostochiensis* resistant plants and the lack of varieties with full resistance to European *G. pallida* pathotypes explains why the latter has become

predominant (Minnis *et al*., 2000; Turner *et al*., 2006). Breeding for a high level of resistance to *G. pallida* has many difficulties and it was only recently that a few potato varieties were commercially accepted. Their effectiveness in controlling *G. pallida* under field conditions is still to be confirmed (Eves-van den Akker *et al*., 2015).

In light of the limited selection of *G. pallida* resistant varieties, use of tolerant varieties might help the growers to achieve an economic yield despite damage caused by PCN. Tolerance is the ability of the plant to tolerate the infestation by the parasite and to support the same amount of the pest as another variety, but with significantly better yield and quality (Trudgill, 1991). It is independent of resistance/susceptibility which describes plant ability to inhibit nematode reproduction through limitation of the feeding site *i.e*. syncytium. Tolerant varieties typically have a larger root system, and by this a higher volume of roots which enable the potato plants to withstand the PCN infestation and still produce the yield (Evans and Haydock, 1990). Ideally potato varieties should be both, resistant and tolerant. Growing tolerant but susceptible varieties increases PCN multiplication and the density of PCN populations to a damaging levels, especially if followed by an intolerant crop (Trudgill, 1991).

1.2.8.3. *Trap crops*

Trap cropping is a management technique where a highly susceptible potato crop is planted and grown for sufficient time to permit nematode invasion and development. Before the completion of the life cycle, the crop is physically removed or destroyed to prevent nematode reproduction. This method requires a potato variety which produces an extensive root system and planting with high density to reach the maximum number of nematodes. Precise timing of plant removal to avoid

the life cycle completion and increase of PCN density is crucial (Viaene *et al*., 2013). Other solanaceous species, such as *S. sisymbriifolium*, may be also used as trap crops. In this scenario the growing plants stimulate and attract nematodes and might even support invading nematodes but, as a poor host, do not permit the life cycle to be completed (Viaene *et al*., 2013). Trap cropping may appear costly, but when carried out properly and in suitable conditions, can reduce PCN population densities by up to 80% (Haydock and Evans, 1998).

1.2.8.4. *Crop rotation*

Crop rotation describes the growing of different crops in succession to avoid exhaustion of the soil and to control weeds, pests, and diseases. The restricted range of PCN hosts, spontaneous hatch and population density decline, due to natural causes, which occur in years when potatoes are not grown, makes crop rotation a practical and an effective control strategy to reduce PCN population densities. Crop rotation brings additional benefits to the land as it generally increases the diversity and stability of microorganisms associated with the rhizosphere, as compared to monocultures (Cook and Baker, 1983). It can also improve soil structure and increase nutrient cycling, which results in higher yield for the main economic crop (Widmer *et al*., 2002). Essentially two types of rotational crops are commonly used for pests management - non-host and resistant host (Widmer *et al*., 2002).

The earliest records of crop rotation are from the Andes in South America where a seven year rotation was introduced to manage PCN (Haydock and Evans, 1998). Evans and Haydock (2000) concluded that a rotation of eight years or more should be employed where *G. pallida* is found even when other crop protection strategies are used. Rotations longer than eight years, unless other effective control

measures were used, was also reported by Trudgill *et al*. (2014) as necessary to decline the *G. pallida* density below the damaging threshold. Traditionally in Europe, potato crops were grown every seven years to prevent yield loss due to PCN infestation but, with help of nematicides and fully or partially resistance varieties, the rotation can be shortened (Phillips and Trudgill, 1998b). Minnis *et al*. (2000) found that most common rotation length applied by growers in the UK was five years. When a decision about the length of rotation is made, the rate of natural decline, which in modelling for PCN population density is typically reported as 30% (Phillips and Trudgill, 1998b), should be taken into account. Evans and Stone, 1977 also reported a decline of PCN populations in temperate climates by about 30% annually when a non-host crop is planted. They assessed that this rate of decline should keep the population density below the damaging threshold (below 20 eggs g^{-1} of air dried soil) when the rotation will be one in five or one in six. In contrast, Lane and Trudgill (1999) suggested that the rotation should be extended if to be successfully used as a management strategy. For the potato production to be economical, a crop should be grown every 4-6 years which is too short to benefit from natural decline, although their assessment was made on the assumption that yearly decline can be even as low as 10%. Whitehead (1995) tested the natural decline of *G. pallida* and *G. rostochiensis* in microplots and found that average annual rates of decline did not differ significantly between these two species and can vary between 12.8 and 40.5%. Variability between the individual field populations might be accounted for by different physical and biological environments of tested fields as well as genetic variation between these populations (Evans and Haydock, 2000). Importantly, volunteer potatoes from unharvested tubers or potato seed might reduce the decline ratio under a non–host crop. That is why it is important to remove weed potato volunteers (den Ouden, 1967).

1.2.8.5. *Biological control*

Biological control (biocontrol) is based on an interaction between a pest species and its natural enemies (antagonists). In nature, nematodes have many antagonists including fungi (Kerry and Crump, 1977), bacteria (Stirling, 1988; Crump, 1989) and predatory nematodes, which have been widely investigated as a method for biological control (Kerry, 1986). Microbial agents pathogenic toward J2, like parasitic bacteria and fungi or nematode-trapping fungi, can reduce crop damage but will not significantly affect PCN density at the end of the season. To provide effective control the agents must attack adult females and/or unhatched eggs (Kerry, 1986). Biological control may be: natural, when the density of parasites naturally present in soil increases to the level effecting the nematode population, or induced, when biological control agents are introduced into the soil where they are naturally absent (Kerry, 1986). Several beneficial fungi have been identified as potential biological control agents against PCN. The second-stage juveniles and males of PCN can be trapped in mycelial structures produced by *Arthrobotrys oligospora*. In the late 1970s, attempts were made to produce commercial products based on this interaction but, due to the inability to synchronize trap production with time of migration of the juveniles and later males, they all failed (Kerry *et al*., 2003). The potential of using *Pochonia chlamydosporia* as a possible tool for biocontrol, and its prospect to be combined with other control measures, was highlighted by Tobin *et al*. (2008). Application of the rhizobacteria *Agrobacterium radiobacter* (strain G12) and *Bacillus sphaericus* (strain B43) can induce systemic resistance in plants and reduce root penetration by *G. pallida* juveniles (Hasky-Günther *et al*., 1998). It appears to be difficult to manipulate these organisms using treatments that growers might consider as practical, in order to generate nematode suppressive

soil. Hence it is suggested that biological control methods should be applied as a part of integrated pest management (Trudgill *et al*., 1992).

1.2.8.6. *Chemical control*

There are two types of pesticides for PCN control: nematostats and fumigant nematicides. It is important to obtain information about the species present before a decision on using chemical control is made as *G. pallida* is less readily controlled by nematostats, possibly due to relatively slower hatch than *G. rostochiensis* (Evans and Haydock, 2000).

Nematostats (sometimes referred to as non–fumigant nematicides) provide sub-lethal effects, used to disturb nematode behaviour, and are applied and incorporated into the soil immediately before the crop is planted (Haydock *et al*., 2013). The active substance must dissolve into soil water (nematostats are in granulate form) to make contact with hatched PCN juveniles migrating through soil particles in search of the roots, and where it is absorbed through the larvae cuticle. The reduction in potato root damage is achieved by the chemical compound disrupting the nervous system (acetylcholinesterase inhibitors) and causing nematode paralysis and also by reducing hatching (Haydock and Evans, 1998). Nematostats approved in the UK include the organophosphates fosthiazate (Nemathorin®) and ethoprophos (Mocap®), and the oxime–carbamate oxamyl (Vydate®) (Lainsbury, 2016).

Fumigant nematicides are lethal to nematodes and can be applied at any time in a rotation when no crop is present and the conditions are suitable, generally in the previous autumn (Haydock *et al*., 2013). Here, as in case of nematostats, the chemical is applied into the soil but it is typically performed in autumn when no crop is present and then the soil surface is sealed. The gas, biocidal methyl–

isothiocyanate, released from liquid or granular form of nematicide, targets encysted eggs and can cause death of up to 80% of still unhatched juveniles (Haydock and Evans, 1998) by degrading their proteins and essential enzymes (Kawakishi and Kaneko, 1985). The only nematicidal substances registered in the UK are metam–sodium (*e.g*. Metam 510®), dazomet (*e.g*. Basamid®) (Lainsbury, 2016) and fluopyram (Velum Prime®) (Crop Science-Bayer, 2019). Nematicides are applied primarily to reduce population's damage to the crop and allowing a higher yield (Tobin *et al*., 2008).

In the UK, many chemical control products are now forbidden, or at least their use is restricted, because of potential health and environmental risks (OJEU, 2009). The use of brassica green manure plant residues in a process known as biofumigation is increasing in popularity (Ngala *et al*., 2015). Biofumigation consists of growing, cutting, chopping and incorporating *Brassica* species which contain the chemical compounds - glucosinolates. Damaging plant tissue prior to green manure incorporation releases endogenous myrosinases which cause hydrolysis of glucosinolates. Final products of this process, primarily isothiocyanates, are biologically active and volatile and can be used to control soil pests (Gimsing and Kirkegaard, 2009). Biofumigation has shown to be successful in the suppression of PCN in *in-vitro*, glasshouse and field based studies (Lord *et al*., 2011; Ngala *et al*., 2015).

1.2.8.7. *Integrated control*

The PCN management methods described have limitations and for that reason using a combination of different but complementary methods should give the best result. The main objectives of integrated pest management, a broad-based approach that integrates practices for economic control of pests, is to prevent yield

loss in the short term and to reduce the population density in the long term (Whitehead, 1986). Integrating resistance, rotation and nematicide use is considered to be the best approach to address both of these objectives (Trudgill *et al*., 1992). The management of *G. pallida* has many more difficulties than that of *G. rostochiensis* due to its greater persistence and absence of fully resistance potato varieties (Evans, 1993).

1.3. Characterisation of potato cyst nematodes

1.3.1. *Potato cyst nematodes detection and determination of species*

The first symptoms of PCN infestation in the potato field are those visible on the above-ground parts of the plant and are described in the section 1.2.7. Excavating the roots of growing plants can be used as a tool to diagnose PCN infestation and to determine species presented thanks to the difference in colouring of *G. pallida* and *G. rostochiensis* cysts at this stage of development. This method can be used only after extensive growth of female body causes the rupture of the root cortex and before cysts lose their distinguishing body colour and can be easy detached from the roots when lifted. Therefore soil testing and further tests on extracted cysts are recommended as the best way to detect and identify PCN (EPPO, 2017) as described in the Chapter 3.

Identification on the basis of morphological features to the genus level can be performed on cysts and second stage larvae (EPPO, 2017) but also, if required, to species level, as described in the section 1.2.2., on cysts, second stage larvae and males.

The polymerase chain reaction (PCR), based on allele-specific amplification, was successfully used by Mulholland *et al*. (1996) to detect and distinguish between *Globodera* species. They developed a method for the species-specific

discrimination of PCN which amplifies a region between the internal transcribed spacer 1 (ITS1) region and the 5.8S ribosomal ribonucleic acid (rRNA) gene and produces fragments of different sizes for *G. rostochiensis* (238 base pair (b.p.)) and *G. pallida* (391 b.p.). Molecular examination of the ribosomal internal transcribed spacer (ITS) to differentiate *G. rostochiensis* from *G. pallida* was also used by Bulman and Marshall (1997) and it is one of the methods recommended by EPPO. The test is designed to the 18S rRNA gene and ITS1 region and produces 434 b.p. fragment for *G. rostochiensis* and 265 b.p. fragment for *G. pallida*. Thiery and Mugniéry (1996) used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of ITS region of the ribosomal DNA to test 26 *Globodera* populations and showed that, even if the intraspecific polymorphism was low, the interspecific polymorphism was sufficient to differentiate between *G. rostochiensis* and *G. pallida* as distinct groups. Clear identification of five *G. rostochiensis* and two *G. pallida* populations was achieved using random amplified polymorphic DNA (RAPD) (Fullaondo *et al*., 1997). Out of eleven primer sets tested eight produced unique RAPD patterns with a high degree of polymorphism and could be used as diagnostic tools. Molecular biology techniques used for detection and identification of *G. rostochiensis* and *G. pallida* were further developed to allow quantification of nematodes in the samples. The real-time polymerase chain reaction (real-time PCR), by using hybridization probes, allows continuous monitoring of the sample and testing for more than one nematode species in one assay. Real-time PCR can be used to measure the quantity of target species DNA in the sample by constructing a standard curve from DNA of known quantity in the same assay - quantitative real-time polymerase chain reaction (qPCR). Nakhla *et al*. (2010) developed a multiplex qPCR which not only simultaneously detected and identified PCN sibling species but also distinguished them from the morphologically similar tobacco cyst nematode.

In their research, DNA was obtained from cysts extracted and collected from infested soil. Real-time PCR developed by Reid *et al*. (2015) simplify this process even further and allowed detection and accurate identification of PCN species without necessity for cysts collection. DNA was extracted directly from float material collected after soil washing.

1.3.2. *Determination of pathotypes and mitotypes*

Observation of *G. pallida* and *G. rostochiensi*s populations on resistant varieties with different resistance genes show that distinct virulent strains occur (Turner and Subbotin, 2013). Fleming *et al*. (1998) suggested that this intraspecific diversity could be due to prolonged coevolution between pest and the host species. Individual PCN populations which could overcome the resistance and reproduce on *Solanum* clones with resistance genes were identified as virulent resistance-breaking pathotypes. Efforts made to categorise and incorporate pathotypes into the taxonomic classification of PCN are described in the section 1.2.3.

A gene-to-gene relationship between pathotypes with recessive virulence genes and potato varieties with corresponding resistance genes was proposed by Howard (1959) and Jones and Parrott (1965). These theories were later tested and confirmed by Parrott (1981) for the relationship between resistance gene H_1 from *S*. *tuberosum* ssp. *andigena* CPC1673 and a gene in *G*. *rostochiensis*, and between H_2 from *S. multidissectum* and a gene in *G. pallida*.

An attempt to differentiate eight European pathotypes of PCN (Pa1 - 3, Ro1 - 5) by analysing their protein and enzyme variations using IEF technique showed no clear relationship between observed groups and pathotypes (Fox and Atkinson, 1984). Isozyme and restriction fragment length polymorphism were tested across sixteen *G. pallida* (classified as Pa2 or Pa3) and one *G. rostochiensis* populations

originating mainly from GB. None of the methods used provided clear and consistent results which allowed populations to be distinguished based on their virulence or geographic origin satisfactorily (Phillips *et al*., 1992). Blok and Phillips (1995) used simple sequence repeat primers to test genetic variability and concluded that further studies are required to establish which biochemical characteristics are linked to virulence after no clear separation between the pathotypes was observed.

The European and Mediterranean Plant Protection Organization recommends that testing any population characterised by new or unusual virulence, which may have overcome an existing resistance in potato varieties, should be a priority (EPPO, 2017).

1.3.3. *Determination of potato cyst nematodes viability*

An appropriate method for distinguishing between dead and live specimens has been investigated over many years and various techniques have been suggested and tested such as visual characterisation (EPPO, 2017), staining methods (Ogiga and Estey, 1974), hatching tests (Fenwick and Widdowson, 1958; Been and Schomaker, 2001), plant infectivity bioassays (Hague and Omidvar, 1962), molecular biology techniques (Lord *et al.,* 2011) and more recently the trehalose assay (van den Elsen *et al.,* 2012).

Some of the early viability assessment methods required individual treatment of every nematode by ejecting the larvae from the egg. This assessment was based on the observation made by Staniland and Stone (1953) that living juveniles straightened out immediately after being removed from the egg while dead ones remained curled. The need of investigating each egg separately greatly increases the time need to process the sample and makes the method unsuitable especially for commercial use.

Methods to test the viability of eggs and juveniles recommended by EPPO (2017) are: visual determination based on morphology (described in the section 1.2.2.), hatching tests, Meldola's blue staining, measurement of trehalose and by real-time PCR.

1.3.3.1. *Hatching test*

In the case of the cyst nematodes, the presence of diffusate from the host roots is predominantly necessary to induces hatching (Perry, 1989a). This characteristic was employed in a hatching test which intended to assess population viability by exposing cysts to root diffusate and enumerating hatched juveniles over the period of time. The eggs of *Globodera* species hatch after stimulation by hatching factors (HFs) contained in the host root diffusate (Perry, 1989b; Devine and Jones, 2001; Devine *et al.,* 2001). Over the years many attempts have been made to characterise HFs. Perry (1989a) discussed their involvement in hatching activation on different levels; some of them could be capable of activating the emergence of the juveniles from the eggs themselves (true hatching factors), others might directly affect unhatched J2 or permeability of the eggshell. But it was not until 1996 that Devine *et al*. (1996) identified multiple HFs in PRD. They concluded, from their investigation of *G. rostochiensis* responses to PRD, that at least 10 HFs are present in the diffusate. Byrne *et al*. (2001) took the investigation on factors a step further and proposed the separation of HFs into three classes based on their selected activity toward PCN. They identify HFs with species-selective (active towards both species but stimulating greater hatch of one species than the other), -specific (active toward only one species) and –neutral (equally active toward both species) activities.

It is important to select the most suitable variety for PRD production prior to conducting the hatching assay as it can have a strong impact on the results. The differences in hatching as a result of treatment by root diffusate obtained from various potato genotypes have been widely reported. Evans (1983) tested the hatching responses of *G. rostochiensis* and *G. pallida* to PRD from 25 potato varieties and showed that some of the diffusate stimulated more eggs to hatch than others. Turner and Stone (1981) tested resistant clones of *S. vernei* against susceptible *S. tuberosum* varieties and found no consistent evidence that varieties with various levels of tolerance and/or resistance might be differently affected by nematodes invasion. The resistance genotypes appeared to induce smaller hatch in some cases but higher in others when compared with a susceptible control. These are, partially, in accordance with the observations by Arntzen *et al.* (1993b) where differences were found in the hatching of *G. pallida* population treated with PRD obtained from thirteen potato varieties differing in tolerance, although no significant correlation between hatchability and tolerance could be drawn. Turner *et al*. (2009) found that treatment of *G. rostochiensis* (except Ro1) and *G. pallida* populations, with root diffusate collected from different *Solanum* species resulted in variation in hatching.

Turner and Stone (1981) did not find the significant differences in the effect of dilutions on hatchability while Evans (1983) observed a direct correlation, whereby a decrease in PRD concentration resulted in a decrease of hatching. A dilution experiment by Arntzen *et al*. (1993b) showed lower hatching in samples treated with 1:4 dilution of PRD when compared with undiluted. More recent work by Turner *et al*. (2009) showed that, for all six potato genotype tested, undiluted and half strength dilutions gave the highest ratio of hatching and dilutions greater than 1:16 displayed a decline in the stimulation of hatch.

Different dynamics and optimal ranges of temperature (described in the section 1.2.6.4.) for the emergence from eggs between sibling species as well as variations in numbers of juveniles, of the same species, hatched at different temperatures indicate how important the choice of the adequate temperature and length of assay are.

The factors which can generate the most error in hatching assays were recognised very early by Fenwick and Widdowson (1958) and were described as the variability between the cysts and variation in the activity of diffusate. A size-grading technique was successfully used by Twomey *et al*. (2000) to reduce the variability of juvenile content of the cysts and to synchronize hatching within the samples, although the results differed between *G. rostochiensis* and *G. pallida* and most likely will differ even across *G. rostochiensis* populations due to variations in cyst size distribution in populations. Determination of the viability by testing hatchability of the population might also be affected by the proportion of the dormant eggs which will not hatch even if they are viable (see section 1.2.6.3.). Cysts obtained from samples collected shortly after potato harvest will most likely contain higher percentage of eggs in obligate diapause then cysts from older populations which overwintered in the field. The European and Mediterranean Plant Protection Organization recommends that to break the dormancy cysts should be exposed to +4°C for at least four months (EPPO, 2017).

1.3.3.2. *Staining methods*

To investigate the viability of the unhatched juveniles, differential staining methods were developed and investigated by a number of authors. The stain will penetrate egg's membrane only in non-viable specimens (Figure 1.8.), due to its degradation, while the intact membrane of alive specimens will act as a protective

barrier preventing stain from penetration. The permeability of the nematode cuticle must be compromised during the event inducing mortality for the stain to correctly detect non-viable individuals (Moriarty, 1964).

Research to identify the most suitable stain for determining the viability of nematodes began by considering a wide range of stains. Doliwa (1954) examined twenty basic stains and thirteen acidic compounds while Hollis (1961) observed the reaction of nematodes to thirty-eight coal-tar dies. Through an elimination process, based on the results, toxicity and invariable results, the number of suitable dyes was reduced by Moriarty (1964) to: chrysoidin (Doliwa, 1954), phloxine B (Fenner, 1962) and new blue R (Shepherd, 1962).

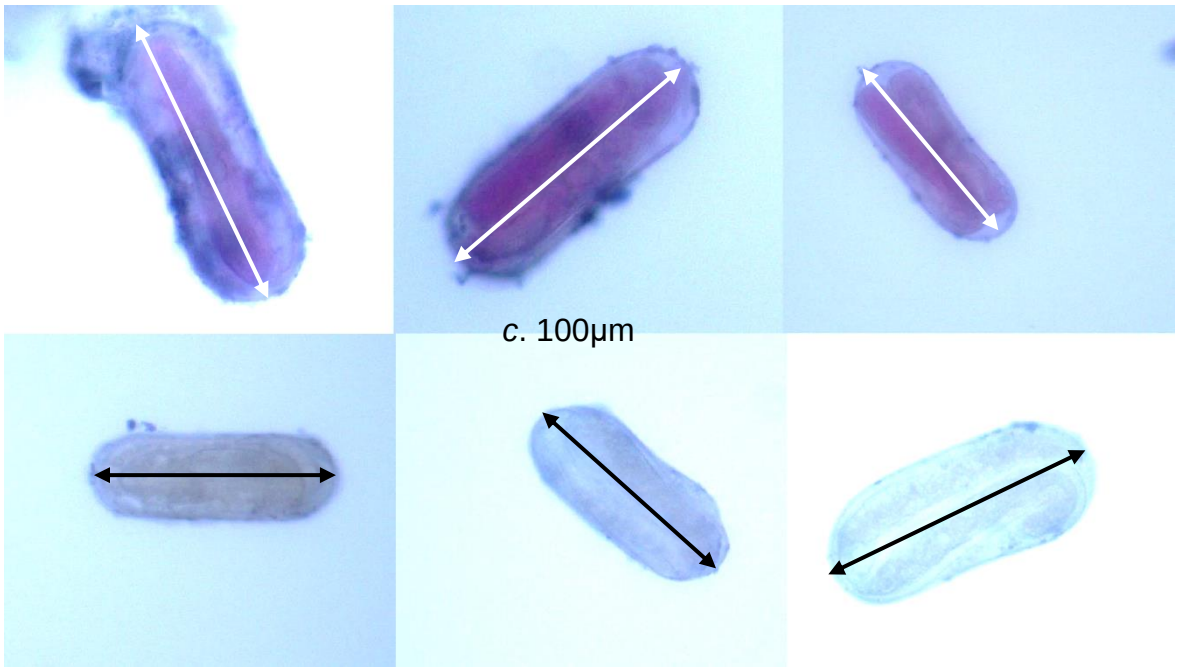


Figure 1.8. *Glodobera* spp. eggs stained-dead (top row) and not stained-live (bottom row) by Meldola's blue dye used for viability testing (Source: Author's own).

The efficacy of phloxine B was also tested by Ogiga and Estey (1974) who, in contrary, described it as not entirely satisfactory. The same authors reported chrysoidin as an effective viability indicator but only after long, normally 24 but in

one case even 48 hours, period of staining. Meyer *et al*. (1988) found that chrysoidin, eosin-Y, new blue R and Nile blue A stains were the most effective at distinguishing between live and dead *Heterodera glycines* eggs regardless of the cause of death. Shepherd (1962) was also successful in distinguishing between live and heat-treated eggs of *G. rostochiensis* (then *Heterodera*) by staining them in a solution of new blue R. She commented that even the differences between lightly stain (dead) and unstained (alive) eggs were efficient for the assessment and that the proportion of doubtful results rarely exceeded 2-3%. She also found no toxicity towards specimens even at highest concentration. Research by Moriarty (1964) confirmed new blue R to be an efficient tool to investigate the viability of cyst nematodes but with the limitation to those killed by heat or chemicals. Staining results on old populations containing eggs that died from natural causes overestimated viability when compared with a hatching assay and suggested that new blue R is unsuitable for ecological work. Goffart (1965) concluded that new blue R is an effective tool to assess viability of plant parasitic nematodes which was later undermined by Ogiga and Estey (1974) who reported that, in some cases, new blue R caused staining of live nematodes while leaving dead ones unstained. Efficacy of new blue R and Meldola's blue was reported equal by Goffart (1965) who compared both stains under the same conditions. This similarity can be partly explained by fact that new blue R and Meldola's blue are regarded as closely related chemicals (Shepherd, 1986; Been and Schomaker, 2001) as their structural formulas differ only in having ethyl or methyl subgroups, respectively (Goffart, 1965). Meldola's blue was also compared with Nile blue A by Ogiga and Estey (1974) who found the mode of action for both stains very similar – uniformly staining dead nematodes but leaving live specimens unstained or with only intestinal granules being stained. In all tests, dead specimens were obtained by application of lethal amounts of heat, cold, dryness or

by the use of nematicides. Ogiga and Estey (1974) concluded that the staining process by Nile blue A and Meldola's blue was simple and rapid and the recognition between stained and unstained specimens could be achieved at low magnification hence both would be suitable for ecological studies.

1.3.3.3. *Trehalose assay*

As an alternative to hatching tests and staining methods, a qualitative viability assessment method, which uses trehalose as an indicator of alive eggs, was proposed by van den Elsen *et al.* (2012). The viability marker used in this technique is trehalose, a non-reducing disaccharide which is mainly, >90% of total cyst trehalose, concentrated in the perivitelline fluid which surrounds the unhatched juveniles within an egg (Clarke and Hennessy, 1976). The investigation of carbohydrate content of *G. rostochiensis* eggs showed that trehalose, α-D-glucopyranosyl-α-D-glucopyranoside, constitutes 6.7% of the weight of whole eggs containing larvae (Clarke and Hennessy, 1976) at a calculated concentration of 0.34 M (Clarke *et al*., 1978). Thanks to the low permeability of the egg shell, the trehalose in perivitelline fluid is retained within the egg until the juvenile hatches. Prior to hatching, an increase in this permeability is required which results in the loss of trehalose from the egg fluid (Clarke and Perry, 1977; Clarke *et al*., 1978). A similar sequence of changes takes place in the event of juvenile death while still in the egg which leaves non-viable eggs trehalose-free and consequently allows the detection of the viable eggs by trehalose hydrolysis into two glucose molecules followed by the detection of glucose (van den Elsen *et al.*, 2012). Comparison with other methods testing viability showed that the trehalose-based method provides a similar estimate with a visual assessment and detected higher numbers of viable eggs when compared with a hatching test (Ebrahimi *et al*., 2015). Beniers *et al*. (2014)

general conclusion was that even if quantification is possible, the method lacks sensitivity. It makes, at this stage, trehalose-based method unsuitable for use as a substitute for visual assessment with further development of the method is required. The described studies and attempts to optimize the trehalose-based method showed that it can be used to determine the number of viable eggs in samples and highlighted its potential as a quantification method.

1.3.3.4. *DNA and RNA based methods*

The development of a molecular based viability tests would be beneficial for the industry through improving assessment accuracy and reducing the time of its completion. Fleming *et al*. (1998) investigated the relationship between number of viable juveniles in cysts, assessed by hatching in PRD, and the amount of DNA that can be extracted from them and found a positive correlation which indicated that qPCR may serve as a viability assessment tool. A qPCR-based method combined with propidium monoazide (PMA), a photoreactive DNA-intercalating dye, method was developed and successfully used by Christoforou *et al*. (2014) to quantify the number of viable eggs in heat-treated samples, newly formed and old cysts.

Another approach to the detection of viable PCN eggs was proposed by Back *et al*. (2004) who exploited the natural phenomenon which occurs upon death and results in a rapid decline of messenger ribonucleic acid (mRNA) in the cell which normally occurs in large quantities. In this study, the presence of the housekeeping protein coding gene glycerol-3-phosphate dehydrogenase (gpd-1) was assessed by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). This method was also developed by Lord *et al*. (2011) to test the effect of biofumigation on *G. pallida* egg viability. Abundance of the target mRNA gene, actin-1, was measured in samples of known proportion between heat-killed and viable cysts and

showed a strong relationship between quantity of investigated mRNA and viable eggs. A similar approach, but targeting mRNA of the elongation factor 1-α gene (EF1-α), was employed by Beniers *et al*. (2014). Reverse transcription and an RNA-specific real-time PCR assay was conducted on mRNA extracted from samples with known eggs densities, however, the assay was concluded to be unsuitable as a tool to estimate population viability. Its limitations were the minimum level of detection and a high variation of single measurements. The problem with the sensitivity was overcome by Mimee *et al*. (2017) who further developed the method initially proposed by Back *et al*. (2004) and found no detection limit for a RT-qPCR by showing the ability to detect a single viable egg.

1.4. Aims

(I) Investigate the occurrence, distribution and species composition of PCN (*G. pallida* and *G. rostochiensis*) in GB and their possible linkages with agricultural factors.

(II) Identify the most suitable viability assessment method for PCN field populations from GB by comparison between classic and novel techniques on natural and six artificially imposed levels of mortality.

(III) Characterise selected *G. pallid*a populations from PCN survey in England and Wales using classic (pathotyping) and novel (mitotyping) techniques.

Chapter 2

National survey documenting the occurrence and distribution of potato cyst nematodes in Great Britain.

2.1. Introduction

Potato cyst nematodes (PCN), composed of two species *Globodera rostochiensis* and *G. pallida*, are destructive pests of potato crops in Great Britain (GB) that cause annual crop losses of approximately £25.9 million (Twining *et al*., 2009).

The Food and Agriculture Organization of the United Nations (FAO) reported that potatoes were the United Kingdom's (UK) 6th most produced crop by quantity and 2nd most valuable by net production value in 2016 (FAO, 2018). This high ranking position highlights the importance of the potato industry for the national economy and the necessity to reach optimal production in the future. This can be partially achieved by understanding *Globodera* species composition and the factors affecting species variability, which would be highly beneficial for sustainable crop production and management.

Potato cyst nematodes were first reported in the UK as early as 1904 when they were described as "potato eelworm" (Strachan and Taylor, 1926). Following this initial report, PCN continued to spread throughout much of the UK potato growing regions and were found to be widespread by the 1970's (Trudgill *et al*., 2003). Even though the pathogenicity of PCN has been known for over a hundred years, it was only in 2000 that a statistically unbiased and systematic survey of potato growing land was conducted (Minnis *et al*., 2002). This survey of England

and Wales identified *G. pallida* as the dominant species, being present in 67% of infested potato fields as a pure population and in 25% as a mixed, comprised of two related species, population. When compared with a previous survey (Hancock, 1996), when 54% of infested land was confirmed as pure *G. pallida* and 41% contained mixed populations, these results indicated that the species distribution of PCN had been changing. *Globodera rostochiensis* was detected in 8% (Minnis *et al*., 2002) of infested samples in 2000 and was confirmed in 5% samples in 1996 (Hancock, 1996). There is a shortage of national information about PCN distribution prior to 1996 but the most likely reason for the *G. pallida* dominance is development of potato genotypes with the H_1 resistance gene. The varieties which are highly effective in controlling *G. rostochiensis* but are susceptible to *G. pallida*. This increases the risk of selection of *G. pallida*, which is more challenging to manage. Since the last national study on PCN occurrence and species composition was completed over 18 years ago (Minnis, 2000), an up-to-date PCN distribution would be useful to help determine the current species composition.

Potato cyst nematodes virulence towards potato genotypes with resistance derived from various sources, and pathotype identification of survey samples are not mentioned in this chapter. Aspects of virulence are discussed in Chapter 4.

2.1.1. *Aim*

Investigate the occurrence, distribution and species composition of PCN (*G. pallida* and *G. rostochiensis*) in GB and their possible linkages with agricultural factors.

2.1.2. *Objectives*

(I) Estimate the proportion and distribution of GB potato fields infested with PCN.

(II) Determine the distribution of the two species, *G. pallida* and *G. rostochiensis* in GB.

(III) Evaluate the possible effect of agricultural factors, *e.g*. length of rotation or soil texture, on abundance and proportions of the PCN sibling species.

2.2. Materials and methods

2.2.1. *Stratified survey*

Based on the total planted ware potato growing area (ha) for 2013 (91,227ha) a stratified survey was designed for England and Wales (Figure 2.1.). Potato growing area information was provided by the Agriculture and Horticulture Development Board (AHDB) Potatoes - Potato Data Centre (data obtained on the 10th of April 2014) for market sectors: fresh bags, fresh chipping, pre-pack, processing and other ware. Counties with a planted potato growing area below 100 hectares (<0.1% of total) were excluded from the survey as being less commercially important, which reduced the number of counties to 34 from the 49 originally listed by the AHDB Potatoes - Grower Panel. The minimum number of fields required to be surveyed for the accurate identification of the proportion of fields that were infested and those free from PCN was calculated by Minnis (2000), from total planted ware potato growing area of 146,200ha, at 96. To achieve a comparable range to that presented in the most recent survey (Minnis, 2000), when 484 sites were sampled, it was decided that 500 soil samples should be collected in England and Wales.

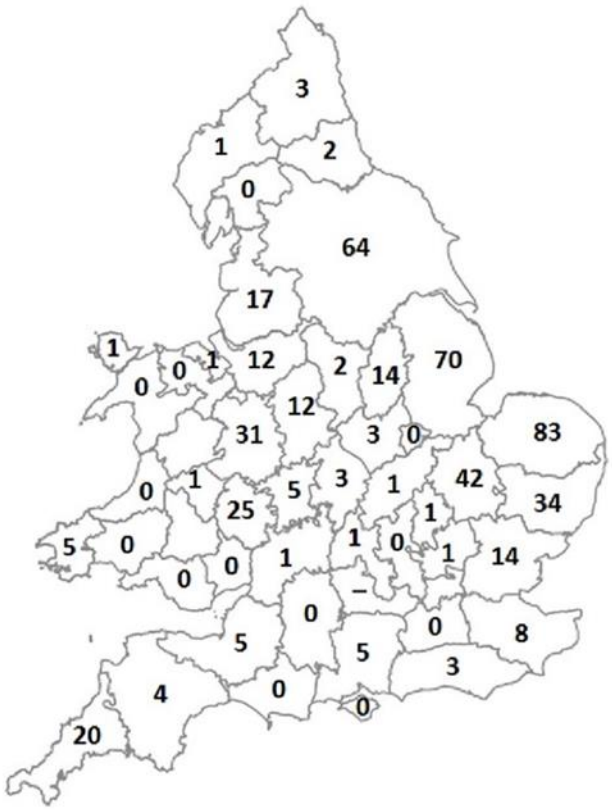


Figure 2.1. Distribution and number of survey samples required from counties in England and Wales determined by a stratified survey based on the total potato growing area (ha).

To express the differences in the production intensity, based on the planted potato growing area, the 500 survey samples were systematically distributed across 34 counties (Table 2.1.) following the calculations below:

$$x = \frac{\text{planted potato growing area (ha) in the county} * 100\%}{\text{planted potato growing area (ha) in England and Wales}}$$

x – planted potato growing area (ha) in the county expressed as a percentage of planted potato growing area (ha) in England and Wales

$$y = \frac{\text{required number of survey samples from England and Wales} * x\%}{100\%}$$

y – required number of survey samples from the county

Table 2.1. Potato planted area in 2013 according to the AHDB Potatoes and number of soil samples required for the stratified survey per county (England and Wales).

Country	Region	County	Planted areas (ha)	Samples required
England				
	East Midlands	Derbyshire	454	2
		Leicestershire	618	3
		Lincolnshire	12787	70
		Northamptonshire	140	1
		Nottinghamshire	2,539	14
		Rutland	62	0
	East of England	Bedfordshire	229	1
		Cambridgeshire	7725	42
		Essex	2,611	14
		Hertfordshire	167	1
		Norfolk	15084	83
		Suffolk	6,133	34
	North East	Durham	281	2
		Northumberland	527	3
	North West	Cheshire	2,150	12
		Cumberland	248	1
		Lancashire	3,135	17
		Westmorland	2	0
	South East	Buckinghamshire	8	0
		Hampshire	804	5
		Isle of Wight	90	0
		Kent	1,463	8
		Oxfordshire	106	1
		Surrey	19	0
		Sussex	620	3
	South West	Cornwall	3,721	20
		Devon	700	4
		Dorset	14	0
		Gloucestershire	233	1
		Somerset	825	5
		Wiltshire	45	0
	West Midlands	Herefordshire	4,591	25
		Shropshire	5,556	31
		Staffordshire	2,089	12
		Warwickshire	1,436	8
		Worcestershire	857	5
	Yorkshire and the Humber	Yorkshire	11,629	64
Wales				
		Anglesey	122	1
		Brecon	24	0
		Caernarvonshire	8	0
		Cardigan	2	0
		Carmarthenshire	28	0
		Denbighshire	17	0
		Flintshire	189	1
		Glamorgan	1	0
		Monmouth	46	0
		Montgomery	29	0
		Pembroke	952	5
		Radnor (Powys)	111	1
Total			91,227	500

2.2.2. *Soil samples collection*

Growers, independent agronomists, and agronomy companies were contacted and invited to contribute to the survey (Appendix 7.1.) by collecting and posting soil samples, providing sub-samples of the soil collected during routine PCN testing or by giving access to field sites for soil sample collection.

Survey sampling methodology used for soil collection was based on sampling procedures recommended by the Official Journal of the European Union (OJEU) in Directive 2007/33/EC on the control of potato cyst nematodes (OJEU, 2007). A total number of 264 soil samples were directly collected from fields previously used for ware potato production. Each sample represented an individual field or four-hectare sampling area, marked out from the main entrance/gate if the field's size exceeded four hectares. A field or sampling area was next visually subdivided into hectare blocks and a soil sample was taken from each block using a "cheese-corer" style auger with a half-cylindrical blade. Individual samples consisted of 25 cores (20cm depth x 2.5cm diameter), which were taken in a rectangular grid pattern and then mixed together. An additional 167 soil samples were collected from potato fields and sent to Harper Adams University (HAU) by external companies, agronomists and growers after being advised to and provided with the sampling guidance recommended by the OJEU in Directive 2007/33/EC. A further 69 fields were randomly selected from a national annual PCN survey conducted by Animal and Plant Health Agency (APHA) following sampling procedures recommended by the OJEU in Directive 2007/33/EC. Due to APHA policy no physical soil samples were provided for 96 fields included in the study and only an electronic data base with the survey results was provided. This latter approach was applied to the counties where an insufficient number of soil samples were collected due to the difficulties with contacting potato growers.

Data for the 500 ware potato fields were collected in the following proportions:

- 33% results were obtained from soil samples sent by agronomy companies originally collected for routine PCN testing;
- 53% results were obtained from soil samples directly collected from individual fields identified by growers and independent agronomists;
- 14% results were obtained (data only) from a national annual PCN survey conducted by APHA.

All soil samples were collected into cotton bags that were clearly labelled and sealed. Field details (agronomic factors) were recorded using a questionnaire (Appendix 7.1.) to investigate their effect on abundance and proportions of the PCN sibling species.

2.2.3. *Cysts extraction and collection*

Soil samples retained in cotton bags were air-dried at 25°C for at least 72 hours before being sieved through a 10mm aperture sieve. Each sample was then mixed and a sub-sample of 500g was taken.

Cyst extraction from the first 395 survey samples was performed at Science and Advice for Scottish Agriculture (SASA) where soil samples were processed using a custom MEKU nematode extraction carousel (Erich Pollähne, Germany); this apparatus allows automated sieving and flotation of organic material. The float materials were then transferred manually from the sieve onto filter papers, dried at 25°C and sent to HAU. Thirty six samples, which were collected in the final stages of the project, were extracted at HAU using the Fenwick can as described by Fenwick (1940) with an additional step of secondary flotation using a conical flask to reduce the volume of organic matter in the sample (Morgan, 1925). The float materials were kept in silk sachets and air-dried at 25°C.

All 431 dry extracts were individually transferred from filter papers and silk sachets onto a channelled aluminium counting tray and manually examined for PCN cysts under a binocular microscope at 30 x magnification (Shepherd, 1986). When present, cysts were hand extracted from float material using forceps and the number of cysts were recorded. The first 20 cysts, or fewer if sufficient number not present, were moved into 2ml safe-lock tubes or 1.5ml Eppendorf tubes for further analysis while remaining cysts were stored in glass vials.

2.2.4. *Potato cyst nematodes detection and species identification (SASA)*

The 2ml safe-lock tubes, with a maximum of 20 cysts, were sent to SASA where PCN species determination was performed using the second assay of the deoxyribonucleic acid (DNA) based method by Reid *et al*. (2015). The first assay, designed to screen all samples for the presence of PCN, was not performed in this study as all samples were suspected to be PCN. In brief, a commercial plant DNA extraction kit (BioSprint 96 DNA Plant Kit, Qiagen) was used to extract total DNA from cysts, before the extracts were subjected to species-specific real-time polymerase chain reaction (real-time PCR) to detect the presence of *G. pallida* and *G. rostochiensis* within each sample. A TaqMan *G. pallida* specific probe (5'-CCGCTATGTTTGGGC-3') labelled with FAM, *G. rostochiensis* specific probe (5'-CCGCTGTGTATKGGC-3') labelled with FAM and the flanking PCN primers, forward (5'-CGTTTGTTGTTGACGGACAYA-3') and reverse (5'-GGCGCTGTCCRTACATT GTTG-3'), were designed in the ITS1 region of the ribosomal deoxyribonucleic acid (rDNA).

2.2.5. *Potato cyst nematodes detection and species identification (HAU)*

Soil samples collected after species testing at SASA, which contained PCN-like cysts, were subjected to DNA extraction, PCN detection and species identification at HAU. To extract DNA, up to 20 cysts, collected from each float, were transferred into 1.5ml Eppendorf tubes. Any soil or organic matter adhering to the cysts was removed by washing in 1000µl of TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0) using a vortex. The buffer was removed and replaced by 150µl of fresh TE buffer and the cysts were homogenised with a sterile micropestle. A small quantity (*c*. 10mg) of activated carbon (Sigma-Aldrich) was applied to each tube and briefly vortexed. The tubes were boiled at 100°C for 5 minutes prior to vortexing for 30 seconds, centrifuged for 5 minutes at 12,000*g* and 100µl of supernatant from each tube was moved into a new tube taking care to not disturb the carbon pellet. All DNA extracts were stored at 4°C until investigated for PCN detection and species composition using a multiplex real-time PCR (Nakhla *et al*., 2010) with the following modifications:

- each sample was tested separately for presence of *G. pallida* and *G. rostochiensis* (tube A contained *G. pallida* specific primers and probe and tube B contained *G. rostochiensis* specific primers and probe);
- Takyon™ No Rox Probe Master Mix dTTP Blue (Eurogentec) was used with a modified PCR mixture composition (Table 2.2.);
- probe for *G. rostochiensis* detection was labelled with Yakima Yellow fluorescent dye;
- cycling parameters were changed (Table 2.3.).

Table 2.2. The composition of real-time PCR reaction mixture used to test survey samples for presence of *G. pallida* and *G. rostochiensis* (Nakhla *et al*., 2010 - modified).

Components	Volume per samples (µl)	Final concentration
Primer PITSpf or PGrtf	0.05	100pmol/µl
Primer PITSp4 or Prostor	0.05	100pmol/µl
Probe GFAMp or GYYp	0.025	100pmol/µl
Takyon Master Mix dTTP Blue	12.5	5.5mM MgCl2
PCR water	7.375	-
DNA template	5	-
Total	25	

Table 2.3. The cycling parameters for real-time PCR used to test survey samples for presence of *G. pallida* and *G. rostochiensis* (Nakhla *et al*., 2010 - modified).

	Temperature (°C)	Time (seconds)
Takyon Master Mix Activation	95	180
	40 cycles	
Denaturation	95	10
Annealing/Extension	58	60

The primers and probes were designed for amplification of rDNA ITS1 region of *Globodera* spp.; primers PITSpf (5'-ACGGACACATGCCCGCTA-3'), PITSp4 (5'-ACAACAGCAATCGTCGAG-3') and TaqMan probe GFAMp (5'-ACATGAGTGTT GGGGTGTAAC-3') labelled with FAM were used for the specific detection of *G. pallida*. Primers PGrtf (5'-TCTGTGCGTCGTTGAGC-3'), Prostor (5'-CGCAGACAT GCCGCAA-3') and TaqMan probe GYYp (5'-CGCAGATATGCTAACATGGA GTGTAG-3') labelled with Yakima Yellow were used for the specific detection of *G. rostochiensis*. Real-time PCR assays were performed in a Bio-Rad CFX-96 and analysed using the associated software. Pure *G. pallida* and *G. rostochiensis* DNA

extracts were provided by Fera Science Limited (Fera) and included in each test as positive controls.

2.2.6. *Potato cyst nematodes detection and species identification (APHA)*

Survey data provided by APHA were generated as follows. Each cyst that was previously extracted from dried soil using the Fenwick can method or the Wye washer method (Winfield *et al*., 1987), was dissected transversely. The eggs, or any J2 that emerged, were assessed visually for viability following the European and Mediterranean Plant Protection Organization (EPPO) diagnostic protocol (EPPO, 2017). The anterior portion of the cyst of those that were deemed to contain viable eggs were sent to Fera for molecular differentiation of *G. pallida* cysts from *G. rostochiensis* cysts. The posterior section was retained in case a molecular result needed to be confirmed by morphological assessment *e.g*. number of cuticular ridges between anus and vulval and basin Granek's ratio (Turner and Subbotin, 2013). If the cyst was deemed to contain no viable eggs or was empty, the material was not submitted for molecular analysis and recorded as *Globodera* spp. (Prior, 2014. Pers Comm. Mr T. Prior is the Senior Plant Nematologist at Fera Science Ltd.).

Deoxyribonucleic acid extractions were performed using QIAGEN DNeasy Blood and Tissue Kit following the manufacture's protocol for animal tissues with the following modifications. The samples were placed in a 1.5ml Eppendorf tube and homogenised with a micropestle in 180µl of ATL buffer. Next, 20µl of proteinase K was added, the samples vortexed and centrifuged briefly. The samples were then incubated at 56°C and 100rpm for at least three hours or overnight. The manufactures protocol was continued from step 3, and for the elution (steps 7 and 8), 60µl of buffer AE was used twice, giving DNA in a total volume of 120µl. Species

confirmation was achieved by performing a real-time TaqMan PCR developed and validated by Fera (EPPO, 2017).

2.2.7. *Scottish data*

A stratified survey was designed for Scotland in 2013 based on the total planted potato growing area (14,799ha) provided by the AHDB Potatoes - Grower Panel for market sectors: fresh bags, fresh chipping, pre-pack, processing and other ware. Counties with a planted potato growing area below 100 hectares (<0.7% of total) were excluded from the survey, which reduced the number of counties from 27 to 12. Based on the ratio between total planted potato growing area (ha) and survey samples collected for England and Wales (91,227ha/500 samples), 81 samples from Scotland were included in the survey. The samples were distributed accordingly across 12 counties (Table 2.4.) to express the differences in the production intensity following the calculations described in section 2.2.1.

Table 2.4. Area of potato plantings in Scotland (2013) as determined by the AHDB Potatoes and the number of samples requested in the stratified survey per county.

Country	County	Planted areas (ha)	Samples required	Samples obtained
Scotland				
	Aberdeenshire	605	3	3
	Angus	4,959	27	27
	Ayrshire	350	2	2
	Banffshire	87	0	0
	Berwickshire	862	5	5
	Caithness	13	0	0
	Clackmannanshire	8	0	0
	Dumfriesshire	51	0	0
	Dunbartonshire	7	0	0
	East Lothian	1,310	8	8
	Fife	2,322	13	13
	Inverness-shire	38	0	0
	Kincardineshire	322	2	0
	Kinross-shire	60	0	0
	Kirkcudbrightshire	33	0	0
	Lanarkshire	44	0	0
	Midlothian	150	1	1
	Moray	304	2	2
	Nairnshire	40	0	0
	Perthshire	2,092	12	12
	Renfrewshire	6	0	0
	Ross and Cromarty	384	2	0
	Roxburghshire	666	4	4
	Stirlingshire	2	0	0
	Sutherland	1	0	0
	West Lothian	11	0	0
	Wigtownshire	72	0	0
Total		14,799	81	77

Results of a statutory survey of ware potato land for years 2010-2015 (sampling years 2011-2016) were provided by SASA and included PCN detection and species identification data. Science and Advice for Scottish Agriculture, a statutory authority in Scotland, routinely tests at least 0.5% of the ware production area following potato harvest (OJEU, 2007). A randomising algorithm was applied to all fields, registered by potato growers, to select fields for inspection. Fields that are selected but located on farms tested in the previous year's survey are excluded

to ensure that any farm is not tested more than once in six years. Each field was sampled by taking 100 cores per hectare from a four hectares area which gives four samples per field (4x400ml/ha). All of these samples were treated individually and, after drying at 25°C, were subject to cysts extraction by using a custom MEKU nematode extraction carousel. The float materials were then transferred manually from the sieve onto filter papers and dried again at 25°C before being analysed by a molecular method to detect presence or absence of each species (Reid *et al*., 2015).

Due to the data protection policy requested results for fields in Ross and Cromarty (2) and Kincardine (2) could not be released and the four samples were not included. The analyses were carried out on remaining 77 data sets (Table 2.4.).

2.2.8. *Statistical analysis*

Survey data was analysed in Genstat (VSN International, 18th Edition) to determine whether agronomic factors (geographic origin, soil texture, previous crop grown, rotation length and history of PCN) had any impact on the occurrence of PCN. To consider the scenario when the response variable was not a scalar but a vector variable, general linear model (GLM) was selected as a most accurate statistical model and performed for each factor separately. Bernoulli distribution was chosen which takes the value 1 with probability p and the value 0 with probability q=1-p (yes-no question). The identity link function could be used as there was no transformation to the data set. Tested relationships between the occurrence of PCN and agronomic factors were always positive hence only strength of the relationships was reported.

2.3. Results

2.3.1. *Potato cyst nematodes detection (England and Wales)*

Analysis of 500 survey samples from England and Wales (Figure 2.2.; Appendix 7.2.) showed that 241 samples (48%) were infested with PCN. Cysts that were visually categorised as PCN, but not yet confirmed to be PCN by molecular assays (PCN-like cysts), were originally found in 250 survey samples (50%). However, real-time PCR assessment, performed on DNA extracts from these samples, did not detect *Globodera* spp. in nine samples, which resulted in the overall reduction in samples where PCN was detected.

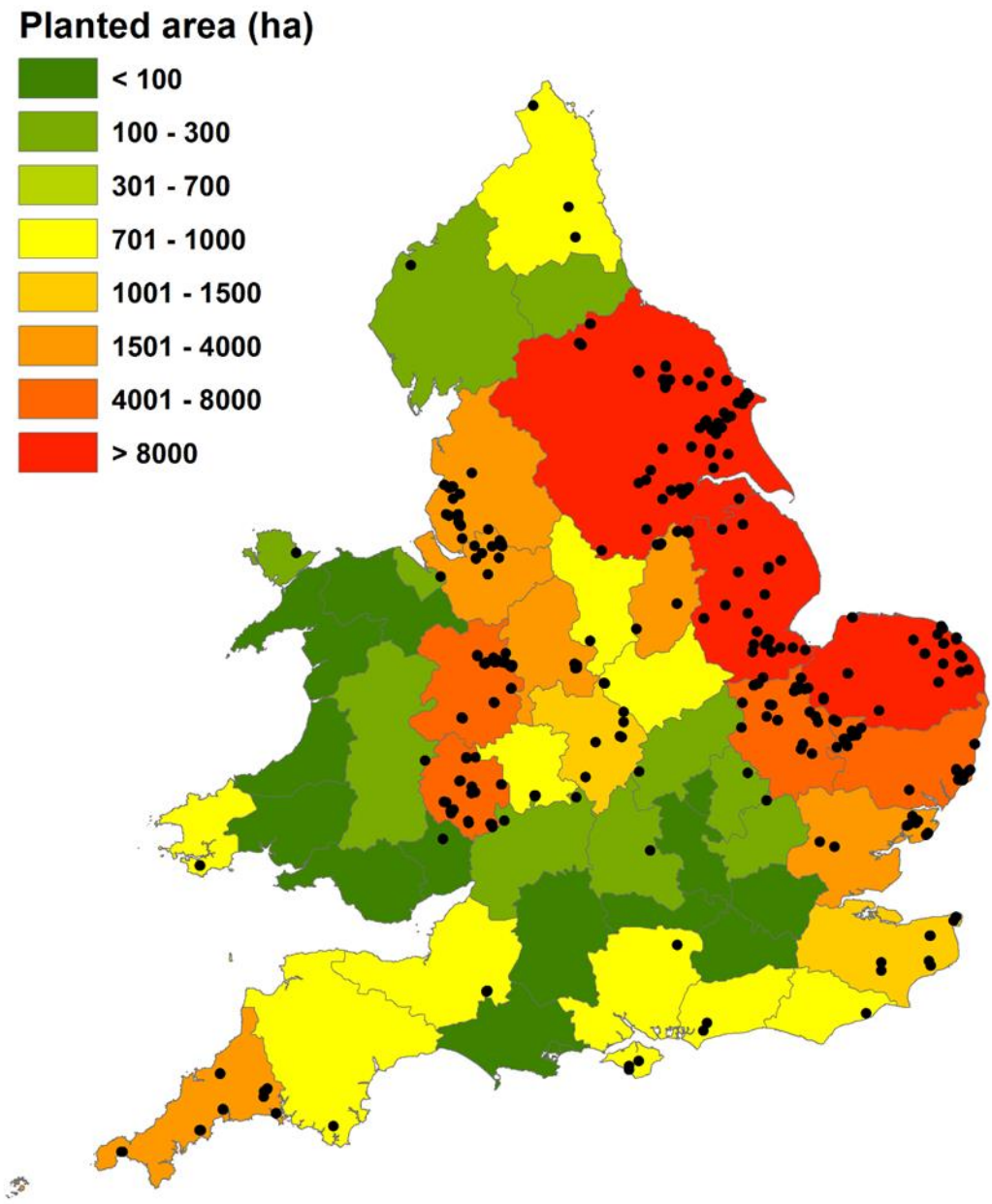


Figure 2.2. Distribution of fields selected for soil sample collection in a stratified PCN survey (England and Wales) based on the total potato growing area (ha).

Total collected survey samples were grouped depending on the location (laboratory) used for analysis (APHA, SASA or HAU) and the submission type (APHA, agronomy companies or growers/independent agronomists). Analyses did not confirm PCN presence in seven samples tested at SASA (1.7% of total analysed) and in two samples tested at HAU (5.5% of total analysed). *Globodera* spp. DNA was not detected in three samples submitted by agronomy companies (1.8% of total submitted) and in six samples submitted by growers/independent agronomists (2.3% of total submitted). Potato cyst nematodes detection was lowest when conducted at APHA (22%) and at similar levels when conducted at SASA and HAU, 53 and 47%, respectively. The proportion of survey samples submitted by agronomy companies and growers/independent agronomists found to contain PCN were 53 and 52%, respectively (Table 2.5.).

Table 2.5. Survey samples containing cysts first visually assessed as PCN (PCN-like) and later confirmed as PCN infested (PCN detected) for survey samples from England and Wales grouped depending on the laboratory used for the analysis and the submission type.

	Total samples	PCN-like	PCN detected (% analysed/submitted)	PCN detected (% total survey samples)
Analysed at:				
APHA	69	15	15 (22)	3
SASA	395	216	209 (53)	42
HAU	36	19	17 (47)	3
Total	500	250	241 (48)	48
Submitted by:				
APHA	69	15	15 (22)	3
Agronomy companies	167	92	89 (53)	18
Growers/independent agronomists	264	143	137 (52)	27
Total	500	250	241 (48)	48

The results from the survey samples were assigned to eight regions of England (East Midlands, East of England, North East, North West, South East, South West, West Midlands and Yorkshire and the Humber) and Wales according to the county grouping presented in Table 2.1. In the East Midlands and North West, PCN was detected in 62 and 70% of the sites tested, respectively. In the remaining regions, the number of infested sites did not exceed 50%. The lowest proportion of infested sites per county occurred in the South West where only 13% of samples were determined to be PCN infested. Across all the eight regions of England and Wales, the mean proportion of PCN infested was 48% (Table 2.6.).

Table 2.6. Survey samples undetected or detected as PCN infested from eight regions of England and Wales.

Country	Region	Total samples	Samples with PCN undetected	Samples with PCN undetected (% total samples)	Samples with PCN detected	Samples with PCN detected (% total samples)
England						
	East Midlands	90	34	38	56	62
	East England	175	90	51	85	49
	North East	5	3	60	2	40
	North West	30	9	30	21	70
	South East	17	10	59	7	41
	South West	30	26	87	4	13
	West Midlands	81	42	52	39	48
	Yorkshire and the Humber	64	39	61	25	39
Wales		8	6	75	2	25
Total		500	259	52	241	48

The highest number of samples by region originated from the East of England and East Midlands, where the PCN infested sites made up 35 and 23% of the total number of infested sites, respectively, although 49 and 62% of samples from these regions were infested. The lowest number of samples by region originated from the North East and Wales, which each contributed 1% to the total number of infested sites (Figure 2.3.).

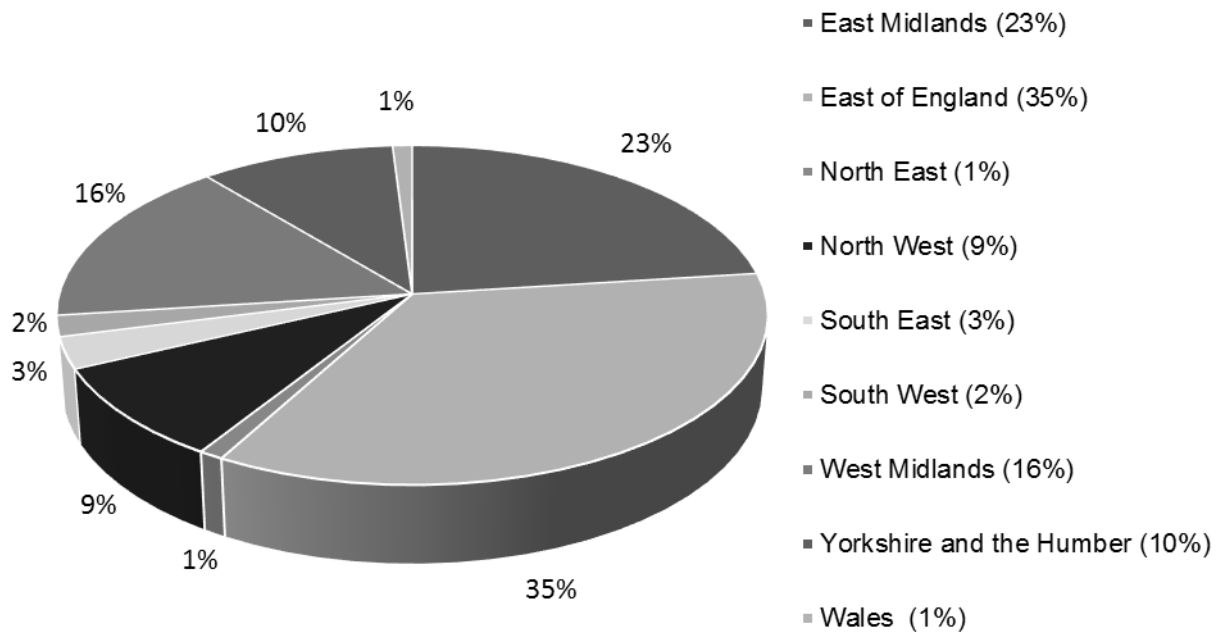


Figure 2.3. The percentage of survey samples detected as PCN infested from eight regions of England and Wales presented as a percentage of the total PCN detected survey samples in England and Wales.

2.3.2. *Species identification (England and Wales)*

The 250 survey samples from England and Wales (Appendix 7.2.) that contained PCN-like cysts were tested to determine the species of PCN. Identification was made by real-time PCR on DNA extracts. The molecular assay did not detect *Globodera* spp. in nine of the samples with PCN-like cysts. These samples were included in the samples not determined as PCN infested. The results for the remaining 241 DNA extracts, where PCN identification was successful, showed that 15 samples (6%) were mixed populations (contained both species), 214 samples (89%) contained pure *G. pallida* and 12 samples (5%) contained pure *G. rostochiensis* populations.

Soil samples containing both species were identified in only four regions of England (East Midlands, East of England, South East and West Midlands). No mixed populations were identified in Wales. Pure *G. pallida* populations were identified in all eight regions of England and in Wales in various proportions. The North East, North West, South West and Yorkshire and Humber were regions where 100% of the infested samples collected were identified as pure *G. pallida* populations. Regions where mixed populations were identified (East Midlands, East of England, South East and West Midlands) were also the only regions where pure *G. rostochiensis* were found. Pure *G. rostochiensis* populations were not found in the samples from Wales (Table 2.7.).

Regional distribution of mixed populations analysed as a percentage of the total survey samples containing mixed populations (Figure 2.4.) shows that the East of England dominates (67%) over the remaining three regions (East Midlands, South East and West Midlands). Over 50% of the pure *G. pallida* populations, when presented as a percentage of total survey samples identified as pure *G. pallida* populations, can be found in the regions of East Midlands and East of England, with 24 and 31% contributions, respectively (Figure 2.5.). Samples identified as pure *G. rostochiensis* populations were mainly identified in samples originating from the region of East of England that, as a percentage of total survey samples identified as pure *G. rostochiensis* populations, contributed 67% to the overall number of sites infested with this species (Figure 2.6.).

Table 2.7. Survey samples containing both species (mixed), pure *G. pallida* (Pa) and pure *G. rostochiensis* populations (Ro) from eight regions of England and Wales.

Country	Region	Total samples	PCN detected	Mixed	Mixed (% total samples)	Mixed (% PCN detected)	Pa	Pa (% total samples)	Pa (% PCN detected)	Ro	Ro (% total samples)	Ro (% PCN detected)
England												
	East Midlands	90	56	3	3	5	52	58	93	1	1	2
	East England	175	85	10	6	12	67	38	79	8	5	9
	North East	5	2	0	0	0	2	40	100	0	0	0
	North West	30	21	0	0	0	21	70	100	0	0	0
	South East	17	7	1	6	14	5	29	71	1	6	14
	South West	30	4	0	0	0	4	13	100	0	0	0
	West Midlands	81	39	1	1	3	36	44	92	2	2	5
	Yorkshire and the Humber	64	25	0	0	0	25	39	100	0	0	0
Wales		8	2	0	0	0	2	25	100	0	0	0
Total		500	241	15	3	6	214	43	89	12	2	5

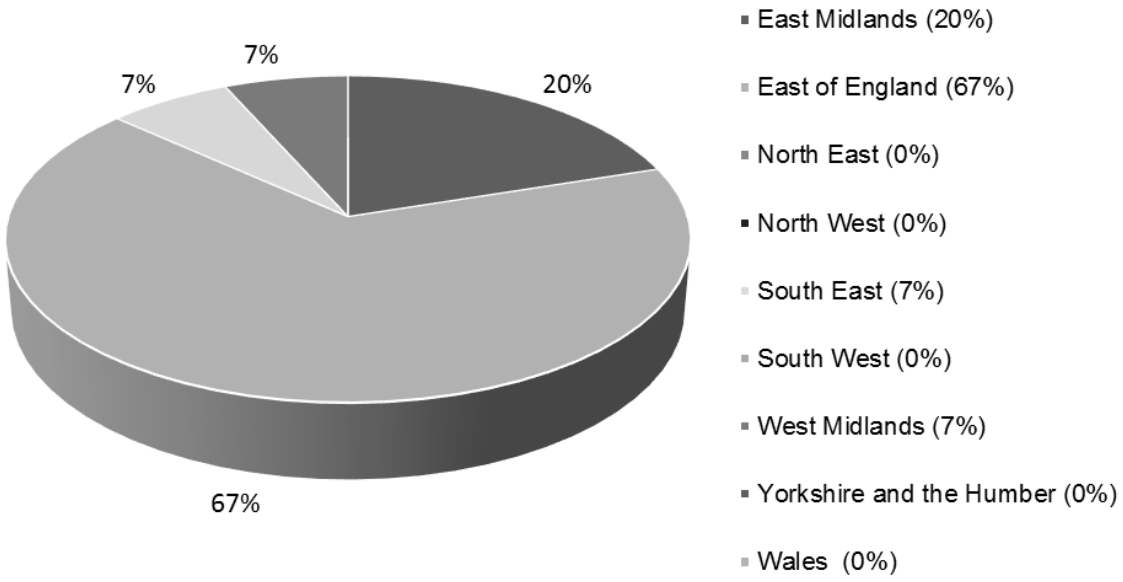


Figure 2.4. Survey samples containing PCN populations with both species (mixed) from eight regions of England and Wales presented as a percentage of the total survey samples identified as mixed populations.

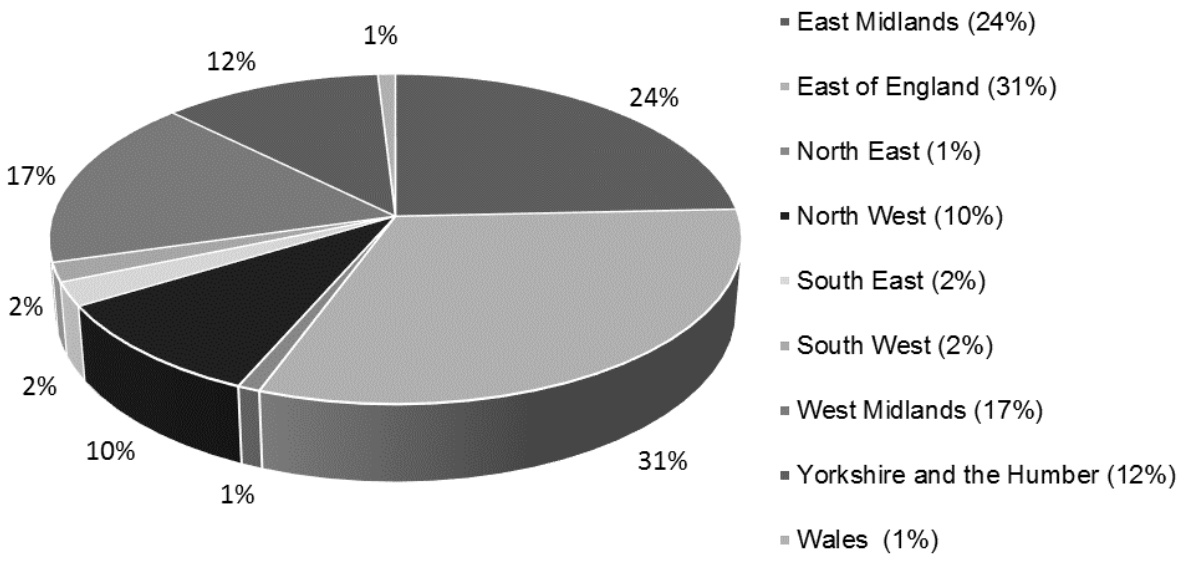


Figure 2.5. Survey samples containing PCN populations with pure *G. pallida* from eight regions of England and Wales presented as a percentage of total survey samples identified as pure *G. pallida* populations.

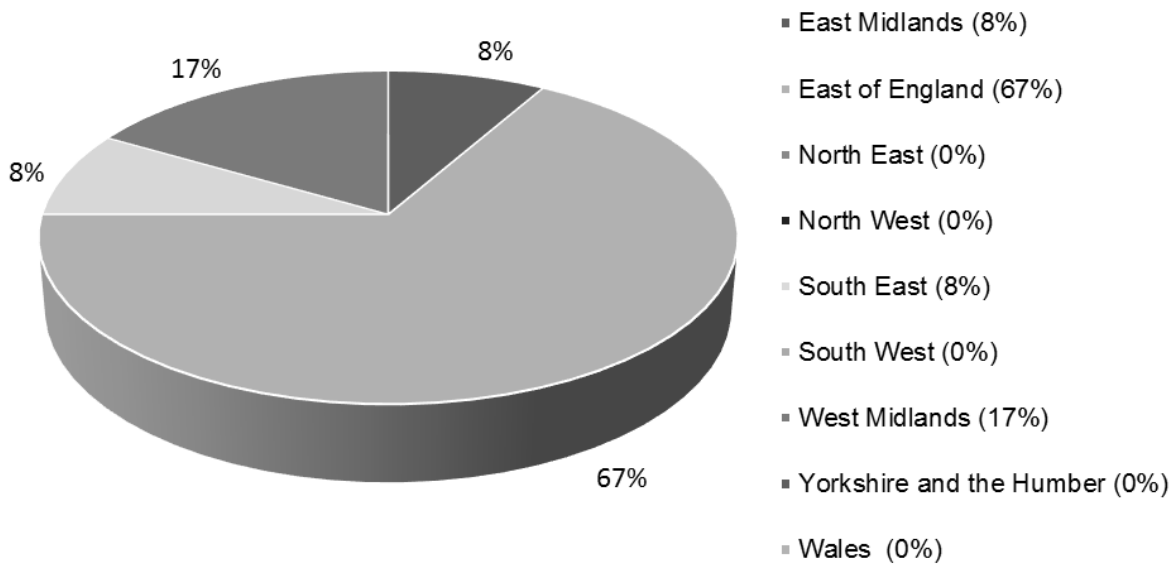
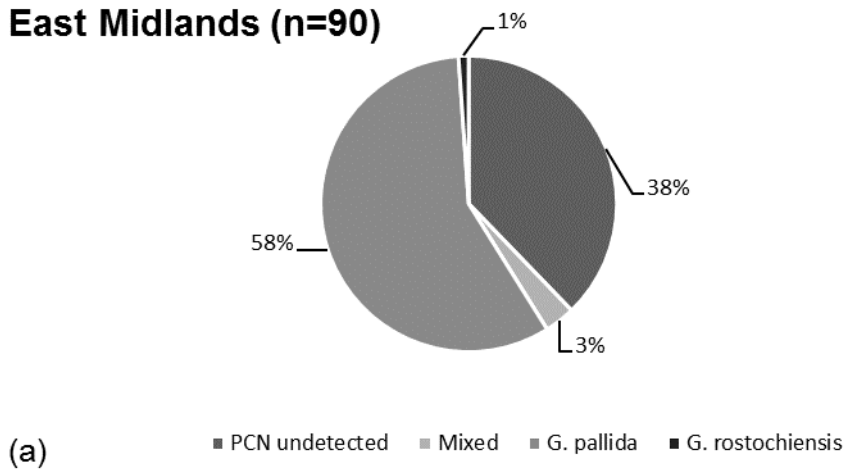
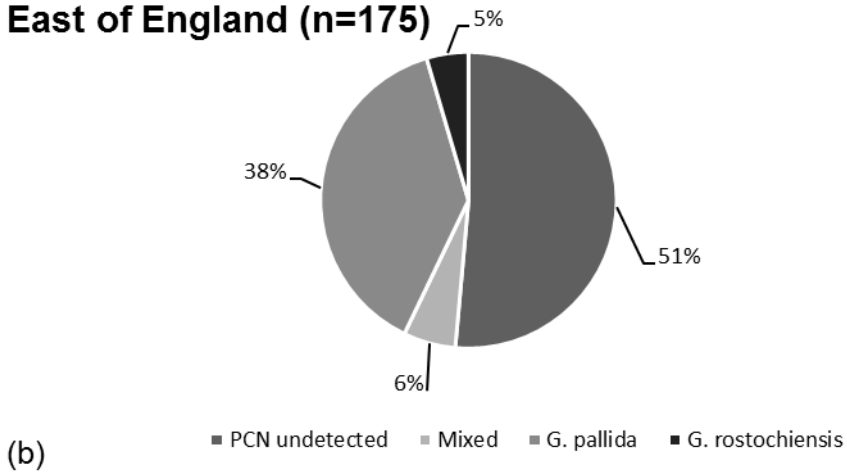


Figure 2.6. Survey samples containing PCN populations with pure *G. rostochiensis* from eight regions of England and Wales presented as a percentage of total survey samples identified as pure *G. rostochiensis* populations.

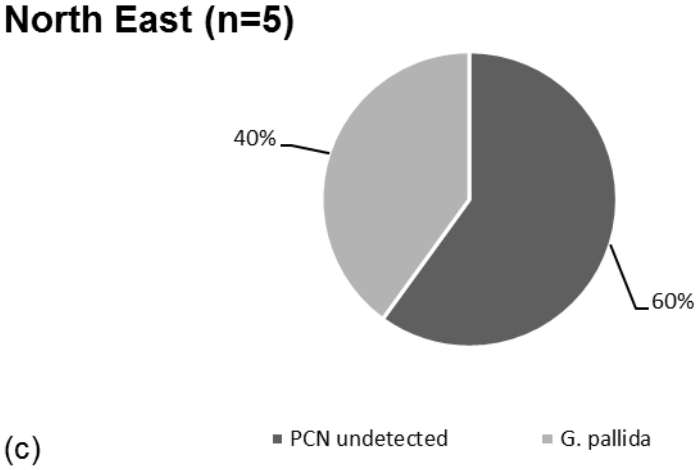
Potato cyst nematodes detection across all English regions and Wales varied from 13% in South West to 70% in North West (average 43%). In all regions, *G. pallida* was the predominant species or, as in North East, North West, South West, Yorkshire and the Humber and Wales, the only species detected. Generally in the regions where *G. rostochiensis* was detected as pure or mixed populations, (East Midlands, East of England, South East and West Midlands) it was found only in a low proportion of sites (1 to 6%). Survey data from regions of England and Wales as a percentage of samples where PCN was undetected and detected, identified as mixed populations, pure *G. pallida* and pure *G. rostochiensis* populations are presented in Figure 2.7.



(a)



(b)



(c)

Figure 2.7. The proportion of survey samples collected from East Midlands (a), East of England (b), North East (c), North West (d), South East (e), South West (f), West Midlands (g), Yorkshire and the Humber (h), Wales (i) and England (eight regions) (j) where PCN was detected or undetected and the proportion of samples containing PCN populations with both species (mixed), pure *G. pallida* and pure *G. rostochiensis*.

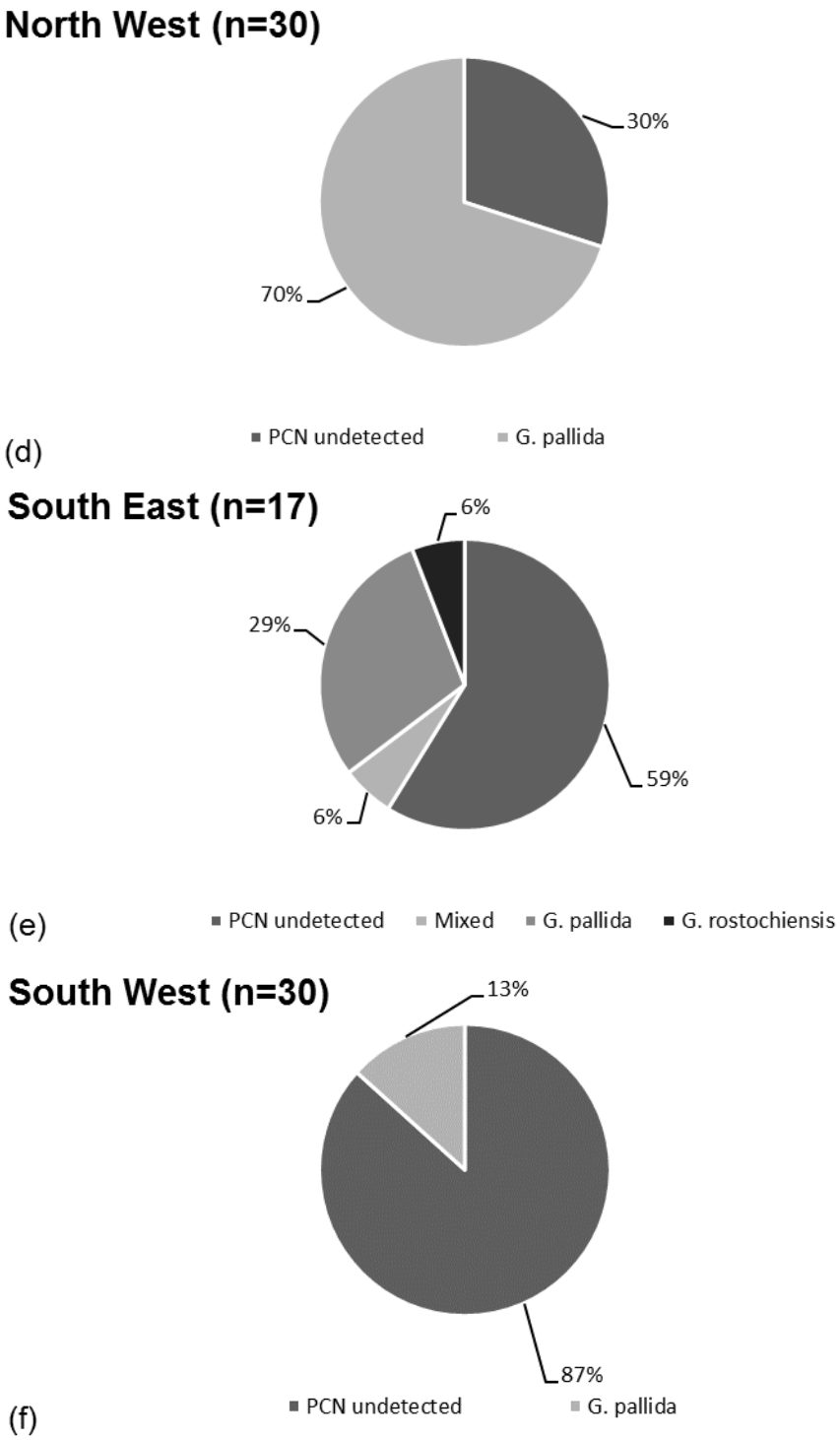


Figure 2.7. The proportion of survey samples collected from East Midlands (a), East of England (b), North East (c), North West (d), South East (e), South West (f), West Midlands (g), Yorkshire and the Humber (h), Wales (i) and England (eight regions) (j) where PCN was detected or undetected and the proportion of samples containing PCN populations with both species (mixed), pure *G. pallida* and pure *G. rostochiensis* (Continuation).

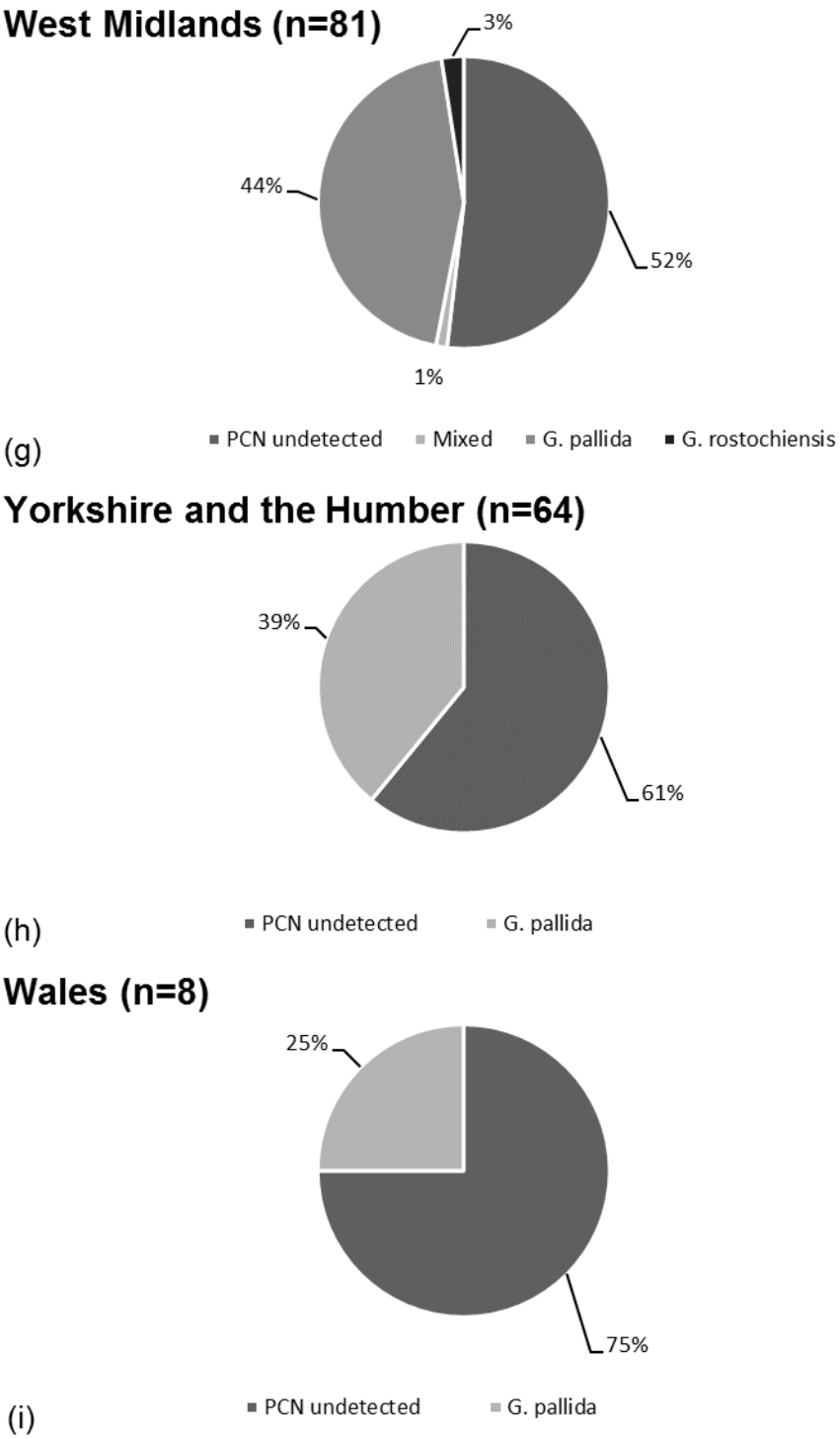
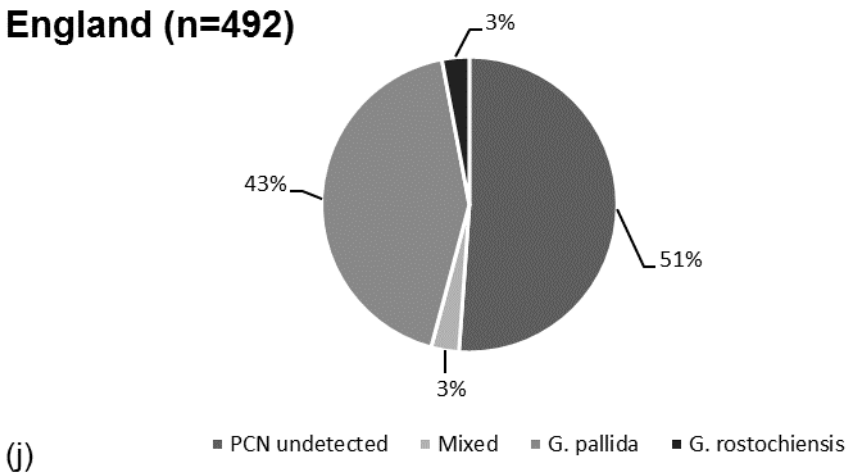


Figure 2.7. The proportion of survey samples collected from East Midlands (a), East of England (b), North East (c), North West (d), South East (e), South West (f), West Midlands (g), Yorkshire and the Humber (h), Wales (i) and England (eight regions) (j) where PCN was detected or undetected and the proportion of samples containing PCN populations with both species (mixed), pure *G. pallida* and pure *G. rostochiensis* (Continuation).



(j)

Figure 2.7. The proportion of survey samples collected from East Midlands (a), East of England (b), North East (c), North West (d), South East (e), South West (f), West Midlands (g), Yorkshire and the Humber (h), Wales (i) and England (eight regions) (j) where PCN was detected or undetected and the proportion of samples containing PCN populations with both species (mixed), pure *G. pallida* and pure *G. rostochiensis* (Continuation).

2.3.3. *Species distribution (England and Wales)*

Table 2.8. provides a summary of the samples collected in each county, the number and proportion of samples where PCN was detected or undetected and the species composition. From the 49 counties originally listed by the AHDB Potatoes - Grower Panel, 34 were investigated for PCN infestation and species distribution (30 in England and 4 in Wales). Counties of Bedfordshire (total number of samples; n=1), Durham (n=2) and Flintshire (n=1) were the only counties where all samples were confirmed to be PCN infested. In contrast, counties of Derbyshire (n=2), Leicestershire (n=3), Northamptonshire (n=1), Hertfordshire (n=1), Northumberland (n=3), Cumberland (n=1), Oxfordshire (n=1), Sussex (n=3), Devon (n=4), Gloucestershire (n=1), Somerset (n=5) in England and Anglesey (n=1) and Radnor (n=1) in Wales were not found to have PCN infested sites. Samples detected as

containing mixed populations were only identified in eight English counties: Lincolnshire (n=70), Nottinghamshire (n=14), Cambridgeshire (n=42), Essex (n=14), Norfolk (n=83), Suffolk (n=34), Kent (n=8) and Worcestershire (n=5). The same counties, except Worcestershire, were additionally confirmed to have fields infested with pure *G. pallida* populations. The counties of Lincolnshire, Suffolk, Kent and Worcestershire were also reported with pure *G. rostochiensis* populations which were also found in Bedfordshire and Shropshire (n=31). As a pure or mixed population *G. pallida* was found in all counties with PCN infested sites with one questionable exception. Bedfordshire was the only county where *G. pallida* was not identified but here only one sample (identified as *G. rostochiensis*) was tested which strongly limited the chance of *G. pallida* detection.

The location of fields from which survey samples were obtained (Figure 2.2.) highlights the differences in the intensity of sampling in various counties that are the result of the stratified survey design based on the intensity of ware potato production. Figure 2.8. shows sites where PCN were detected and Figure 2.9. shows sites where PCN were not detected. The distribution of the fields identified with pure *G. pallida* or containing *G. pallida* as a mixed population is presented in Figure 2.10. Subsequently Figure 2.11. shows the distribution of the fields with pure *G. rostochiensis* or containing *G. rostochiensis* as part of a mixed population. Finally, Figure 2.12. shows the distribution of the fields with mixed populations. Figures 2.8. – 2.12. serve as a graphical visualisation of the numerical results presented in Table 2.8. and discussed above.

Table 2.8. Survey samples collected from counties in England and Wales where PCN was detected or undetected and the number of samples containing PCN populations with both species (mixed), pure *G. pallida* (Pa) and pure *G. rostochiensis* (Ro).

Country	Region	County	Total samples	PCN undetected	PCN undetected (% total	PCN detected	PCN detected (% total samples)	Mixed	Pa	Ro
England										
	East Midlands	Derbyshire	2	2	100	0	0	0	0	0
		Leicestershire	3	3	100	0	0	0	0	0
		Lincolnshire	70	20	29	50	71	2	47	1
		Northamptonshire	1	1	100	0	0	0	0	0
		Nottinghamshire	14	8	57	6	43	1	5	0
		Rutland	0	—	—	—	—	—	—	—
	East of England	Bedfordshire	1	0	0	1	100	0	0	1
		Cambridgeshire	42	18	43	24	57	1	23	0
		Essex	14	9	64	5	36	1	4	0
		Hertfordshire	1	1	100	0	0	0	0	0
		Norfolk	83	43	52	40	48	6	34	0
		Suffolk	34	19	56	15	44	2	6	7
	North East	Durham	2	0	0	2	100	0	2	0
		Northumberland	3	3	100	0	0	0	0	0
	North West	Cheshire	12	2	17	10	83	0	10	0
		Cumberland	1	1	100	0	0	0	0	0
		Lancashire	17	6	35	11	65	0	11	0
		Westmorland	0	—	—	—	—	—	—	—
	South East	Buckinghamshire	0	—	—	—	—	—	—	—
		Hampshire	5	2	40	3	60	0	3	0
		Isle of Wight	0	—	—	—	—	—	—	—
		Kent	8	4	50	4	50	1	2	1
		Oxfordshire	1	1	100	0	0	0	0	0
		Surrey	0	—	—	—	—	—	—	—
		Sussex	3	3	100	0	0	0	0	0
	South West	Cornwall	20	16	80	4	20	0	4	0
		Devon	4	4	100	0	0	0	0	0
		Dorset	0	—	—	—	—	—	—	—
		Gloucestershire	1	1	100	0	0	0	0	0
		Somerset	5	5	100	0	0	0	0	0
		Wiltshire	0	—	—	—	—	—	—	—
	West Midlands	Herefordshire	25	21	84	4	16	0	4	0
		Shropshire	31	6	19	25	81	0	24	1
		Staffordshire	12	5	42	7	58	0	7	0
		Warwickshire	8	7	88	1	13	0	1	0
		Worcestershire	5	3	60	2	40	1	0	1
	Yorkshire and the Humber	Yorkshire	64	39	61	25	39	0	25	0
Wales										
		Anglesey	1	1	100	0	0	0	0	0
		Brecon	0	—	—	—	—	—	—	—
		Caernarvonshire	0	—	—	—	—	—	—	—
		Cardigan	0	—	—	—	—	—	—	—
		Carmarthenshire	0	—	—	—	—	—	—	—
		Denbighshire	0	—	—	—	—	—	—	—
		Flintshire	1	0	0	1	100	0	1	0
		Glamorgan	0	—	—	—	—	—	—	—
		Monmouth	0	—	—	—	—	—	—	—
		Montgomery	0	—	—	—	—	—	—	—
		Pembroke	5	4	80	1	20	0	1	0
		Radnor (Powys)	1	1	100	0	0	0	0	0
Total			500	259	52	241	48	15	214	12

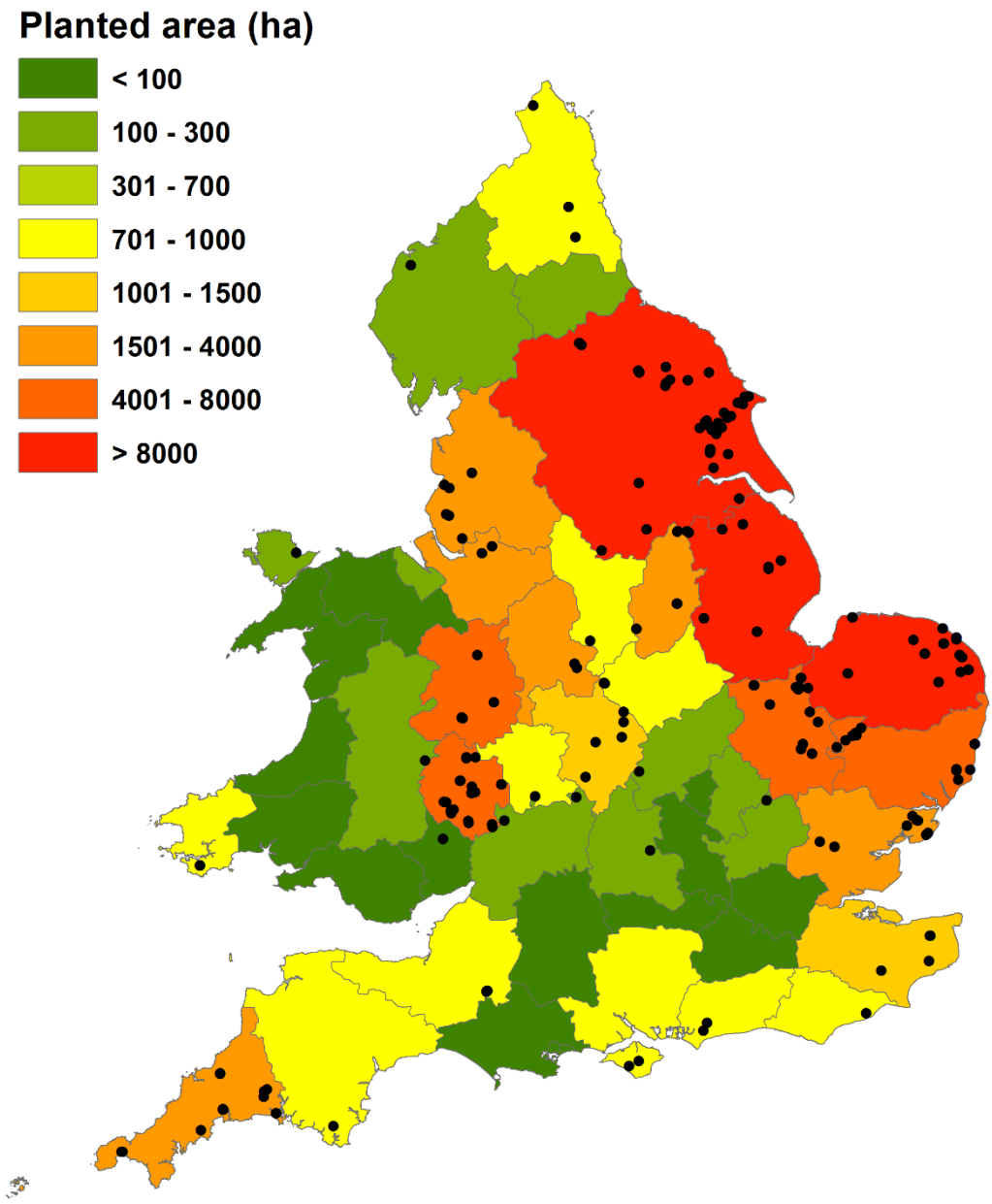


Figure 2.8. Distribution of the fields undetected as PCN infested in a stratified PCN survey (England and Wales) based on the total potato growing area (ha).

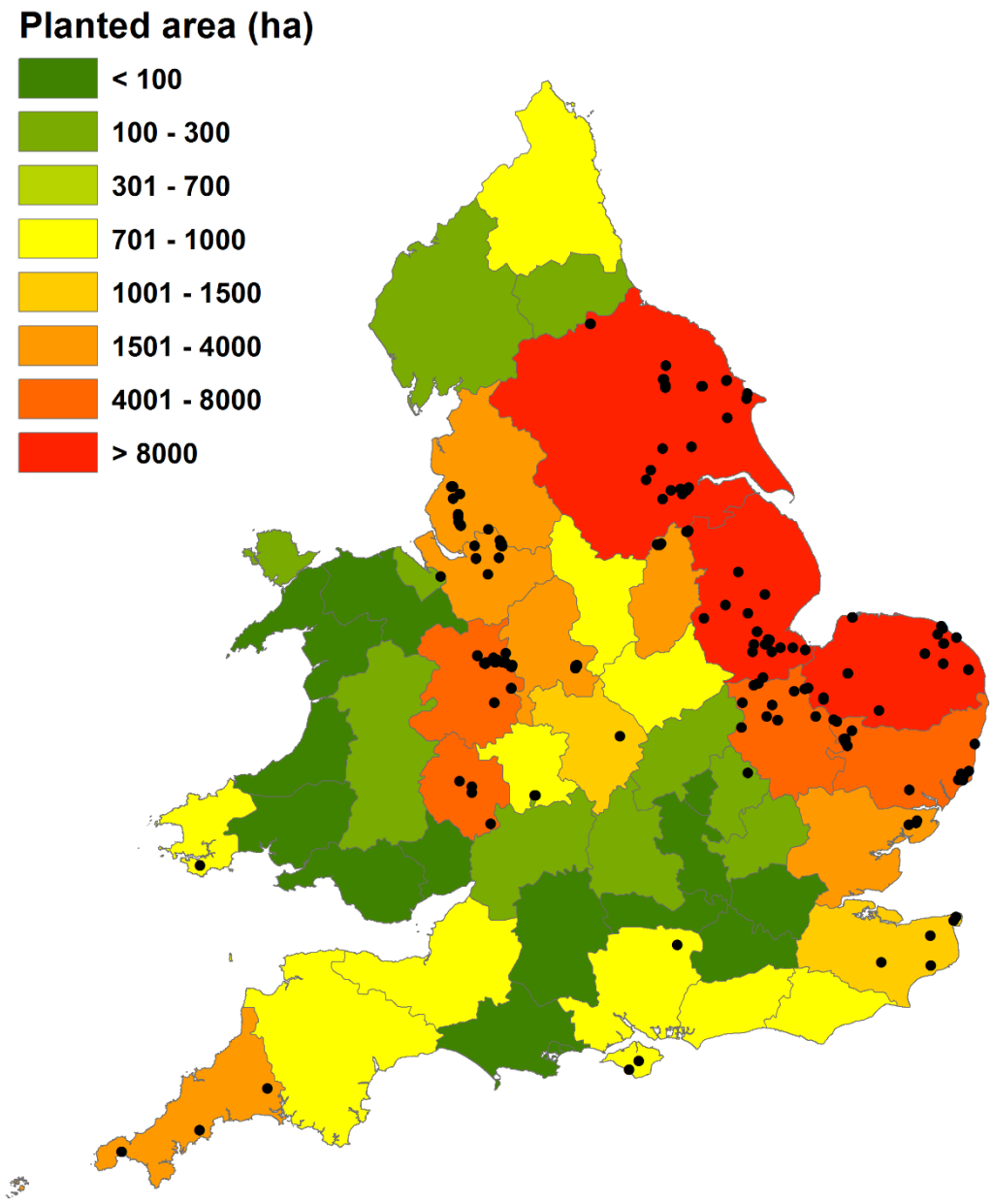


Figure 2.9. Distribution of the fields detected as PCN infested in a stratified PCN survey (England and Wales) based on the total potato growing area (ha).

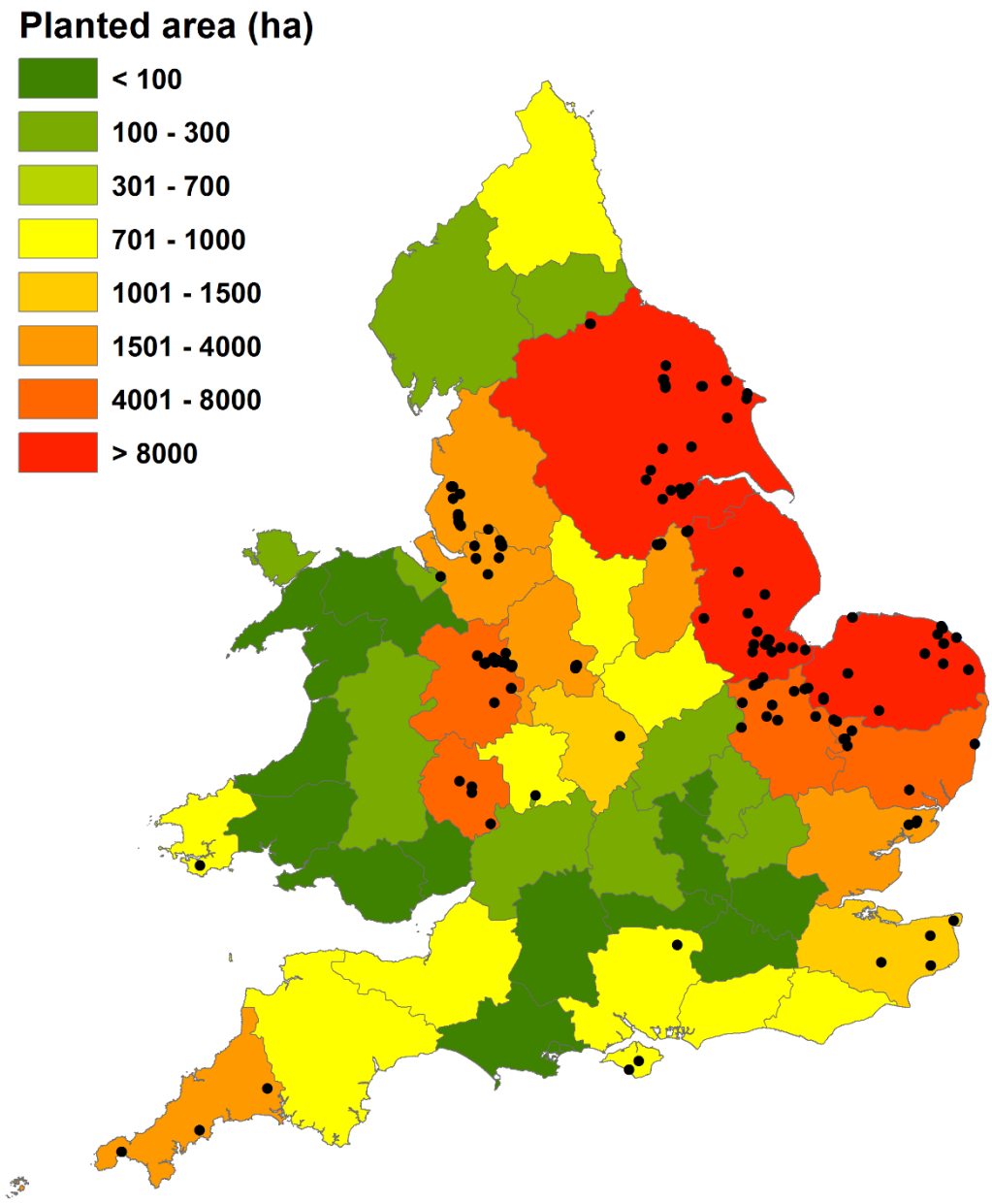


Figure 2.10. Distribution of the fields detected as PCN infested containing *G. pallida* (as a pure and mixed) populations in a stratified PCN survey (England and Wales) based on the total potato growing area (ha).

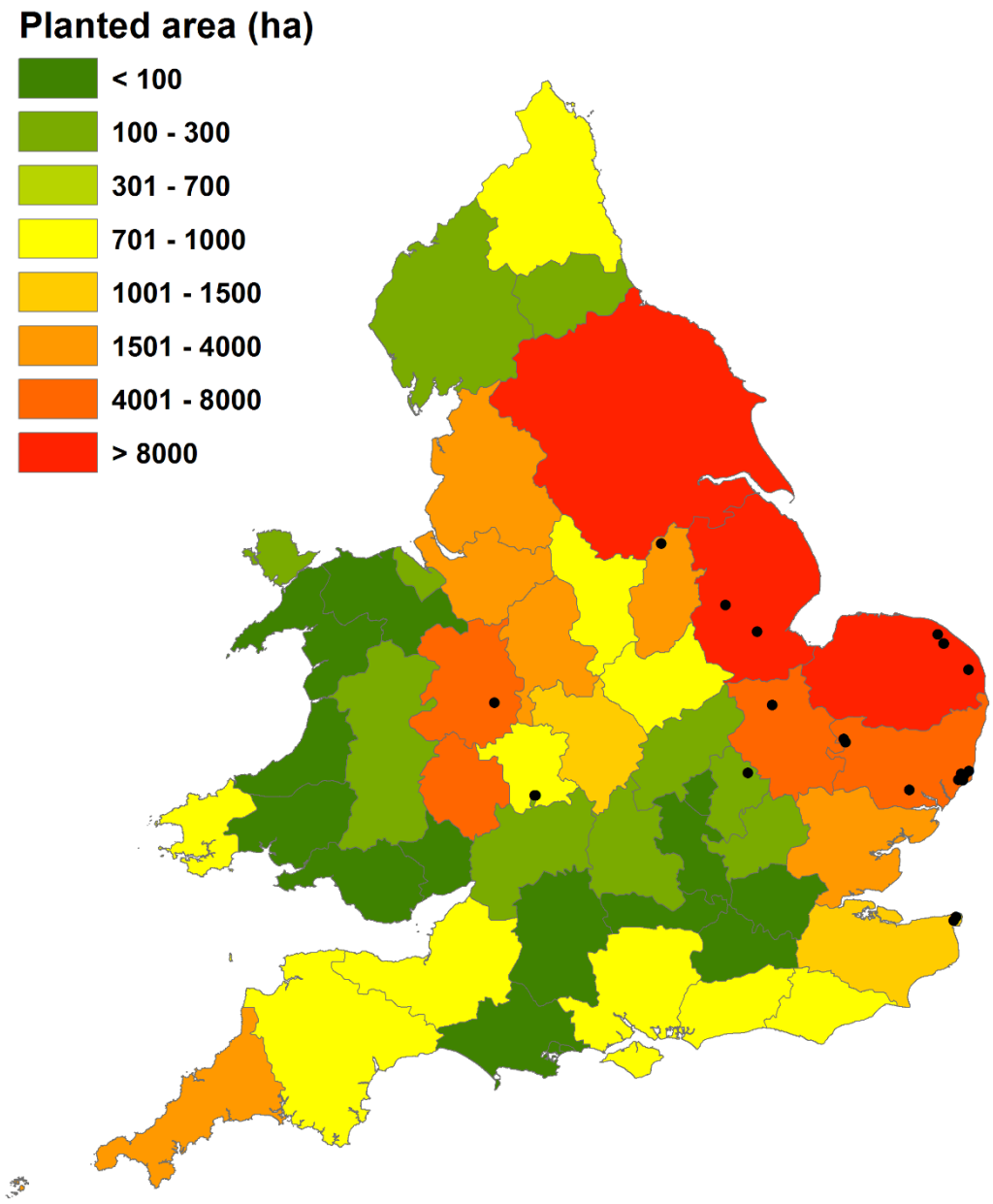


Figure 2.11. Distribution of the fields detected as PCN infested containing *G. rostochiensis* (as a pure and mixed) populations in a stratified PCN survey (England and Wales) based on the total potato growing area (ha).

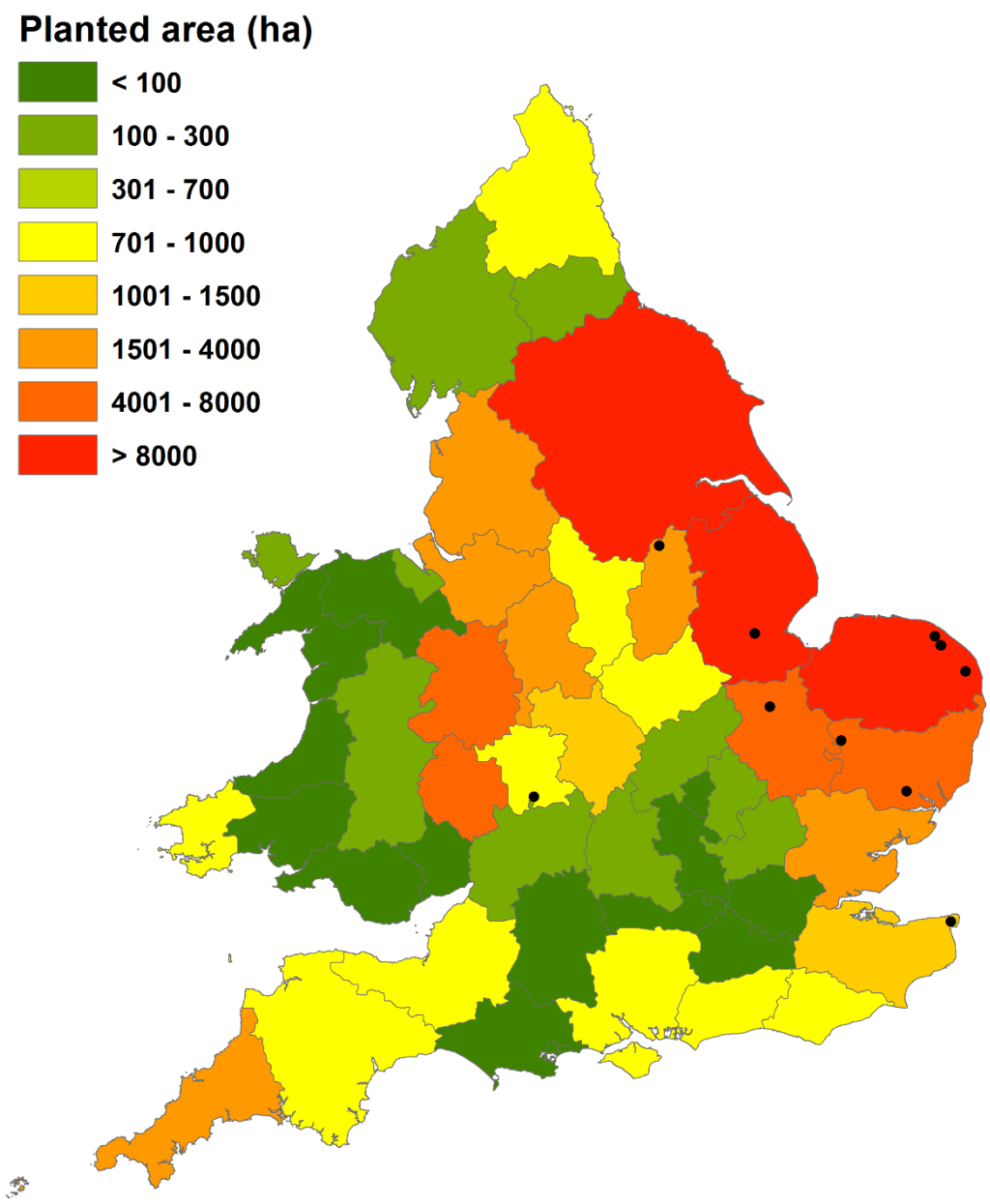


Figure 2.12. Distribution of the fields detected as PCN infested containing mixed (*G. pallida* and *G. rostochiensis*) populations in a stratified PCN survey (England and Wales) based on the total potato growing area (ha).

2.3.4. *Potato cyst nematodes detection and species identification (Scotland)*

Data provided by SASA for 77 soil samples from Scotland showed that 29 samples (38%) were determined to be PCN infested.

Angus was shown to provide the highest proportion of PCN infested samples (34% of total infested) while the lowest number of samples originated evenly between counties of Aberdeenshire and Roxburghshire (3%) (Figure 2.13.). The 29 samples found to contain PCN were comprised of four samples (14%) with mixed populations, 17 samples (59%) containing pure *G. pallida* and eight samples (27%) containing pure *G. rostochiensis* populations.

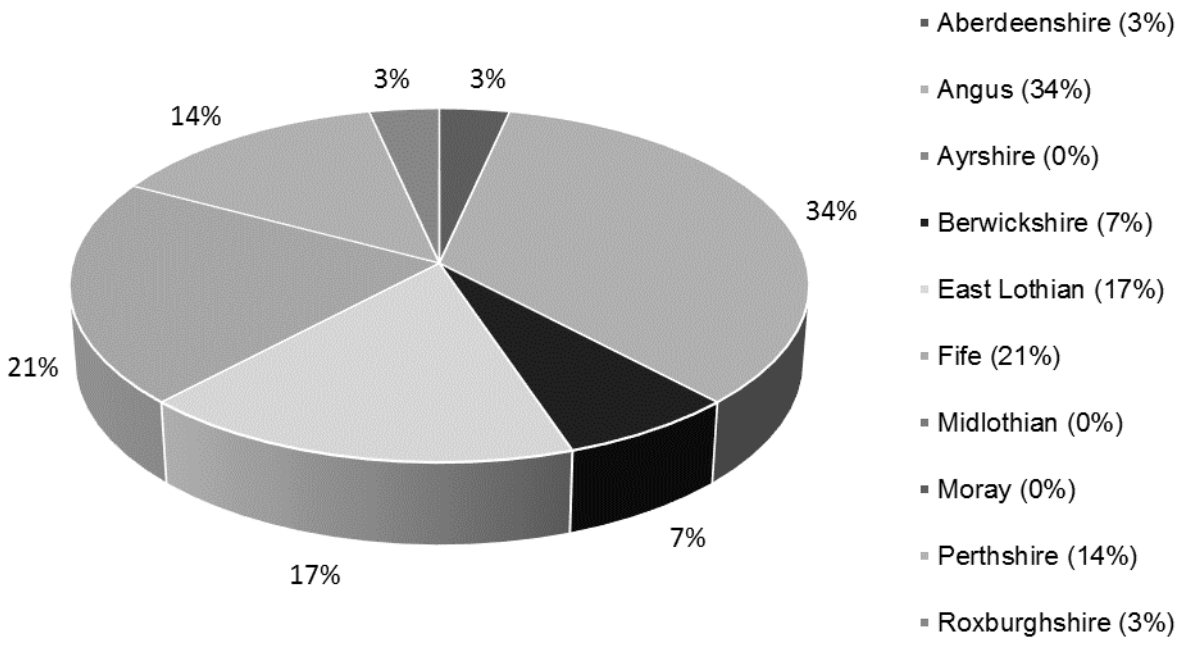


Figure 2.13. Survey samples determined to be PCN infested from counties of Scotland presented as a percentage of the total survey samples containing PCN in Scotland.

All tested samples were collected from 10 counties of Scotland but PCN infested samples were only detected from sites originating in seven counties where detection varied from 63% in East Lothian to 25% in Roxburghshire. The counties of Ayrshire, Midlothian and Moray were found to be PCN free. Samples from Aberdeenshire contained only mixed populations. Counties with samples containing single species only were Roxburghshire (*G. pallida*), Perthshire (*G. rostochiensis*) and Berwickshire (*G. pallida* and *G. rostochiensis*). Samples containing pure *G. pallida* populations were found in East Lothian, but there were also mixed populations present. Samples from two counties, Angus and Fife, were identified with all three population types (Table 2.9.).

Table 2.9. Survey sample data from counties in Scotland where PCN was detected or undetected and the number of samples containing PCN populations with both species (mixed), pure *G. pallida* (Pa) and pure *G. rostochiensis* (Ro).

Country	County	Total samples	PCN undetected	PCN undetected (% total samples)	PCN detected	PCN detected (% total samples)	Mixed	Pa	Ro
Scotland									
	Aberdeenshire	3	2	67	1	33	1	0	0
	Angus	27	17	63	10	37	1	8	1
	Ayrshire	2	2	100	0	0	0	0	0
	Banffshire	0	—	—	—	—	—	—	—
	Berwickshire	5	3	60	2	40	0	1	1
	Caithness	0	—	—	—	—	—	—	—
	Clackmannanshire	0	—	—	—	—	—	—	—
	Dumfriesshire	0	—	—	—	—	—	—	—
	Dunbartonshire	0	—	—	—	—	—	—	—
	East Lothian	8	3	38	5	63	1	4	0
	Fife	13	7	54	6	46	1	3	2
	Inverness-shire	0	—	—	—	—	—	—	—
	Kincardineshire	0	—	—	—	—	—	—	—
	Kinross-shire	0	—	—	—	—	—	—	—
	Kirkcudbrightshire	0	—	—	—	—	—	—	—
	Lanarkshire	0	—	—	—	—	—	—	—
	Midlothian	1	1	100	0	0	0	0	0
	Moray	2	2	100	0	0	0	0	0
	Nairnshire	0	—	—	—	—	—	—	—
	Perthshire	12	8	67	4	33	0	0	4
	Renfrewshire	0	—	—	—	—	—	—	—
	Ross and Cromarl	0	—	—	—	—	—	—	—
	Roxburghshire	4	3	75	1	25	0	1	0
	Stirlingshire	0	—	—	—	—	—	—	—
	Sutherland	0	—	—	—	—	—	—	—
	West Lothian	0	—	—	—	—	—	—	—
	Wigtownshire	0	—	—	—	—	—	—	—
Total		77	48	62	29	38	4	17	8

2.3.5. *Potato cyst nematodes occurrence and agronomic factors (England and Wales)*

Survey data (Appendix 7.2.) was analysed by general linear model (GLM) to determine whether agronomic factors (geographic origin, soil texture, previous crop grown, rotation length and history of PCN) had any impact on the occurrence of PCN. There were highly variable numbers of samples for each factor level and as such, some factor levels with few samples had high levels of calculated variance compared to those with many samples.

2.3.5.1. G*eographic origin*

All survey samples were allocated, according to their geographic origin, to eight regions of England (East Midlands, East of England, North East, North West, South East, South West, West Midlands and Yorkshire and the Humber) and Wales. The data were analysed by general linear model (GLM) to determine whether there was a relationship between the occurrence of PCN and the geographic origin. The results showed that there was a highly significant relationship ($F=4.24$, $P<0.001$), but only accounted for 6% of the variance, between the detection of PCN and the geographic origin. Due to the stratified design of the survey based on ware potato production, some regions have low numbers of samples and consequently have large standard errors (*e.g*. North East and Wales) (Figure 2.14.). The North West had the highest, 70% PCN occurrence, in contrast with South West where this occurrence was only 13%. Other regions exhibited levels between these two values.

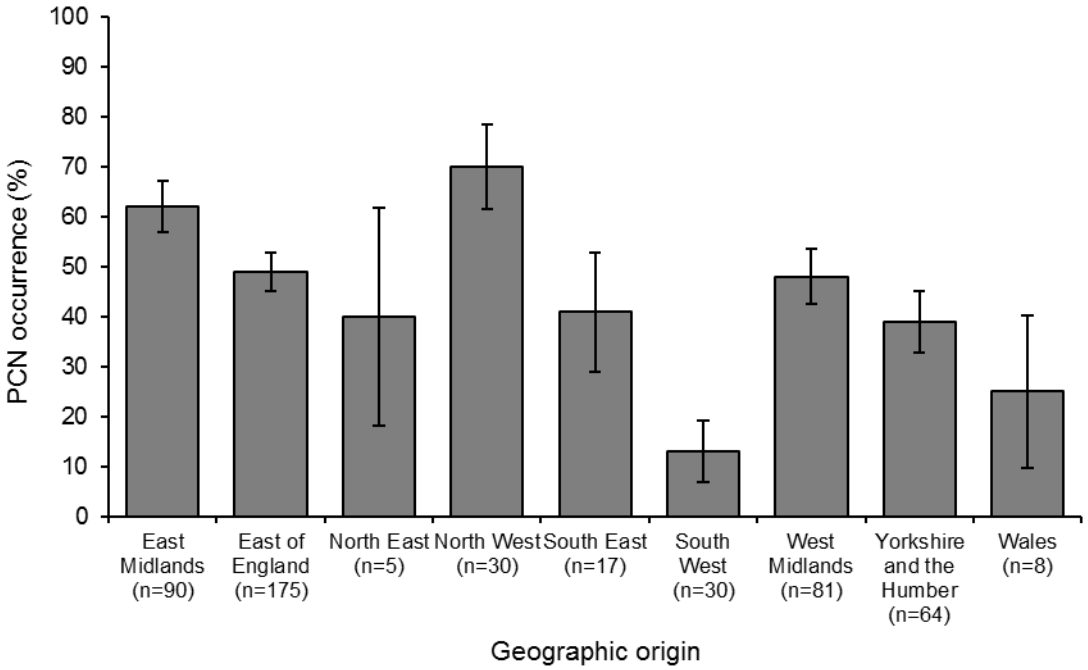


Figure 2.14. Occurrence of PCN in survey samples from eight regions of England and Wales (geographic origin). n= number of observations. Error bars represent the standard error of the mean.

2.3.5.2. *Soil texture*

Developed by Cranfield University and sponsored by the Department for Environment, Food and Rural Affairs (DEFRA) “The Cranfield Soil and Agrifood Institute - Soilscapes” is a simplified soils dataset, created from the more detailed National Soil Map, covering England and Wales. Soilscapes soil textures (Table 2.10.) were assigned to all survey samples using field coordinates (latitude and longitude) in this freely available resource that offers soil overview information accessible online at http://www.landis.org.uk/soilscapes/.

Table 2.10. Soilscapes soil textures used to allocate survey samples into groups based on the field coordinates (latitude and longitude).

Soilscapes	Soil texture
Saltmarsh soils	loamy
Shallow very acid peaty soils over rock	peaty
Shallow lime-rich soils over chalk or limestone	chalky
Sand dune soils	sandy
Freely draining lime-rich loamy soils	loamy
Freely draining slightly acid loamy soils	loamy
Freely draining slightly acid but base-rich soils	loamy
Slightly acid loamy and clayey soils with impeded drainage	loamy
Lime-rich loamy and clayey soils with impeded drainage	clayey
Freely draining slightly acid sandy soils	sandy
Freely draining sandy Breckland soils	sandy
Freely draining floodplain soils	loamy
Freely draining acid loamy soils over rock	loamy
Freely draining very acid sandy and loamy soils	sandy
Naturally wet very acid sandy and loamy soils	sandy and loamy
Very acid loamy upland soils with a wet peaty surface	peaty
Slowly permeable seasonally wet acid loamy and clayey soils	loamy and clayey
Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils	loamy and clayey
Slowly permeable wet very acid upland soils with a peaty surface	peaty
Loamy and clayey floodplain soils with naturally high groundwater	loamy and clayey
Loamy and clayey soils of coastal flats with naturally high groundwater	loamy and clayey
Loamy soils with naturally high groundwater	loamy
Loamy and sandy soils with naturally high groundwater and a peaty surface	peaty
Restored soils mostly from quarry and opencast spoil	loamy
Blanket bog peat soils	peaty
Raised bog peat soils	peaty
Fen peat soils	peaty

The data were analysed by GLM to determine if there was a relationship between the occurrence of PCN and the soil texture. The results show that there was a highly significant relationship ($F=8.14$, $P<0.001$), but only accounted for 10% of the variance, between the detection of PCN and the soil texture. Potato cyst nematodes occurrence was the highest in samples with the ‘sandy and loamy’ soil texture (75%) but was also high in samples with the ‘sandy’ and ‘peaty’ soil textures (70 and 72%, respectively). The lowest occurrence was in samples with the ‘chalky’ soil texture (25%) (Figure 2.15.).

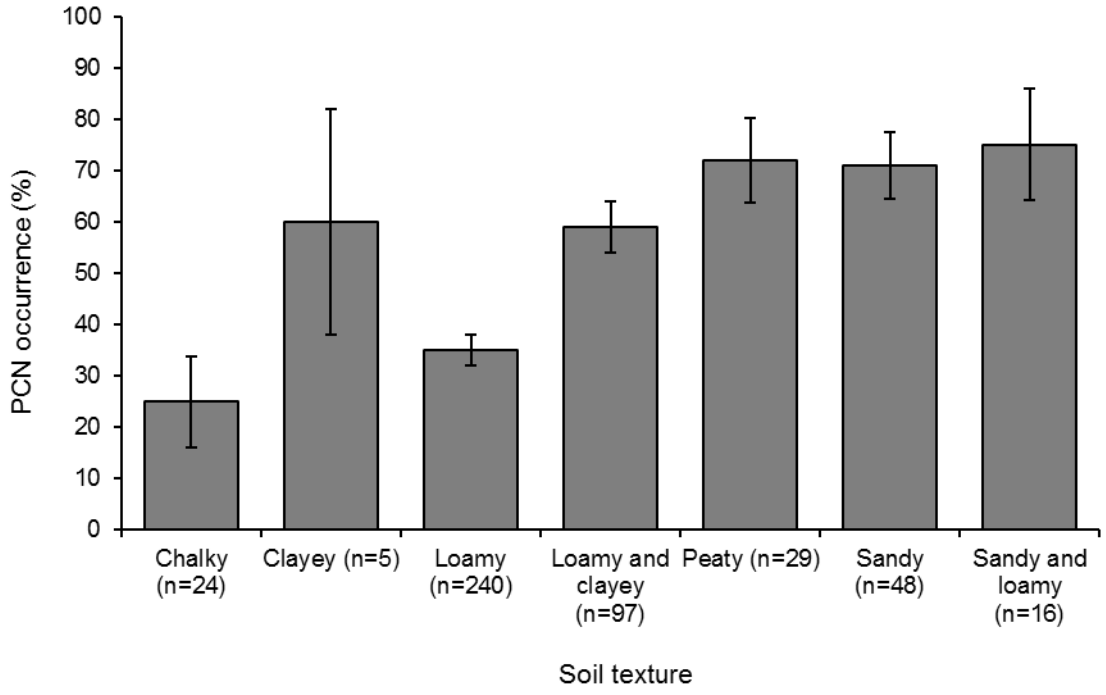


Figure 2.15. Occurrence of PCN in survey samples with different soil textures. n= number of observations. Error bars represent the standard error of the mean.

2.3.5.3. *Previous crop grown*

Information about the previous crop grown in the field prior to PCN sampling was provided for 209 fields. Based on this data samples were allocated to following groups:

- cereals - any crop cultivated for the edible components of its grain: wheat, barley, maize, oat, rye, (78 samples);
- non cereals – any other crops reported excluding potato crop: grass, Brussels sprout, cabbage, broccoli, peas, oilseed rape, onion, sugar beet and fallow (29 samples);
- potato – all potato crops (102 samples).

General linear model analysis was applied to investigate whether there was a relationship between the occurrence of PCN and the previous crop grown. No significant relationship ($F=1.34$, $P=0.26$) was found.

2.3.5.4. *Rotation length*

The rotation length data were provided for 82 fields and survey samples were grouped as shown in Table 2.11.

Table 2.11. The rotation length in fields used for survey samples collection.

Rotation length (years)	Number of survey samples
1	2
2	2
3	3
4	6
5	24
6	17
7	12
8	5
10	5
11	2
13	4
Total	82

The data were analysed by GLM to determine if there was a relationship between the occurrence of PCN infestation and the length of the rotation. No significant relationship (F=1.39, P=0.18) was found.

2.3.5.5. *History of potato cyst nematodes*

The survey questionnaire investigated knowledge about the history of PCN infestation (presence/absence) for sampled fields. Based on the 212 responses, survey samples were allocated into 3 groups:

- No, when the field was known to be PCN free;
- Yes, when the field was known to be PCN infested;
- Unknown, when presence/absence of PCN was unknown.

General linear model analysis was applied to investigate whether there was a relationship between the occurrence of PCN and the history (presence/absence) of

PCN. The results show that there was a highly significant relationship (F=30.32, P<0.001), accounted for 27% of the variance, between the detection of PCN and the information about presence/absence of PCN provided. Potato cyst nematodes occurrence was the highest in the group reported as known to be PCN infested (75%) and the lowest in the group reported as known to be PCN free (22%). The probability of PCN occurrence in samples of unknown status of PCN presence fell between these (33%) (Figure 2.16.).

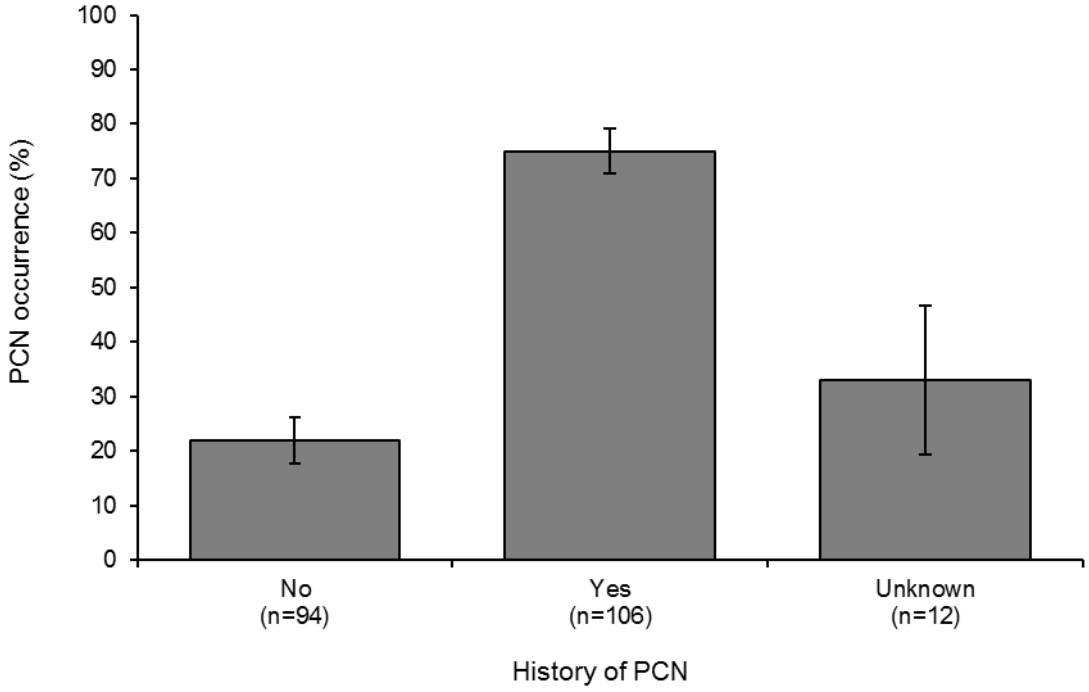


Figure 2.16. Occurrence of PCN in survey samples for categories of field history relating to PCN (known presence/absence). n = number of observations. Error bars represent the standard error of the mean.

2.3.5.6. *Potato cyst nematode species identification and agricultural factors*

Potato cyst nematode species identification gave evidence that 95% of the samples containing PCN were *G. pallida* either pure or in a mixture with *G. rostochiensis*. Due to the high percentage of overlapping data, GLM analysis of the relationship between PCN species occurrence (*G. pallida*) and agricultural factors

would result in replication of the results obtained already when testing a relationship between the occurrence of PCN and agricultural factors (reported in the section 2.3.5.). In contrast, *G. rostochiensis* occurrence was low and it was found, as pure and mixed populations in only 11% samples detected as PCN infested. General linear model analysis between PCN species occurrence (*G. rostochiensis*) and agricultural factors were not possible due to the limited quantity of data.

2.4. Discussion

2.4.1. *Potato cyst nematodes detection and species identification*

Potato cyst nematodes were detected in 48% of samples obtained from ware potato growing land in England and Wales. The result is lower than infestation levels reported by Minnis (2000) and by Hancock (1996) by 16% and 19%, respectively. In the most recent stratified survey (Minnis, 2000), soil samples were collected from 484 randomly selected ware fields from which 64% were reported to contain PCN cysts. In that study, PCN presence confirmation by species identification was reported for 261 samples only, combined from 254 positive results from PCR and 7 positive results from IEF analysis. Isoelectric focusing was performed to re-examine samples which failed to be detected by PCR. The total number of samples tested by PCR was 269 which indicates that PCR failed to detect PCN species in 15 samples. As mentioned previously, 7 samples were confirmed to be PCN infested when retested by IEF but the outcome of the 8 samples, for which both methods failed to produce the results, is unclear. Confirmation of PCN presence, by testing species composition, was only reported for 54% of the survey samples. It appears that the remaining 10% (50 samples) reported as PCN infested were evaluated by visual assessment of the cysts found in the soil after Fenwick can extraction (Fenwick, 1940). Eight samples were reported as PCN infested even though neither

were confirmed by PCR or IEF. It is also not clear, in case of the remaining 42 samples, if they were tested for PCN presence by other methods. It is very unlikely that morphological assessment was carried out on the cysts or juveniles as no such data were presented. Additionally, from 311 samples containing cysts, only 263 samples are reported to contain eggs and these, most likely, were tested by PCR and IEF. As no evidence was provided, other than visual assessment which itself could be misleading due to morphological similarities between cysts of PCN and other nematodes, for PCN presence in 50 samples it is possible that actual infestation level was closer to 54% and not 64% as reported (Minnis, 2000).

In the current study, the cysts visually assessed as PCN (PCN-like) were found in 250 samples. Real-time PCR assays confirmed the presence of *G. pallida* and/or *G. rostochiensis* in 241 samples whereas nine PCN-like samples were not confirmed to be PCN. It is possible that PCN-like cysts collected from these samples were mistaken for other cyst nematodes. The distinction between cysts of *Globodera* and *Heterodera* spp., *e.g*. *H. avenae* or *H. schachtii*, extracted from field soil can be challenging as they are often misshapen (Figure 2.17.).

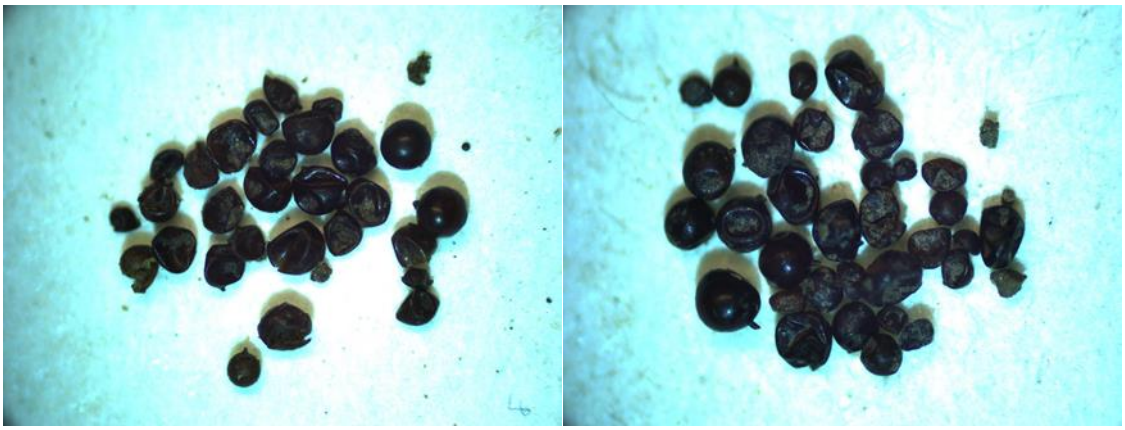


Figure 2.17. An example of misshapen PCN cysts extracted from field soil.

These samples may represent an old population which would be composed of mostly empty cysts (no eggs) which would reduce the chance of DNA detection.

Testing for PCN presence at HAU and SASA did not include investigation of cyst content as they were directly used for DNA extraction. Samples submitted to APHA were dissected transversely and tested for PCN eggs and hatched juvenile content before being submitted for species detection. This pre-detection would be the reason why all samples with PCN-like cysts were later confirmed as PCN infested by molecular assessment while some of the samples, at HAU and SASA, initially recognised as PCN-like were not confirmed to originate from PCN infested fields.

In the earlier survey of ware land (Hancock, 1996) the results of infestation (67%) were obtained using soil samples sent to ADAS for advisory purpose in years 1994-1995. A non-random selection of tested fields could result in the survey being biased towards positive identification of PCN given that many of the samples were received from growers who knew about the infestation and wished to investigate the species composition of the population. The importance of investigating the land used for ware potato production (advisory reason) independently from seed production (statutory reason) was indicated (Hancock, 1996) when analysis of samples submitted to ADAS (years 1993-1995) for both reasons showed a decrease of infestation level to 36%. Growers who submitted their samples for statutory reasons and aimed to export their crop were likely to submit samples from fields they believe to be free from PCN. Taylor and Hockland (2010) also showed the necessity to separate the analyses for these two production types. The authors tested soil samples originating from ware land planted with crops for export (48%) and from prospective seed potato land (52%). From the total number of 2474 samples, only 35 samples were positive for PCN and from these 91.5% originated from land growing a ware crop which, as authors agreed, could be expected.

The direct comparison between surveys conducted by various researchers is difficult as they most likely did not use the same methodology for sample collection,

cysts extraction, PCN detection and species identification. This is not only due to differences in facilities available at the institutions conducting the research, or decisions made on experimental design, but also because of the development of new and improvement of existing methods. More sensitive, time and labour effective methodology become available between the PCN surveys of ware potato land in GB.

For example, in the survey conducted by Minnis (2000), the author used, as in this study, Fenwick can extraction (Fenwick, 1940) to detect the presence of cysts. Here, the method was used on samples collected in fields selected for the survey only. Minnis (2000) also performed the first extraction directly on the survey soil samples but then again on the sub-set of 83 samples. These samples were found to have no PCN cysts but to confirm this assessment were next subjected to a bait plant test after which the extraction was repeated. This combination of bait plant test and the second extraction revealed infestations in 22 samples which were earlier assessed as PCN free. The same author used visual assessment, PCR and IEF as a methods for PCN infestation confirmation. In this study visual assessment was not definitive and all samples containing PCN-like cysts were tested for *G. rostochiensis* and *G. pallida* DNA presence. Additionally, the PCR method used here was more recently developed (Nakhla *et al*., 2010; Reid *et al.*, 2015) than the method used by Minnis (2000) (Bulman and Marshall, 1997) which, with the molecular biology tools becoming more sensitive and reliable, could possibly improve the detection in samples with *e.g*. low level of PCN infestation.

The reduction in the proportion of fields found to be infested with PCN, between surveys in England and Wales, might be partly explained by the proceeding decline in area (ha) planted for potato production. Figure 2.18. shows a comparison of the planted area (ware and seed) (AHDB, 2017) and proportion of PCN infested

land for 1996, 2000 and 2016, in correspondence with the results from the last two surveys of ware potato land in England and Wales (Hancock, 1996; Minnis, 2000). The key outcomes are:

- A 3% decrease in the planted area from 1996 to 2000;
- A 20% decrease in the planted area from 2000 to 2016;
- A 23% decrease in the planted area from 1996 to 2016

and

- A 3% decrease in PCN infestation from 1996 to 2000;
- A 16% decrease in PCN infestation from 2000 to 2016;
- A 19% decrease in PCN infestation from 1996 to 2016.

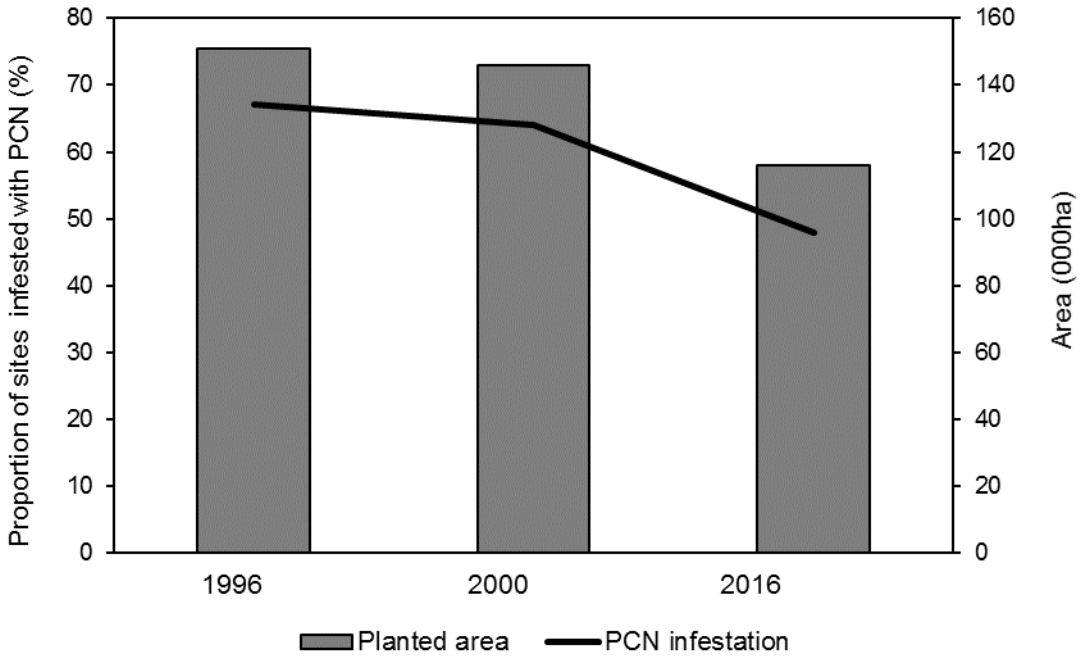


Figure 2.18. Ware potato planted area and proportion of sites found to be infested with PCN by the AHDB Potatoes - Potato Data Centre in years 1996, 2000 and 2016 which correspond with surveys by Hancock (1996), Minnis (2000) and current.

For 1996, 2000 and 2016 (Figure 2.19.) the number of potato growers registered to crop areas of 3 or more hectares was 13,400, 4,550 and 1,900,

respectively, but the area available for ware potato production per grower (ha) increased to 11, 29 and 56, respectively (AHDB, 2017). When informed about presence, and if positive, about population density, growers might avoid using infested land and move production onto land with no or lower PCN populations. In this scenario, PCN infestation is less likely to be detected if the survey is targeting potato fields recently used for ware production. Survey reports often simplify the results by describing fields as PCN free or infested which, in case of the former, should be only understood as the lack of the detection of *Globodera* spp. in the soil sample examined. Usage of distinctive machinery, requirement of soil texture suitable for a potato crop and farm design typical for potato production, followed by the high cost of crop management (*e.g*. pathogens and diseases control) resulted in potato production becoming more specialised, growers with knowledge and experience expanding their production rather than moving to the production of another crop. This trend can be seen in the reduction in the number of registered growers by 86%, between 1996 and 2016, which is accompanied by only 23% decline in total planted area (AHDB, 2017). The number of smaller-scale growers is in decline, largely due to higher capital investment, and organisations within the potato sector are becoming more highly specialised in order to compete. Nevertheless, consolidation of the industry is slowing with the average area per grower remaining at around 53ha in three consecutive years (2013-2015) (AHDB, 2018c).

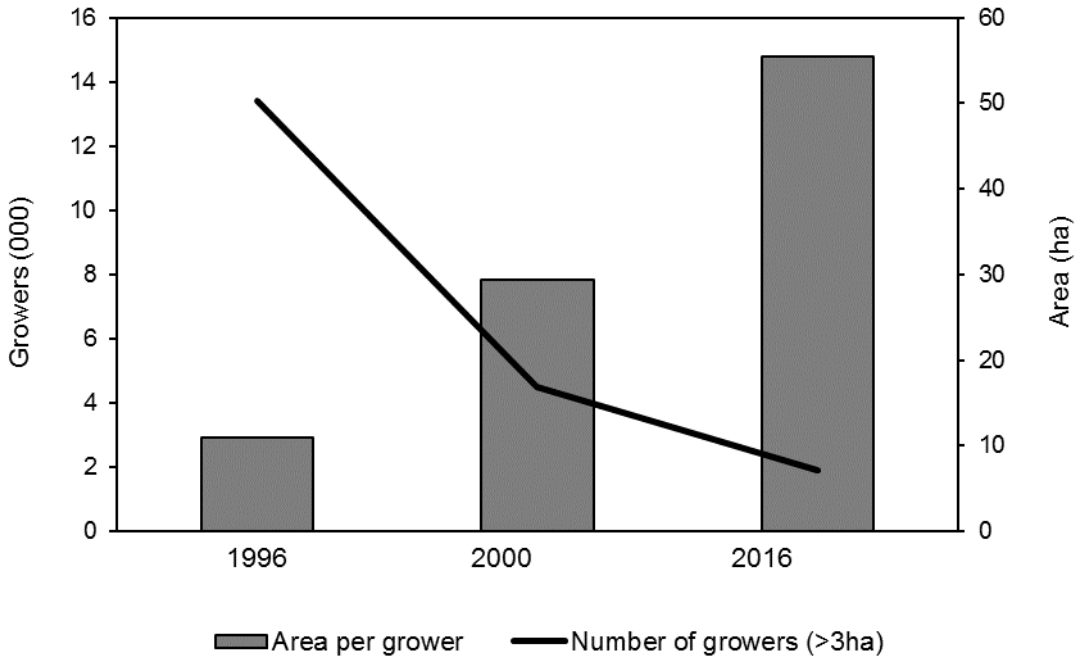


Figure 2.19. Number of potato growers and average area of potato land per grower (AHDB Potatoes - Potato Data Centre) in the years 1996, 2000 and 2016 which correspond with the surveys by Hancock (1996), Minnis (2000) and current.

Average rotation length between 2000 and 2016 has shortened only marginally (7 to 6 years), although the number of fields used for this estimation varied greatly and in this study was almost 5 times lower than the figures used in the survey conducted by Minnis (2000). Over 50% of fields sampled in the survey by Minnis (2000) had a rotation length of 1 in 5 or shorter. The author suggested that this more frequent use of fields could be explained by potato industry specialisation. He also highlighted that this shortening of the rotation would result in reduction of PCN population decline.

Potato cyst nematodes occurrence for regions showed that the East Midlands and North West had the highest proportion of PCN infested sites of 62 and 70%, respectively. Some of the first UK reports of crop damage caused by PCN are from Lincolnshire (East Midlands, 1924) (Morgan, 1925) and from Lancashire and

Cheshire (North West, 1927) (Smith and Prentice, 1929). In East Midlands in 1967 82% of infested potato fields were reported as a pure *G. pallida* populations and 7% as *G. rostochiensis* populations (Guile, 1967). The remaining 11% fields were found to be mixed populations. The current proportion of infestation in East Midlands reported in this study confirmed a shift towards *G. pallida* with 93% samples being pure *G. pallida*, 2% pure *G. rostochiensis* and 5% a mixed population. A lack of literature on the proportion of PCN infested field sites found in individual counties and regions make comparisons between the current survey and previous surveys not possible.

Identification of PCN species from positive survey samples collected in England and Wales showed that 6% populations contained both species (mixed), 89% populations were pure *G. pallida* and 5% populations were pure *G. rostochiensis*. The change in species ratio is particularly notable when comparing the current survey findings for mixed populations with the results of the survey conducted by Minnis (2000), who reported that 25% of PCN positive samples contained both species. Hancock (1996) reported 41% of mixed populations being found in samples analysed by ADAS, which shows an even greater reduction in mixed PCN populations for England and Wales. In contrast, the proportion of sites with pure *G. pallida* populations has increased by 22 and 35% compared to the results reported by Minnis (2000) and Hancock (1996), respectively. Finally, pure *G. rostochiensis* populations were found in fewer samples when compared with the figure of 8% reported by Minnis (2000), but were in line with the findings of Hancock (1996) where 5% of sites surveyed were found to contain pure *G. rostochiensis* populations.

Globodera pallida was found, as pure or mixed populations, in 95% of sites while *G. rostochiensis* was found in only 11% of the samples found to contain PCN.

Currently the difference in proportions between sibling species is greater than in the survey reported by Minnis (2000) who found, including mixed populations, *G. pallida* in 92% and *G. rostochiensis* in 33% of positive samples. However, it is important to note that an assessment of species was conducted only on 54% of the total survey samples which is 84% of samples reported as PCN infested in England and Wales. When Minnis (2000) reported the occurrence of PCN at 64% he referred to 311 samples reported as PCN infested by visual assessment on the cysts extracted directly from field soil and from field soil in which bait plants had been grown prior an extraction of cysts. The PCN species were only determined for 261 populations where sufficient material was available, while the remaining 50 samples were not subjected to species identification. The gap in the occurrence between the sibling species was even less radical in samples analysed by ADAS (Hancock, 1996). By combining pure and mixed populations, *G. pallida* was recognised in 95% while *G. rostochiensis* in 46% of all PCN infested samples.

The current study showed that *G. pallida* is a predominant species in all regions of England and in Wales. The regions where pure *G. rostochiensis* populations were detected are also the only regions where mixed populations were detected (East Midlands, East of England, South East and West Midlands). The first information about species distribution reported in England dates back to 1963 where South-east England and East of England were described as regions mostly infested with *G. rostochiensis*. *Globodera pallida* populations were commonly found in Yorkshire and the Humber and East Midlands. Other regions were represented by mixture of these populations (Jones and Pawelska, 1963). Another study on PCN species distribution from the East Midlands reported 11% of sites with mixed species, 82% with pure *G. pallida* and 7% with pure *G. rostochiensis* populations (Guile, 1967). These results approximately correspond with the findings in this study

which showed that 5% of sites contained mixed species, 93% contained pure *G. pallida* and 2% of contained pure *G rostochiensis* populations. *Globodera rostochiensis* was previously recognised as a predominant species in the South East, East of England and West Midlands (Jones and Kempton, 1978). This original predominance could explain why South East, East of England and West Midlands and East Midlands are regions where *G. rostochiensis*, as a pure and mixed population, can be still be found (12, 11, 4 and 4%, respectively), although West Midlands has a higher proportion of pure *G. rostochiensis* over East Midlands. Although, when pure and mixed populations are considered together, the occurrence of *G. pallida* has remained at a similar level - 95% in 1996, 92% in 2000 and 95% in 2016. On the other hand the occurrence of *G. rostochiensis* has declined and was 46% in 1996, 33% in 2000 and 11% in 2016. These figures strongly indicate a shift towards *G. pallida* as the predominant PCN species (Figure 2.20.). For example, in this study all survey samples from Lancashire (North West) were found to be *G. pallida* infested while the distribution of PCN in this county reported previously (Dixon *et al*., 1968) was 70% *G. pallida* - 30% *G. rostochiensis*. Separate analysis of species distribution as pure and mixed populations highlights that the main difference between PCN surveys is the strong reduction in occurrence of mixed populations (Figure 2.20.). This observation, combined with increased occurrence of pure *G. pallida* populations, could indicate that by successfully controlling *G. rostochiensis,* populations previously reported as mixed are now identified as pure *G. pallida*.

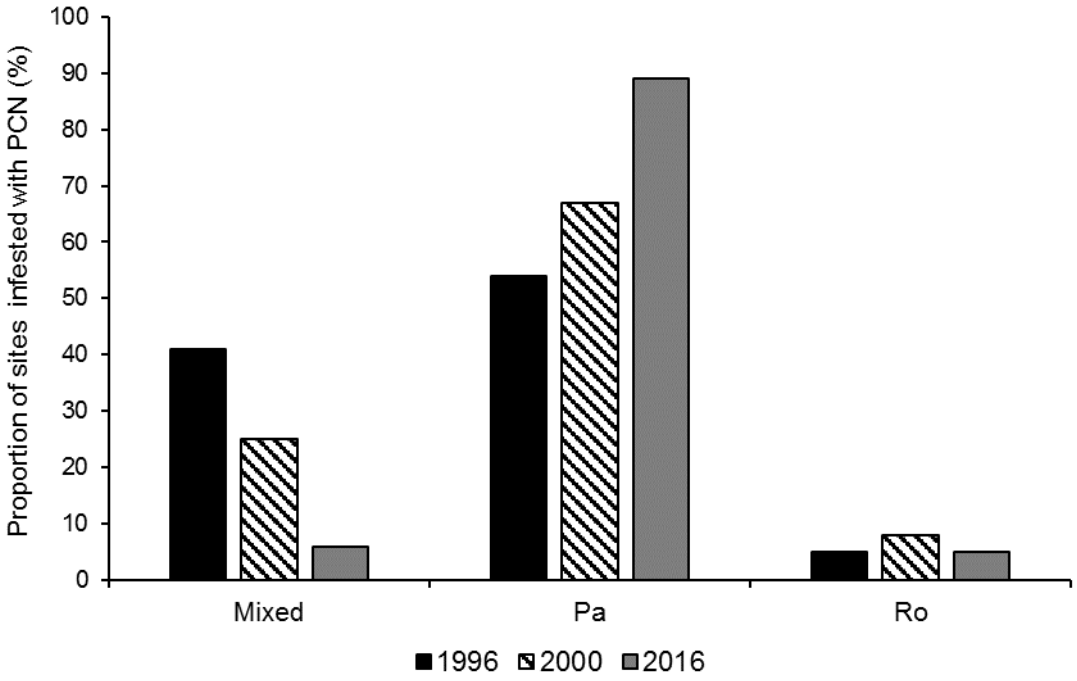


Figure 2.20. The proportion of PCN populations containing both species (mixed), pure *G. pallida* (Pa) and pure *G. rostochiensis* populations (Ro) as a percentage of the total survey samples confirmed as PCN infested in England and Wales for surveys conducted in 1996 (Hancock, 1996), 2000 (Minnis, 2000) and current.

The results of the Scottish survey are not comparable, as some aspects of methodology used differ from techniques used in the survey for England and Wales, and are presented separately.

Using a database provided by SASA for a statutory survey of ware potato land, PCN were reported in 38% of the soil samples extracted. This shows a ~15% increase in occurrence of PCN from an approximate infestation level of ~23% from 1996/1997 (Evans, 2003. Pers Comm. in Haydock, 2003). Species characterisation showed that 14% populations contained both PCN species (mixed), 59% populations were pure *G. pallida* and 28% populations were pure *G. rostochiensis*. *Globodera rostochiensis* was found to be the predominant species in 1978 (Jones and Kempton, 1978) and again reported to be present in 1986 (Stone *et al*., 1986).

The current survey, when compared with the 1996/1997 survey (Evans, 2003. Pers Comm. in Haydock, 2003), indicates a 9% decrease of mixed populations, 35% increase of pure *G. pallida* and 25% decrease in the occurrence of pure *G. rostochiensis*.

The selection of resistant and susceptible potato varieties may have played a significant role in the observed changes in the prevalence of PCN species. The potato varieties Maris Piper, Markies, Lady Rosetta and Melody are in the top five most grown varieties and accounted for 33% of total planted area in England and Wales in 2016 (AHDB, 2018a). These main crop varieties are resistant to *G. rostochiensis* (pathotypes Ro1 and Ro4) but susceptible to *G. pallida* (pathotype Pa1 and Pa2/3) and are widely used in the pre-pack and processing market sectors; the top two sectors which together in 2016 accounted for 72% of total planted area in England and Wales. On a regional level, the pre-pack market sector is leading in East Midlands, East of England, South West and Wales, while the processing sector is mainly associated with the North East, North West, South East, West Midlands and Yorkshire and the Humber (AHDB, 2018a). Maris Piper, the first variety with resistance towards *G. rostochiensis*, is the most widely grown variety since its introduction to UK's potato industry in 1966, despite its susceptibility to *G. pallida,* due to high market demand (Evans and Haydock, 2000). Other varieties with the H_1 resistance gene soon followed (Turner *et al*., 2006). In GB, between 2009 and 2017, by planted area (ha) Maris Piper was continuously ranked first, Markies ranked fourth in 2009 and then due to increasing popularity was ranked second in 2017, Lady Rosetta ranked third in 2009 slowly lost popularity but was still ranked fifth in 2017. Finally, Melody which ranked fourteenth in 2009, has received lots of interest over the last years and was ranked fourth in 2017 (AHDB, 2018a). The influence of the market dictates the lasting popularity of potato varieties with full resistance to *G.*

rostochiensis and susceptible to *G. pallida* and by this, indirectly selected the more problematic species *G. pallida* from mixed populations. Evans and Haydock (2000) summarised that management strategies, which combine crop rotation, the use of nematicides and varieties with H_1 resistance gene, *e.g*. Maris Piper, Cara and Saturna, affected the ratio between PCN sibling species.

Another factor which could possibly contribute to the reduction of mixed PCN populations are physiological differences between sibling species which benefit *G. pallida*. Literature previously discussed in Chapter 1 (section 1.2.6.4.) indicated that *G. rostochiensis* generally hatched quicker than *G. pallida*. This biological disparity between species it thought to be responsible for unequal effectiveness of control by nematostats. These chemicals, sometimes referred to as non–fumigant nematicides were very popular and often included in the PCN management strategy, before their use was forbidden or restricted (OJEU, 2009), and were suggested to act as a selective factor favouring *G. pallida* in mixed populations (Evans and Haydock, 2000). When both species were present, *G. rostochiensis* would be affected to a greater extent by an active substance that, after being dissolved into soil water, would with time lose toxic activity (Whitehead, 1992). Physiological differences were also reported when investigating optimum hatching temperatures described in Chapter 1 (section 1.2.6.4.) with results indicating that *G. rostochiensis* has a preference for a higher temperature range than *G. pallida*.

The general trend which showed increases in the number of fields being infested with pure *G. pallida* populations act as an additional factor itself. As discussed in Chapter 1 (section 1.2.8.1.), the spread to non-infested land might occur due to cross contamination between fields (*e.g*. machinery not being properly cleaned after use on infested field). If for any of the reasons described above *G. rostochiensis* was suppressed in the population earlier assessed as mixed, the

contamination from such a fields would result in a further shift towards *G. pallida* as the predominant GB's PCN species.

The most recent directive 2007/33/EC on the control of PCN took effect from 1 July 2010, and implemented more intensive sampling (an increase from a previous standard rate of pre-planting sampling of 600ml per four hectares) and a requirement for official pre-planting soil testing on land used for potato seed production (OJEU, 2007). In this respect, the directive addressed the necessity to ensure that no PCN are found in fields for the production of seed potatoes which is a top market sector in Scotland (AHDB, 2018a).There is no requirement to test land before planting a crop of ware potatoes, where none of the crop will be retained as seed for further planting. The increased chance of detection, especially in the early stages of PCN population establishment, allows for the infestation to be treated sooner, reducing the risk of its multiplication over the following season to an even more damaging level *e.g*. nematostats are known to be more effective at lower population densities (Haydock and Evans, 1998b). In 2016, the Scottish planted area (ha) for the pre-pack and processing market sectors was almost 6 times lower than the area in England and Wales. It is possible that less intensive use of varieties with resistance to *G. rostochiensis,* which are very popular in those two sectors, reduced the selection pressure for *G. pallida* (AHDB, 2018a).

2.4.2. *Potato cyst nematodes occurrence and agronomic factors*

During the collection of survey samples, site details such as the geographic location, soil texture, cropping history, rotation length and history of PCN infestations (presence/absence) were recorded. Inconsistencies between the descriptions provided by land owners or agronomists made it challenging to analyse individual

factors, e.g. common names of soil textures. Testing for an interaction between factors was not possible due to the low numbers sampled.

A highly significant relationship was found between the detection of PCN and the geographic region. Occurrence of PCN varied from 70% in the North West to only 25% in Wales and 13% in South West. These last two regions are more geographically isolated than any other regions which could partly benefit a slower process of infestation. Also both have lower potato planted areas (ha), second and fourth lowest respectively, to regions with high detection like East Midlands (62%) and East of England (49%), first and second highest respectively. The highly significant relationship between this factor and occurrence of PCN might suggest the necessity for a more intensive sampling regime which could improve detection in regions where it was shown to be low.

A similar conclusion could be drawn for the highly significant relationship between the detection of PCN and the soil texture. One of the highest detected occurrences of PCN, above 70%, was in fields with a peaty soil texture. Devine and Jones (2001) observed much slower decrease of viable eggs $cyst^{-1}$ in peaty soil which might partly explain the high level of detection. The same authors also reported over twice as fast a decrease in the clay and sandy soils which, does not support findings in this study. The potato can be grown almost on any type of soil, except saline and alkaline soils. Naturally loose soils, which offer the least resistance to enlargement of the tubers, are preferred, and loamy and sandy loam soils that are rich in organic matter, with good drainage and aeration, are the most suitable (FAO, 2008). Sandy, clayey, peaty, sandy/loamy and loamy/clayey soil textures showed high occurrence of PCN, above 50%. Conversely, detected PCN occurrence in chalky and loamy soils, which accounted for 5 and 52% of total survey samples, was low.

A highly significant positive relationship was found between the occurrence of PCN and PCN field history (absence or presence) provided by the growers. Worryingly 22% of growers from the group that believed their field sites were uninfested, do actually have PCN infested sites. On the other hand, 75% of growers, from the group are aware of the infestation, were correctly informed about the existing PCN problem on their fields. From the last group of growers who previously did not test their fields for PCN, 33% of samples were identified as PCN infested. Due to insufficient amount of information provided in the questionnaires, the investigation into whether there was a relationship between the PCN species identification results from the survey and the history of PCN species occurrence was not possible. No significant relationship was found between other factors and PCN occurrence.

Difficulties in finding sufficient individual contacts for growers or agronomists resulted in the necessity to obtain samples by three different approaches: direct field sampling, receiving soil samples collected by external companies, agronomists and growers and using data from a national annual PCN survey database provided by the APHA. For the same reason, the original strategy to collect no more than one sample from each farm could not be fulfilled. In some cases, several samples were collected at the same farm which reduced the geographic spread of survey samples. In future studies, it would be helpful to have greater access to information about the individual field sites. Ideally, to monitor changes in PCN populations, it would be useful to revisit fields or, as a minimum, the farms tested in the current study. Due to confidentiality of data from the survey conducted by Minnis (2000) this approach could not be undertaken.

2.4.3. *Key findings*

- Potato cyst nematodes were detected in 48% of samples obtained from ware potato growing land in England and Wales.
- Identification of PCN species from positive survey samples collected in England and Wales showed that: 6% populations contained both species (mixed), 89% populations were pure *G. pallida* and 5% populations were pure *G. rostochiensis.*
- Potato cyst nematodes were detected (data provided by SASA) in 38% of samples from ware potato growing land in Scotland.
- Identification of PCN species (conducted by SASA) from positive Scottish samples included in survey showed that: 14% populations contained both species (mixed), 59% populations were pure *G. pallida* and 27% populations were pure *G. rostochiensis*
- The 29 samples found to contain PCN were comprised of four samples with mixed populations, 17 samples containing pure *G. pallida* and eight samples containing pure *G. rostochiensis* populations.
- The results showed that there was a highly significant relationship ($P<0.001$) between the detection of PCN and a) the geographic origin; b) the soil texture and c) the information about presence/absence of PCN provided.
- No significant relationship ($P=0.3$) was found between the occurrence of PCN and the previous crop grown.
- No significant relationship ($P=0.2$) was found between the occurrence of PCN infestation and the length of the rotation.

Chapter 3

Comparison of methods used to assess the viability of potato cyst nematode populations from Great Britain.

3.1. Introduction

Potato cyst nematodes (PCN) are obligate parasites of Solanaceous plants and the most destructive pests of potato crops in the United Kingdom (UK) causing significant annual crop losses (Twining *et al*., 2009). The assessment of PCN population densities for field samples is important for the selection of adequate management strategies, although egg and cyst counts may not precisely estimate the actual viable population. To fully understand a possible infestation level it is necessary to include a measurement of the viability of the eggs.

In the past the density of the infestation was assessed as number of cysts presented in a given amount of soil, without considering the number of eggs or their viability. Currently, commercial laboratories report the level of infestation as number of eggs per gram of soil. For routine advisory soil samples the assessment of egg viability, if requested, is based on visual examination only. The eggs are considered as viable if they do not show physical damage/discolouration or are not empty (Keer, 2014. Pers. Comm. Mr J. Keer is the Trials and Research Director at Richard Austin Agriculture Limited). Damaged or discoloured eggs may still be able to hatch and cause damage to the crop. Conversely, healthy looking eggs are not necessarily fit to hatch and infest the roots. The absence of a precise viability assessment might lead to inaccurate determination of field populations and inappropriate use of control measures. At the moment viability tests are not often commercially performed as

they are still too expensive and time consuming to be widely accessible for the growers and presence or absence is often all that is requested.

Turner (1996) showed that PCN cysts were still present in land not used for potato production for over 42 years. Even though a number of juveniles could still be detected within these cysts, further testing revealed that they were no longer viable. The empty egg shells left behind after spontaneous hatch can remain in cysts and if not diagnosed properly, could lead to overestimation of PCN densities and therefore overuse of agrochemicals *e.g*. nematicides. This highlights how important the assessment of PCN population viability is. It is challenging for commercial potato growers to keep infested land crop-free for the extended period of time, often needed for a population to decline. More detailed sample analysis, with a viability assessment, may reveal a lower infestation level and therefore a shorter period of time needed to achieve an acceptable level of infestation (Evans and Stone, 1977). Studies conducted on PCN populations support the hypothesis that natural decline takes place and it is important to develop the methodology to measure it accurately which then requires and relates to studies on viability assessment.

3.1.1. *Aim*

Identify the most suitable viability assessment method for PCN field populations from GB by comparison between classic and novel techniques on natural and six artificially imposed levels of mortality.

3.1.2. *Objectives*

(I) Investigate and improve the suitability of the trehalose assay (van den Elsen *et al*., 2012) as a method to assess the viability of *G. pallida* field populations.

(II) Evaluate the comparative effectiveness of hatching in PRD assay (assay I) with hatching in PRD followed by Meldola's blue staining assay (assay II), Meldola's blue staining assay (assay III) and the trehalose assay (assay IV) to assess natural viability of *G. pallida* field populations.

(III) Evaluate the comparative effectiveness of hatching in PRD assay (assay I) with hatching in PRD followed by Meldola's blue staining assay (assay II), Meldola's blue staining assay (assay III) and the trehalose assay (assay IV) to assess viability of *G. pallida* field populations on six artificially imposed levels of mortality.

3.2. Materials and methods

3.2.1. *Potato root diffusate*

Potato root diffusate (PRD) was collected from potato plants four weeks after emergence according to the procedure described by Widdowson (1958). Chitted tubers were planted in coarse horticultural sand in 16.5cm diameter pots approximately 10cm below the surface of the growing medium. Plants were grown in the glasshouse not under climate-controlled conditions and watering daily as required. The variety 'Estima' was chosen based on it susceptibility to both PCN species (AHDB, 2018b). The diffusate was leaked out with 200ml of tap water which was allowed to drain through the pot completely, made up to 250ml. and then put through a further four times. The potato root diffusate was next filtered through a Whatman No. 1 filter paper and stored at 4°C. An aliquot of 1:1 (v/v) PRD:distilled water, used across all experiments, was prepared directly prior to use.

3.2.2. *Potato cyst nematode populations*

Potato cyst nematode populations (Table 3.1.) were obtained from five ware potato producing fields, previously reported to be infested with *G. pallida* and included in the PCN survey undertaken in this study (populations HAU152, 164, 165, 166, 167 – from this point referred to as populations A–E respectively). The soil samples were stored in linen bags, air-dried at 25°C and used for cyst extraction using a Fenwick can flotation method (Fenwick, 1940). The residues were air-dried at 25°C before PCN cysts were collected under a binocular microscope at 30x magnification. Egg viability was assessed by four methods: hatching in PRD (assay I), hatching in PRD followed by Meldola's blue staining of unhatched eggs (assay II), solely by Meldola's blue staining (assay III) and the trehalose assay (assay IV). Results were recorded as the number of viable eggs per cyst (viable eggs $cyst^{-1}$) and as percentage viable eggs of total eggs in the sample (viability %). *Globodera* species composition was tested by the molecular diagnostic method described in Chapter 2 (section 2.2.5.). All populations were confirmed as *G. pallida* with no trace of *G. rostochiensis* DNA.

Table 3.1. Information about *G. pallida* field populations used for the comparison between various viability assessment techniques.

G. pallida population	Survey code	Geographic orgin	Soil texture	Previous crop grown	Years since potatoes grown
A	HAU 152	East of England	sandy	non cereal	6
B	HAU 164	West Midlands	loamy and clayey	cereal	1
C	HAU 165	West Midlands	sandy	non cereal	3
D	HAU 166	West Midlands	loamy and clayey	cereal	6
E	HAU 167	West Midlands	sandy	potato	5

3.2.3. *Hatching in potato root diffusate - assay I*

The protocol was based on the plate assay method described by Ngala *et al*. (2015). Cysts of five *G. pallida* populations (see sections 3.2.7. and 3.2.8. for

experimental design) were soaked in distilled water for seven days then transferred to 1:1 (v/v) PRD:distilled water solution. The number of newly emerged second-stage juveniles (J2) was enumerated weekly, using a binocular microscope at 60x magnification over a period of eight weeks with PRD dilution being changed after each count. Samples were kept at 16°C (Turner and Subbotin, 2013). After the final counts, cysts were crushed between an aluminium block and a glass slide before the released eggs were rinsed into a 100ml Nessler tube. Distilled water was added to make a 50ml egg suspension from which, after thorough mixing, a 1ml aliquot was assessed using a binocular microscope at 60x magnification. The number of hatched viable eggs cyst^{-1} was calculated using equation (1).

$$\frac{\text{hatched J2}}{\text{total cysts used}} = \text{hatched viable eggs cyst}^{-1} \quad \text{.....(1)}$$

The percentage viability was calculated using equation (2).

$$\frac{\text{hatched J2 } x\ 100\%}{\text{hatched J2+ unhatched eggs}} = \text{viability (\%)} \quad \text{.....(2)}$$

3.2.4. *Hatching in potato root diffusate followed by Meldola's blue staining - assay II*

Cysts of five *G. pallida* populations (see sections 3.2.7. and 3.2.8. for experimental design) were treated as described in section 3.2.3. until the last count of newly emerged J2 was recorded. After eight weeks of exposure to PRD the cysts with their unhatched eggs were stained as described in section 3.2.5. to differentiate between non-hatched but viable and non-viable eggs. The number of viable eggs cyst^{-1} was calculated using equation (3).

$$\frac{\text{hatched J2 + viable eggs}}{\text{total cysts used}} = \text{viable eggs cyst}^{-1} \quad \text{.....(3)}$$

The percentage viability was calculated using equation (4).

$$\frac{(\text{hatched J2} + \text{viable unhatched eggs})\ x\ 100\%}{\text{hatched J2} + \text{total unhatched eggs}} = \text{viability}\ (\%) \quad \text{.....(4)}$$

3.2.5. *Meldola's blue staining - assay III*

The viability of five *G. pallida* populations was assessed following the technique described by Shepherd (1986). Cysts (see sections 3.2.7. and 3.2.8. for experimental design) were soaked in distilled water for seven days then transferred to 0.05% (w/v) aqueous Meldola's blue solution (Aldrich Chemicals, Poole, Dorset, UK) for a following seven days. To remove excess stain cysts were then placed in distilled water for 24 hours before being gently crushed between an aluminium block and a glass slide. The released eggs were rinsed into a 100ml Nessler tube and distilled water was added to make a 50ml egg suspension from which, after thorough mixing, a 1ml aliquot was assessed using a binocular microscope at 60x magnification. The quantity of unstained (viable) and stained (non-viable) eggs were recorded. The number of viable eggs cyst^{-1} was calculated using equation (5).

$$\frac{\text{viable eggs}}{\text{total cysts used}} = \text{viable eggs cyst}^{-1} \quad \text{.....(5)}$$

The percentage viability was calculated using equation (6).

$$\frac{\text{viable eggs}\ x\ 100\%}{\text{total eggs}} = \text{viability}\ (\%) \quad \text{.....(6)}$$

3.2.6. *Trehalose assay – assay IV*

The quantity of trehalose in eggs of five *G. pallida* populations was tested using a detection kit (K-TREH, Megazyme International Ireland Ltd., Wicklow, Ireland) following protocol by van den Elsen *et al*. (2012) but including some modifications. In brief, quantification of trehalose is based on the change in the concentration of nicotinamide adenine dinucleotide phosphate ($NADPH^+$) which is a product of trehalose hydrolysis to two molecules of D-glucose caused by reaction with trehalase enzyme. Increase in the $NADPH^+$ concentration was measured

spectrophotometrically by recording change in absorbance (∆A) at 340 nanometer (nm) wavelength (λ) before and after the hydrolysis reaction (van den Elsen *et al*., 2012).

3.2.6.1. *Trehalose assay amendments*

Due to the difficulty experienced when a high number of cysts (50) was maintained in a small volume of reaction mixture recommended by van den Elsen *et al*. (2012), three alternative compositions of reaction mixture were tested (Table 3.2.).

Table 3.2. Composition of mixtures, recommended by van den Elsen *et al*. (2012) and the modifications, tested to determine the optimal proportion of reagents required for the trehalose assay to assess the viability of *G. pallida* field populations.

Reagents	µl per sample			
	van den Elsen *et al* . (2012)	Test mixture 1	Test mixture 2	Test mixture 3
Distilled water	12	60	120	1000
Imidazole buffer	2	10	20	100
$NADP^+$/ATP solution	1	5	10	50
Hexokinase and and glucose-6-phosphate dehydrogenase	0.2	1	2	10
Subtotal	3.8	76	152	1160
Sample	10	50	100	100
Trehalase	1	5	10	10
Total	26.2	131	262	1270

The reagent volume used in test mixtures 1 and 2 was 5 and 10 times, higher respectively than that recommended by van den Elsen *et al*. (2012). Test mixture 3 had the reagent proportions reduced by 50% from the suggested in the suppliers' manual for the trehalose detection kit (K-TREH, Megazyme International Ireland Ltd., Wicklow, Ireland). Details of the data used for justifying selection of the trehalose assay mixture components used in this study are provided in Appendix 7.3.

The method published by van den Elsen *et al*. (2012) was developed on PCN glasshouse populations. The authors reported that the extracted cysts were first kept at 4ºC for four months (to complete diapause) and after viability assessment, stored at -80ºC until being used. In this study method suitability for *G. pallida* field populations freshly extracted from soil was investigated. Measurement of absorbance was monitored in samples containing cysts heated (30min, 99°C) or unheated, kept dry or soaked in water 24 hours prior the experiment. Each treatment, replicated six times, was assessed on 30 cysts originating from field populations. The results are provided in Appendix 7.4.

Comparison of methods for the extraction of trehalose by van den Elsen *et al*. (2012) suggested that crushing cysts does not improve the process. Due to the character and aim of the research egg counts were necessary prior to the assay (ΔA viable egg^{-1}). The success of trehalose extraction between crushed and non-crushed cysts was investigated. The results are provided in Appendix 7.5.

To identify upper and lower assay limitations and change of absorbance representing a single viable egg (ΔA viable egg^{-1}) sets of 1, 3, 5, 10, 25 and 50 cysts for each population (A-E) were tested. Cysts were transferred to a 1,5ml Eppendorf tubes and soaked in 300µl distilled water 48 hours before being gently crushed to release eggs. Each egg suspension was used to prepare serial dilutions in a 1:3 dilution (100µl egg suspension and 200µl distilled water) to obtain 200µl of a final suspension to secure sufficient volume for the reaction. Number of eggs in series dilution samples was recorded post assay using a binocular microscope at 60x magnification.

To test for the number of cysts suitable for the assessment of the field population's viability sets of 1, 3, 5, 10, 25 and 50 cysts, in three replicates for each population (A-E), were treated as described above but no serial dilutions were

prepared. Number of eggs was also registered post assay using a binocular microscope at 60x magnification.

3.2.6.2. *Trehalose assay viability assessment*

Cysts (see sections 3.2.7. and 3.2.8. for experimental design) kept in a 1.5ml Eppendorf tubes were soaked in 200µl distilled water 48 hours prior the assay. These samples were used to assess population's natural viability and its change on artificial viability levels. Eppendorf tubes containing cysts or egg suspensions (serial dilutions) were next incubated for 30 min at 99°C to release trehalose. Cysts were then gently crushed with sterile pestle, homogenized for 5 seconds using a vortex and the egg suspensions were spun at 12,000rpm for 10 seconds to remove debris that could interfere with the reaction or instrument used to measure trehalose content. Samples with egg suspension were only homogenized for 5 seconds using a vortex. Following the preparation steps, samples were transferred into 96-well plates by pipetting 100µl of each sample to individual wells. Each plate included a negative control (blank) containing 100µl of distilled water instead of the sample. Volume of reagents used per sample was: 120µl distilled water, 20µl imidazole buffer (2M, pH7.0) with 100mM $MgCl_2$, 10µl $NADP^+$/ATP solution (12.5 + 36.7µg/µl) and 2µl hexokinase (0.425U/µl) and glucose-6-phosphate dehydrogenase (0.212U/µl). The mixture of reagents was prepared in a volume adequate for the number of samples tested and to increase uniformity between samples 152µl of reaction mixture was pipetted into each sample and mixed thoroughly. After 5 minutes, the 96-well plate was transferred into spectrophotometer (FLUOstar Omega, BMG Labtech, Aylesbury, UK) and shaken for 30s before the absorbance (A1) at λ = 340nm was measured. Subsequently the plate was removed and 10µl of trehalase solution (0.49U/µl) was added individually to each reaction well and mixed

thoroughly. After 10 minutes the plate was replaced in the spectrophotometer and, after 30s of gentle shaking, the absorbance was measured again (A2) at λ = 340nm.

The number of viable eggs was obtained for each individual sample. Each egg was described as viable by visual morphological determination (Table 3.3.) using a binocular microscope at 60x magnification following EPPO guidance (EPPO, 2017).

Table 3.3. Visual morphological determination of potato cyst nematodes egg viability. Source: EPPO, 2017.

Viable eggs	Dead eggs
a. Whole egg is intact	a. Egg may be damaged/broken and empty
b. Egg shell is smooth	b. Egg shell often not smooth
c. Egg is clear/transparent with distinct contents or a dark line down the middle of the egg	c. Contents have black/grey granular appearance with no structure
d. Curled juvenile fill up against the egg shell	d. Shrivelled disintegrated juvenile in egg
e. Sometimes clear lip region and stylet present	e. No clear lip region or stylet present

The specimen's background noise was assessed by measuring ∆A in samples containing only heat-treated cysts (section 3.2.8.). Change of absorbance per viable egg was calculated from samples containing between 100 and 5,000 viable eggs with ∆A greater than the specimen's background noise calculated for each population individually using equation (7).

$$\frac{\Delta A - \text{specimen's background noise}}{\text{total viable eggs}} = \Delta A \text{ viable egg}^{-1} \quad \text{.....(7)}$$

3.2.7. *Experimental design - natural viability*

Five replicates of 25 cysts (assay I, II and IV) and five replicates of 50 cysts (assay III), from each population (A-E), were used to assess populations natural viability. All experiments were conducted three times in April, May and July (from this point referred to as experiments 1, 2 and 3 respectively) to test the variability between experiments within the same technique.

3.2.8. *Experimental design – artificially imposed levels of mortality*

A range of artificial viability levels were created by mixing cysts placed in distilled water and heated for 30 minutes at 99°C (Evans, 1991) with the unheated cysts in various ratios (Table 3.4.). The supernatant from heated samples prepared for the trehalose assay was removed and replaced by fresh distilled water directly after heat treatment and 24 hours later to remove possible trehalose residues. Three replicates of 40 cysts were used for each level and each population (A-E).

Table 3.4. Artificially imposed levels of mortality comprised of a mixture of unheated and heat-treated *G. pallida* cysts.

Population	Viability level	Number of heat-treated cysts	Number of unheated cysts
A-E	1	0	40
	2	20	20
	3	30	10
	4	35	5
	5	39	1
	6	40	0

3.2.9. *Statistical analysis*

Within each assay (I-IV) the differences in variance between experiments was examined by Microsoft Excel (Microsoft Corporation, 2016) using paired F-tests (F-test two-sample for variances). Each combination of experiments 1, 2 and 3 was tested to confirm or reject the null hypothesis that there was no difference (unequally) in variances. As a result of significant differences in variation between the datasets, the three datasets were not combined and analysed individually. Viability data (%) obtained by assays I, II, III and IV were compared using simple linear regression analysis. Tested relationships were always positive hence only strength of the relationships was reported (Fowler *et al*., 2013). The percentage

viability results were subjected to logit transformation before being analysed by general ANOVA in Genstat (VSN International, 18th Edition). Where all eggs were detected as viable (100%) the result was reported as 99.9% to allow logit transformation of data. Tukey's multiple range test (95% confidence intervals) was used to confirm where differences occurred between tested groups.

3.3. Results

3.3.1. *Trehalose assay - number of eggs to be used for* G. pallida *field population viability assessment*

Serial dilutions of egg suspensions obtained from 1, 3, 5, 10, 25 and 50 cysts of *G. pallida* populations (A-E) produced a wide range of samples containing 0 to 11,947 eggs.

All samples detected with viable eggs, regardless of their population or initial cysts number origin, were divided in four groups containing: 1 to 99; 100 to 999; 1,000 to 3,999 and 4,000 to 12, 000 *G. pallida* eggs. A modest relationship (R=0.45) between ΔA and samples with 1 to 99 eggs (Figure 3.1.) and a very weak relationship (R=0.04) for samples with 4,000 to 12,000 eggs (Figure 3.4.) suggested that these two extremes of the scale should be avoided, when preparing samples, as they could not provide reliable reads. When ΔA measured in samples containing between 100 and 999 eggs (Figure 3.2.) and between 1,000 and 3,999 eggs (Figure 3.3.) were plotted against egg counts a strong relationship (R=0.7) was found.

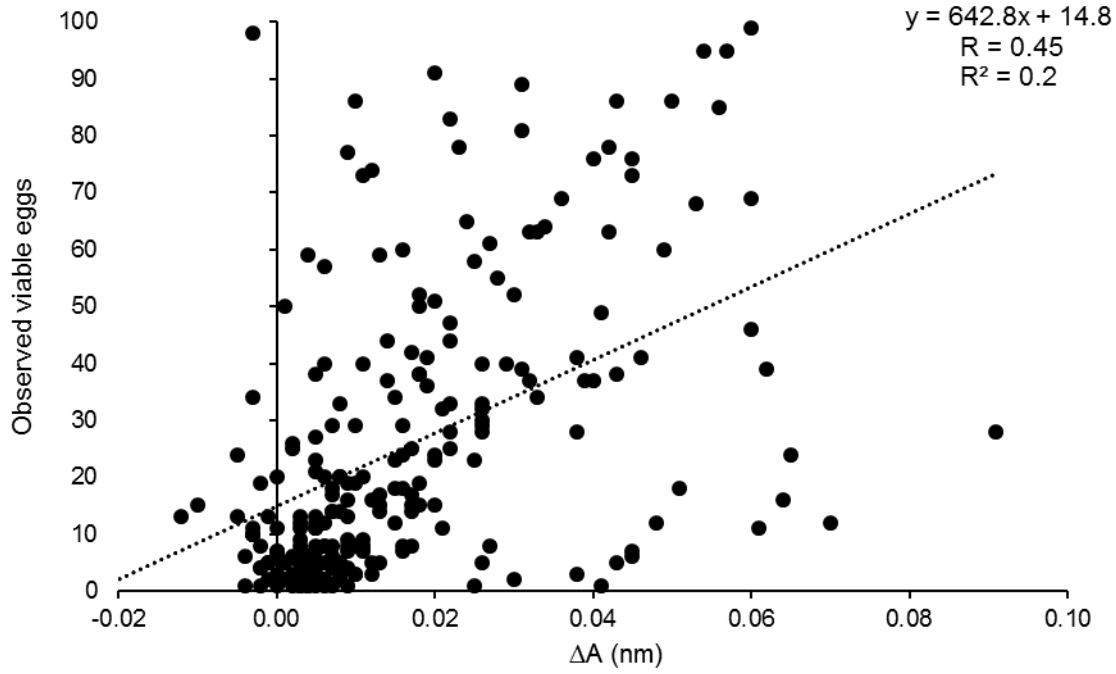


Figure 3.1. Relationship between change of absorbance (ΔA) and number of observed viable *G. pallida* eggs (populations A-E) between 1 and 99.

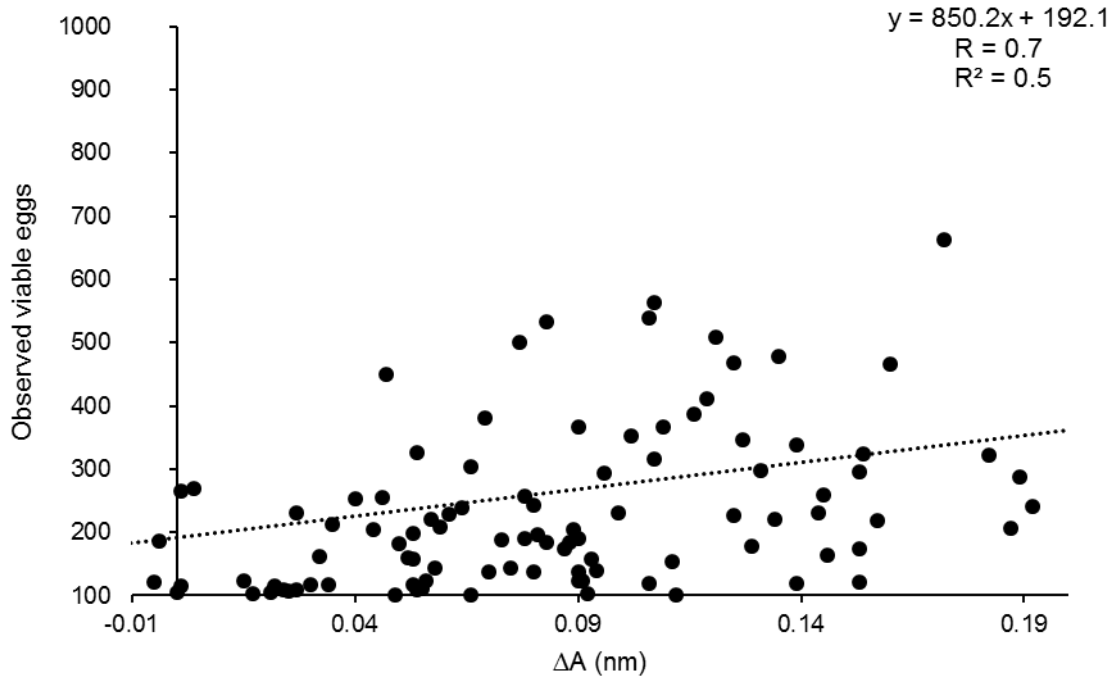


Figure 3.2. Relationship between change of absorbance (ΔA) and number of observed viable *G. pallida* eggs (populations A-E) between 100 and 999.

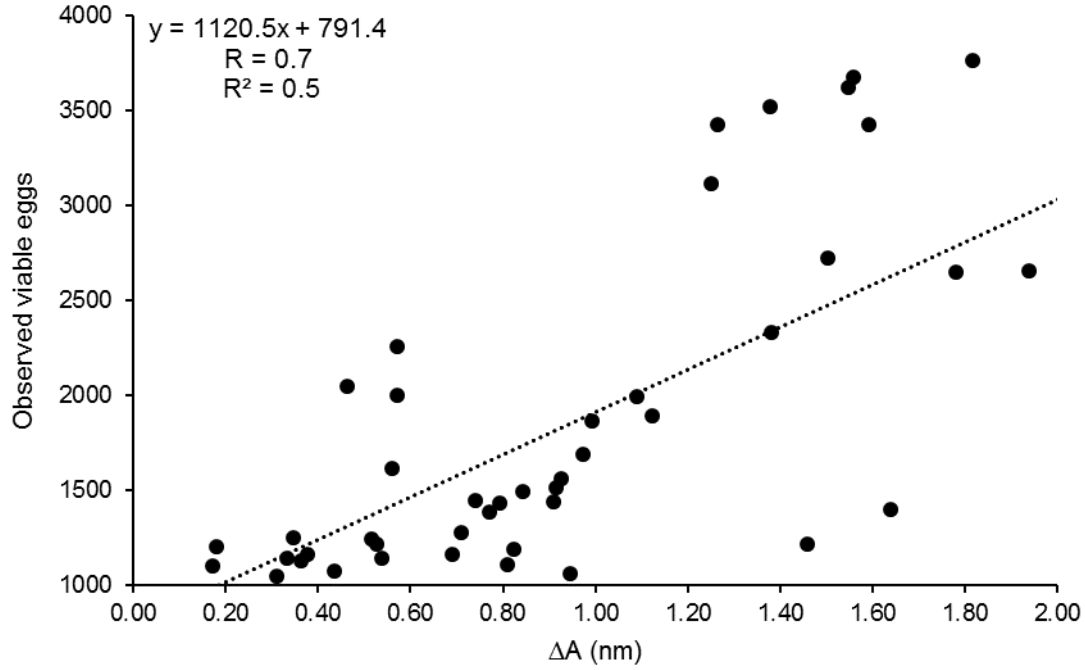


Figure 3.3. Relationship between change of absorbance (ΔA) and number of observed viable *G. pallida* eggs (populations A-E) between 1,000 and 3,999.

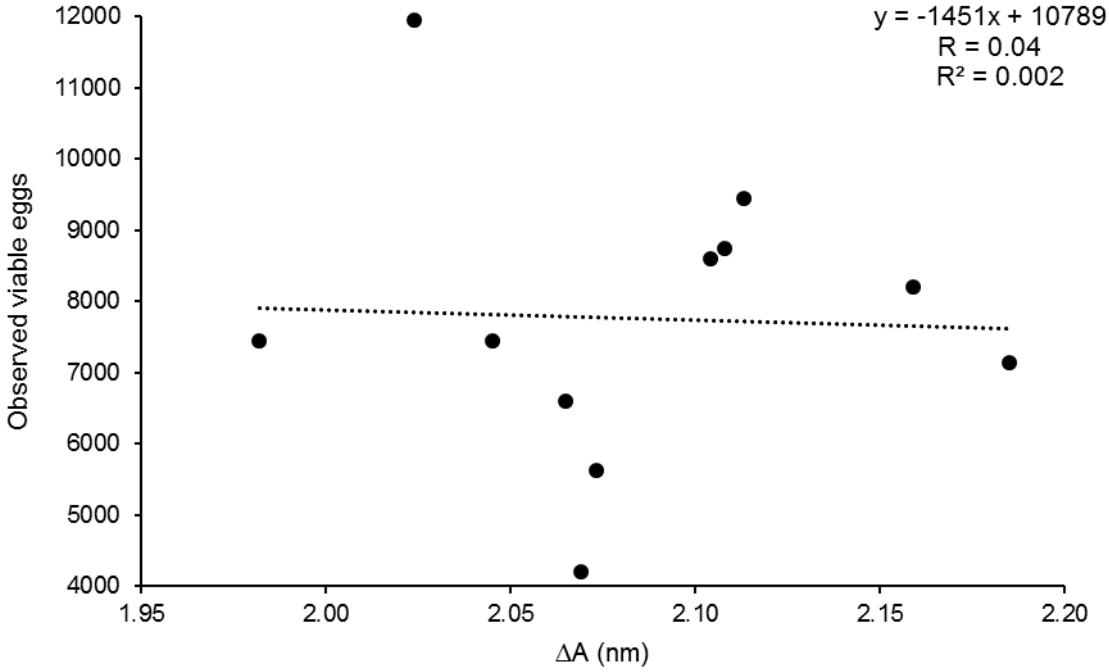


Figure 3.4. Relationship between change of absorbance (ΔA) and number of observed viable *G. pallida* eggs (populations A-E) between 4,000 and 12,000.

To closer investigate the assay's upper threshold, individual samples with the highest numbers of observed viable eggs (3,000 – 12,000) were plotted individually (Figure 3.5.). The upper threshold was reached at approximately 5,000 viable eggs at ΔA just above 2nm. There was a marginal increase in ΔA between 4,204 and 5,631 eggs, from 2.069 to 2.073nm, but ΔA for 6,601 eggs showed no further increase and was even lower (2.065nm).

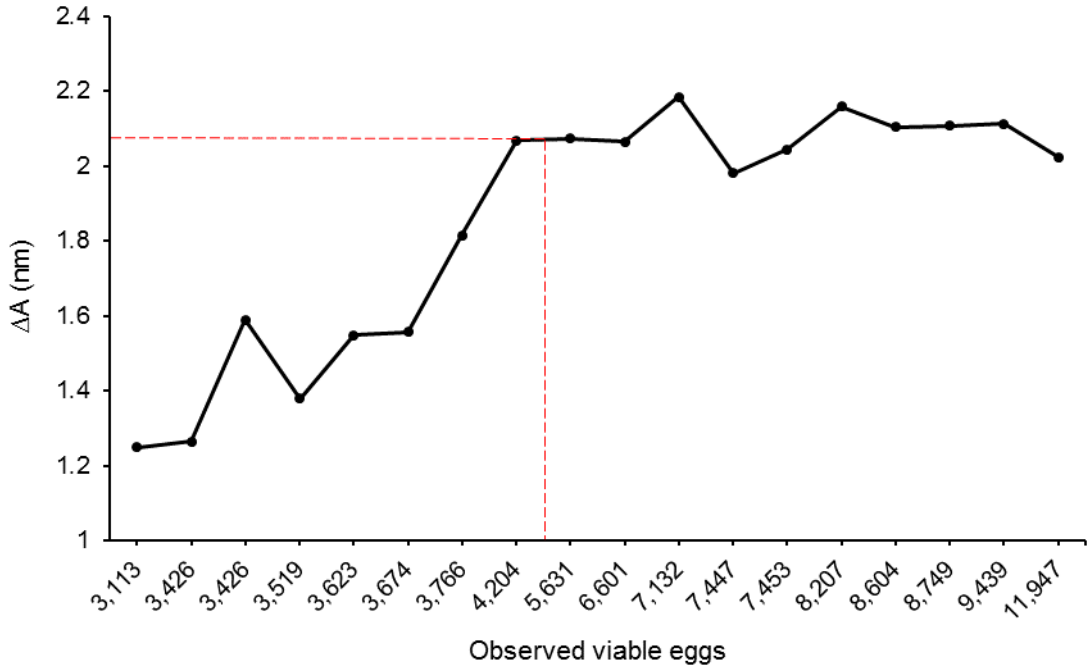


Figure 3.5. The change of absorbance (ΔA) measured in samples with a number of observed viable *G. pallida* eggs (populations A-E) greater than 3,000. Red dashed lines highlight the conditions when assay's upper threshold was reached.

The same approach was taken to assess the assay's lower threshold (Figure 3.6.). The ΔA gradually stabilized, with only a few exceptions, when number of observed viable eggs in sample exceeded 116. These observations suggested that the assay should be conducted on samples containing between 100-5,000 *G. pallida* eggs.

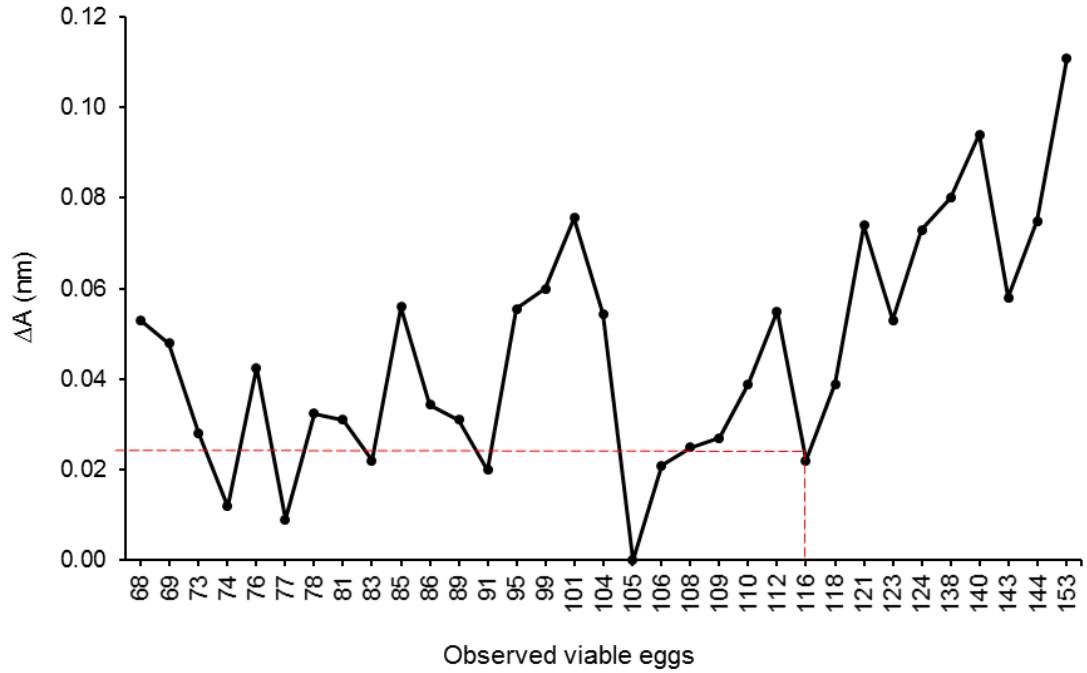


Figure 3.6. The change of absorbance (ΔA) measured in samples with number of observed viable *G. pallida* eggs (populations A-E) lesser than 200. Red dashed lines highlight the conditions when assay's lower threshold was reached.

A very strong relationship (R=0.9) was observed between ΔA and number of eggs on samples containing more than 100 and less than 5,000 eggs (Figure 3.7.).

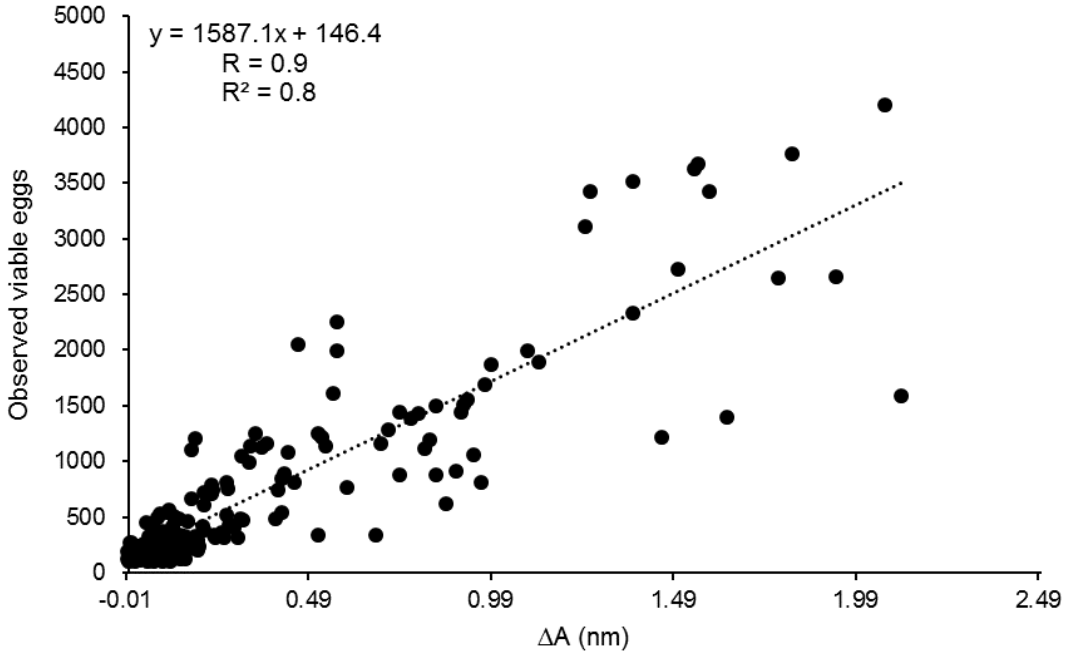


Figure 3.7. Relationship between change of absorbance (ΔA) and number of observed viable *G. pallida* eggs (populations A-E) between 100 and 5,000.

A positive change in absorbance was detected in 32% samples without observed viable eggs. A value of zero was observed in 10% of these samples, while 58% of the samples had a negative ΔA value. The mean value for background noise from samples with no eggs was -0.0002nm. Some samples with apparently viable eggs produced zero or negative ΔA reads. This was seen in samples with 1 to 10 eggs (53%), 11 to 100 eggs (32%) and more than 100 eggs (16%). In the last group only one sample contained more than 200 eggs (n=713). Samples with an increasingly higher number of eggs had a gradually lower probability of having zero or negative absorbance readings.

3.3.2. *Trehalose assay - number of cysts to be used for* G. pallida *field population viability assessment*

Variability in egg contents observed in samples with same number of cysts (1, 3, 5, 10, 25 and 50) but originating from different populations were found to have a strong relationship (R=0.7) (Figure 3.8.). Variability in egg content between samples with the same number of cysts was lower when the relationship was tested for individual populations. Populations A, B, C were described by a very strong (R=0.9) and population D by strong relationship (R=0.86). A modest relationship (R=0.6) was observed for population E.

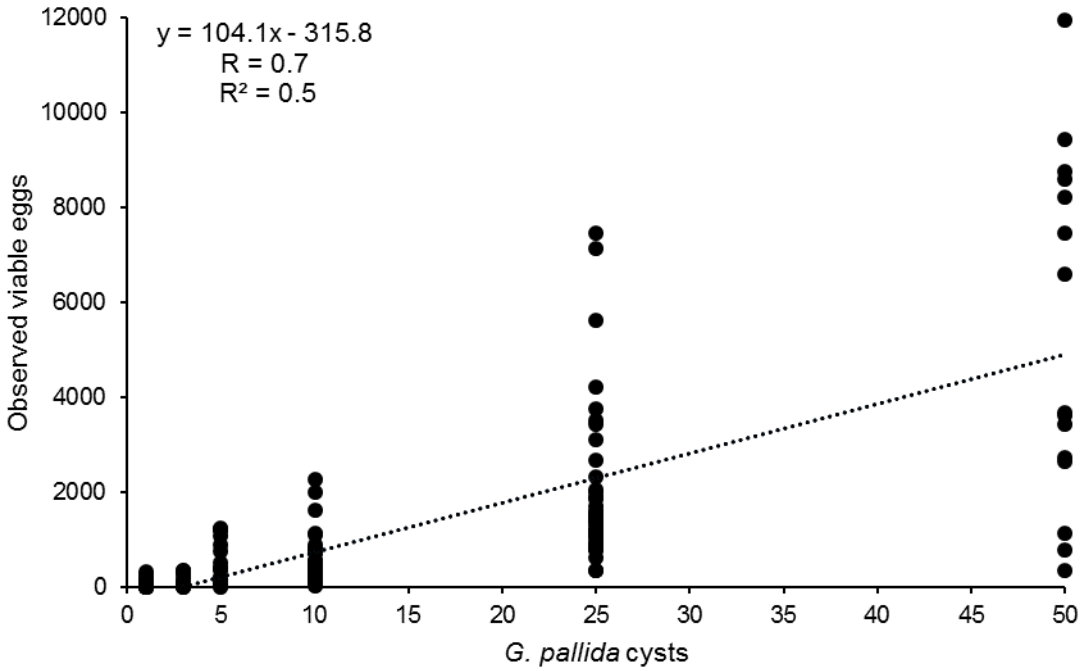


Figure 3.8. Relationship between number of *G. pallida* cysts (1, 3, 5, 10, 25 and 50) and number of eggs within these cysts (populations A-E combined).

When the trehalose assay was performed on samples with one cyst, on populations (A-E) combined, 40% contained no eggs and only 60% could be included in the viability assessment. Of these samples, 44% gave a negative ΔA value. Additionally, the optimal number of viable eggs for the assay (100 – 5,000) was only found in 33% of these samples. This variability between individual egg suspensions was represented by a modest relationship (R=0.67) seen between number of eggs and ΔA.

A very strong relationship (R=0.95) was seen between the number of eggs and ΔA (Figure 3.9.) in samples obtained from three cysts, populations (A-E) combined. Nevertheless, the optimal number of viable eggs for the assay (100 – 5,000) was only present in 47% of the samples.

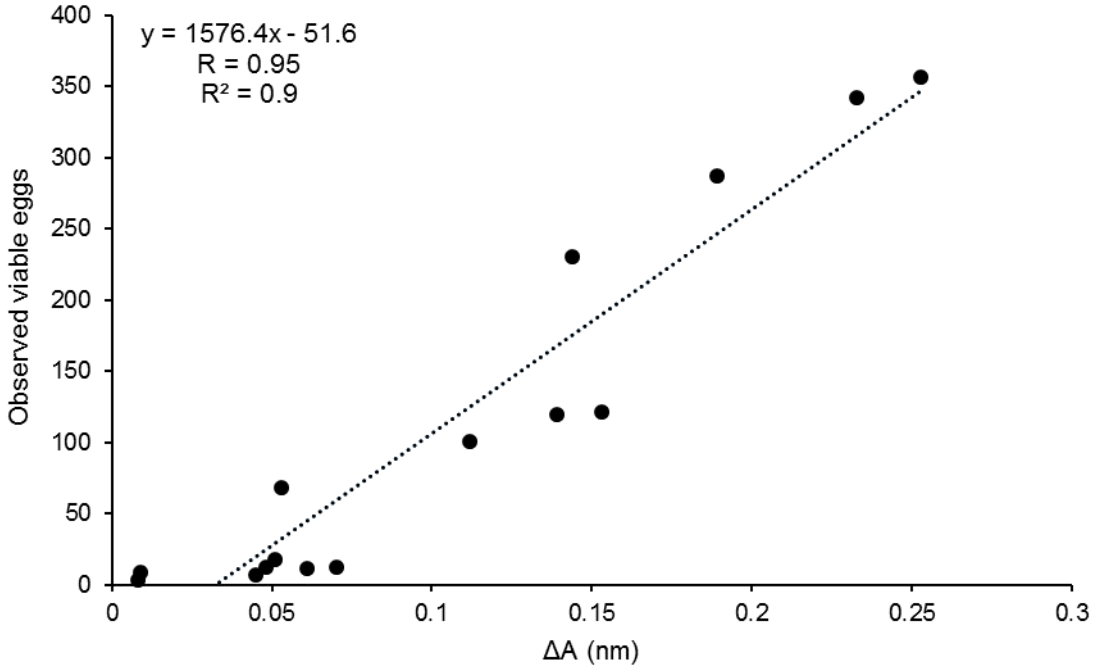


Figure 3.9. Relationship between change of absorbance (∆A) and number of observed viable *G. pallida* eggs released from three cysts (populations A-E).

The optimal number of viable eggs (100 – 5,000) for the assay was observed in 80, 100 and 87% of the samples originating from 5, 10 and 25 cysts, respectively. Only one, negative ∆A value, in each group, was seen for samples obtained from 5 and 10 cysts. When the number of eggs was plotted against ∆A, a very strong relationship was seen for the egg suspensions obtained from 5, 10 and 25 cysts (R=0.98, 0.95 and 0.92 respectively). (Figure 3.10. - 3.12.).

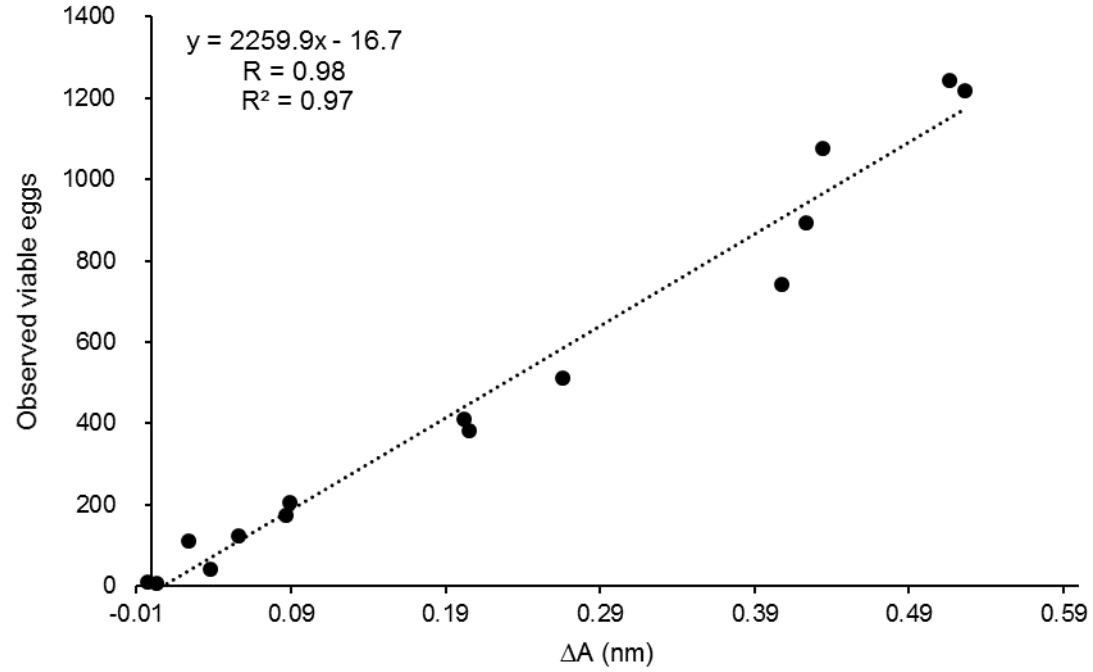


Figure 3.10. Relationship between change of absorbance (ΔA) and number of observed viable *G. pallida* eggs released from five cysts (populations A-E).

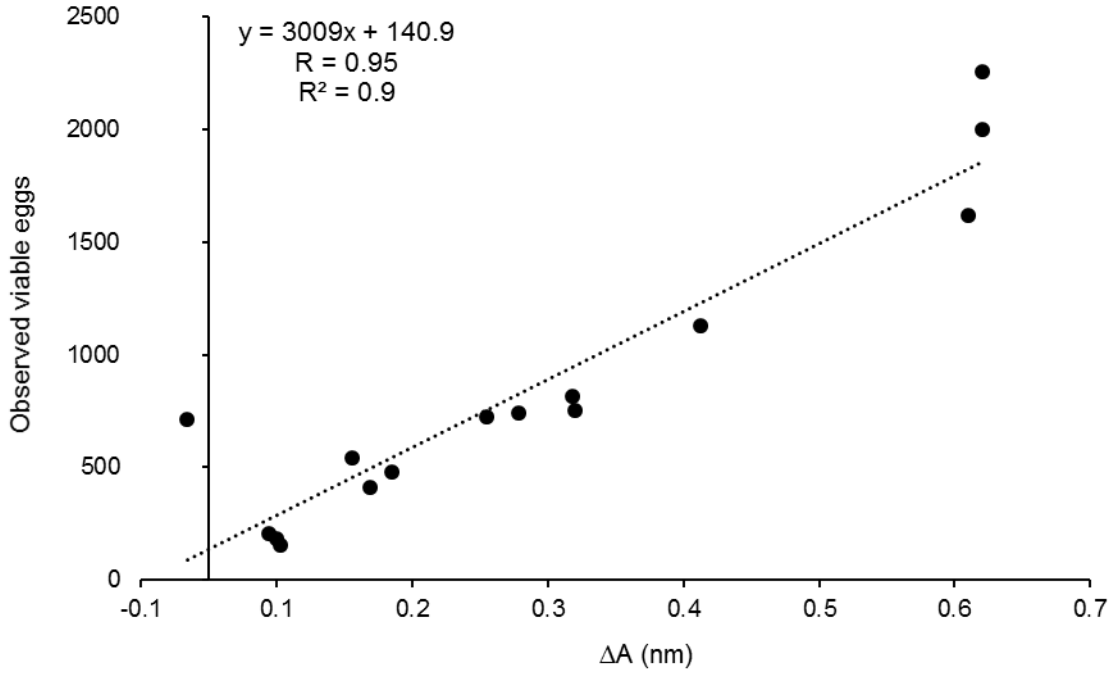


Figure 3.11. Relationship between change of absorbance (ΔA) and number of observed viable *G. pallida* eggs released from 10 cysts (populations A-E).

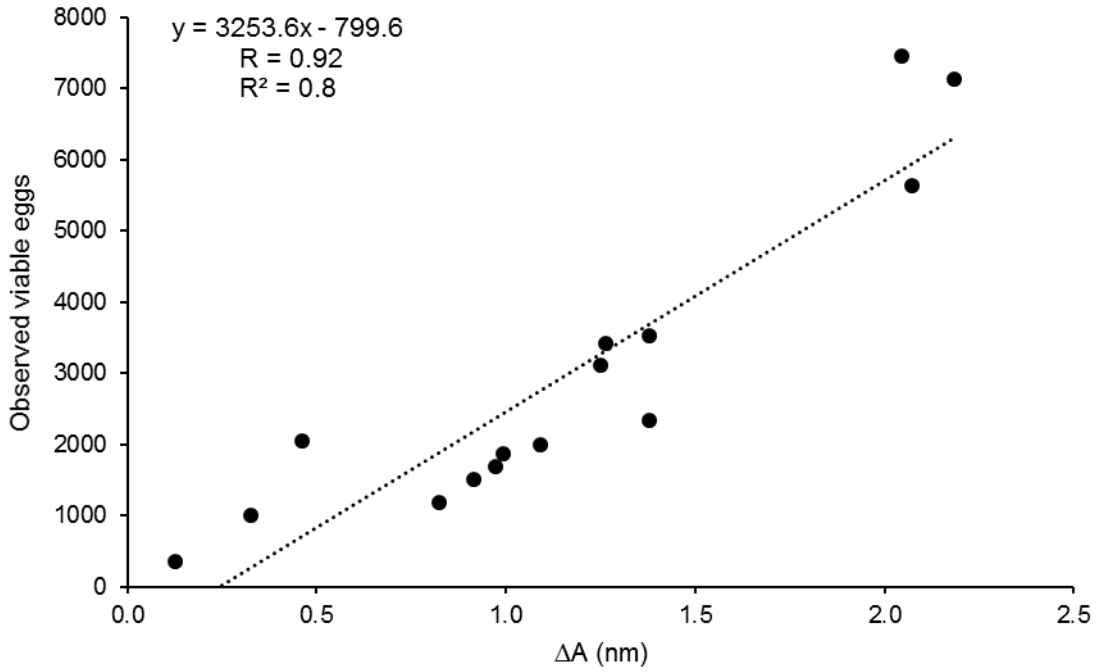


Figure 3.12. Relationship between change of absorbance (ΔA) and number of observed viable *G. pallida* eggs released from 25 cysts (populations A-E).

The optimal number of viable eggs for the assay (100 – 5,000) was observed in only 53% of the samples obtained from 50 cysts, populations (A-E) combined. There were no samples without viable eggs nor with negative ΔA calculated value. A modest relationship (R=0.84) was observed between number of eggs and ΔA (Figure 3.13.). Samples with more than 6,000 observed viable eggs with a ΔA value above 2nm exceeded the assay's upper threshold (Figure 3.13.).

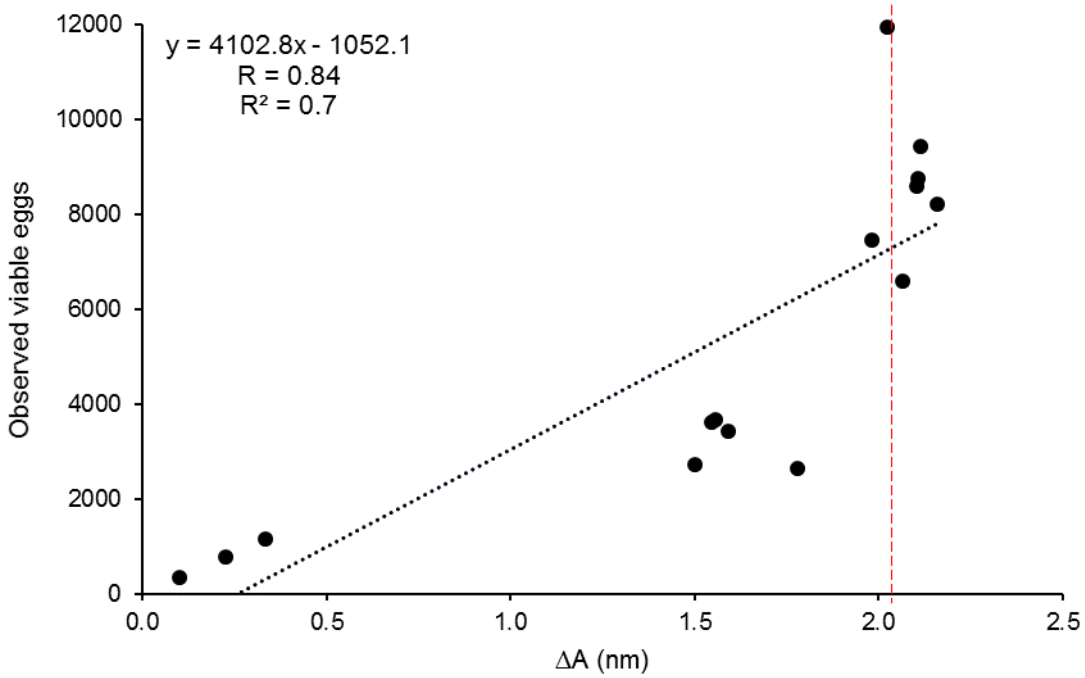


Figure 3.13. Relationship between change of absorbance (ΔA) and number of observed viable *G. pallida* eggs released from 50 cysts (populations A-E). Red dashed line highlights the ΔA measuring threshold.

The spectrophotometer measuring limit was observed at approximately 2.7nm when A2 was measured. This value was reduced to 2.4nm when the measurement was adjusted by the deduction of the negative (blank) control value (Figure 3. 14.).

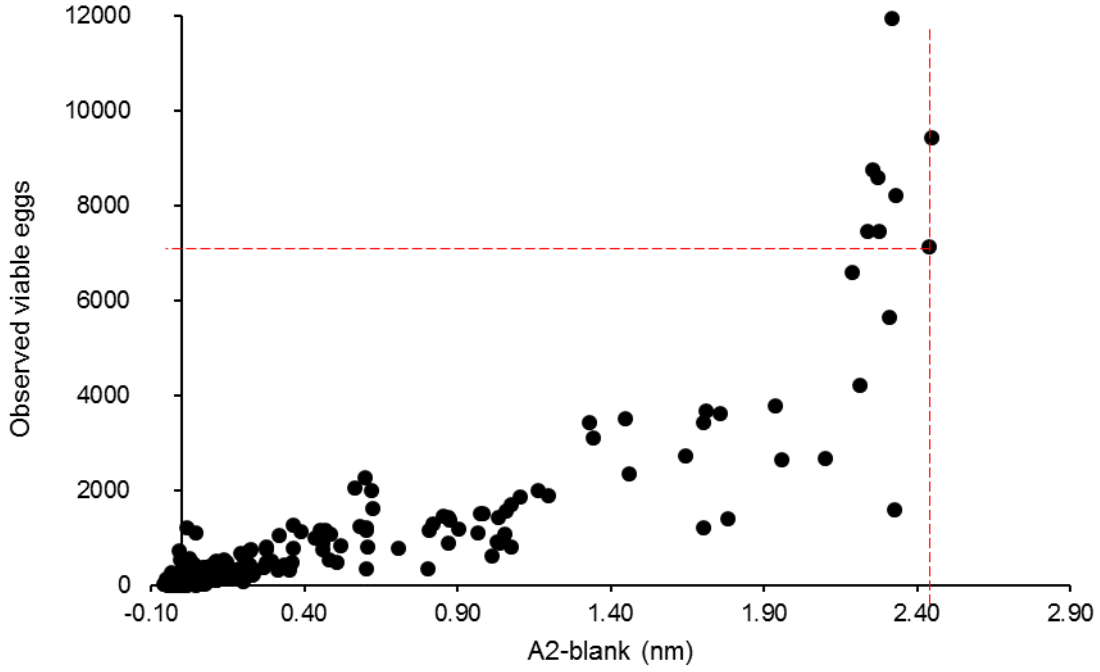


Figure 3. 14. Distribution of absorbance measured after trehalose hydrolysis reaction (A2) uniformed by deduction of negative control value (blank) measured in samples with observed viable *G. pallida* eggs between 1 and 12,000 (populations A-E). Red dashed lines highlight the conditions when assay's upper threshold was reached.

Based on the experiments conducted, it was decided that the trehalose assays of *G. pallida* field populations should be performed on 25 cysts. This number was chosen over 5 and 10 cysts as it would provide greater representation of the population.

Number of eggs released from the same number of cysts varied between *G. pallida* field populations (Figure 3.15.). It was especially noticeable when 10 and 25 cysts were used to assess the viability of five populations (A-E) where a highly significant ($P<0.001$) difference in the number of eggs was observed. Significant ($P<0.05$) difference was detected between the populations when the number of eggs released from five cysts was tested.

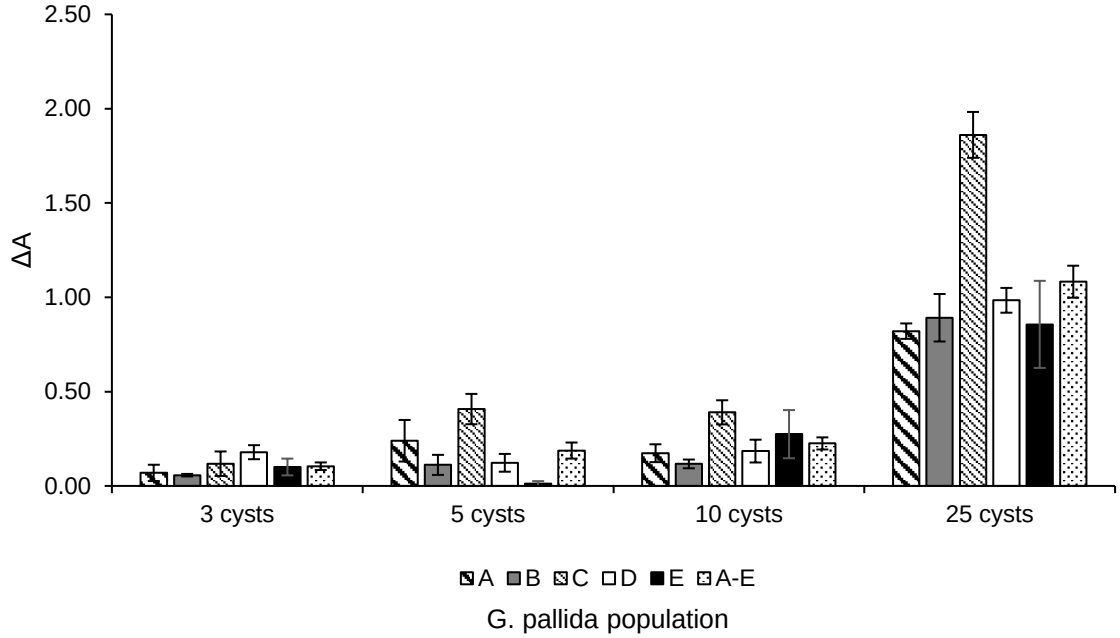


Figure 3.15. Number of viable eggs released from 5, 10 and 25 cysts of *G. pallida* field populations (A-E). Error bars represent the standard error of the mean.

3.3.3. *Trehalose assay – change of absorbance per viable egg*

The number of eggs per cyst (eggs cyst^{-1}) for populations used in this study was on average 52, 80, 171, 58 and 42, populations (A-E) combined, respectively when 25 cysts were tested. High significant difference ($P<0.001$) was observed when eggs cyst^{-1} data for all populations (A-E), subjected to square root transformation, were analysed (Figure 3.16.). This variability highlighted the need to study the *G. pallida* field populations individually to investigate ΔA per individual viable egg.

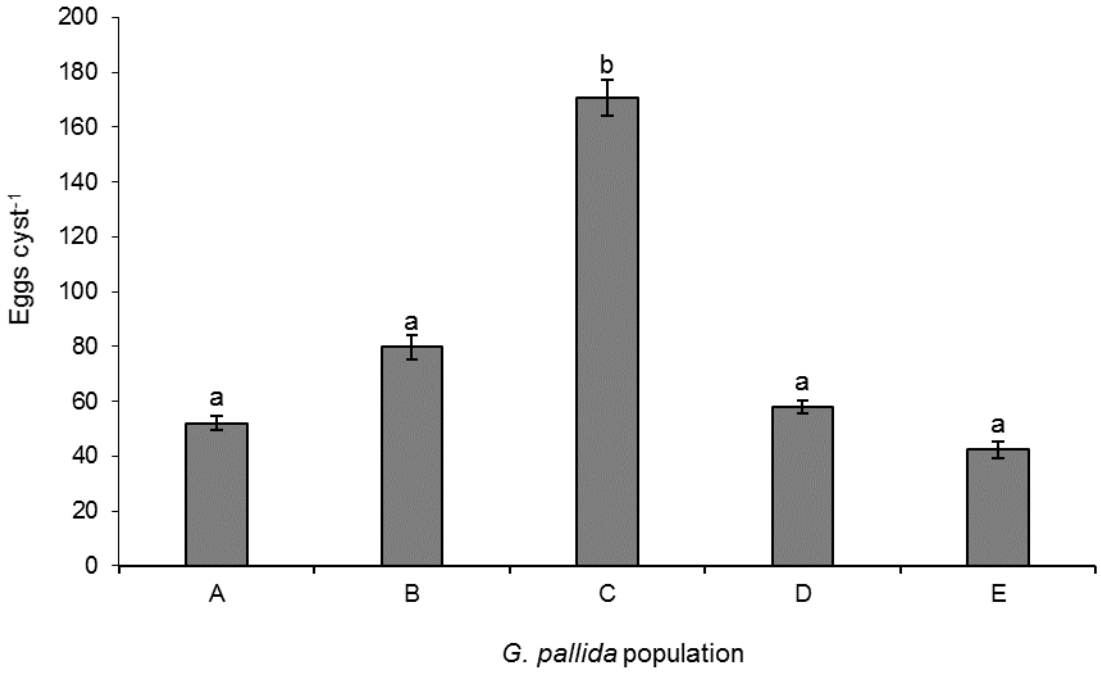


Figure 3.16. Number of eggs per cyst (eggs cyst^{-1}) in samples containing 25 cysts of *G. pallida* field populations (A-E). Error bars represent the standard error of the mean. Letters above data bars represent differences determined by Tukey's test (P>0.05).

Assessment of viability on *G. pallida* eggs with artificially imposed levels of mortality, discussed later in detail (section 3.3.5.), revealed that absorbance was still detectable in samples containing heat-killed eggs. Change of absorbance detected in samples containing dead specimens was 0.008, 0.004, 0.046, 0.021 and 0.017nm for populations A-E, respectively. It was important to report this finding here as the specimen's background noise needed to be accounted for when calculating ΔA viable egg^{-1}. A significant difference (P<0.05) was observed between ΔA of populations (A-E) from samples containing dead eggs (Figure 3.17.) suggesting that the specimen's background noise should be deducted individually for ΔA of the population tested.

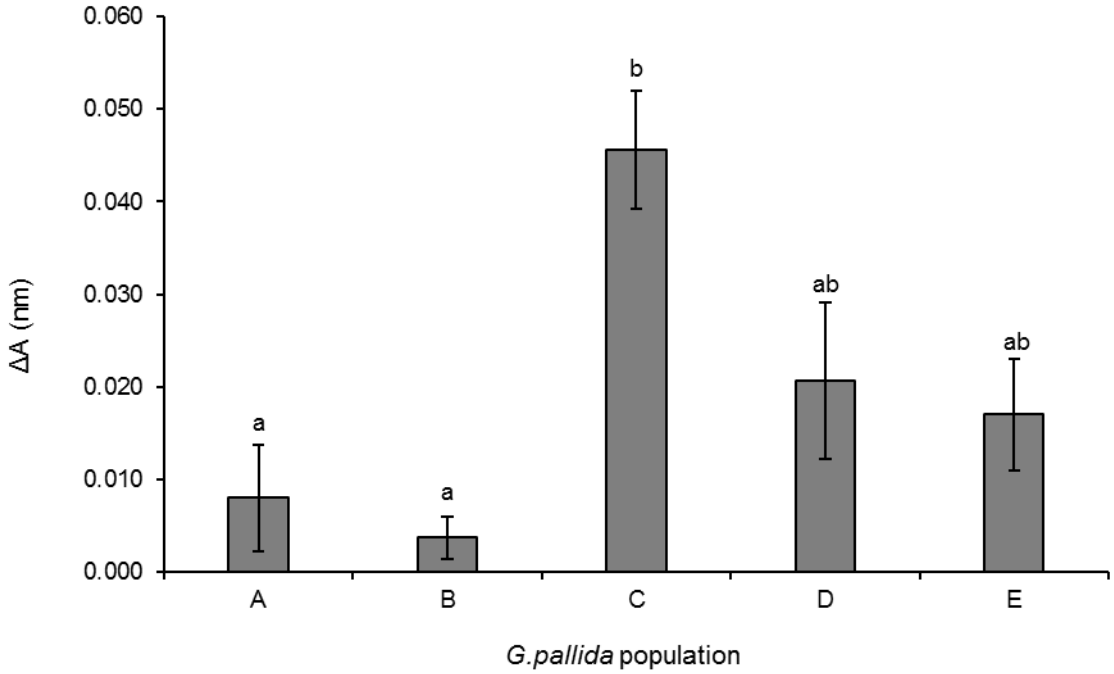


Figure 3.17. Change of absorbance (∆A) detected in samples containing dead (heat-killed) cysts of *G. pallida* field populations (A-E). Error bars represent the standard error of the mean. Letters above data bars represent differences determined by Tukey's test (P>0.05).

Change of absorbance per viable egg calculated from samples containing between 100 and 5,000 viable eggs (Figure 3.18.), after reduction of the specimen's background noise calculated individually for each population, was 0.005 for population A and D, 0.004 for populations B and C and 0.006 for population E. The background noise, observed in samples with no eggs, was not included in this calculation. It accounted for only 0.4% of ∆A measurements when obtained from samples containing egg content near the assays lower threshold (100-150eggs) and 0.01% of ∆A measurement when obtained from samples containing egg content near assays higher threshold (2,000-5,000 eggs).

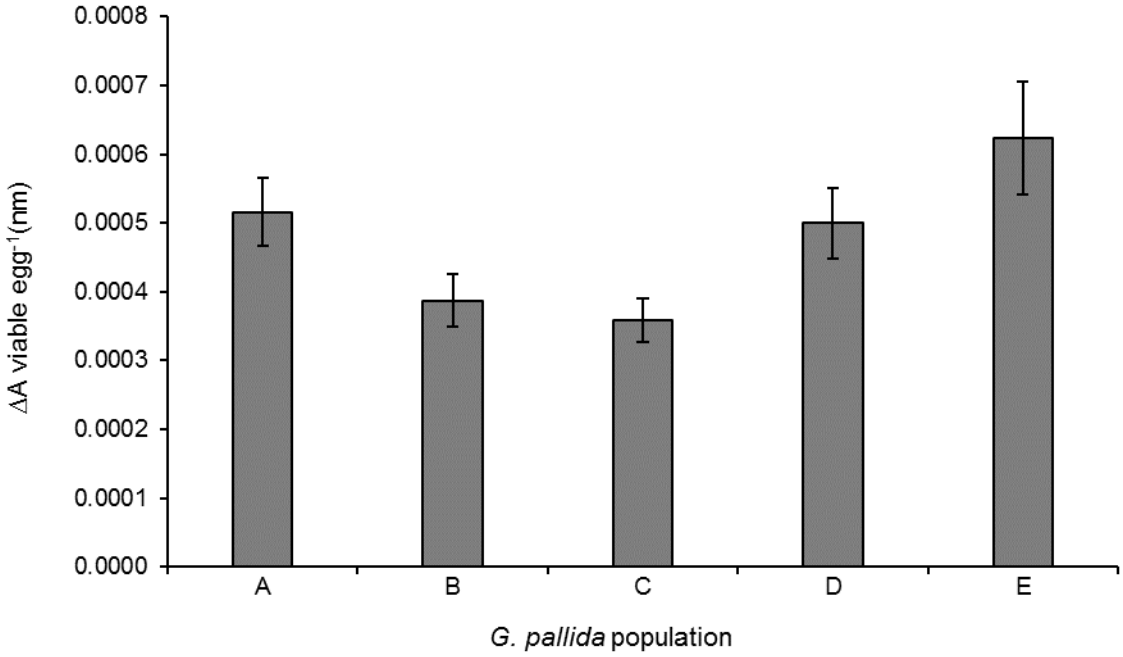


Figure 3.18. Change of absorbance (ΔA) per viable egg calculated from samples containing between 100 and 5,000 viable eggs, after reduction of the specimen's background noise, of *G. pallida* field populations (A-E). Error bars represent the standard error of the mean.

Only 2, 7, and 11% samples from populations A, B and D, respectively had ΔA lower than the specimen's background noise and at the same time contained more eggs than the lowest recommended number of eggs to be used for testing (100). Population C had a higher proportion of such samples – 28%, while population E had no samples outside recommended eggs ratio which showed ΔA below specimen's background noise.

3.3.4. A*ssessment of natural viability on* G. pallida *eggs*

Variance in viability (%) between experiments 1, 2 and 3, for assay I-IV individually, was tested (F test) and showed that variances of the experiments are unequal. The null hypothesis was rejected and the experiments could not be combined.

The natural viability of *G. pallida* field populations (A-E) assessed by hatching in PRD assay (assay I), hatching in PRD followed by Meldola's blue staining assay (assay II), Meldola's blue staining assay (assay III) and the trehalose assay (assay IV) in experiments 1, 2 and 3 are presented as percentage viability and number of viable eggs cysts^{-1}. Viable eggs per cysts as a proportion of total eggs cyst^{-1} are presented to highlight the variability between the populations and between the assays. Analysis were performed on viability percentage data.

3.3.4.1. *Hatching in potato root diffusate assay - assay I*

The results of natural viability of *G. pallida* field populations (A-E) assessed by hatching in PRD assay (assay I) in experiments 1, 2 and 3 are presented in Figure 3.19.

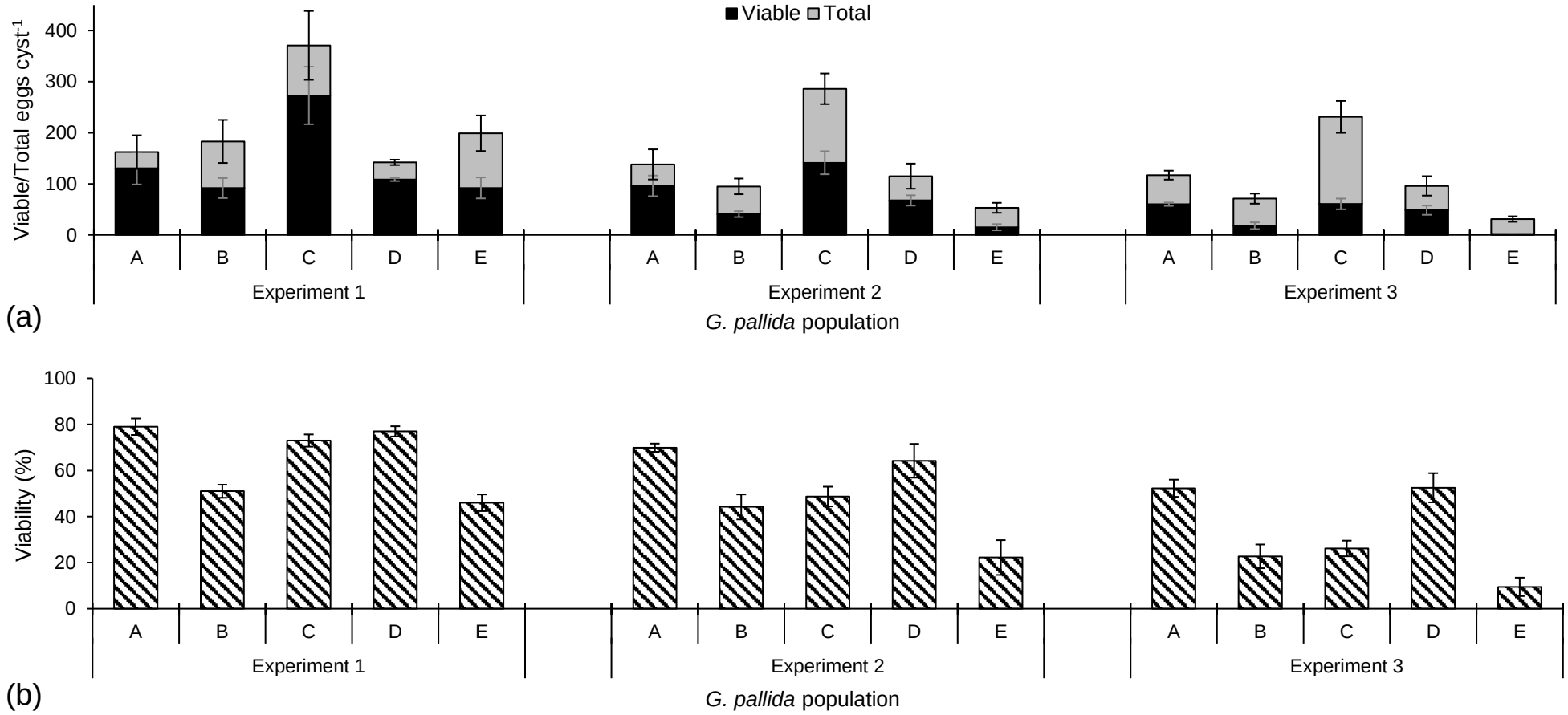


Figure 3.19. Viability of *G. pallida* field populations (A-E) assessed by hatching in PRD assay (assay I) reported as (a) number of viable eggs of total eggs per cysts (viable/total eggs cyst^{-1}) and as (b) a percentage viability (%). Error bars represent the standard error of the mean.

Natural viability assessed by assay I in experiments 1, 2 and 3 was greatest in experiment 1. The assessments of experiment 2 were lower by comparison with experiment 1 and again lower in experiment 3, by comparison with experiment 1, for all *G. pallida* populations (A-E). The reduction was consistently greater across all populations in experiment 3 than in experiment 2. This shows that even if experiments were found to be significantly different from each other they do follow the pattern of reduction from one experiment to another across all populations.

After seven days of exposure to PRD, population B, across all three experiments, had the lowest score of total juveniles hatched. This population was followed by population A and populations D. Populations C and E, were similar to population B, having a very low hatch in experiment 1 and a higher hatch in experiment 2 and experiment 3. Fourteen days after being exposed to PRD, almost all populations in experiments 1, 2 and 3 showed an increase of hatching, reaching their peak which ranged from 80 (population C in experiment 1) to 29% (population B in experiment 3) of total hatching. Only population D (experiment 2) and population E (experiment 3) had a lower number of juveniles hatched after 14 days compared with the hatch observed after seven days of treatment. Twenty-one days after exposure, a reduction in hatching was observed for all populations in experiments 1, 2 and 3, and ranged from 20 (population B, experiment 2) to 5% (population D, experiment 2). A further gradual decline of hatching was observed on the following obeservation dates: - 28, 35, 42, 49 and 56 days of exposure to PRD. The only exception was population B (experiment 3) where hatching increased 5% following 28 days of exposure of PRD when compared to the hatch observed after 21 days (Figure 3.20.).

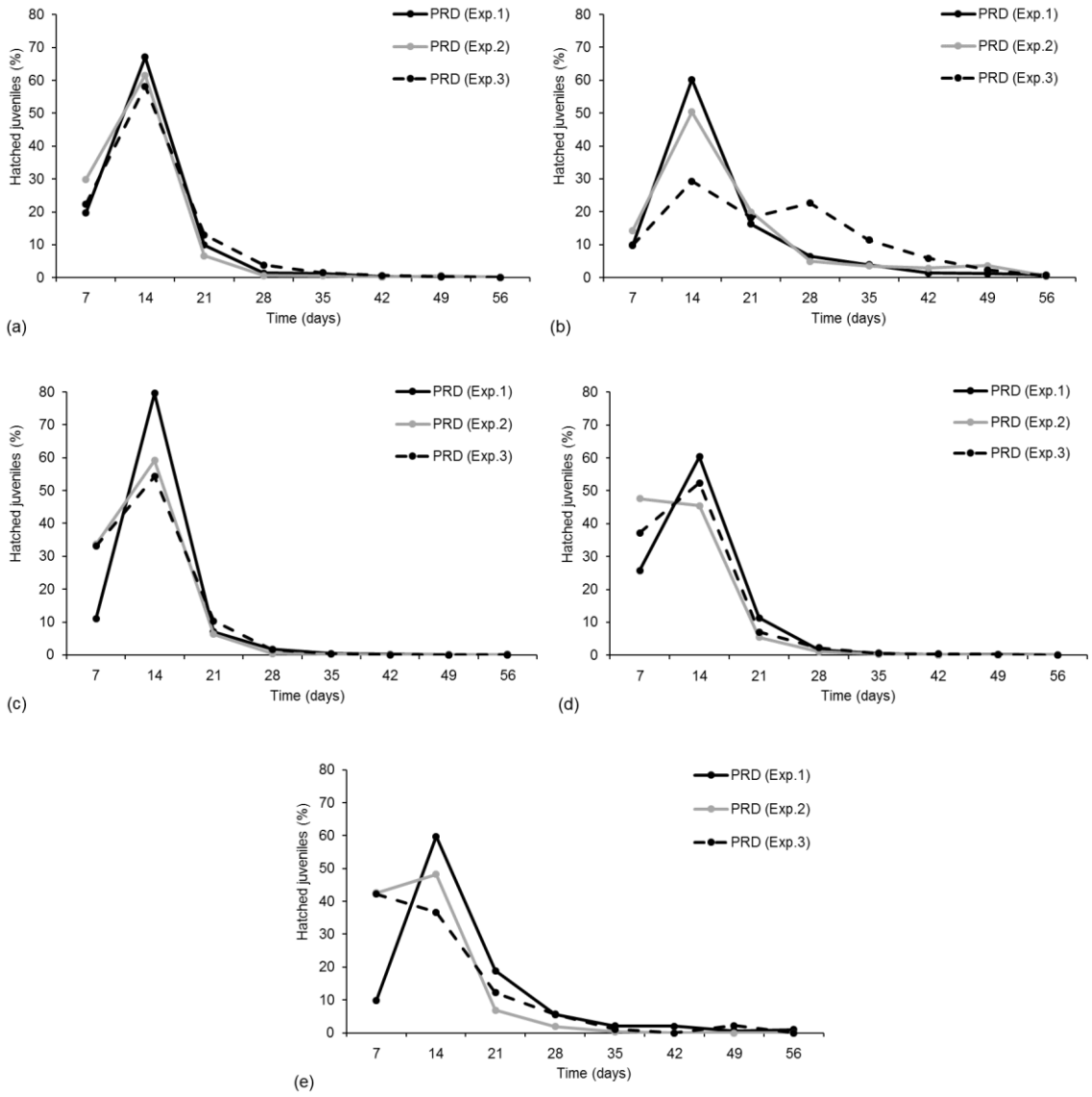


Figure 3.20. Hatching (assay I) of *G. pallida* field populations A (a), B (b), C (c), D (d) and E (e) after 7, 14, 21, 28, 35, 42, 49 and 56 days of exposure to PRD. Data presented as the percentage of total juveniles that hatched.

3.3.4.2. *Hatching in potato root diffusate followed by Meldola's blue staining assay - assay II*

The results of natural viability of *G. pallida* field populations (A-E) assessed by hatching in PRD followed by Meldola's blue staining assay (assay II) in experiments 1, 2 and 3 are presented in Figure 3.21.

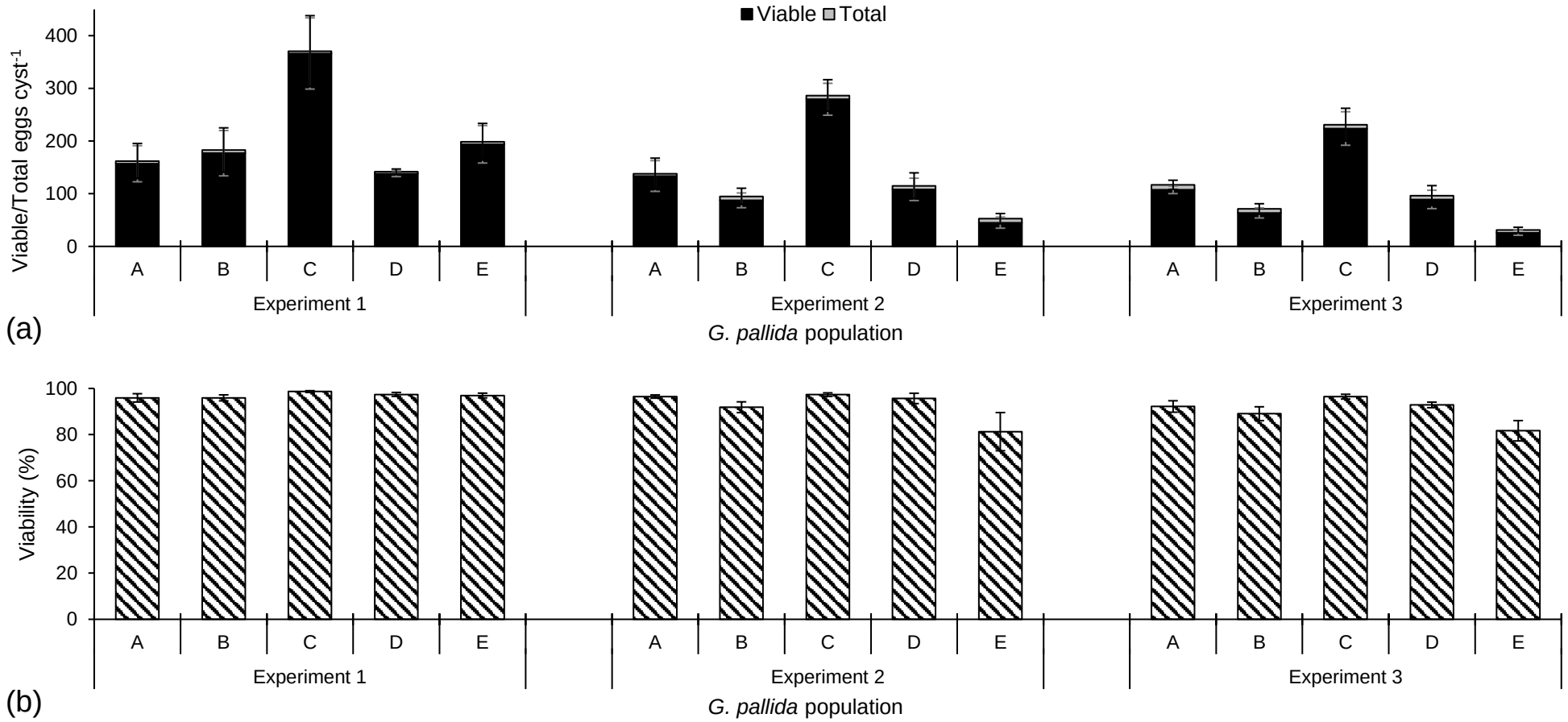


Figure 3.21. Viability of *G. pallida* field populations (A-E) assessed by hatching in PRD followed by Meldola's blue staining assay (assay II) reported as (a) number of viable eggs of total eggs per cysts (viable/total eggs cyst^{-1}) and as (b) a percentage viability (%). Error bars represent the standard error of the mean.

Natural viability assessed by assay II was highest, with only one exception, in experiment 1. The assessments of experiment 2 were lower than in experiment 1 for *G. pallida* field populations B-E. Only population A had higher viability (by 1%) when assessed in experiment 2. The assessments of experiment 3 were lower for all *G. pallida* field populations (A-E), by comparison with experiment 1. The reduction of viability was greater in experiment 3 then in experiment 2 when populations B, C and D were tested. Population E showed similar reduction in both experiments.

Secondary assessment by Meldola's blue staining after eight weeks (56 days) of exposure by PRD showed high viability of the unhatched eggs (Figure 3.22.). The highest viability of unhatched eggs was shown for popualtion C where 95% was reported as alive (viable). Populations B, D and E also had a high egg viability. The viability assessment of unhatched eggs was lowest, but still substantial, for population.

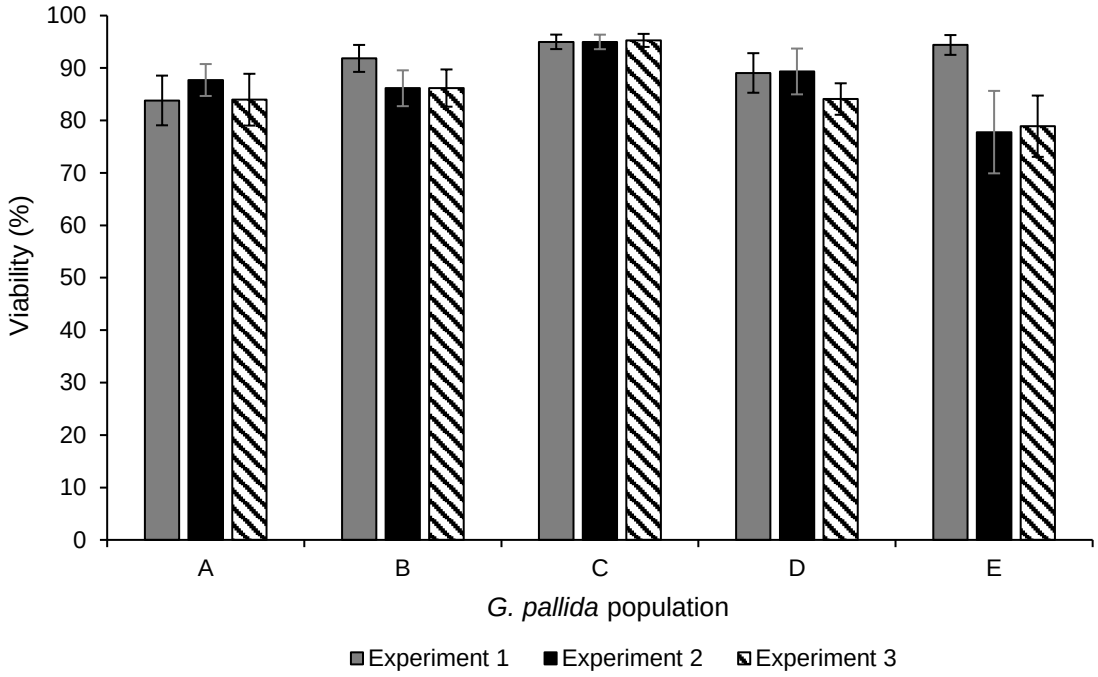


Figure 3.22. Viability (%) of unhatched *G. pallida* eggs assessed by Meldola's blue staining following eight weeks of exposure to PRD (assay II). Error bars represent the standard error of the mean.

3.3.4.3. *Meldola's blue staining assay - assay III*

The results of natural viability of *G. pallida* field populations (A-E) assessed by Meldola's blue staining assay (assay III) in experiment 1, 2 and 3 are presented in Figure 3.23.

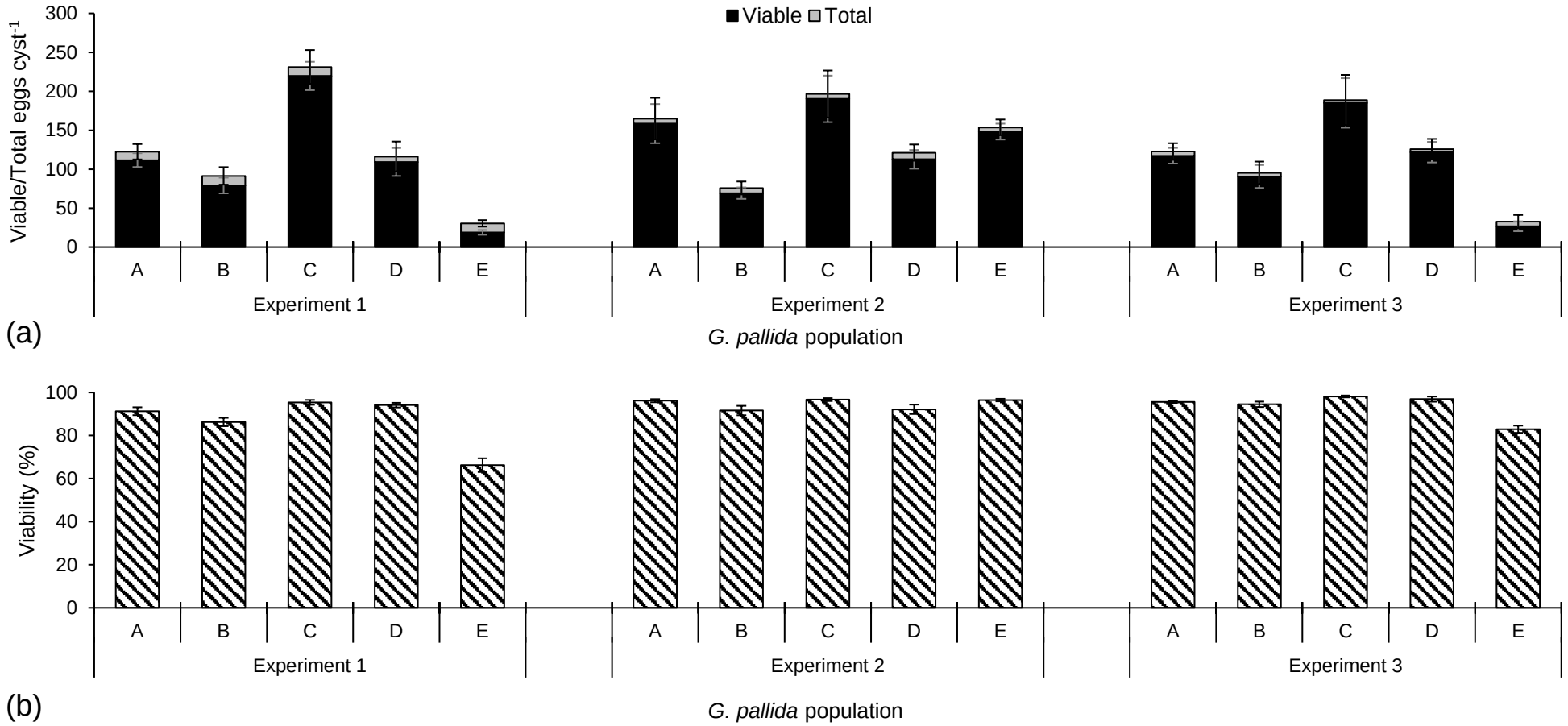


Figure 3.23. Viability of *G. pallida* field populations (A-E) assessed by Meldola's blue staining assay (assay III) reported as (a) number of viable eggs of total eggs per cysts (viable/total eggs cyst^{-1}) and as (b) a percentage viability (%). Error bars represent the standard error of the mean.

Natural viability assessed by assay III was, with one exception, lowest in experiment 1. Comparison with experiment 1 showed that assessments of experiment 2 were lower, by 2%, only for population D. The remaining populations A, B, C and E showed an increase in viability. Experiment 3 assessed viability of all populations (A-E) higher than in experiment 1. The increase of viability was greater in experiment 3 for populations B and C and greater in experiment 2 for population E. Population A showed similar increase in both experiments.

3.3.4.4. *Trehalose assay - assay IV*

The results of natural viability of *G. pallida* field populations (A-E) assessed by the trehalose assay (assay IV) in experiments 1, 2 and 3 are presented in Figure 3.24.

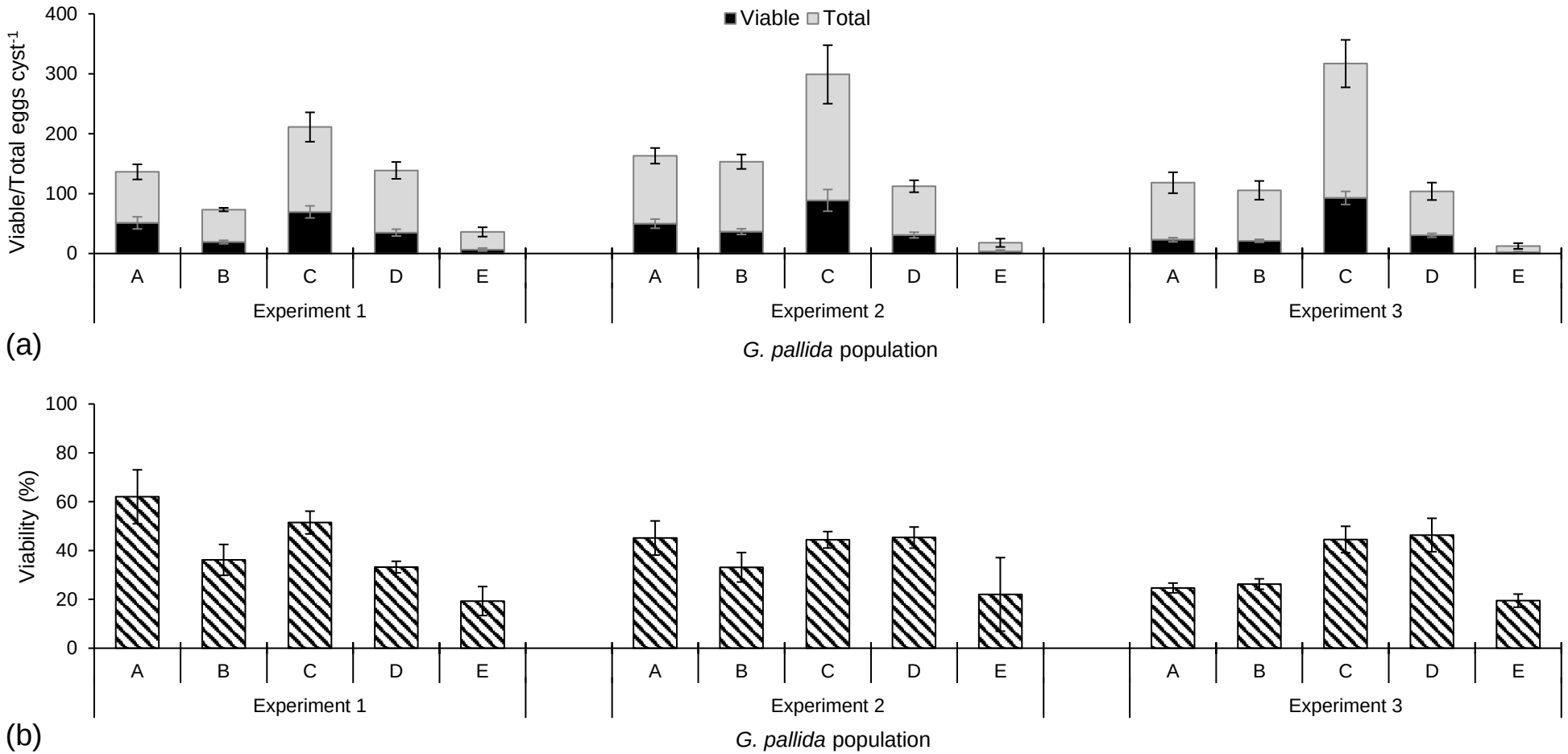


Figure 3.24. Viability of *G. pallida* field populations (A-E) assessed by the trehalose assay (assay IV) reported as (a) number of viable eggs of total eggs per cysts (viable/total eggs cyst^{-1}) and as (b) a percentage viability (%). Error bars represent the standard error of the mean.

Natural viability assessed by assay IV in experiment 1 was highest for populations A, B and C but lowest for population D and E. Lower estimation of viability in experiment 3 than in experiment 2, by comparison with experiment 1, was observed for populations A and B. Population C showed equal reduction in viability assessment in experiments 2 and 3 when compared against results from experiment 1. The increase of the viability of populations D was greater, by comparison with experiment 1, in experiment 3 than in experiment 2. The same comparison for population E shower greater viability increase in experiment 2.

3.3.4.5. *Natural viability – experiment 1*

General analysis of variance showed highly significant difference ($P<0.001$, 73% variance accounted for), between the viability estimates provided by the four viability assays (assays I-IV) for experiment 1 based on natural field populations. Also highly significant differences ($P<0.001$, 10% variance accounted for), were found between populations A-E, assays I-IV combined. The difference between the means for viability assessment methods (I-IV) and field populations (A-E) was tested and found to be significantly different ($P=0.03$) and accounted for only 4% of the variance (Figure 3.25.).

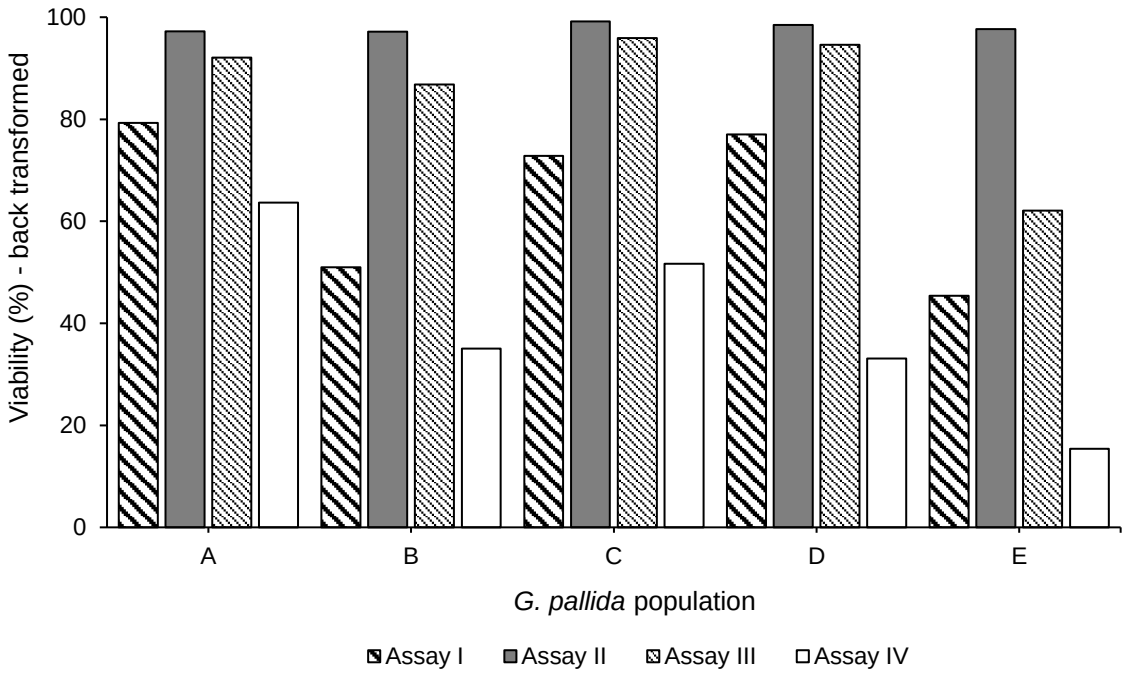


Figure 3.25. Viability (% - back transformed) assessed in experiment 1 by hatching in PRD assay (assay I), hatching in PRD followed by Meldola's blue staining assay (assay II), Meldola's blue staining assay (assay III) and the trehalose assay (assay IV) for *G. pallida* field populations (A-E).

3.3.4.6. *Natural viability – experiment 2*

General analysis of variance showed highly significant difference ($P<0.001$), that accounted for 81% of the variance, between the viability estimates provided by the four viability assays (assays I-IV) for experiment 2 based on natural field populations. A highly significant differences ($P<0.001$), but accounted for only 9% of the variance, were also found between populations A-E, assays I-IV combined. Significant differences ($P=0.04$), accounted for only 5% of the variance, were found when the difference between the means for viability assessment methods (I-IV) and field populations (A-E) was tested (Figure 3.26.).

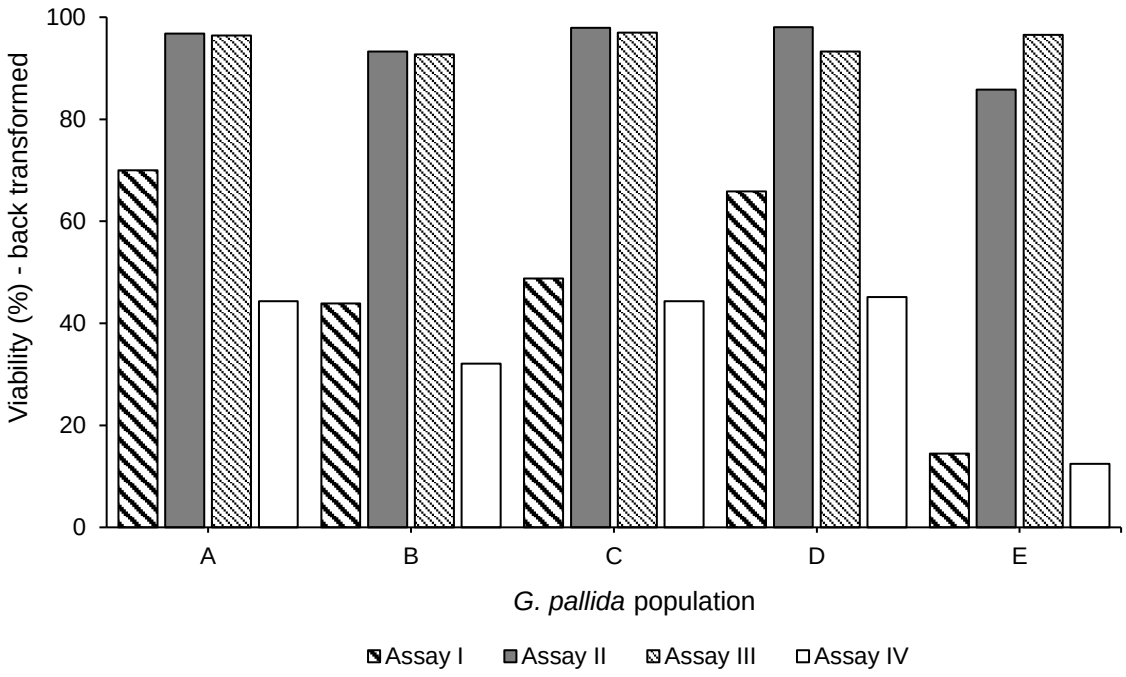


Figure 3.26. Viability (% - back transformed) assessed in experiment 2 by hatching in PRD assay (assay I), hatching in PRD followed by Meldola's blue staining assay (assay II), Meldola's blue staining assay (assay III) and the trehalose assay (assay IV) for *G. pallida* field populations (A-E).

3.3.4.7. *Natural viability – experiment 3*

General analysis of variance showed highly significant difference ($P<0.001$), and accounted for 79% of the variance, between the viability estimates provided by the four viability assays (assays I-IV) for experiment 3 based on natural field populations. A highly significant differences ($P<0.001$), but accounted for only 10% of the variance, were found between populations A-E, assays I-IV combined. In experiment 3 highly significant differences ($P<0.001$), accounted for only 4% of the variance, were also found when the difference between the means for viability assessment methods (I-IV) and field populations (A-E) was tested (Figure 3.27.).

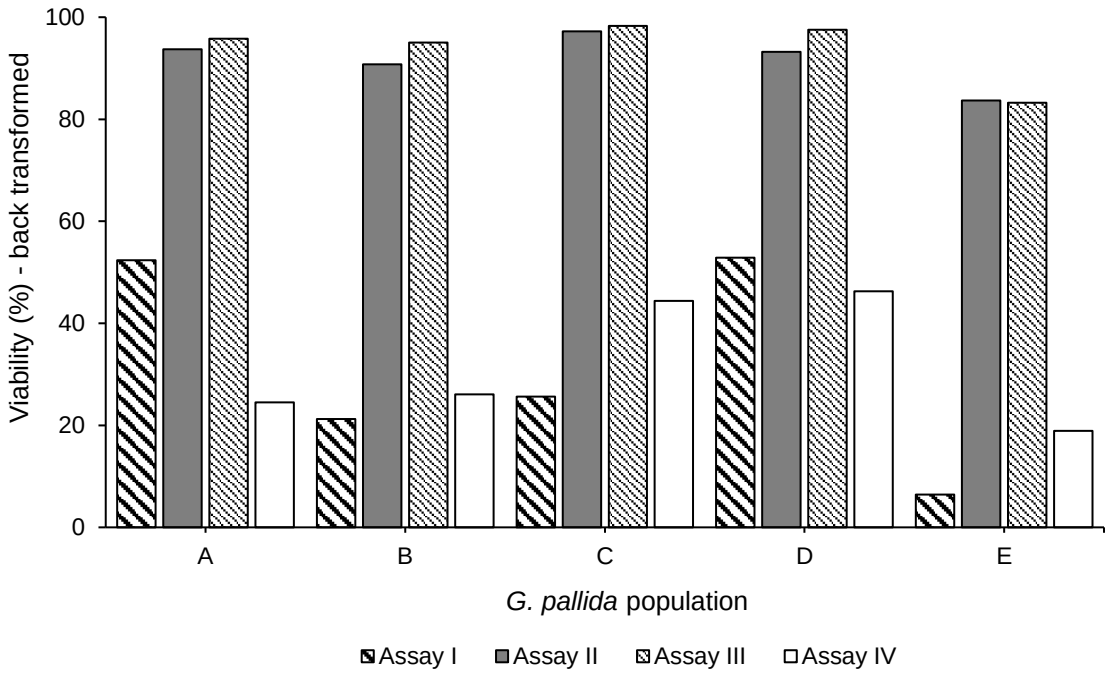
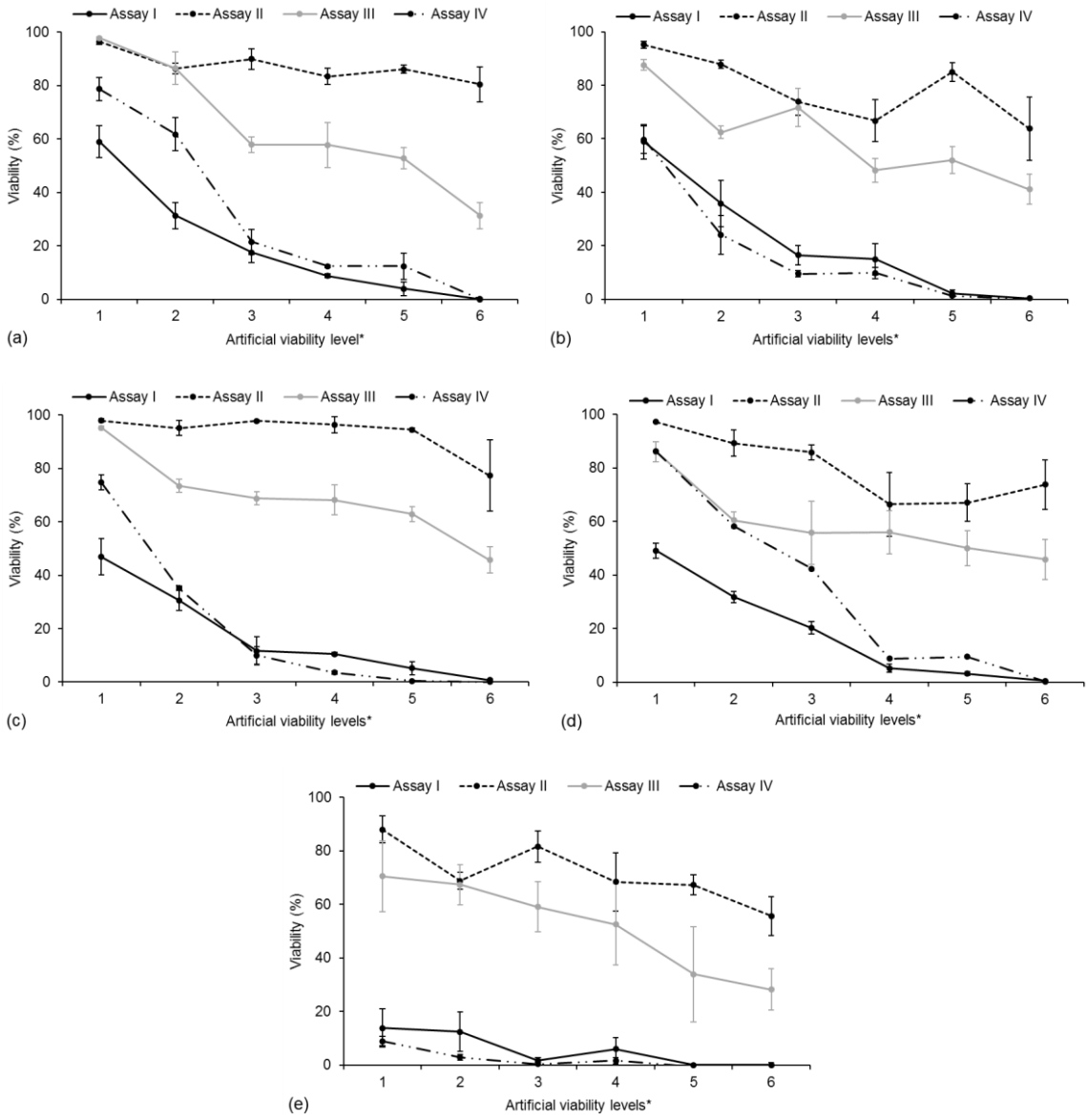


Figure 3.27. Viability (% - back transformed) assessed in experiment 3 by hatching in PRD assay (assay I), hatching in PRD followed by Meldola's blue staining assay (assay II), Meldola's blue staining assay (assay III) and the trehalose assay (assay IV) for *G. pallida* field populations (A-E).

3.3.5. *Assessment of viability on* G. pallida *eggs with artificially imposed levels of mortality*

Globodera pallida eggs (populations A-E) with six imposed levels of artificial viability gradually reduced viability in step with the number of heat-treated cysts in the samples, regardless of the initial viability used. Both assays II and III provided a higher estimate of viability than assay I and IV on all levels for all populations. A comparison between assay II and III showed that in case of 93% assessments (all levels/all populations) assay II detected higher number of viable eggs. The viability estimates by assay IV were higher than assay I for population A and D and lower for population B and E. Assessment for population C was higher when tested by

assay IV in samples with low proportion of heat-treated cysts (level 1 and 2) but lower on remaining levels (Figure 3.28.).



* Artificial viability levels comprised of a mixture of heat-treated and unheated *G. pallida* cysts. The cysts (heat-treated/unheated) proportion on each level was – 1: 0/40, 2: 20/20, 3: 30/10, 4: 35/5, 5: 39/1 and 6: 40/0 (Table 3.4.).

Figure 3.28. Viability (%) distribution assessed by hatching in PRD assay (assay I), hatching in PRD followed by Meldola's blue staining assay (assay II), Meldola's blue staining assay (assay III) and the trehalose assay (assay IV) for *G. pallida* field populations (a) A, (b) B, (c) C, (d) D and (e) E on six imposed levels of artificial viability. Error bars represent the standard error of the mean.

Differences between assays were clearly visible when viability level 6 (100% heat-treated cysts) was compared. Assay I provided an accurate estimate of non-viable nematodes in populations A, B, D and E. This assay also provided an acceptable estimate of viability for population C where level 6 had a viability estimate of less than 1%. A similar level of accuracy was observed with assay IV where populations A, B and C, at level 6, were correctly measured as 100% non-viable and populations D and E were determined to be <99% viable. In contrast, assays II and III incorrectly estimated viable eggs in samples where 100% of the cysts had been heat-treated (level 6). Meldola's blue staining assay (assay III) incorrectly produced estimates of percentage viability ranging from 28 (population E) to 46% (population C and D). The assessment of viability was even higher when assess by assay II. Here detection of the viability ranged from 81 (population A) to 56% (population E) (Figure 3.29.).

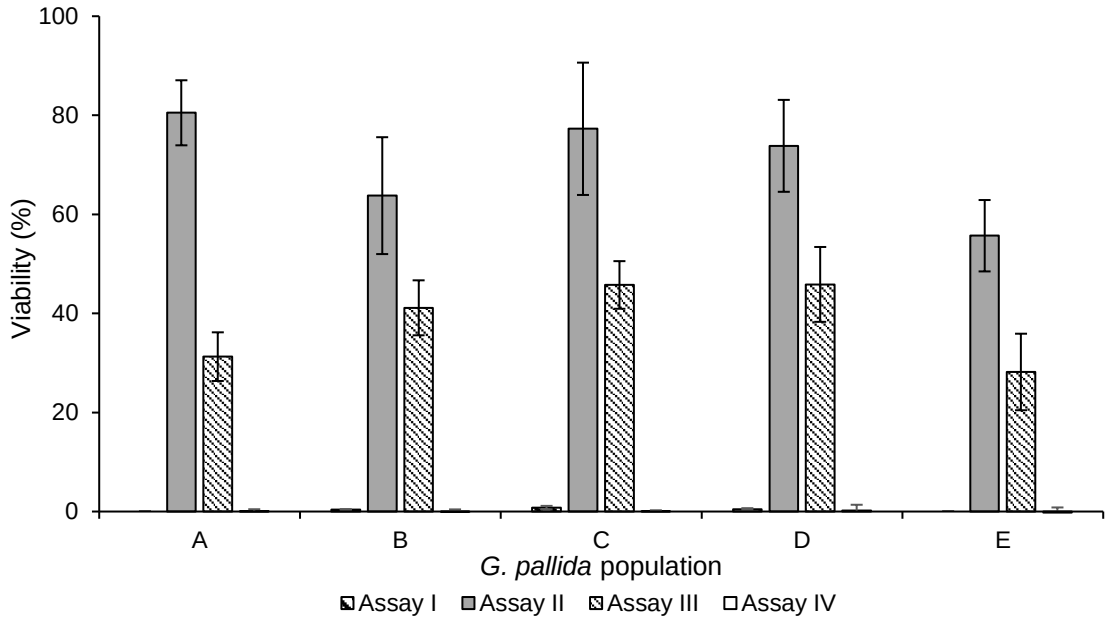


Figure 3.29. Viability (%) assessed by hatching in PRD assay (assay I), hatching in PRD followed by Meldola's blue staining assay (assay II), Meldola's blue staining assay (assay III) and the trehalose assay (assay IV) in samples containing 100% heat-treated cysts (6th imposed level of artificial viability) of *G. pallida* field populations (A-E). Error bars represent the standard error of the mean.

A very strong relationship (R=0.9) was found between the estimated viability (%) and the six levels of imposed artificial viability for population A when tested by assays I, III and IV. However, the estimated viability provided by assay II had a modest relationship (R=0.55) with the artificial levels of viable *G. pallida* eggs. This relationship was also modest for population B and C when tested by assay II (R=0.55 and 0.45, respectively) but strong when assessed by assay III (R=0.8 and 0.84, respectively) and IV (R=0.8 and 0.84, respectively) and a very strong for assay I (R=0.9, both). The relationships between the assessed viability and the levels of imposed artificial viability for population D were very strong (R=0.9) in case of assessment by assay I and IV, strong (R=0.7) by assay III and modest (R=0.6) by assay II. Population E had strong relationship only when assessed by assay IV (R=0.7) while remaining methods had modest relationship between artificial and assessed viabilities, R=0.5, 0.6 and 0.6, for assays I, II and III respectively (Figure 3.30.).

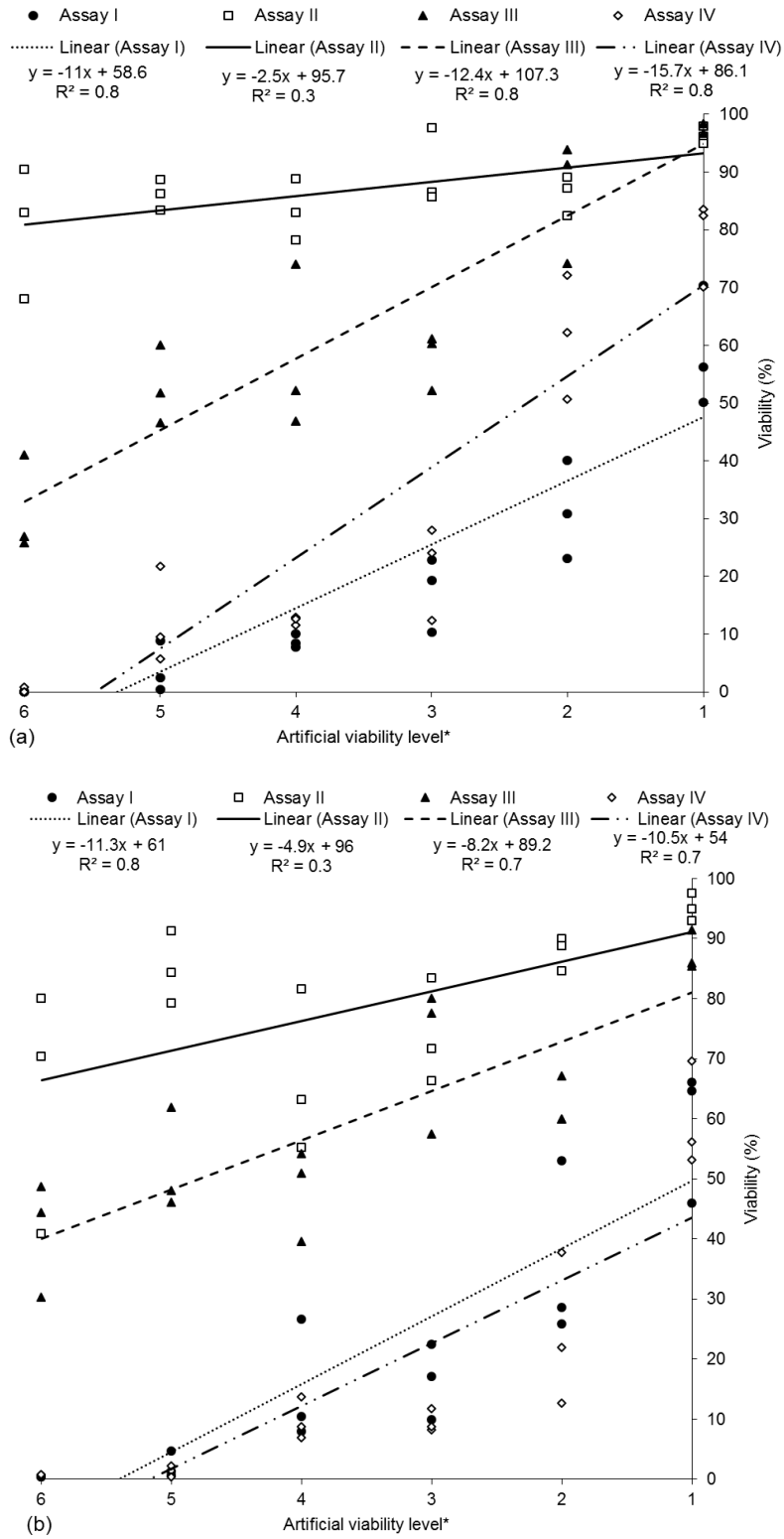


Figure 3.30. Relationships between viability (%) assessed by hatching in PRD assay (assay I), hatching in PRD followed by Meldola's blue staining assay (assay II), Meldola's blue staining assay (assay III) and the trehalose assay (assay IV) and six artificial viability levels for *G. pallida* field populations (a) A, (b) B, (c) C, (d) D and (e) E.

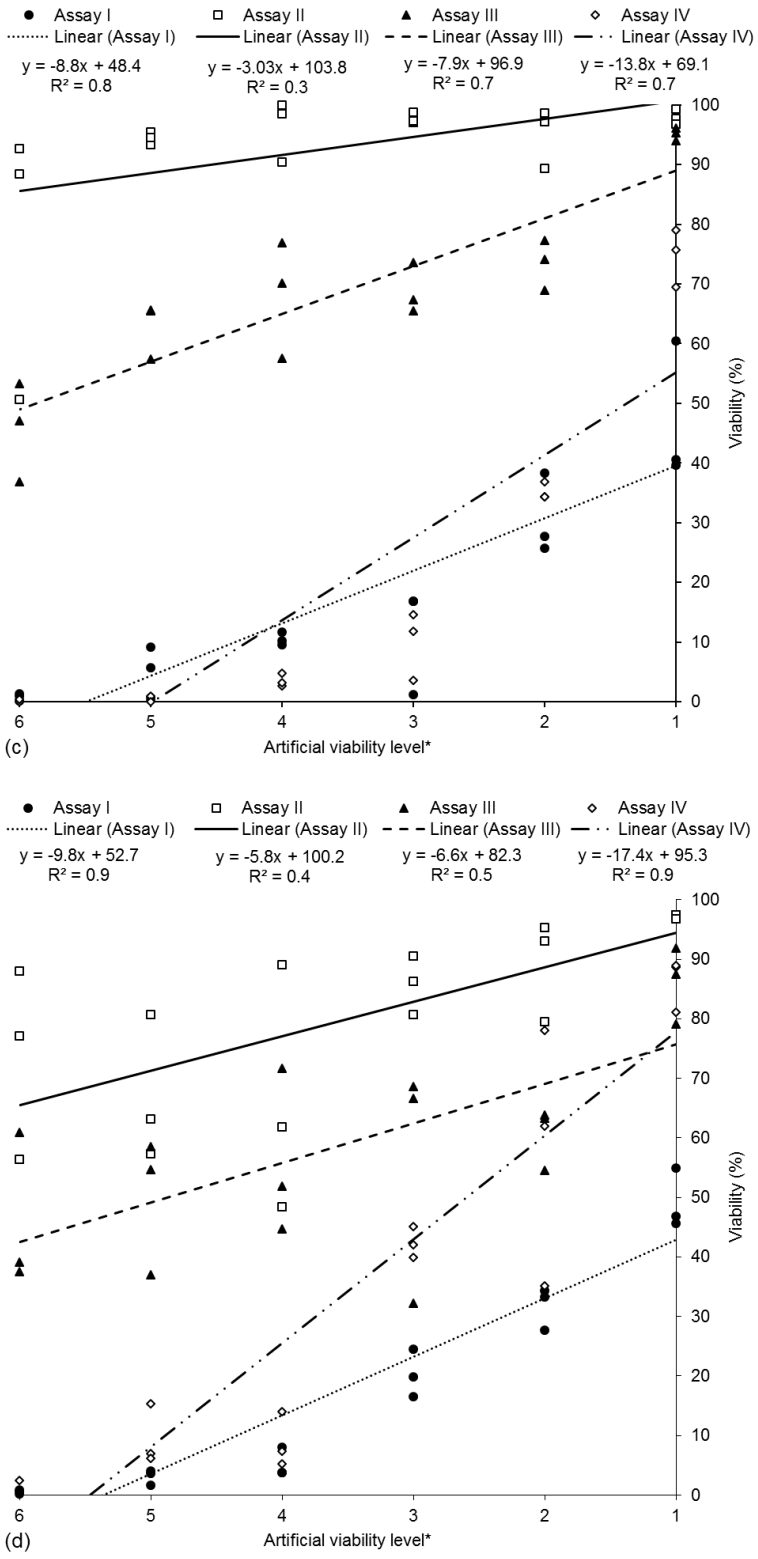


Figure 3.30. Relationships between viability (%) assessed by hatching in PRD assay (assay I), hatching in PRD followed by Meldola's blue staining assay (assay II), Meldola's blue staining assay (assay III) and the trehalose assay (assay IV) and six artificial viability levels for *G. pallida* field populations (a) A, (b) B, (c) C, (d) D and (e) E (Continuation).

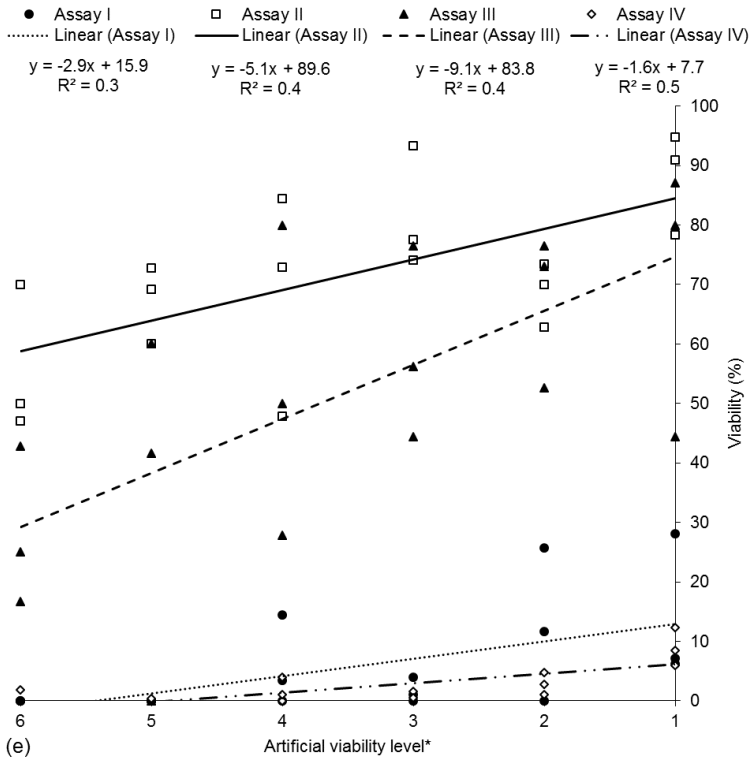


Figure 3.30. Relationships between viability (%) assessed by hatching in PRD assay (assay I), hatching in PRD followed by Meldola's blue staining assay (assay II), Meldola's blue staining assay (assay III) and the trehalose assay (assay IV) and six artificial viability levels for *G. pallida* field populations (a) A, (b) B, (c) C, (d) D and (e) E (Continuation).

3.4. Discussion

3.4.1. *Introduction*

Access to a more cost effective and less time-consuming viability assessment method could be beneficial to both research and industry. Existing viability assessment methods are often criticised as being too laborious and not reliable enough to be included in routine testing for determining PCN field population density. Methodology proposed by van den Elsen *et al*. (2012) uses trehalose as a viability marker and was recommended as an alternative for assessing the viability of PCN. In their study, a *G. pallida* glasshouse population characterised by 100%

hatchability was used as a reference population, which in the case of this study was not desired as the aim of this research was to test viability assessment methods on PCN field populations. The highest hatchability recorded in this study was 67% (population A) while the lowest was 26% (population E). Investigation of the viability for field populations based on the comparison with results obtained from glasshouse population may be biased. van den Elsen *et al*. (2012) measured the trehalose detection threshold on *G. pallida* population reared on a susceptible potato genotype in the glasshouse under controlled environment, which again would not have been suitable for this study.

3.4.2. *Trehalose assay amendments*

A reaction volume of 26.2µl, including egg suspension obtained from maximum of 25 cysts, was proposed by van den Elsen *et al*. (2012). Increasing the volume as a way to improve the detection of trehalose, was not tested. In their study, increased numbers of cysts did not increase the detection of trehalose when assay was conducted in 26.2, 52.4 and 78.6µl reaction volumes, but a linear relationship between cyst number and trehalose was observed with a volume of 104.8µl. Consequently, 26.2µl was used as a reaction volume only for egg suspensions and in samples with low numbers of eggs (maximum 30) whilst work on cysts (maximum 30) was conducted in a 104.8µl reaction volume (Ebrahimi *et al*., 2015). van den Elsen *et al*. (2012) highlighted that a small extraction volume limited the optimal extraction of trehalose. To find the best assay condition for work with a higher number of cysts (n=50), three alternative mixtures of reagents and corresponding extraction volumes were tested. The results proved that increasing the reaction volume (262µl) was necessary for satisfactory measurement, but not indefinitely, since the 1270µl reaction volume gave lowest value of ΔA. In this study, all samples,

regardless of number of eggs, were quantified in reaction volume of 262µl including 100µl extraction volume. Even if the volume used was much higher than the others reported in the literature, the detection limit was reached. It will be beneficial to continue testing samples containing high number of eggs in different reaction and extraction volumes to improve the absorbance detection.

A positive (32%) and negative (58%) change of absorbance was detected in samples without observed viable eggs. In the remaining 10% of these samples, a value of zero was observed. The mean value for background noise from samples with no viable eggs was -0.0002nm (range from -0.013 to 0.011). Higher average ΔA for cysts with dead eggs originating from field populations, 0.002nm (range from -0.003 to 0.007), was observed by van den Elsen *et al*. (2012). A positive change in absorbance was detected in 57% samples without observed viable eggs. A value of zero was observed in 20% of these samples, while 23% of the samples had a negative ΔA value. As in this study, the authors categorised eggs as non-viable by microscopic assessment (EPPO, 2017) but the condition of the cysts did not allow for a species identification. The proposed detection threshold at ΔA=0.0094 was calculated from samples with empty cysts, cysts with unidentifiable content and cysts with dead larvae (van den Elsen *et al*., 2012). The trehalose level in non-viable samples, confirmed by visual assessment and hatching test, was also investigated by Ebrahimi *et al*. (2015). Change of absorbance in samples originating from old populations was still detectable and on average was equal 0.005nm which is again higher than ΔA seen in this study. No effect on the trehalose measurement by addition of cysts with dead content was observed by Beniers *et al*. (2014). Positive but low trehalose signal from samples with non-viable eggs may originate from the small amount of trehalose detected in the cyst wall (Clarke and Hennessy, 1976).

Change of absorbance detected in samples containing heat-killed specimens was 0.008, 0.004, 0.046, 0.021 and 0.017nm for populations A-E, respectively, and it was statistically proven that the results should not be averaged. Identical to population B, results from heat-killed samples were reported by Ebrahimi *et al*. (2015). In their study, only one population was tested which makes the comparison difficult. It is recommended that a viability assessment of individual populations should be supported by the inclusion of heat-treated (dead) samples to ensure accurate background noise detection for a tested population. Samples used in this study were collected in East of England and West Midlands, originating from fields with sandy and loamy/clayey soil texture but no grouping factor was observed.

Samples with a higher number of eggs had a gradually lower probability of having zero or negative absorbance readings. The ∆A gradually stabilized, with only a few exceptions, when number of observed viable eggs in sample exceeded 116. These observations suggested that the assay should be conducted on samples containing between 100 (0.4 viable egg per one microliter of reaction volume - viable egg μl^{-1}) - 5,000 (19 viable egg μl^{-1}) *G. pallida* eggs. The attempt to detect one viable egg resulted in positive value of ΔA but as it never reached the detection threshold was considered unsuccessful (van den Elsen *et al*., 2012). The same researchers successfully detected ΔA above the detection threshold in samples with only 10 viable eggs (0.4 viable egg μl^{-1}). Their findings agree with results reported in this study, nevertheless, ΔA from 10 and 20 viable eggs was almost equal, 0.02 and 0.021nm, respectively. This confirms the conclusion from this study that low (<100) number of viable eggs in samples may be misleading. Higher sensitivity in the assay was observed by Ebrahimi *et al*. (2015) when ΔA above the threshold was measured in the samples with only 5 viable eggs (0.2 viable egg μl^{-1}). In their study, a sample with 10 viable eggs did not achieve the expected increase in ΔA rising by only 24%.

The assay's upper detection threshold was reached at approximately 5,000 viable eggs (19 viable eggs μl^{-1}) at ΔA just above 2nm. Beniers *et al*. (2014) reported that the correlation between number of viable eggs and trehalose measurement lost its linear character when egg suspension concentration was greater than 10 viable eggs μl^{-1}, which, in this study, was represented by 2620 viable egg μl^{-1}. The ΔA reached a plateau at approximately 3nm which is higher than observed in this study (2.4nm) but the difference could be expected taking into account that in both studies different instruments were used to measure ΔA. Ebrahimi *et al*. (2015) tested a dilution series and reached the upper detection threshold at approximately 11 viable eggs μl^{-1}, when reaction volume was 26.2µl. In reaction volume of 52.4µl threshold was reached when suspension density was 9 viable eggs μl^{-1}. Detection of the upper threshold for the remaining tested reaction volumes, 78.6 and 104.8µl, was not observed. This may be due to the low concentration of the eggs in the suspension, which did not exceeded 11 viable eggs μl^{-1} for 78.6µl and 8 viable eggs μl^{-1} for 104.8µl. The variation in the assay's upper threshold could be party explained by the difference in the change of absorbance per viable egg between *G. pallida* populations. The findings described above suggest that lower and upper detection thresholds can be population-dependent. Additionally the number of viable eggs in each individual sample was assessed by visual observation, which has high probability of human error even if performed by trained stuff.

Based on the experiments conducted, it was decided that the trehalose assays conducted on *G. pallida* field populations should be performed on 25 cysts. This number was chosen over 5 and 10 cysts as it would provide greater representation of the population. When feasible, the number of cysts used in the assay should be based on a population's fecundity which could be tested prior the trehalose-based viability assessment. Use of cysts with very low number of eggs or

large proportion of empty cysts (<4 eggs $cyst^{-1}$) might not provide sufficient signal to be detected. Similarly cysts from a population with high fecundity may reach the assay's upper threshold and underestimate the infestation level. When 50 cysts were used 47% of samples exceeded upper detection threshold. A greater number of cysts per sample might provide a better representation of a given population but as indicated here, further research is needed to extend the upper detection threshold. Such an improvement could be achieved by further gradual increase of the reaction volume and/or testing alternative spectrophotometers to improve sensitivity. Preparation of the egg suspension is time consuming and makes the process more laborious. To simplify the protocol, it is preferable to use encysted eggs if feasible. Comparison of methods for the extraction of trehalose by van den Elsen *et al*. (2012) suggested that crushing does not improve the process and, as a manual treatment difficult to standardize, resulted in low consistency between individual samples. In contrary, significantly lower measurement in non-crushed compared to crushed cysts was observed in research conducted by Beniers *et al*. (2014) and Ebrahimi *et al*. (2015). This trend was also observed in this study which showed that viability assessment by the trehalose assay should be performed on the egg suspension and highlighted necessity for methodology suitable for encysted eggs.

Change of absorbance per viable egg calculated from samples containing between 100 and 5,000 viable eggs, after reduction of the specimen's background noise (heat-killed samples) calculated individually for each population, was 0.005 for population A and D, 0.004 for populations B and C and 0.006 for population E. Research on viability assessments by trehalose-based methods completed to date did not aim for the investigation on ΔA viable egg^{-1}, nevertheless some experiments conducted by van den Elsen *et al*. (2012) and Ebrahimi *et al*. (2015) included

samples of known number of viable eggs. Both research groups investigated one population only and in both cases ΔA viable egg^{-1} was calculated as 0.001 which is lower than those reported for populations A-E. However, the results were obtained from samples containing 5, 10 or 20 viable eggs, which in the light of the research discussed here fell below lower detection threshold and for that reason cannot be treated as a reliable.

The suppliers' manual for the trehalose detection kit (K-TREH, Megazyme International Ireland Ltd., Wicklow, Ireland) recommends to perform a test in 2540µl reaction volume (including 200 µl of tested sample) which allows 100 assays to be completed. As shown in this study the use of that high reaction volume did not benefit the detection on trehalose and the reduction to 262µl was possible. Further detailed calculations need to be undertaken nevertheless, with current cost of the trehalose detection kit of €220, proven here 10-fold reduction of volumes used drastically reduce the cost from €2.2 (approx. £1.97) to €0.22 (approx. £0.2) per reaction which, if test to be commercialized, will be much more appealing to the growers. Additionally the research published to date was conducted on the glasshouse populations. The results found in this study showed that the trehalose assay has the potential to serve as a commercial test for the viability assessment on the samples originating from field. In conclusion, the trehalose viability assay may provide a more sensitive and reliable method for determining soil population densities of PCN.

3.4.3. *Viability assessment methods*

In general, all populations showed the same pattern of response to PRD (assay I). A low number of hatched juveniles was observed after seven days of exposure to PRD was followed by the peak of hatchability at 14 days, after which

there was a gradual decline in the weekly hatching assesments. Only population D in experiment 2 and populations B and E in experiment 3 did not exactly follow this pattern.

The estimates by assay I (populations A-E), II (populations B-E) and IV (populations A-C) were highest in the first experiments (experiment 1) performed to assess natural viability. The reduction in the following experiments was consistently greater in experiment 3 than in experiment 2. It is possible that the viability of the eggs was reduced due to natural causes during the period of sample storage between the experiments which were performed in April, May and July, experiment 1, 2 and 3 respectively. This shows that these three methods, even if they were found to be highly significantly ($P<0.001$) different from each other, successfully detected viability decline. In contrast, viability assessed by assay III, which was also found to be highly significanly ($P<0.001$) different, was lowest in experiment 1. Further investigation is necessary to explain why reduction in viability (%) between experiments was not detected when tested by Meldola's blue staining assay.

No clear conclusion could be drawn when relationships between natural viabilities from assays I-IV were analysed. None of the relationships were described with the same strength across all experiments (1-3). A strong relationship ($R=0.7$) between assay I and assay II, in experiment 2, could be explained by the fact that hatching provided a significant proportion of the estimate for assay II. But this strength of the relationship was not confirmed in experiments 1 and 3 when modest ($R=0.6$) and weak ($R=0.3$) relationships were found. The explanation for strong relationship between assay I and II could be supported by the weaker relationship between assay I and assay III which was observed only in experiment 2 ($R=0.07$). The relationship between assays II and III was described as strong (experiment 1) but then as very weak (experiment 2) and modest (experiment 3) even if the viability

determined by assay II is only slightly different in individual populations (*c*. 3%) when compared to assay III. The lack of correlation between assays II and III is explained by the relatively higher proportion of values nearing the upper limit of the viability scale as well as by the large variation between replicates within some of the populations. Closest to uniformity between experiments were the relationships between assay IV and remaining assays but the strength of the relationship was never higher than modest (R≤0.6).

Where *G. pallida* eggs (populations A-E) natural viability was investigated, assays II and III provided a higher estimate of viability than assays I and IV as reported in experiments with six imposed levels of artificial viability. Natural viability assessments by assay II were higher, compared with assay III, in 60% of the samples. A comparison between assays II and III showed that in the case of 93% artificial viability assessments (all levels/all populations) assay II detected higher number of viable eggs. This makes the relationship between assays II and III much clearer than in the case of natural viability. Higher estimation of viable eggs by assay II is due to secondary staining with Meldola's blue where *c*. 88% of unhatched eggs are detected as viable. This was previously observed by Kroese *et al*. (2011) who reported that the majority, also on average 88%, of the eggs from a field population which did not hatch when exposed to PRD did stain when subsequently exposed to Meldola's blue dye. The same treatment on glasshouse population revealed that 98% of unhatched eggs were viable. This highlights that further tests should be implemented to gain a more robust assessment as the remaining eggs described as non-viable may contribute to the damage potential. Natural viability assessments by assay IV were lower, compared with assay I, in 80% of the samples. Ebrahimi *et al*. (2015) reported a lower estimate of viability from hatching tests in comparison with the trehalose assay which was reported in only 20% of the samples in natural

viability studies. Viability assessment on six artificial viability levels was lower by assay IV in 53% assessments.

Kroese *et al*. (2011) support the use of Meldola's blue stain (assay III), which is less time and resource intensive as there is no need to produce hatching factors, as an alternative to a hatching assay (assay I). As showed in this study testing *G. pallida* populations by staining in Meldola's blue stain may result in overestimation of viable eggs. This is partly due to difficulties related to visual assessment of stain and non-stain juveniles. If nematode body is lightly stain the decision whether juvenile is dead or alive depends on personal judgment. Additionally, Meldola's blue dye deeply stains PCN eggshells and, if not enough time is allowed for washing, the assessment of the dye intake by juveniles might be impossible unless the juveniles are dissected.

Both, assays II and III incorrectly estimated viable eggs in samples where 100% of the cysts were heat-treated (level 6). On average 38% remained unstained when only staining was used to examine egg viability (assay III). There was no hatching detected over the period of exposure to PRD in assay II, which means that overestimation, on average 70%, was entirely from secondary staining by Meldola's blue. It is possible that exposure to PRD affected the permeability of the egg-shell and hindered staining by Meldola's blue dye hence the higher percentage of unstained eggs in assay II. On the same level, assays I and IV correctly measured sample as non-viable (>99%). Hatchability (assay I) reported in this study was observed when all populations were tested against PRD collected from variety 'Estima'. The differences in hatching, as a result of treatment by root diffusate obtained from various potato genotypes, can have a strong impact on the results (Evans, 1983; Turner and Stone, 1981; Arntzen *et al*., 1993b; Turner *et al*., 2009). It is crucial to select, based on literature review or preliminary experiments, suitable

variety for PRD production prior to conducting the hatching assay and use it consecutively.

Ebrahimi *et al*. (2015) found no difference between the visual assessment of *G. pallida* eggs and the trehalose assay when testing the viability of cultured cysts. The comparison between these two methods was not included in this research as viability testing by microscopic observation was part of investigation for ΔA viable egg^{-1} value.

Each *G. pallida* field population used in this study had different natural viability *e.g*. when tested by assay I it range from 67 to 26% for populations A and E, respectively, which could cause lack of clarity in the comparison between viability assessment methods. Introduction of levels and standardization of all natural viabilities as 'level 1' helped provide a more uniformed comparison between the assays on field *G. pallida* populations. The variability between the experiments can be also partly explained by the origin of *G. pallida* populations. The aim of this study was to test viability of field populations directly after collection, which made this research more agronomically practical but also required work with non-uniform populations. The difference in results obtained from glasshouse and field populations was already noted by van den Elsen *et al*. (2012). When some of the samples, from glasshouse population, did not reach the threshold level, the authors highlighted that the detection cut-off was calculated from samples originating from field with considerable background noise. Kroese *et al*. (2011) reported higher viability estimates when using Meldola's blue stain but only for a glasshouse population, considered to be in diapause. When a field population, considered not to be in diapause, was tested no significant difference was detected. In this study, population B (1 year since potatoes grown) was consider to have higher percentage of eggs in diapause and had viability estimated by assay III 52% higher compare to

assay I. Notably, this population had also second lowest hatchability from tested field population. Greater viability estimation by staining with Meldola's blue was also reported for populations collected from fields not used for potato production for 6 years but the differences were smaller. Viability for populations A and D by assay III was 27 and 30%, respectively, higher than by assay I. These two populations were also reported with higher hatchability between tested populations. Therefore, it is important to know the background of the PCN population when deciding on the most suitable method for given population. If we assess the viability for PCN populations collected from land recently used for potato production, it is not appropriate to use Meldola's blue staining as the test will be biased due to high proportion of eggs in diapause.

Further comparison between viability assessment techniques is discussed in Chapter 5. In brief, as demonstrated here, hatching in PRD (assay I) cannot be performed independently if actual population's viability is required, and therefore, must be supported by a following test for determining the viability of the remaining eggs. Secondary staining with Meldola's blue dye (assay II) was proven to greatly overestimate number of viable eggs. To lesser degree also staining solely by Meldola's blue staining (assay III) detected alive eggs in unviable samples. Both, assay I and assay IV correctly detected unviable samples but to unless time consuming hatching in PRD trehalose assay can be completed in a day. It is necessary to test trehalose assay on wider range of field populations and further investigate the value of ΔA viable egg^{-1} to consider it development to a commercial test.

3.4.4. *Key findings*

- Under the described conditions, the trehalose assays of *G. pallida* field populations should be performed on 25 cysts or 100-5,000 viable *G. pallida* eggs. A very strong relationship (R=0.9) was observed when the number of eggs was plotted against ΔA.
- The mean value for background noise from samples with no eggs was -0.0002nm.
- Ideally, preliminary tests were required to investigate cyst contents, which would aid with the decision making for experimental design.
- Change of absorbance detected in samples containing dead specimen was statistically significantly different between populations and was 0.008, 0.004, 0.046, 0.021 and 0.017nm for populations A-E, respectively.
- The specimen's background noise should be deducted individually for ΔA of the population tested.
- Change of absorbance per viable egg calculated from samples containing between 100 and 5,000 viable eggs, after reduction of the specimen's background noise calculated individually for each population, was 0.005 for population A and D, 0.004 for populations B and C and 0.006 for population E.
- The reduction in natural viability (assay I, II and IV) from experiment 1 to 2 and even greater reduction from experiment 1 to 3 could be due to natural causes during the period of sample storage between the experiments.
- In general, all populations showed the same pattern of response to PRD overall, when assessed by assay I.
- High significant differences ($P<0.001$) were also found between viability assessments produced by assay I-IV in experiments 1, 2 and 3.

- Comparison between natural viabilities (%) in experiment 1 clearly showed that assay IV produced the lowest figures. The second lowest output was produced by assay I followed by assay III and II. This was only partly confirmed in experiment 2 (populations A and D) but was not confirmed in experiment 3.
- Investigation of the relationship between the estimated viability (%) and the six levels of imposed artificial viability showed the strongest relationship when assessed by assay I in populations A, B, C and D.
- Testing *G. pallida* populations by staining in Meldola's blue dye may result in viable eggs overestimation. Test on heat-killed samples showed that, on average, 38% remained unstained when examined by assay III and 70% when examined by assay II. An overestimation was entirely from secondary staining by Meldola's blue.
- Hatching in PRD cannot be performed independently if actual population's viability is required, and therefore, must be supported by a following test for determining the viability of the remaining eggs.

Chapter 4

Assessment of established and novel methods for the characterisation of the virulence of potato cyst nematode populations from Great Britain.

4.1. Introduction

Pathogenicity refers to the ability of an organism to cause disease. It is a qualitative term and an organism can be defined as being pathogenic or non-pathogenic. Virulence is a term that quantifies pathogenicity of specific genotypes of a species towards varieties of a specific species and may exhibit a wide range of levels depending on the conditions present (Shapiro-Ilan *et al*., 2005). The extent of the virulence is usually correlated with the ability of the pathogen to multiply within the host.

The separation of potato cyst nematodes (PCN) into two species, *Globodera pallida* and G*. rostochiensis*, did not fully account for the variability in virulence between PCN populations. Consequently, further methodology was required to measure and describe the virulence (pathotypes) within each PCN species. Attempts to introduce the system for pathotype identification and classification were made by several research groups and are described in Chapter 1 (section 1.2.3.).

The complexity of virulence found in PCN field populations is not well defined by the methodology used in the Kort *et al*. (1977) pathotype scheme which includes a limited selection of resistance genes and lacks an important genotype currently used in potato breeding programs, although the ideology used to develop this scheme is still in use (CABI and EPPO, 2018). This makes the search for virulence markers that can be used to monitor populations even more important. Recently,

the European and Mediterranean Plant Protection Organization (EPPO) produced a protocol for testing potato varieties to assess resistance to *G. rostochiensis* and *G. pallida* (EPPO, 2006) of new or unusual populations. The virulence of populations overcoming the resistance in varieties currently used by potato industry should be tested by assessment of their multiplication ability on a set of different potato genotypes (EPPO, 2017).

The future use of synthetic pesticides in agriculture is uncertain due to potential health and environmental risks (OJEU, 2009). One sustainable alternative for PCN management is to employ host resistance for population density management. To exploit the full potential of this method, it is important to know the virulence composition of populations found in a given field. Since the virulence status of PCN populations found within the United Kingdom (UK) was last assessed several decades ago (Phillips and Trudgill, 1998a) there is a need to reassess this characteristic with current field populations. If an unknown pathotype or mixture of pathotypes occurs, the performance of the resistant potato variety chosen might be different than previously observed. Also, there is evidence that populations of *G. pallida* can become more virulent over successive generations when exposed to selective pressure from rearing on particular potato genotypes (Turner *et al*., 1983; Phillips and Blok, 2008) indicating that ongoing monitoring of population virulence is needed.

In animals, mitochondrial DNA (mtDNA) is typically passed on through generations from mothers to their offspring. Mitochondrial DNA is an important tool used in population genetics because it evolves five to ten times faster than nuclear DNA (Castro *et al*., 1998). The cytochrome *b* gene (cyt-*b*) is a commonly used region of mtDNA for determining phylogenetic relationships between organisms, due to its sequence variability. It is considered to be most useful in determining relationships

within families and genera. Work by Picard *et al*. (2007) and Plantard *et al*. (2008) showed that Peruvian and Western European PCN populations can be assigned into the same clades (groups of populations that have a common ancestor) when genotyped with a partial sequence of cyt-*b*. Using this approach Plantard *et al*. (2008) observed that three individual populations from Peru can be considered as potential sources of *G. pallida* introduction into Europe. Recent work by the James Hutton Institute (JHI) has found that certain Scottish populations of *G. pallida* contain mixtures of genotypes, which are likely to represent these three distinct introductions from the Andes (Eves-van den Akker *et al*., 2015). These haplotypes were renamed by authors as mitotypes.

The implications of these results with regard to virulence are not known and the potential for the formation of novel hybrids between these putative introductions has also not been evaluated. In this study the virulence of PCN field populations from England and Wales and their characterisation as geographically distinct mitotypes was investigated.

4.1.1. *Aim*

Characterise selected *G. pallid*a populations from PCN survey in England and Wales using classic (pathotyping) and novel (mitotyping) techniques.

4.1.2. *Objectives*

(I) Assess the virulence of selected *G. pallid*a populations from the PCN survey conducted in England and Wales (Chapter 2) by testing their ability to reproduce on a range of different potato genotypes in the glasshouse.

(II) Assess the resistance of different potato genotypes to selected *G. pallid*a populations from the England and Wales PCN survey (Chapter 2) by testing their relative susceptibility in the glasshouse.

(III) Investigate genetic variability of *G. pallida* DNA extracts obtained from PCN survey samples (Chapter 2) in relation to potential sources of PCN introductions into Europe by use of a metagenetic approach to next-generation sequencing.

4.2. Materials and methods

4.2.1. *Potato cyst nematode populations*

Field populations from the survey samples collected in Great Britain (GB) in 2013-16, characterised for species composition and confirmed to be pure *G. pallida* populations were selected for a resistance test (Table 4.1.). The chosen survey populations represent a range of geographical locations, in addition to unknown reproductive abilities. The number of populations included was limited by the necessity of obtaining a sufficient number of cysts from the collected soil samples and the feasible size of the experiment. Methodology used for field sample collection, cyst extraction and species identification is described in Chapter 2 (sections 2.2.2.-2.2.4.). The virulence assessment included three reference populations of *G. pallida* pathotypes from the JHI collection (Table 4.1.); Lindley (pathotype Pa2/3) population was originally collected from a location in England, whilst Luffness (Pa3) and Pa1 (Pa1) were collected in Scotland. To obtain the required number of cysts for the assessment, control populations were multiplied on susceptible genotypes at the JHI (Gartner, 2018. Pers. Comm. Ms U. Gartner).

Table 4.1. Test (survey) and control (the JHI collection) *G. pallida* populations included in the glasshouse experiment for virulence assessment using four different potato genotypes.

PCN population	Species	Pathotype	Source	Geographic orgin	Soil texture	Previous crop grown	Years since potatoes grown
HAU 152	*G. pallida*	unknown	survey	East of England	sandy	non cereal	6
HAU 164	*G. pallida*	unknown	survey	West Midlands	loamy and clayey	cereal	1
HAU 165	*G. pallida*	unknown	survey	West Midlands	sandy	non cereal	3
HAU 166	*G. pallida*	unknown	survey	West Midlands	loamy and clayey	cereal	6
HAU 167	*G. pallida*	unknown	survey	West Midlands	sandy	potato	5
HAU 178	*G. pallida*	unknown	survey	North West	sandy	cereal	4
HAU 298	*G. pallida*	unknown	survey	Yorkshire and the Humber	loamy	cereal	2
HAU 351	*G. pallida*	unknown	survey	East Midlands	sandy	potato	1
HAU 356	*G. pallida*	unknown	survey	North West	peaty	potato	9
Pa1	*G. pallida*	Pa1	JHI collection	Scotland	unknown	unknown	1
Luffness	*G. pallida*	Pa3	JHI collection	Scotland	unknown	unknown	1
Lindley	*G. pallida*	Pa2/3	JHI collection	England	unknown	unknown	1

4.2.2. *Host plants*

The virulence assessment included the susceptible potato variety ‘Desiree’ and genotypes representing the main sources of partial resistance towards *G. pallida* pathotypes Pa1, Pa2/3 and Pa3 (Table 4.2.).

Table 4.2. The potato genotypes (susceptible and partially resistant to *G. pallida*) used in the glasshouse experiment for virulence assessment of *G. pallida* field populations from England and Wales.

Genotype	Source of resistance	Resistance to Pa1	Resistance to Pa2/3	Resistance gene
'Desiree'	None	Susceptible	Susceptible	None
'Vales Everest'	*S. tuberosum* spp. *andigena* CPC2802	High	Moderate	Quantitative trait locus (*H3*)
'P55/7'	*S. multidissectum* PH1366	High	Partial	Major gene (*H2*)
'Innovator'	*S. vernei*	High	High	Polygenes (*GpaV*$_{vrn}$)

4.2.3. *Glasshouse experiment*

A spherical piece was cut from a chitted seed tuber of the potato genotypes ‘Desiree’, ‘Innovator’, ‘P55/7’ and ‘Vales Everest’ before being planted in pots containing 550g sand:loam (50:50) previously autoclaved (1 hour at 121°C) and mixed. Just before planting, a sealed silk sachet with 20 cysts was individually buried at a depth of about 5cm below the surface of the growing medium for each *G. pallida* population (initial population - Pi cysts). The pots were nested in a layer of autoclaved sand in a raised bed in the glasshouse (Figure 4.1.). The virulence assay was replicated with four replicates per *G. pallida* population/potato genotype combination and randomised (Appendix 7.6.).

Figure 4.1. Arrangement of the glasshouse experiment for virulence assessment of *G. pallida* field populations from England and Wales (1 DAP).

Plants were grown in the glasshouse (Figure 4.2.) for 10 weeks (70 days after planting (DAP)) under climate-controlled conditions (16h light at 20ºC/8h dark at 16ºC) before watering (daily as required) was stopped. The experiment was established on the 30th of April 2016. Due to unsuccessful germination in a few pots, seed tuber pieces needed to be replaced by new ones on the 20th of May and the 6th of June 2016. The assay was terminated on the 9th of July 2016, the 29th of July and on the 15th of August, respectively. Dry soil was collected individually from each pot and sent to HAU where newly formed cysts were extracted, collected and recorded (final population - Pf cysts) as described in Chapter 2 (sections 2.2.3.).

Figure 4.2. Potato plants development in the glasshouse experiment in the virulence assessment of *G. pallida* field populations from England and Wales (38 DAP).

Following extraction, 50 cysts or less (if fewer cysts were available) from each sample were placed in distilled water 24h before being gently crushed between an aluminium block and a glass slide. Released eggs were rinsed into a 100ml Nessler tube and distilled water was added to make a 50 (sample containing 26 to 50 cysts) or 25ml (sample containing 11 to 25 cysts) egg suspension. After thorough mixing, a 1ml aliquot was assessed using a binocular microscope at 60 x magnification. The fecundity of each population (Pf eggs $cyst^{-1}$) was calculated using equation (1).

$$\frac{\text{eggs in 1ml x volume of egg suspension (ml)}}{\text{total number of cysts used}} = \text{Pf eggs cyst}^{-1} \quad \text{.....(1)}$$

For the remaining samples (containing 1 to 10 cysts), released eggs were rinsed into a 5ml watch glass and the total volume of egg suspension was assessed

using a binocular microscope at 60 x magnification. The fecundity of these populations (Pf eggs cyst^{-1}) was calculated using equation (2).

$$\frac{\text{total number of eggs in the suspension}}{\text{total number of cysts used}} = \text{Pf eggs cyst}^{-1} \quad \text{.....(2)}$$

Total number of eggs (Pf eggs) was recorded directly during fecundity assessment (samples containing 1 to 10 cysts) or calculated using equation (3) (sample containing 11 to 50 cysts).

$$\text{Pf eggs cyst}^{-1} \text{ x Pf cysts} = \text{Pf eggs} \quad \text{.....(3)}$$

To assess the initial population density, four replicates of 20 cysts (except of population HAU178 for which only one sample was used due to insufficient number of available cysts) were placed in distilled water 24 hours before being gently crushed between an aluminium block and a glass slide. Released eggs were rinsed into a 5ml watch glass. Total volume of egg suspension was assessed using a binocular microscope at 60 x magnification (Pi eggs) and number of eggs cyst^{-1} (Pi eggs cyst^{-1}) was calculated using equation (4).

$$\frac{\text{total number of eggs in the suspension}}{\text{total number of cysts used}} = \text{Pi eggs cyst}^{-1} \quad \text{.....(4)}$$

4.2.4. *The resistance test*

An assessment of reproduction (population multiplication ability – Pf/Pi ratio) expressed as number of cysts was calculated using equation (5).

$$\frac{\text{Pf cysts}}{\text{Pi cysts}} = \text{Pf/Pi ratio} \quad \text{.....(5)}$$

Populations that produced more cysts than were used for inoculation and had a Pf/Pi ratio larger (>) than 1 were characterised as virulent and potato genotype as susceptible. Analogously a Pf/Pi ratio smaller or equal (≤) 1 indicated avirulent population and resistance potato genotype (Kort *et al*., 1977).

To assess the resistance of the four potato genotypes to *G. pallida* field populations (EPPO, 2006) the relative susceptibility was calculated using equation (6).

$$\frac{\text{Pf cysts (test variety)}}{\text{Pf cysts (susceptible control variety)}} * 100\% = \text{relative susceptibility} \quad \text{.....(6)}$$

When relative susceptibility calculated on cysts was less than 3%, eggs counts were used and relative susceptibility was calculated using equation (7) (EPPO, 2006).

$$\frac{\text{Pf eggs (test variety)}}{\text{Pf eggs (susceptible control variety)}} * 100\% = \text{relative susceptibility} \quad \text{.....(7)}$$

Relative susceptibility was used to assess resistance according to the standard scoring notation (Table 4.3.).

Table 4.3. Standard scoring notation to assess resistance of potato varieties to *G. pallida* (EPPO, 2006).

Relative susceptibility (%)	Score*
< 1	9
1.1–3	8
3.1–5	7
5.1–10	6
10.1–15	5
15.1–25	4
25.1–50	3
50.1–100	2
> 100	1

*A score of 9 indicates the maximum level of resistance.

4.2.5. *Next-generation sequencing - DNA samples*

Mitotype diversity was determined in 101 of the survey samples collected between 2013 and 2016 from fields in England, previously used for ware potato production, and confirmed to be *G. pallida*. Methodology used for field sample

collection, cyst extraction and species identification is described in Chapter 2 (sections 2.2.2.-2.2.5.).

To extract DNA, up to 20 cysts, collected from each float, were transferred into 2ml Eppendorf tubes with 500µl GeneScan lysis buffer (Neogen Europe Ltd.) and homogenised with a sterile micropestle. Samples were centrifuged for 15s at 15,600*g* and then 5µl of 10 mg ml^{-1} RNase A (Thermo Fisher Scientific) and 5µl of 20mg ml^{-1} proteinase K in 40% (v/v) glycerol (Sigma-Aldrich) was added and incubated for 1h at 65°C. After incubation, 500µl of chloroform:isoamyl alcohol (24:1) (Sigma-Aldrich) was added and mixed by inverting the tubes several times. Samples were centrifuged for 10min at 15,600*g* and the upper aqueous phase (450µl) was transferred into a new 2ml Eppendorf tubes to which 360µl of ice-cold propan-2-ol (VWR) was added. Samples were next mixed thoroughly, incubated for 30min at 20°C, centrifuged for 10min at 15,600*g* and the pellets were retained. The pellets were washed twice with 500µl of 75% ethanol and centrifuged for 5min at 15,600*g* and then re-suspended in 100µl sterile distilled water. To further purify DNA extracts, PVPP spin columns were used. A suspension of 10% PVPP (Sigma-Aldrich) was made up with sterile distilled water and 600µl was transferred into each spin column (NBS Biologicals) positioned in a 2ml Eppendorf tube. Subsequently, PVPP columns were centrifuged at 11,000*g* for 1min, the catch-tubes were emptied and, after the spin columns were rotated 180° within the centrifuge, the centrifugation was repeated. The re-suspended DNA samples were transferred to the spin columns and centrifuged at 11,000*g* for 1min (Reid *et al*., 2011). Purified eluates were transferred to a new sterile 1.5ml Eppendorf tubes and stored at -20°C until further stages of metagenetic next-generation sequencing (NGS) were undertaken.

4.2.6. *Next-generation sequencing - polymerase chain reaction and library preparation*

Primers that amplify a 310bp region of cyt-*b*, which is descriptive of each mitotype and identifiable by their unique sequences that differed from one another by no less than two descriptive single nucleotide polymorphism (SNPs) (Table 4.4.) were designed (Eves-van den Akker *et al*., 2015). Next, a library of these primers with the individual addition of distinctive 4bp 'barcodes' to the 5' end of the primer was generated. Primer pairs with unique combinations of barcodes could be used for each sample such that when these amplicons were sequenced, reads containing specific barcode pairs could be attributed to their correct samples. Two 5' adenosines were also added before the barcode and the actual forward or reverse primer sequences to act as a protecting buffer for the barcode in the sample sequencing process.

Table 4.4. Comparison between descriptive single nucleotide polymorphism in region of cytochrome *b* gene used to investigate *G. pallida* field populations for the presence and composition of mitotypes.

SNP position	61	118	137	158	228
Mitotype 1	G	A	A	A	T
Mitotype 2	G	G	A	A	C
Mitotype 3	A	G	G	C	T

Deoxyribonucleic acid obtained from 101 survey samples was used to investigate the presence and composition of cyt-*b* mitotypes. Following methodology by Eves-van den Akker *et al*. (2015), the mixtures of ratios of three plasmids encoding known partial sequences of cyt-*b*, from this point referred to as Type 1, Type 2 and Type 3, were also included as controls The number of control mixtures, each replicated with 5 unique barcode pairs, was increased to nine and the proportions (%) of either two or three plasmids were slightly modified (Table 4.5.).

Table 4.5. Proportions used to prepare control mixtures of plasmids encoding known partial sequences of cyt-*b* (Type 1, 2 and 3), used in the next-generation sequencing for mitotypes assessment of *G. pallida* field populations from England.

	Type 1 (%)	Type 2 (%)	Type 3 (%)
Mix 1	10	90	0
Mix 2	10	80	10
Mix 3	25	50	25
Mix 4	25	75	0
Mix 5	33	66	0
Mix 6	50	50	0
Mix 7	33	33	33
Mix 8	10	45	45
Mix 9	1	99	1

All DNA samples and control mixtures were subject to PCR with 5µl of template. The polymerase chain reaction mixtures were prepared using Phusion High-Fidelity DNA Polymerase (New England BioLabs) in the proportions shown in Table 4.6. (Eves-van den Akker *et al*., 2015).

Table 4.6. Polymerase chain reaction mixture composition used in next-generation sequencing for mitotype assessment of *G. pallida* field populations from England (Eves-van den Akker *et al*., 2015).

Components	Volume per samples (µl)	Final concentration
Forward primer	2.5	0.5µM
Reverse primer	2.5	0.5µM
5X Phusion HF buffer	5	1X
Phusion DNA polymerase	0.25	0.5 unit
dNTPs mix	0.5	200µM
PCR water	9.25	—
DNA template	5	<250ng
Total	25	

The polymerase chain reaction cycle conditions were as described by Eves-van den Akker *et al*. (2015) (Table 4.7.). The resultant products were analysed by gel electrophoresis to assess the strength of the amplification. Based on this assessment, all PCR products were pooled together: 20µl of samples when the amplification appeared to be low (weak or not visible fluorescence) or 2µl of samples when amplification appeared to be high (strong fluorescence).

Table 4.7. Polymerase chain reaction cycle conditions used in the next-generation sequencing for mitotype assessment of *G. pallida* field populations from England (Eves-van den Akker *et al*., 2015).

	Temperature (°C)	Time (seconds)
Denaturation	98	20
Annealing	64	30
Elongation	72	60
	37 cycles	
Final extension	72	300

Total pooled PCR products were precipitated at -80°C overnight with the addition of 1 volume isopropanol and 0.2 volumes 3M sodium acetate. Precipitated DNA was pelleted by centrifugation at 13,000*g* for 20min at 4°C. The pellet was washed once in 70% ethanol, allowed to air dry, and re-suspended in 180µl of 10mM Tris-Cl, pH 8.5. Concentrated PCR products were additionally purified by size selection using MagJET NGS Cleanup and Size Selection beads (Thermo Fisher Scientific) following the manufacturer's instructions for adapter removal for an amplicon of 300bp. Size selected blunt ended DNA was eluted from the beads in 50µl of buffer and analysed by gel electrophoresis.

Following these steps, cleaned and purified PCR products were sent to Edinburgh Genomics (University of Edinburgh, Edinburgh, UK) for library preparation and sequencing as described in Eves-van den Akker *et al*. (2015).

4.2.7. *Next-generation sequencing - sequencing, bioinformatic and data analyses*

The sequencing of the 310bp amplicons was carried out at Edinburgh Genomics (University of Edinburgh, Edinburgh, UK). Analyses were conducted on the101 survey samples, - an additional four replicates of five selected survey samples (HAU152, 164, 165, 166 and 167) acting as internal controls, a sample containing PCR water instead of DNA template acting as a negative control, and four replicates of nine mixes of ratios of plasmids containing known partial sequences of cyt-*b* acting as positive controls (Table 4.5.). After sequencing, obtained reads were available in an electronic version as two files, one containing reads with the forward primer and the other with reads from the reverse primer.

The bioinformatic and data analyses were performed at the University of Cambridge (Cambridge, Cambridgeshire, UK) under the supervision of Dr Sebastian Eves-van den Akker following the pipeline described in Eves-van den

Akker *et al*. (2015), which is presented in greater detail under the results section in this chapter (section 4.3.5.).

4.2.8. *Statistical analysis*

The data from the glasshouse virulence experiment, fecundity (eggs cyst^{-1}) and multiplication ratio (Pf/Pi), were subjected to square root transformation before being analysed by general ANOVA in Genstat (VSN International, 18th Edition). Tukey's multiple range test (95% confidence intervals) was used to confirm where differences occurred between tested groups.

To test if the observed values of the ratios (%) of plasmids containing known partial sequences of cyt-*b* (Type 1, 2 and 3) were significantly different from the expected values the data were subjected to chi-square goodness of fit test (χ^2 P value cut-off 0.05 in Excel (Microsoft Corporation, 2016).

4.3. Results

4.3.1. *Reproduction assessment - genotype*

The population data (cysts, total eggs and eggs cyst^{-1}) used in this chapter are presented in Appendix 7.8. for initial, and in Appendix 7.9. for final population. Tested and control populations showed the best reproduction on the susceptible variety 'Desiree'. On average, across all populations, the number of newly formed cysts (cysts pot^{-1}) on this genotype was 270 and the average number of eggs cyst^{-1} was 318 (Figure 4.3.). The other three genotypes, 'Innovator', 'P55/7' and 'Vales Everest', showed lower reproduction rates with average number of cysts pot^{-1} at 4, 42 and 7, respectively, and average numbers of eggs cyst^{-1} of 183, 230 and 164, respectively (Figure 4.3.).

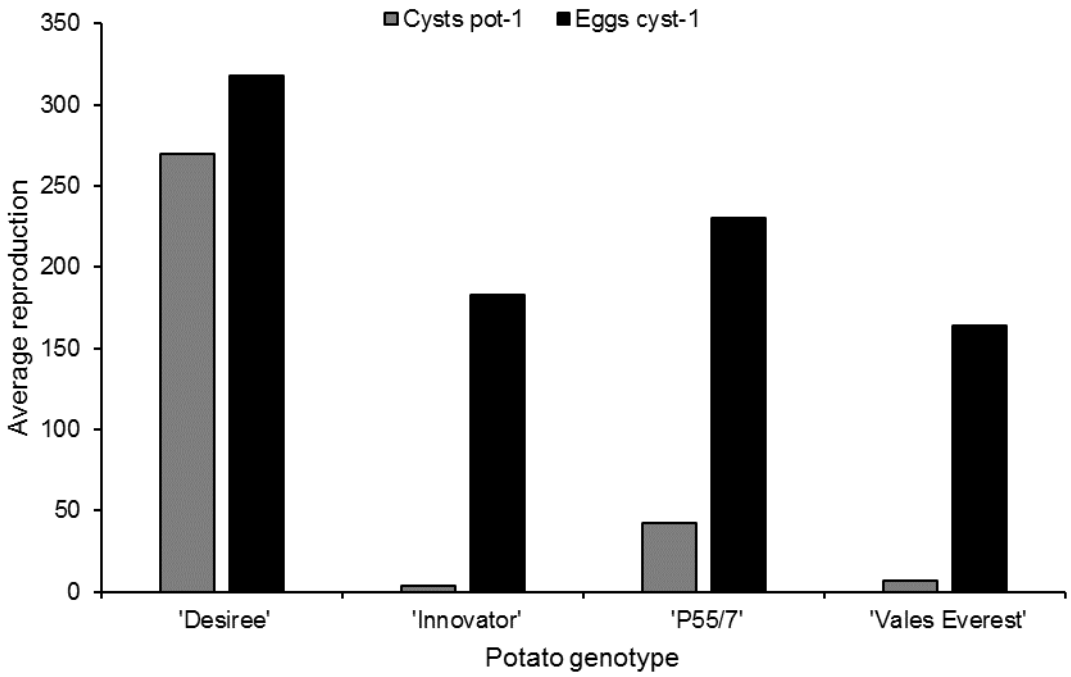


Figure 4.3. Average reproduction (cysts pot^{-1} and eggs $cyst^{-1}$) of *G. pallida* field and control populations on four different potato genotypes in the glasshouse experiment for virulence assessment.

On average, partially resistant genotypes showed a strong reduction in reproduction (cysts pot^{-1}) when compared with 'Desiree', by 98% for 'Innovator', 82% for 'P55/7' and 96% for 'Vales Everest'. When reported as numbers of eggs $cyst^{-1}$ the reproduction on 'Innovator', 'P55/7' and 'Vales Everest' was 48, 27 and 46%, respectively, lower than reproduction on 'Desiree' (Figure 4.4.).

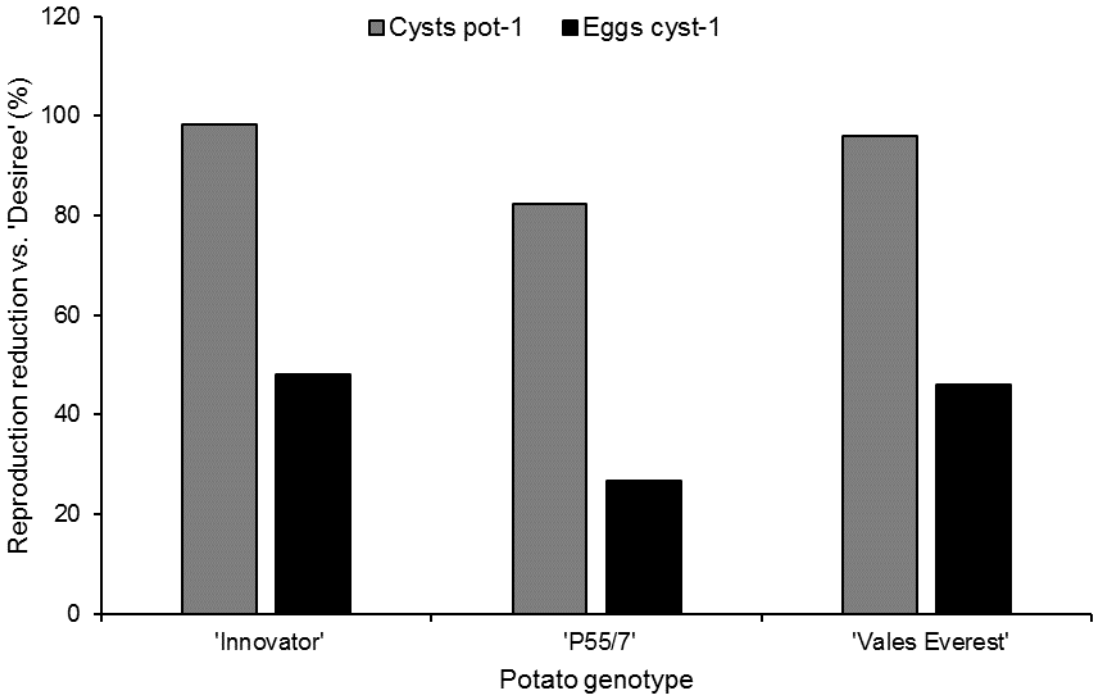


Figure 4.4. Average reduction in reproduction (%) of three different potato genotypes in the glasshouse experiment for virulence assessment relative to reproduction on susceptible genotype 'Desiree' expressed as cysts pot^{-1} and eggs cyst^{-1}.

4.3.2. *Reproduction assessment* – G. pallida *population*

Reproduction of field and control *G. pallida* populations on potato genotypes was calculated and presented in two ways: number of cysts pot^{-1} and number of eggs cyst^{-1}.

Reduction in multiplication, based on number of cysts pot^{-1} (Figure 4.5.a), for Lindley and Luffness populations was strongest with 'Innovator' where multiplication was 98 and 99% lower than on 'Desiree', respectively. 'Innovator' was closely followed by 'Vales Everest' which reduced the multiplication of Lindley and Luffness populations by 94 and 97%, respectively. The number of newly formed cysts pot^{-1} for these two populations was also lower, relative to reproduction on 'Desiree', on partially resistant genotype 'P55/7", although the inhibition of multiplication for the

Lindley population was more pronounced (91%) than Luffness (71%) (Table 4.8.). The number of eggs cyst^{-1} (Figure 4.5.b) was also lower on all genotypes for Lindley and Luffness populations, when compared with multiplication on 'Desiree'. The reduction was higher for the Luffness population (80%) compared to Lindley (20%) with 'Innovator'. In contrast, when tested on 'P55/7', the reduction was higher for the Lindley population (47%) than for Luffness (15%). Similar reductions were observed for Luffness and Lindley when tested on 'Vales Everest', 61 and 63%, respectively (Table 4.9.). The number of cysts pot^{-1} of population Pa1 (Pa1), post reproduction, was strongly reduced (99%) on both, 'P55/7' and 'Vales Everest' (Table 4.8.). The eggs cyst^{-1} reduction on these two potato genotypes for the Pa1 population was 62 and 36%, respectively. No multiplication was recorded for population Pa1 (100% reduction) on 'Innovator' (Table 4.9.).

Reproduction (cysts pot^{-1}) from the field populations (HAU) was, with one exception, lowest on 'Innovator'. Cysts pot^{-1} counts were between 92 and 99% lower relative to multiplication on 'Desiree'. 'Vales Everest' reduced cysts pot^{-1} by between 80 and 99%. Populations HAU165, 298 and 351, had the same response to 'Innovator' and 'Vales Everest' with multiplication 99% lower than 'Desiree'. These three populations were also, along with HAU164, best controlled by 'P55/7', with reductions of 87, 92, 88 and 87%, respectively. Reduction in multiplication of population HAU166 was 97% on 'Innovator' and 99% on 'Vales Everest', which was different to the responses from the other field populations. Across all field populations, the weakest reduction in cysts pot^{-1} was observed on 'P55/7' with a range between 62 and 92% (Table 4.8.).

Table 4.8. Reduction in reproduction (%) of *G. pallida* field populations from England and Wales on different potato genotypes relative to reproduction on the susceptible genotype ‘Desiree’ (cysts pot^{-1}).

Population	Potato genotype		
	'Innovator'	'P55/7'	'Vales Everest'
HAU 152	98.0	80.3	97.2
HAU 164	98.9	86.8	98.3
HAU 165	99.1	87.4	98.7
HAU 166	97.2	83.3	98.8
HAU 167	91.9	62.1	79.5
HAU 178	98.5	83.2	94.3
HAU 298	99.3	92.3	99.0
HAU 351	99.3	88.2	99.3
HAU 356	98.4	63.1	97.0
Pa 1	100.0	98.9	99.0
Luffness	99.2	70.9	97.2
Lindley	98.4	91.1	94.2
Average	98.2	82.3	96.0

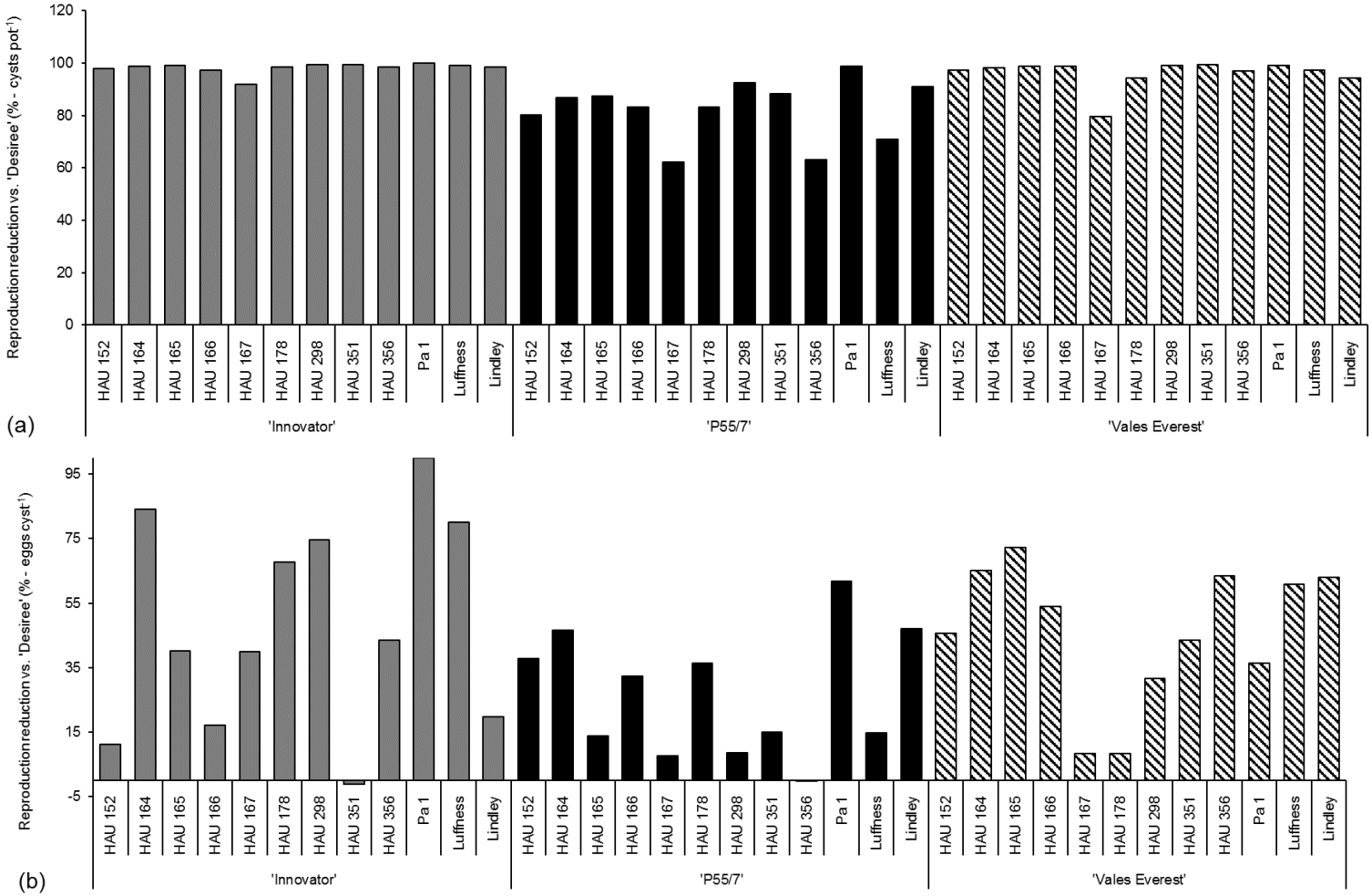


Figure 4.5. Reduction in reproduction (%) of *G. pallida* field populations from England and Wales on the three potato genotypes relative to reproduction on susceptible genotype 'Desiree' expressed as (a) cysts pot^{-1} and (b) eggs cyst^{-1} for comparison.

Table 4.9. Reduction in reproduction (%) of *G. pallida* field populations from England and Wales on different potato genotypes relative to reproduction on susceptible genotype 'Desiree' (eggs cyst^{-1}).

Population	Potato genotype		
	'Innovator'	'P55/7'	'Vales Everest'
HAU 152	11	38	46
HAU 164	84	47	65
HAU 165	40	14	72
HAU 166	17	32	54
HAU 167	40	8	8
HAU 178	68	36	8
HAU 298	75	9	32
HAU 351	-1	15	43
HAU 356	44	0	64
Pa 1	100	62	36
Luffness	80	15	61
Lindley	20	47	63
Average	48	27	46

4.3.3. *Reproduction (Pf/Pi) ratio*

Populations producing more cysts than were used for the inoculation resulted in a Pf/Pi ratio >1, which indicates susceptibility according to the Kort *et al*. (1977) pathotype scheme and analogously Pf/Pi ratio ≤ 1 indicates resistance. 'Innovator' and 'Vales Everest' contain genes that confer high resistance to Pa1, Pa2/3 and Pa3, which suggests that these pathotypes should have a low Pf/Pi ratio on these varieties. In contrast, 'P55/7' has high resistance to Pa1 and partial resistance to Pa2/3 and Pa3 which suggests that the multiplication ratio of *G. pallida* population of pathotype Pa2/3 might be higher on this genotype than on 'Innovator' and 'Vales Everest'. As described above, all genotypes were expected to prevent the multiplication of pathotype Pa1. Consistently low Pf/Pi ratio (≤1) (*i.e.* a Pf of less than 20 cysts) across the genotypes indicates the presence of *G. pallida* population

with a Pa1 pathotype as observed with the control population Pa1 which had Pf/Pi's of ≤1.

All control and field populations had the highest Pf/Pi ratio on 'Desiree'. The control and seven out of the nine field populations had the second highest Pf/Pi ratio on 'P55/7', followed by 'Vales Everest' and 'Innovator' (Figure 4.6.). Population HAU351 had identical reproduction ratios on both 'Innovator' and 'Vales Everest', while population HAU166 was the only one which reproduced better on 'Innovator' than on 'Vales Everest'. 'Innovator' was resistant, according to the Kort *et al*. (1977) scheme, to all 12 populations. With one exception, this was also the case for the genotype 'Vales Everest'. Population Lindley had a Pf/Pi of 1.3 on 'Vales Everest' indicating that it was weakly virulent on this genotype. Genotype 'P55/7' was resistant to the control population Pa1 and to field population HAU167 (Pf/Pi=0.2 and 0.8 respectively), however the multiplication of HAU167 on Desiree was much lower than other populations and controls. Based on the characteristics described, population HAU 167 was classified as pathotype Pa1. All remaining field populations, were assigned to pathotype Pa2/3 (Table 4.10.). Further differentiation and separation between Pa2 and Pa3 was not possible as the differential genotype (62.33.3) used to define Pa3 (see Kort *et al*., 1977) was not included in the experiment.

Table 4.10. Reproduction ratios (Pf/Pi) on different potato genotypes and pathotype designation of field populations from England and Wales.

Population	Genotype				Pathotype designation
	'Desiree'	'Innovator'	'P55/7'	'Vales Everest'	
HAU 152	16.7	0.3	3.3	0.5	Pa2/3
HAU 164	7.9	0.09	1.0	0.1	Pa2/3
HAU 165	22.9	0.2	2.9	0.3	Pa2/3
HAU 166	12.5	0.4	2.1	0.2	Pa2/3
HAU 167	2.0	0.2	0.8	0.4	Pa1
HAU 178	6.8	0.1	1.1	0.4	Pa2/3
HAU 298	13.2	0.09	1.0	0.1	Pa2/3
HAU 351	10.2	0.1	1.2	0.1	Pa2/3
HAU 356	14.1	0.2	5.2	0.4	Pa2/3
Pa 1	18.5	0.0	0.2	0.1	Pa1 control
Luffness	13.8	0.1	4.0	0.4	Pa3 control
Lindley	23.3	0.4	2.1	1.3	Pa2/3 control
Average	13.5	0.2	2.1	0.4	

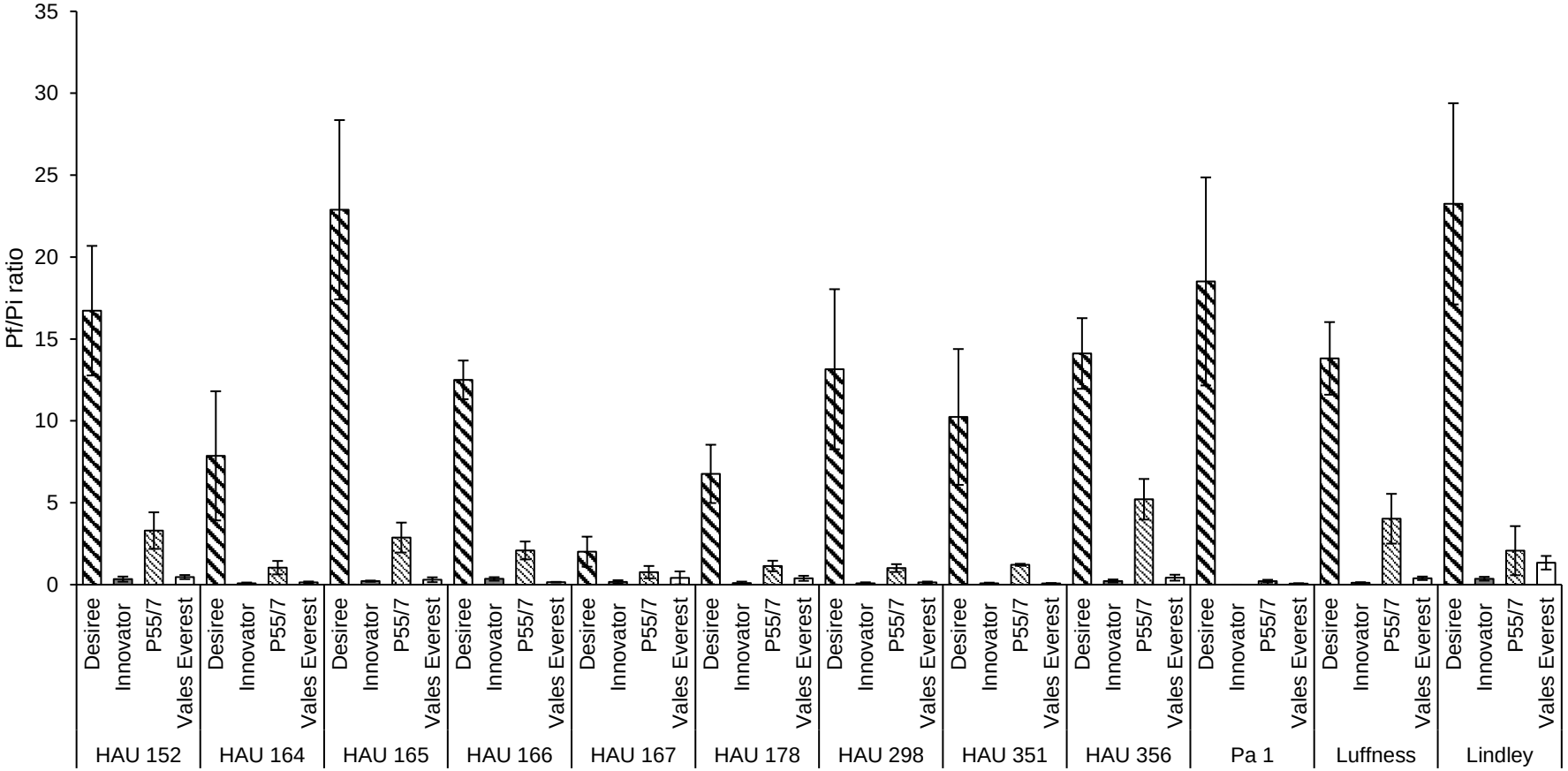


Figure 4.6. Reproduction ratio (Pf/Pi) of *G. pallida* field populations from England and Wales on the four potato genotypes in the glasshouse experiment. Populations Pa1 (Pa1), Luffness (Pa3) and Lindley (Pa2/3) were used as controls. Error bars represent the standard error of the mean.

4.3.4. *Relative susceptibility and resistance score*

Relative susceptibility (%) calculated from data obtained from cyst counts was higher than 3% (EPPO, 2006) in 44% of the samples from the glasshouse experiment. Following the EPPO protocol, egg counts were obtained for the remaining samples (Table 4.11.). A score of 9, indicating the maximum level of resistance, was observed with 'Innovator' with six field populations (HAU164, 165, 178, 298, 351, 356) and all three control populations. The same score was noted on 'Vales Everest' for five field populations (HAU164, 165, 166, 298, 351) and one control population, Pa1, which was also the only population that scored 9 on 'P55/7'. Genotype 'Innovator' had the second highest resistance score (8) when tested against populations HAU152 and 166, as did 'Vales Everest' for HAU152, 356 and Luffness. The lowest resistance score (6) on 'Innovator' was for population HAU167 which also produced the lowest resistance scores on 'Vales Everest' (4) and on 'P55/7' (3). Second lowest resistance score (6) on 'Vales Everest' was for populations HAU178 and Lindley. In contrast, when tested on genotype 'P55/7', the resistance score of 6 was the highest (populations HAU298 and Lindley). Resistance score on 'P55/7 was 5 for populations HAU164, 165, 351, 4 for populations HAU152, 166, 178 and 3 for populations HAU356 and Luffness (Figure 4.7.).

Table 4.11. Resistance scores for four different potato genotypes tested against field populations from England and Wales.

Population	Genotype			
	'Desiree'	'Innovator'	'P55/7'	'Vales Everest'
HAU 152	-	8	4	8
HAU 164	-	9	5	9
HAU 165	-	9	5	9
HAU 166	-	8	4	9
HAU 167	-	6	3	4
HAU 178	-	9	4	6
HAU 298	-	9	6	9
HAU 351	-	9	5	9
HAU 356	-	9	3	8
Pa 1	-	9	9	9
Luffness	-	9	3	8
Lindley	-	9	6	6
Average	-	8.6	4.8	7.8

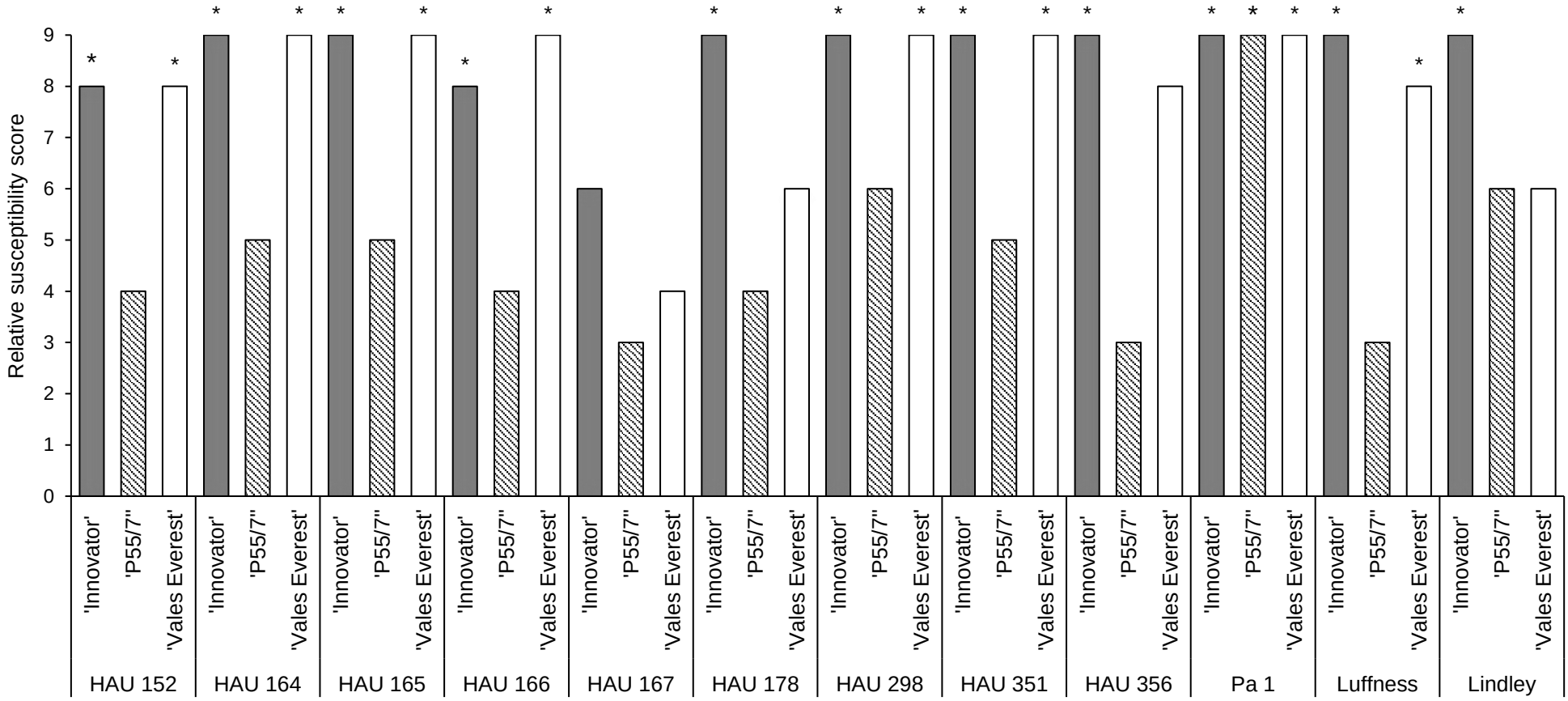


Figure 4.7. Resistance scores of three potato genotypes for *G. pallida* field populations from England and Wales in the glasshouse experiment. Populations Pa1 (Pa1), Luffness (Pa3) and Lindley (Pa2/3) were used as controls. Asterisks indicate genotypes when relative susceptibility calculated with cysts was less than 3% (*i.e*. a score of 8 or above) and eggs counts were obtained.

The data from the glasshouse experiment were analysed by general ANOVA to determine whether potato genotype, *G. pallida* population and the interaction between these two variables had a significant effect on fecundity (eggs $cyst^{-1}$) of cysts and Pf/Pi ratio. The results showed that the potato genotypes used have a highly significant effect ($P<0.001$) on fecundity of reproduced cysts ($P<0.001$) and Pf/Pi ratio ($P<0.001$). Additionally, there was a highly significant difference between *G. pallida* populations, including control populations, for the fecundity of reproduced cysts ($P<0.001$) and Pf/Pi ratio ($P<0.001$). The interaction between potato genotypes and *G. pallida* populations was significant for fecundity of reproduced cysts ($P=0.004$) and Pf/Pi ratio ($P=0.04$).

A significant difference between *G. pallida* populations when Pf/Pi values were tested individually for 'Desiree', 'P55/7', 'Innovator' and 'Vales Everest' ($P=0.002$, 0.001, 0.004 and 0.02 respectively) (Figure 4.8.).

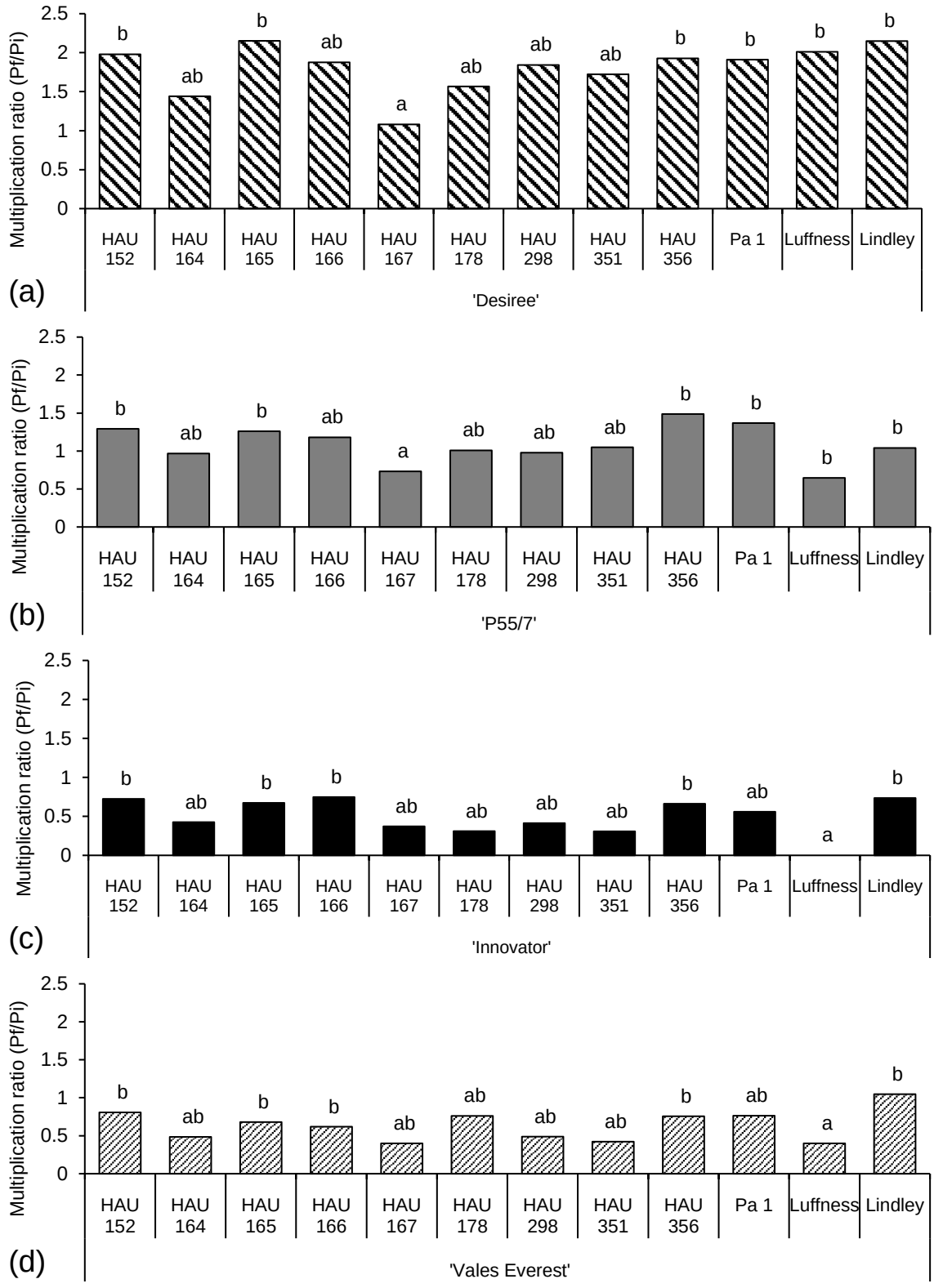


Figure 4.8. Reproduction ratio (Pf/Pi) of *G. pallida* field populations from England and Wales on potato genotypes (a) ‘Desiree’, (b) ‘P55/7’, (c) ‘Innovator’ and (d) ‘Vales Everest’ in the glasshouse experiment. Populations Pa1 (Pa1), Luffness (Pa3) and Lindley (Pa2/3) were used as controls. Bars with the same letter are not significantly different according to Tukey's multiple range test (P>0.05).

4.3.5. *Next-generation sequencing*

The paired-ends reads (over 14 million) were subjected to bioinformatic processes, which included trimming of the leading and trailing low quantity bases (Q-score 28) and selection for the minimum lengths of 150bp. The remaining 8.2 million reads (58.5%) were assembled using pair-end read merger (Pear-0.9.6) to select for overlapping pairs of reads that were then converted to Phred64 coding and next into FASTA format. The remaining 7.9 million (96.5%) reads were uniformed by changing the orientation to 5'-3' and reads from file with forward barcode and file with reverse barcode were concatenated. This reduced the number of reads to 7.8 million. Next, reads of a minimum length of 265bp containing the forward and reverse primer binding sites and barcodes no shorter than 4bp (but up to 6bp to account for the additional 5' adenosines) were selected, which further reduced surviving reads to 7.6 million. All steps described above were performed to present the reads in a uniform format and could be compared with data from Eves-van den Akker *et al*. (2015).

It was noted that one of the previously described SNPs that was used to distinguish between types, lies within a stretch of homo-polymers, and coincided with a sequencing error (deletion) in this dataset at the position 118 of the 265bp cyt-*b* fragment. This region was deemed to be uninformative, and was excluded from further analyses by replacing with letter N. This correction to the data did not affect further analyses as the SNP at the position 118 is not the only descriptive SNP of mitotype 1, others can be used to distinguish it from types 2 and 3.

The data taken for further analyses, now reduced to 7.4 million reads, was found to contain, after the barcode sequences were excluded, 143,028 unique sequences (100% of all analysed reads) which were sorted from most to least replicated. From this selection, the top 11 most common unique sequences, which

contained 72.7% of all analysed reads (Figure 4.9.) but represent only 0.008% of the unique sequences, were selected and extracted from the file containing all sequences. The remaining unique sequences are expected to mostly contain very rare PCR and/or sequencing errors.

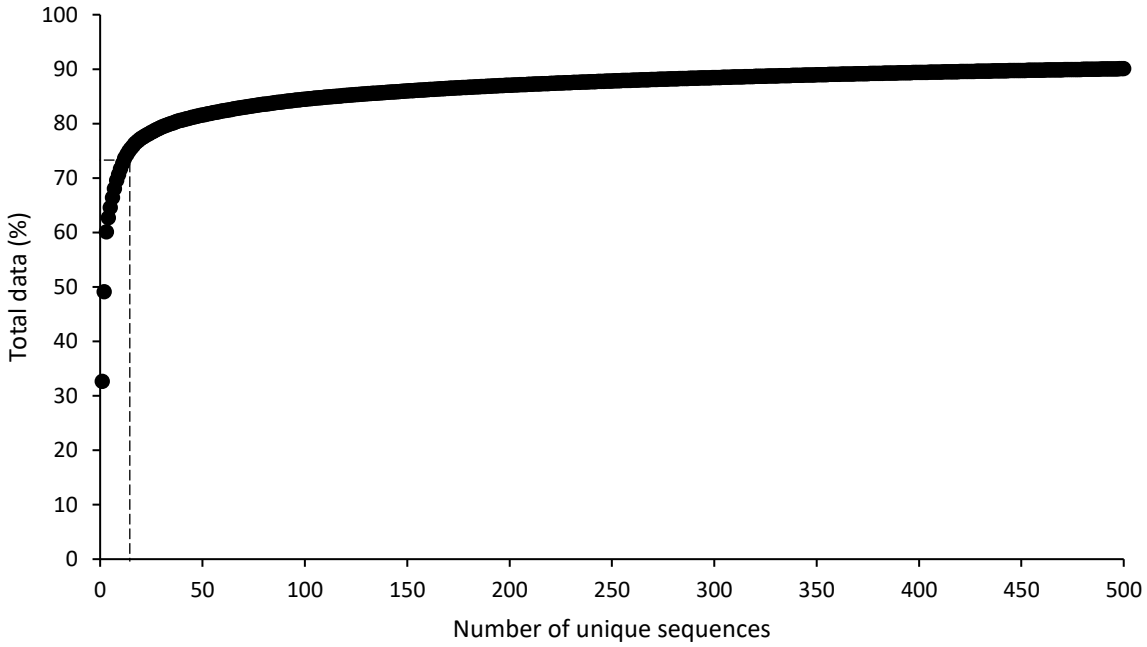


Figure 4.9. The top 500 most common unique sequences and their abundance as a percentage of the total data. The vertical dashed line highlights the proportion (%) of sequences accounted for by the top 11 most common unique sequences.

The top 11 most common unique sequences were numbered in order of frequency and compared to sequences describing the fragment of cyt-*b* gene from the three *G. pallida* mitotypes (plasmid Type 1, 2 and 3) (Appendix 7.7.). The first most replicated unique sequence was identified as mitotype 3, which was also detected in the 4th, 6th, 7th and 8th most replicated unique sequences. The second most replicated unique sequence was identified as mitotype 2, which was also recognised in the 9th and 10th most replicated unique sequences. The third most

replicated unique sequence was identified as mitotype 1, which was also detected in the 5th and 11th most replicated unique sequences (Figure 4.10.).

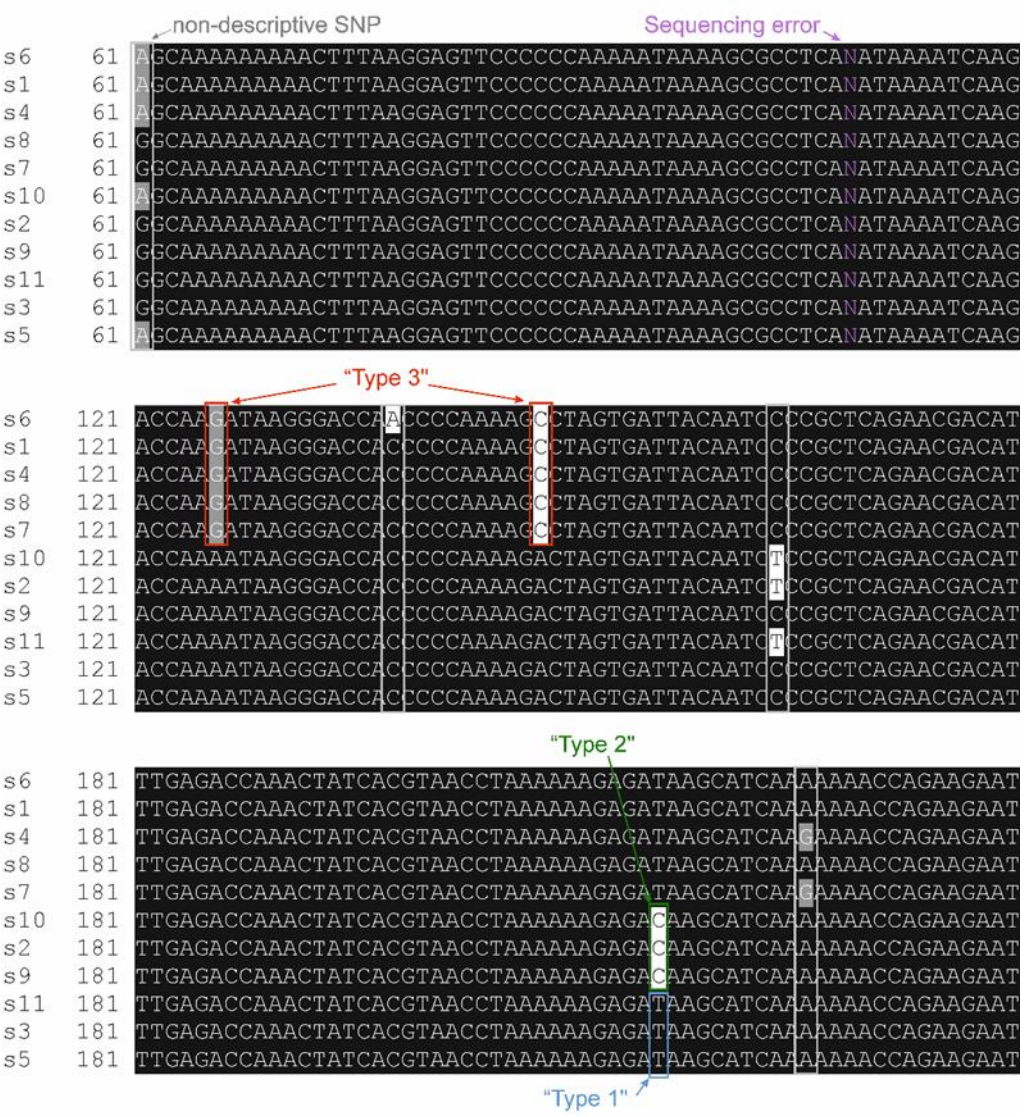


Figure 4.10. The fragment of the top 11 most common unique sequences showing the descriptive SNPs of each mitotype. A blue box indicates SNP descriptive of mitotype 1, a green box indicates SNP descriptive of mitotype 2, a red box indicates SNPs descriptive of mitotype 3 and a grey box indicate non-descriptive SNPs. Letter 'N' indicates corrected sequencing error.

To test method sensitivity for each control mixture of the plasmids, reads corresponding to the plasmid sequences with 100% identity were considered a signal, while all other reads were considered noise. The percentage of the unique sequences recognised as a noise, within a given barcode pair, indicated the level of noise. Based on the noise level detected in the control mixtures, a cut-off point was imposed for all other barcode pairs and unique sequences that contributed less than 2.6% to the total number of sequences within barcode pair were removed. Additionally, an integer cut-off was applied by counting the number of reads in samples containing PCR water instead of DNA template (negative control - 1,357) and subsequently samples with number of reads below this threshold, four survey samples (HAU140, 144, 145 and 150) and one replicate of internal control (HAU167-control 4), were removed.

To test method specificity, nine mixtures of ratios (%) of two or three mitotypes encoded by plasmids (Type 1, Type 2 and Type 3) were included as control amplifications. The comparison of expected and actual observed proportion (%) of Type 1, Type 2 and Type 3 for each of nine control mixes is presented in Figure 4.11. The observed ratios were compared with the expected ratios and the null hypothesis that there was no significant difference between the observed and the expected value was accepted (χ^2 P value cut-off 0.05).

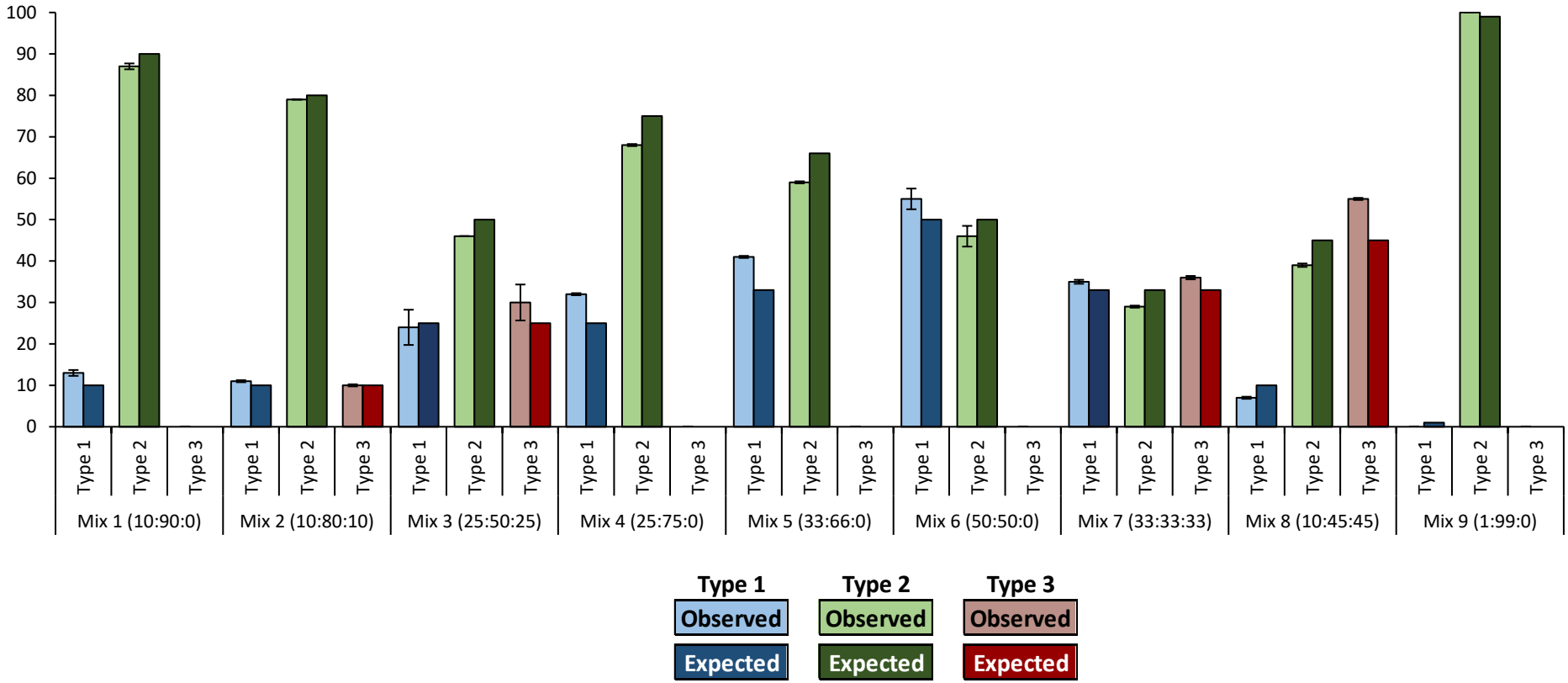


Figure 4.11 Ratios (%) of plasmids containing known partial sequences of cyt-*b* (Type 1, 2 and 3), used in the next-generation sequencing for mitotype assessment of *G. pallida* field populations from England in nine mixtures (control amplifications). The observed ratios (light coloured bars) are not significantly different from the expected ratios (dark coloured bars) (χ^2 P value cut-off 0.05). Error bars represent the standard error of the mean.

To test method reliability, an additional four replicates (control 1-4) of five selected survey samples (actual HAU152, 164, 165, 166 and 167) were included to act as internal controls and compared with results from the actual samples (Figure 4.12.). Populations HAU152 and HAU166 were identified as mitotype 3 in actual samples and confirmed by all four control replicates. Population HAU164 was also identified as mitotype 3 in actual samples but this result was confirmed only by three control replicates. The control 2 for this population was found to have a low proportion (0.1) of mitotype 1 mixed with mitotype 3 (0.9). The control replicates for population HAU167 were found to contain a mixture of mitotypes 1 and 3 and confirmed assessment of the actual sample, although the proportion of mitotype 1 was lower by 10% for controls 1 and 2 and higher by 30% for control 3. The control replicate 4 was removed from further analyses as the number of reads for that sample was below the negative control (PCR water) threshold. Comparison between the actual sample and its control replicates for population HAU165 showed differences in the mitotype detection. Whilst the actual sample was characterised as mitotype 1, control 1 was characterised as mitotype 1 and controls 2, 3 and 4 were a mixture of mitotypes 1 and 3.

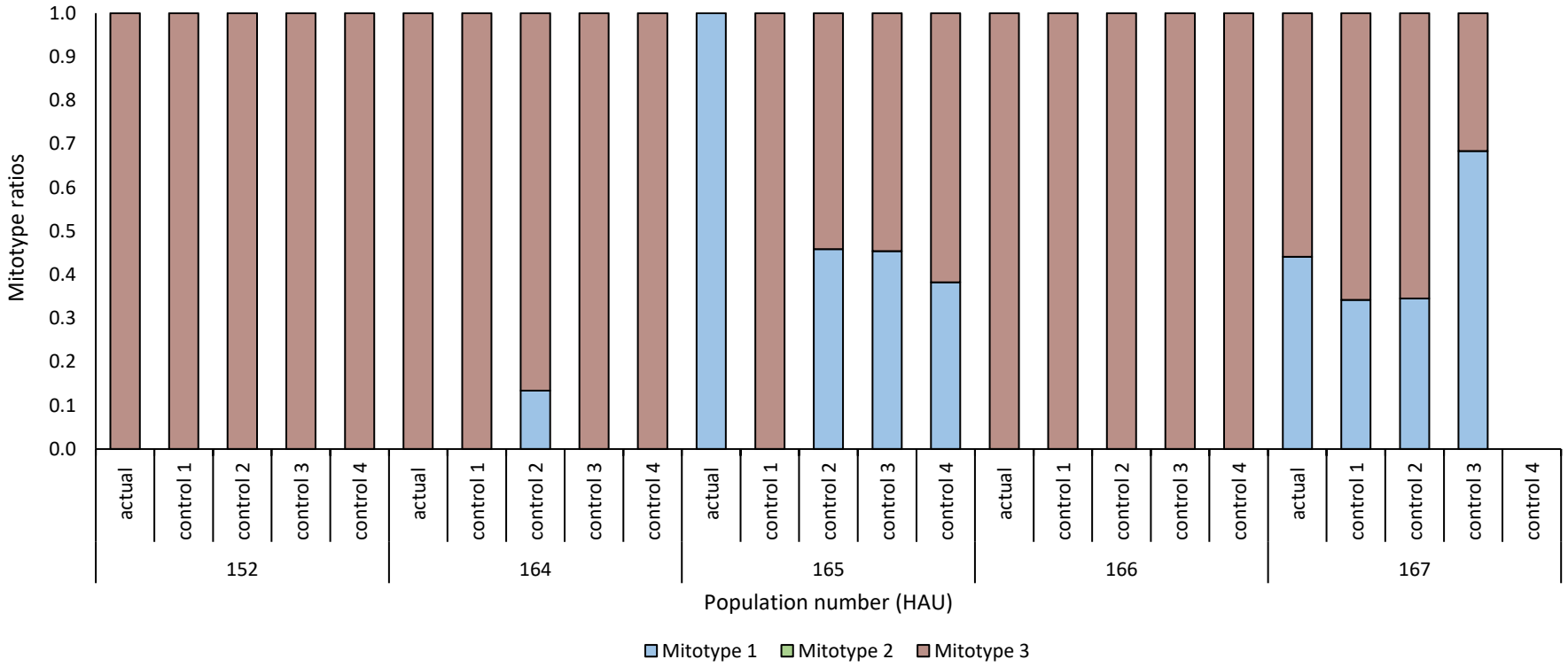


Figure 4.12. The presence and ratios of sequences descriptive of mitotypes 1, 2 and 3 in five *G. pallida* field populations from England (actual) and their replicates (controls 1-4). Replicate 4 for the HAU167 control was removed from further analyses as its number of reads was below the negative control (PCR water) threshold.

4.3.6. *Mitotype distribution*

Using a distinctive 4bp barcode pair (forward and reverse primer barcodes) assigned to each DNA sample, individual survey samples were identified and investigated for the presence of reads identified within the top 11 most common unique sequences descriptive of mitotypes 1, 2 and 3. From the survey samples, 61% contained a single mitotype and 39% contained a mixture of two mitotypes. Two survey samples were found to contain mitotype 1, mitotype 3 was detected in 57 survey samples and the remaining 38 survey samples were described as a mixture of mitotypes 1 and 3 (Figure 4.13.). Mitotype 2 was not found.

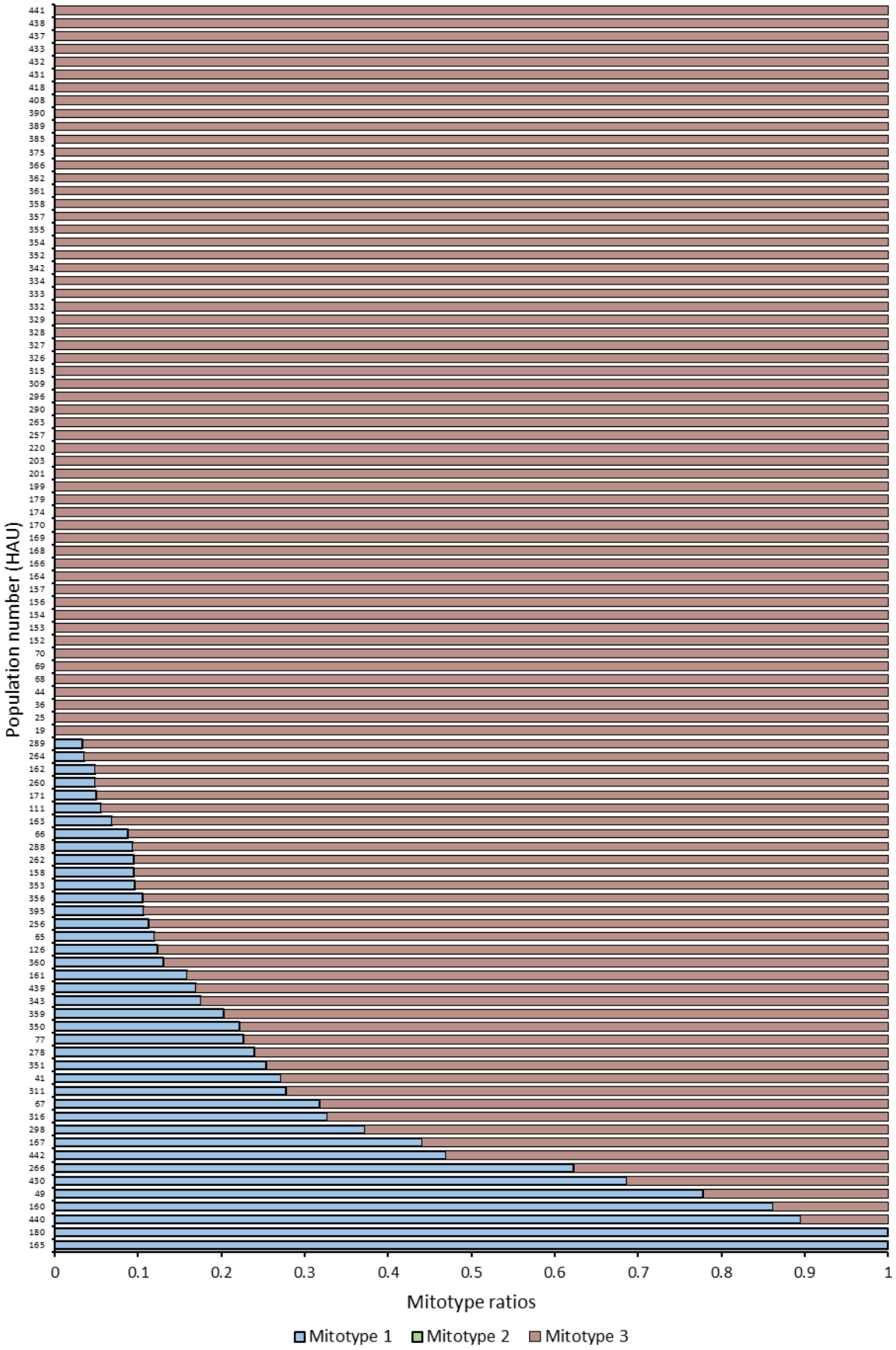


Figure 4.13. The presence and ratio of sequences descriptive of mitotypes 1, 2 and 3 in individual *G. pallida* field populations from England.

Information recorded in the survey's questionnaires showed that samples included in the NGS analysis originated from East Midlands, East of England, North West, South East, South West, West Midlands and Yorkshire and the Humber. North East and Wales were not represented by survey samples included in NGS (Figure 4.14.).

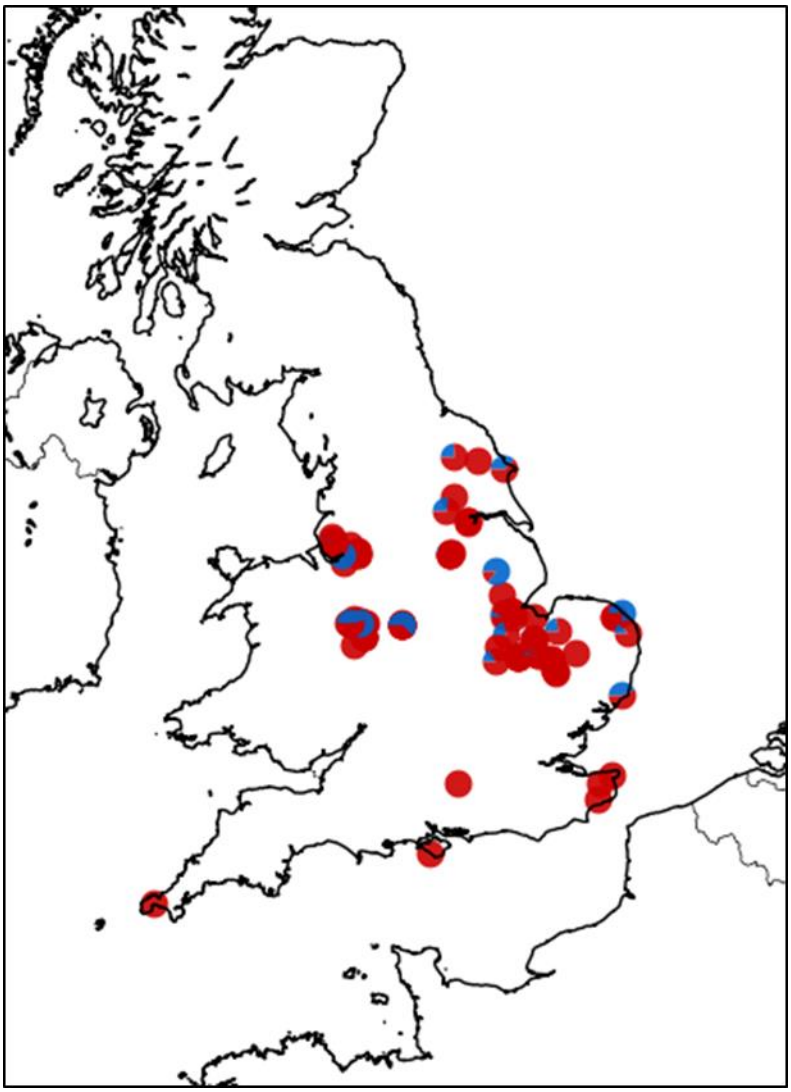


Figure 4.14. Distribution of *G. pallida* field populations from England characterised as mitotype 1 (blue shaded area of pie charts) or mitotype 3 (red shaded area of pie charts).

Potato cyst nematode populations identified as mitotype 3 were found in soil samples originating from all seven regions of England included in this study while populations identified as mitotype 1 were detected solely in North West and West Midlands. The South East and South West were the only regions where populations described as a mixture of mitotypes 1 and 2 were not found (Table 4.12.).

Table 4.12. Field *G. pallida* populations characterised as mitotype 1, mitotype 3 or a mixture of mitotypes 1 and 3 from seven regions of England.

Country	Region	Total samples	Mitotype 1	Mitotype 3	Mix of mitotypes 1 and 3
England					
	East Midlands	19	0	11	8
	East England	25	0	15	10
	North West	13	1	8	4
	South East	5	0	5	0
	South West	1	0	1	0
	West Midlands	26	1	14	11
	Yorkshire and the Humber	8	0	3	5
Total		97	2	57	38

4.4. Discussion

4.4.1. *Data reporting*

All partially resistant potato genotypes, when compared with the susceptible genotype, showed a stronger reduction in the number of cysts pot^{-1} (average of 92%) than in the number of eggs cyst^{-1} (average of 40%). This indicates that the results from tests on resistant/susceptible genotypes, if reported in this format, should contain both figures. As shown here, reporting reduction in number of cysts pot^{-1} only, if not supported by other analysis, does not describe the effect on multiplication completely. It provides information about number of fertilized females but does not define their fertility. This characteristic can be provided by reporting number of eggs cyst^{-1}. When using reproduction as a tool for screening potato genotypes for resistance against PCN reduction, assessment of both cysts pot^{-1} and eggs cyst^{-1} is important in some circumstances. Whitehead and Russell (1994) argued that PCN multiplication in pot experiments is best assessed by counting eggs cyst^{-1} rather than the number of cysts pot^{-1}. They asserted that the assessment of only the number of newly formed cysts does not take into account differences between number of eggs cyst^{-1} produced by the same population on susceptible (higher eggs cyst^{-1}) and resistant (lower eggs cyst^{-1}) genotypes. Use of cyst reproduction as useful measure of resistance was supported by Schouten and Beniers (1997) they reported that increase in reproductive ability, under the selective pressure form genotypes with resistance gene, was due to a greater proportion of individual nematodes becoming female rather than an increase in fecundity.

The survey populations tested in this research were collected from various fields under different management practices and infested by PCN populations of different age. Younger populations would, most likely, have a higher percentage of diapaused eggs while older populations would be under a greater effect of natural

mortality (described in Chapter 1 - sections 1.2.6.2. - 1.2.6.3.). This variability might influence the multiplication ability of such populations. To provide a standardised comparison of the results, the calculations of the reduction in multiplication for each population were expressed as a fraction of the corresponding multiplication on a susceptible genotype (Whitehead and Russell, 1994).

4.4.2. *Multiplication on potato genotypes*

Multiplication of field and control *G. pallida* populations was highest, with one exception, on genotype 'Desiree' regardless if number of cysts pot^{-1} or fecundity of reproduced cysts was measured. Population HAU351, on genotype 'Innovator' compared to 'Desiree', had lower cyst counts (2 and 205, respectively), but higher eggs cyst^{-1} counts (366 and 362, respectively). This small (1%) but unpredicted increase in fecundity observed on cysts produced on 'Innovator' by population HAU351 was compensated by 99% reduction in cysts pot^{-1}. The cysts pot^{-1} reproduction on the partially resistant genotype 'Innovator' was relative to reproduction on susceptible genotype 'Desiree' and ranged from 1 to 8%. These results are lower than previously reported for UK *G. pallida* populations for the genotype 'Morag', with resistance also derived from *S. vernei*, which ranged from 6 to 44% (Phillips and Trudgill, 1998a) and 9 to 52% (Phillips *et al*. 1998a). The range was broader, from 1 to 20%, on 'Vales Everest' and was in accordance with previous studies on resistance derived from *S. tuberosum* ssp. *andigena* CPC2802 which reported a range of virulence from 2 to 21 % (Phillips and Trudgill, 1998a) and from 2 to 25% (Phillips *et al*. 1998a). Variability in multiplication of *G. pallida* populations on genotypes with resistance derived from these two sources and differences in reproductivity between the genotypes reported by Phillips and Trudgill (1983) was confirmed.

4.4.3. *Resistance assessment*

All control and tested field populations had the highest Pf/Pi ratio on ‘Desiree’ (rating from 2.0 to 23.3). Genotype ‘P55/7’, with full resistance against pathotype Pa1, but partial resistance to Pa2/3 derived from *S. multidissectum* PH1366, was classified as a resistant (Pf/Pi≤1) to control population Pa1, which is in accordance with its classification as pathotype Pa1 and avirulent to the H_2 resistance gene in ‘P55/7’ (Blok *et al*., 1997). Populations Luffness and Lindley, pathotypes Pa3 and Pa2/3 respectively, had Pf/Pi ratios larger than one when tested on ‘P55/7’ with Luffness having almost twice the reproduction ratio compared with Lindley. These characteristics of genotype ‘P55/7’ were confirmed when relative susceptibility was calculated. In both tests, the genotype ‘Innovator’, with high resistance to pathotype Pa1, Pa2/3 and Pa3 derived from *S. vernei*, had a high multiplication reduction (Pf/Pi≤1, score 9) for all three control *G. pallida* populations. Genotype ‘Vales Everest’, with high resistance to pathotype Pa1 and moderate resistance to Pa2/3 derived from *S. tuberosum* spp. *andigena* CPC2802, was found to be resistant to control populations Pa1 (Pf/Pi≤1, score 9) and Luffness (Pf/Pi≤1, score 8) but partially resistant to Lindley (Pf/Pi>1, score 6).

Only one field population, HAU167, had a similar pattern seen for control population Pa1, with a Pf/Pi ratio below 1 on all resistant genotypes. This contrasts with the results by relative susceptibility assessment - population HAU167 had the lowest score on 'Vales Everest', ‘P55/7’ and ‘Innovator’ suggesting it had the highest virulence of the all tested PCN populations. However, population HAU167 also had the lowest Pf/Pi of 2 on genotype ‘Desiree’ and one of the HAU167 replicates on ‘P55/7’ failed to produce which, if this occurred due to environmental causes, could give an incorrect Pf/Pi score assessment. The second lowest resistance score on ‘Vales Everest’ was observed for populations HAU178 and control Lindley. Both also

had the largest difference in response, assessed by resistance score, to resistance derived from *S. tuberosum* spp. *andigena* CPC2802 and *S. vernei*. This characteristic was confirmed by the Pf/Pi ratio only for the Lindley population. The multiplication ratio on 'Vales Everest' was higher than on 'Innovator' but still below 1. A low score for relative susceptibility on 'P55/7' (ranging from 3-6) was observed for populations Luffness, HAU152, 166 and 356 but these populations were confirmed to be virulent against this genotype derived from *S. multidissectum* PH1366 by their Pf/Pi ratios (>1). Populations HAU152, 166 and 356 also, like Luffness, had very high resistance scores (9 and 8) and Pf/Pi ratios below 1 on 'Innovator' and 'Vales Everest'. As the differential genotype *S. vernei* hybrid 62.33.3 used in the Kort *et al*. (1977) scheme to distinguish between Pa2 and Pa3 pathotypes was not included in the glasshouse experiment, the distinctness of the Luffness population from Pa2/3 British populations that was found by genetic fingerprinting (Blok *et al*., 1997), was not observed in this study. Population HAU166 was the only population with higher, but only marginally, virulence towards the genotype originating from *S. vernei* than towards the genotype derived from *S. tuberosum* spp. *andigena* CPC2802. The remaining field populations HAU164, 165, 298 and 351 had the maximum resistance (score 9) for both 'Innovator' and 'Vales Everest', which was in line with very low Pf/Pi (≤0.3) assessments. These populations also had the highest resistance scores on genotype 'P55/7' amongst the field populations, which was supported by the lowest (except of population HAU165), but still greater than 1, Pf/Pi ratios.

4.4.4. *Origin of the resistance*

Phillips and Trudgill (1998a) reported Luffness as being more virulent on 'Morag', 'Sante' and '62.33.3' (with resistance originating from *S. vernei* CPC2488,

CPC2487 and a German clone 796-84De) than on *S. tuberosum* ssp. *andigena* CPC2802 (clones 11415 and 12674). The same trend was observed when the cyst reproduction was assessed for the Lindley population but too a much less extent. South American populations were characterised by the opposite trend. In the present study, genotype 'Innovator' was found to have higher, with one exception (HAU166), resistance to all *G. pallida* populations when compared with 'Vales Everest'. To confirm this response of current field populations to *S. vernei*, further testing with genotypes 'Morag', 'Sante' and '62.33.3' is needed as resistance in 'Innovator' was derived from different *S. vernei* source (Rouppe van der Voort *et al*., 2000).

In this study, an interaction between potato genotypes and *G. pallida* populations had a significant effect on reproduction of cysts, fecundity of the reproduced cysts and the multiplication ratio. Highly significant interactions between the potato genotype and English *G. pallida* populations was suggested to partly explain the variation in nematode population responses to resistance genes (Phillips and Trudgill, 1983). Phillips and Trudgill (1998a) also observed a significant interaction between genotypes and European PCN populations. They concluded that it was mainly due to differences between the two sources of resistance. A highly significant relationship between sub-populations and susceptible genotypes was also confirmed by Phillips *et al*. 1998a. When differences in multiplication rates between sub-populations produced from *G. pallida* field populations were examined, sub-populations reared on 'Maris Piper' consistently returned higher values than reared on 'Pentland Crown'.

Turner *et al*. (1983) investigated the selection pressure on genotypes with resistance originating from *S. vernei* and observed an increase in virulence (Pf/Pi ratio) after five generations. The potential for increasing *G. pallida* virulence through

the use of potato genotypes derived from *S. vernei* and *S. tuberosum* ssp. *andigena* CPC2802 was evaluated by Phillips and Blok (2008). Whilst differences between these two sources of partial resistance were observed, it was found that the reproduction ability of *G. pallida* populations increased when reared on both partially resistant varieties. The Newton population, for example, showed an increase in reproductive ability, measured as cysts produced relative to reproduction on the susceptible genotypes; 58% on genotypes derived from *S. vernei* and 79% on genotypes derived from *S. tuberosum* ssp. *andigena* CPC2802.

Phillips *et al*. (1998a) also evaluated the multiplication of sub-populations of a field population of *G. pallida*, reared over a six generations, by multiplication of single cysts on three susceptible genotypes. The authors observed differences in the general reproductive capability between these three groups of sub-populations. This variation could be back-related to the genotype originally used to produce the sub-populations. It was suggested that the susceptible genotypes had an influence on the sub-population's virulence. *Globodera pallida* control populations, from the collection at the JHI, are multiplied on susceptible genotypes, one year on 'Desiree' and one year on 'Maris Piper' (Gartner, 2018. Pers Comm. Ms U. Gartner – the JHI). In the light of findings by Phillips *et al*. (1998a) new generations of control populations should be tested against original populations to ensure that virulence characteristics do not change.

4.4.5. *Next-generation sequencing - mitotypes occurrence and distribution*

It was suggested that three Peruvian cyt-*b* gene haplotype groups (mitotypes), describing the three main introductions into Europe (Plantard *et al*., 2008), represent a promising opportunity to study the diversity of *G. pallida* populations. A distinct geographical origin might be reflected in the population's

particular characteristics *e.g*. virulence (Phillips and Trudgill, 1998a). In this study, an attempt was made to test for the presence of these introductions and to assess the complexity of the English field populations.

Overall, *G. pallida* field populations from England tested by next generation sequencing were found to be mitotype 1 (2% of samples), mitotype 3 (59% of samples) or a mixture of mitotypes 1 and 3 (39% of samples). Mitotype 2 was not detected in any of the populations tested, either solely or as a mix. In comparison, data obtained from a similar study conducted in Scotland (Eves-van den Akker *et al*., 2015), confirmed the presence of mitotype 2, albeit at a lower prevalence. Eves-van den Akker *et al*. (2015) also found that mitotype 3 was the most prevalent mitotype and mitotype 1 was the second most common, similar to the results presented here (Figure 4.15.). Furthermore, the Scottish study indicated that *c*. 79% of *G. pallida* populations contained a single mitotype, *c*. 18% contained a mixture of two mitotypes, and *c*. 2.3% contained a mixture of all three mitotypes. In this study, *G. pallida* populations belonged to either a single mitotype (61%) or a mixture of mitotypes 1 and 3 (39%). The combined detection of mitotypes 1 and 3 could be partly expected based on the finding by Eves-van den Akker *et al*. (2015) who reported that a mixture of mitotypes 1 and 3 was approximately twice as likely to occur than mixtures of 1 and 2, or 2 and 3. The authors, following the hypothesis by Plantard *et al*. (2008) of geographically isolated original introductions from Peru, also concluded that the presence of mixed populations of *G. pallida* mitotypes within a field could give rise to novel hybrids.

Due to the limited number of samples (n=93), available from the English field sites, the presence of mitotype 2 cannot be completely excluded. The possibility of the mitotype not being detected becomes more likely when the low occurrence of mitotype 2 in Scotland, is taken into account. The absence of mitotype 2 in England

may be due to (i) *G. pallida* mitotype 2 currently having a limited distribution following an introduction to Scotland or (ii) mitotypes being influenced by particular environmental conditions and/or management practices such as cultivar choice. Further tests are required on a larger scale to investigate this phenomenon and the biological significance of mitotype 2 and if confirmed measures need to be implemented to avoid the introduction of mitotype 2 into England.

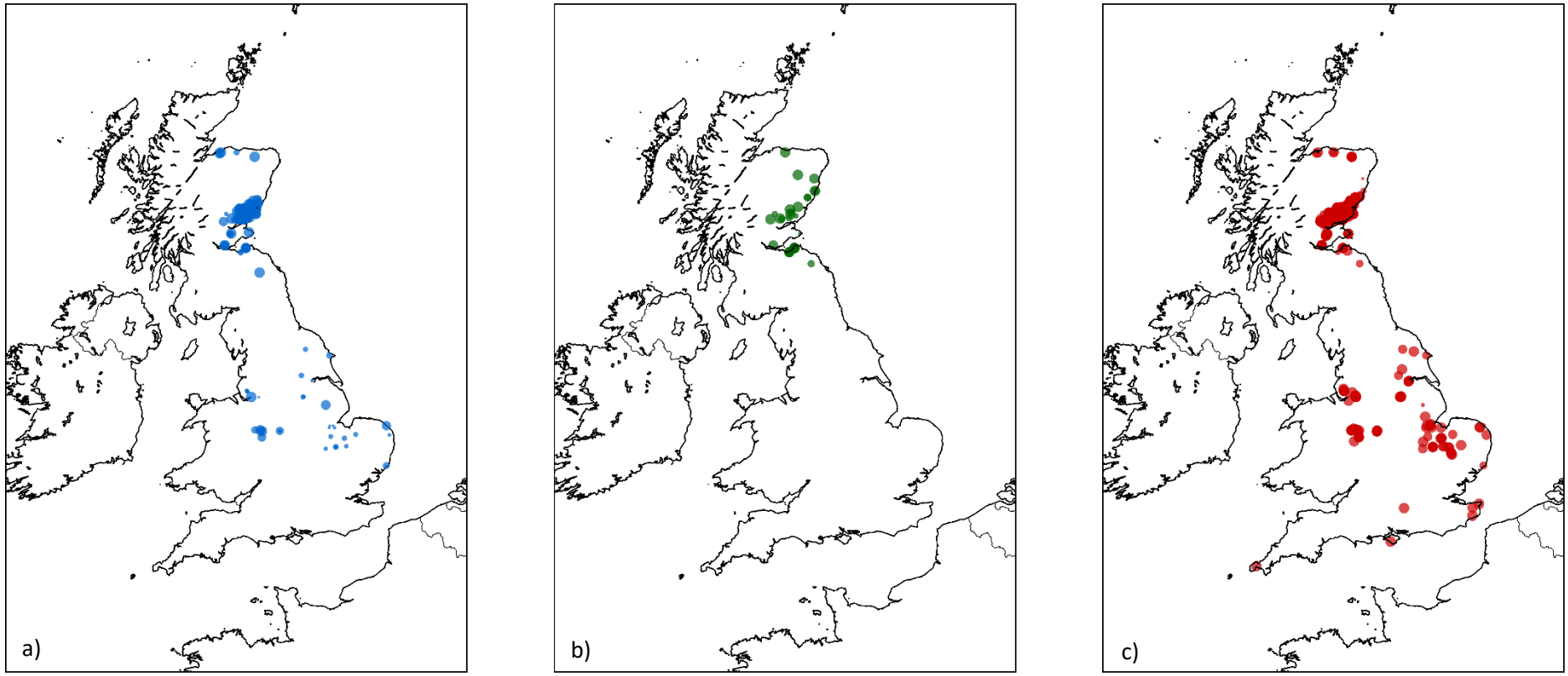


Figure 4.15. Distribution of *G. pallida* mitotypes from England and Scotland characterised as a) mitotype 1 (blue dots), b) mitotype 2 (green dots) and c) mitotype 3 (red dots).

Eves-van den Akker *et al*. (2015) concluded that mitotyping might have potential to aid the selection of appropriate potato cultivars when single mitotypes are present. However, when two or three mitotypes occur together, cultivar selection may prove to be challenging.

The method used for mitotyping is prone to errors and every stage requires at least one measure to minimize the risk of error (Eves-van den Akker *et al*., 2015). The method specificity was controlled by including nine mixtures of two or three mitotypes encoded by plasmids (Type 1, Type 2 and Type 3). The comparison of expected and actual observed results showed no significant differences, indicating that the ratio of reads corresponds to each mitotype within a barcode pair is a true reflection of ratio of mitotypes in the analysed survey samples. Furthermore, the reliability of the method was tested by including internal controls, which consisted of four additional replicates of five selected survey samples. The difference in mitotype detection seen between survey samples (actual) and the replicates (control), particularly apparent in population HAU165 is likely to be due to the variation in *G. pallida* mitotypes found at field-scale resolution as reported by Eves-van den Akker *et al*. (2015). In this publication, the authors demonstrated that the distribution of the individual mitotypes across mixed fields is not even and local peaks of single mitotypes were often present. The existence of such variations suggest that population HAU165 is likely to be a mixture of mitotypes, highlighting the necessity for an intensive sampling across specific field sites.

4.4.6. *Current situation*

Based on the lower variability in virulence between *G. rostochiensis* populations, when compared with *G. pallida* populations, Burrows *et al*. (1996) hypothesised that initial introductions of *G. pallida* to the UK were more genetically

diverse. In the UK, pathotypes Pa1 and Pa2/3 have been already identified but possible additional “founder” populations such as the unusually virulent Luffness population also have been described (Blok *et al*., 1997). There is a great need for improved knowledge about current virulence of PCN field populations and continually monitoring to determine if the resistance of currently used potato varieties is durable (EPPO, 2017). The often disputed pathotype identification scheme, the lack of consistency between tests conducted by independent research groups and technical challenges of the assessment, like limited genotype availability, labour intensity and requirement for large glasshouse space, caused the scheme proposed by Kort *et al*. (1977) to be rarely used and the results to be treated with uncertainty. The methodology used for testing the resistance of potato varieties to *Globodera* spp. devised by EPPO (EPPO, 2006) to investigate populations of unknown virulence (EPPO, 2017), to standardise the assessment and to refine how susceptibility was determined has now been adopted. Alternatively, the hypothesis was proposed that mitotypes could be used as a proxy of pathotypes (Eves-van den Akker *et al*., 2015).

The aim of the glasshouse experiment was to assess virulence of current field populations towards sources of resistance being used in the UK breeding programs. When resistance/susceptibility of control glasshouse populations was assessed based on Pf/Pi values (Kort *et al*., 1977) the results were supported by observations based on relative susceptibility (EPPO, 2006). When the virulence of *G. pallida* field populations was tested on different potato genotypes, generally both assessment methods agreed although this was not always the case *e.g*. HAU167. ‘Innovator’ showed very high levels of resistance to the field populations. Slightly lower but still very satisfactory was the resistance level in ‘Vales Everest’. Finally, especially when compared to the multiplication ratio on ‘Desiree’, genotype ‘P55/7’

showed partial resistance to all of the field populations tested. This indicates that using these genotypes in the field is likely to be highly effective to control *G. pallida* populations in the short-term at least. In future, the durability of the resistance of these genotypes should be monitored and broad-spectrum resistance achieved by combining different sources of resistance should be undertaken. The described differences observed with multiplication of PCN populations on genotypes with partial resistance and differences in the schemes that have been developed to evaluate virulence of field populations highlights the complexity and difficulty in assigning a pathotype to unknown field populations.

Next generation sequencing revealed that no mitotype 2 was found in any of the *G. pallida* field populations from England. All tested populations were found to be mitotype 1, mitotype 3 and a mixture of mitotypes 1 and 3. The mitotypes assessment used in this study followed methodology described in Eves-van den Akker *et al*. (2015). Their research was conducted on Scottish soil samples where all three mitotypes were found.

4.4.7. *Key findings*

- Tested and control populations showed the best reproduction on the susceptible variety ‘Desiree’.
- On average, partially resistant genotypes showed a strong cysts pot^{-1} reduction in reproduction when compared with ‘Desiree’ - 98%, 96% and 82% for ‘Innovator’, ‘Vales Everest’ and ‘P55/7’, respectively.
- On average, the eggs cyst^{-1} reduction in reproduction was lower - 48, 46 and 27% for ‘Innovator’, ‘Vales Everest’ and ‘P55/7’, respectively.
- Reduction in reproduction was always lowest on ‘P55/7’.

- All control populations, and seven out of nine tested populations had the highest Pf/Pi ratio on ‘Desiree’ followed by ‘P55/7’, ‘Vales Everest’ and ‘Innovator’.
- Genotype ‘Innovator’ was found to be resistant (Pf/Pi≤1) to all field and control populations (no indication of resistance breaking).
- Genotype ‘Vales Everest’ was found to be resistant (Pf/Pi≤1) to all field and control populations, except Lindley (no indication of resistance breaking).
- Genotype ‘P55/7’ was found to be resistant (Pf/Pi≤1) to the Pa1 control population and one field population (HAU167).
- Only population HAU167 could be classified as pathotype Pa1 based on its Pf/Pi ratio, however it reproduced poorly on ‘Desiree’.
- Multiplication ratio (Pf/Pi) was confirmed by resistance score for all control *G. pallida* populations but not for all field populations, *e.g*. HAU167.
- An interaction between potato genotypes and *G. pallida* populations had a significant effect on reproduction of cysts, fecundity of reproduced cysts and multiplication ratio.
- As resistant cultivars are grown more frequently, then the virulence and composition of field populations should be revisited.
- *G. pallida* field populations from England were found to be mitotype 1 (2%), mitotype 3 (59%) and a mixture of mitotypes 1 and 3 (39%).
- Mitotype 2 was not found in any of the populations tested, solely or as a mix.

Chapter 5

General discussion on occurrence, distribution, viability and virulence of potato cyst nematode populations from Great Britain.

5.1. *Introduction*

Potato cyst nematodes (PCN), comprised of two related species (*Globodera rostochiensis* and *G. pallida*), are the most destructive pests of potato crops in Great Britain (GB) and other temperate regions. It is estimated that PCN are the second, after late blight, greatest cause of losses in potential yield and quality worth nearly £26 million per year, mainly affecting the processing and fresh market sectors (Twining *et al*., 2009). The management of these pests is expensive and becoming more challenging due to the pressure to replace chemical control products (OJEU, 2009), because of potential health and environmental risks, and the lack of potato genotypes fully resistant to *G. pallida* (Turner *et al*., 2006; AHDB, 2018b).

5.2. *Discussion of chapters*

Understanding PCN populations is of paramount importance to the selection of appropriate alternative management methods and to indicate the direction of the future research. This study aimed to characterise current PCN populations by conducting a PCN survey of ware potato growing land in GB. Collected survey samples were further investigated to provide up-to-date information about the regional prevalence of PCN, the ratio between PCN species, the virulence and mitotype composition of selected populations and determined the viability using a test validated as suitable for field samples of *G. pallida* populations. The results

indicated *G. pallida* to be the predominant species mainly due to the reduction in occurrence of mixed populations (Chapter 2). When the viability of field populations were compared, it was clear that Meldola's blue stain overestimated viability while the trehalose assay showed good potential as an alternative to the existing less accurate methods (Chapter 3). Next generation sequencing revealed that mitotype 2 was not present in any of the *G. pallida* field populations from England. All tested populations were found to be mitotype 1, mitotype 3 and a mixture of mitotypes 1 and 3. Finally, the partial resistance of tested potato genotypes provided a satisfactory level of resistance against the selected group of *G. pallida* field populations (Chapter 4).

A survey conducted in 1994-95 reported that 5% of infested land was confirmed as pure *G. rostochiensis*, 54% as pure *G. pallida* and 41% contained mixed populations (Hancock, 1996). In the PCN survey of England and Wales (1998-99), pure *G. pallida* populations were found in 67% of the infested potato fields, 25% contained mixed populations and 8% were identified as pure *G. rostochiensis* (Minnis *et al*., 2002). Although *G. pallida* was again identified as being the dominant species the overall results indicated that the species distribution of PCN has been changing. Given that the last survey of PCN was undertaken nearly 20 years ago it was critical that the current occurrence and distribution of this pest in GB were investigated.

The most recent stratified survey (Chapter 2) showed that potato cyst nematodes were present in 48% of sites sampled and of the populations found, 89% were *G. pallida*, 5% were *G. rostochiensis* and 6% contained both species. The results found a decrease in the incidence of PCN compared with previous surveys (Hancock, 1996; Minnis, 2000) and when pure and mixed populations were considered together, the occurrence of *G. pallida* has remained at a similar level.

Continuous drift towards *G. pallida* as the predominant species in all regions of England and in Wales was confirmed when the occurrence of *G. rostochiensis*, pure and mixed populations combined, showed a decline between 1996, 2000 and 2016. This is especially noticeable when reviewing the long-term changes to the occurrence of mixed populations. Between 1996 and 2016 there was a 35% decrease in the number of mixed population while the occurrence of pure *G. rostochiensis* populations remained on the similar level. The intensive cropping of varieties resistant to *G. rostochiensis* but susceptible to *G pallida e.g*. 'Maris Piper' (AHDB, 2018a) contributed to strong selection towards *G. pallida* as the predominant species. The popularity of these varieties is dictated by the market and, as a result, grower's selection of varieties being planted on infested fields is restricted. In East Midlands almost 80% of the potato production is for pre-pack and processing, industry sectors focusing mainly on varieties like 'Maris Piper' (AHDB, 2018a). Selection of these 'preferred' varieties by the growers in this region resulted in reduction of pure *G. rostochiensis* and mixed populations between 1967 and 2016 and bring the infestation by pure *G. pallida* to 93% (Guile, 1967). As for potato production market, continuous popularity of the varieties with the H_1 resistance gene and decrease in the planted area, 23% from 1996 to 2016 (AHDB, 2018a), means more intensive cultivation and possibly further increase in infestation by *G. pallida*. Based on the prevalence of *G. pallida* in England and Wales, PCN density assessments should include a molecular test for species ratio as a routine practice.

A decline in PCN viability due to factors such as in-egg mortality can often leave intact, but non-viable eggs, which may be included in the estimate of population density. The assessment of PCN population viability is an additional expense for potato growers and for this reason not often requested for field samples. Furthermore, viability assessment by commercial laboratories, if undertaken, is only

based on visual examination (Keer, 2014. Pers Comm. Mr J. Keer at Richard Austin Agriculture Ltd.). This type of assessment may not fully describe the problem as damaged or discoloured eggs can be viable whilst healthy looking eggs may not be capable of hatching. Other currently available tests are not commercially accessible. Staining with Meldola's blue dye (Ogiga and Estey, 1974), and hatching in potato root diffusate (PRD) (Fenwick and Widdowson, 1958), used separately or combined, are the assessment methods most often used by researchers to investigate viability. Recently the trehalose assay was developed as an alternative method for distinguishing live and dead PCN eggs (van den Elsen *et al*., 2012) but was not yet amended and investigated on field populations. The comparative effectiveness of all these methods on *G. pallida* field population under identical conditions was also unknown.

Experiments investigating the suitability of the trehalose assay as a viability test for *G. pallida* field populations, highlighted that modifications of the protocol originally proposed by van den Elsen *et al*. (2012) are required for the methodology to be suitable (Chapter 3). Change of absorbance per viable egg (ΔA viable egg^{-1}) was effectively calculated from samples containing between 100 and 5,000 viable eggs (25 cyst), after reduction of the specimen's background noise (heat-killed samples) when the assay was performed in 262µl reaction volume with 100µl extraction volume. Successful completion of the assay by following protocol with modification highlights methodology flexibility, which may be very helpful when working with living organism, and opportunity for further improvement. A significant difference ($P<0.05$) was observed between populations when the absorbance from heat-killed samples was measured. Additionally, assessment of absorbance per viable egg for samples related by the geographical origin did not reveal grouping factor. These results, due to limited number of populations being compared, cannot

be treated as final, although at this stage of the method development calculations should be performed individually for each population.

Highly significant differences were found between viability assessments by hatching in PRD assay, hatching in PRD followed by Meldola's blue staining assay, Meldola's blue staining assay and the trehalose assay when field populations were tested (Chapter 3). Soil samples submitted for PCN testing by growers and/or agronomists are usually expected to be processed in a short time frame. Therefore, if viability test required, hatching bioassays or staining with Meldola's blue may not be suitable. Viability assessment by hatching in PRD is time consuming, and even if extended to 10 weeks, holds the possibility of some J2 remaining unhatched, providing a slightly lower viability estimation (Ebrahimi *et al*., 2015). Additionally, hatching assay does not take into account the viability of the remaining, possibly dormant eggs. On average (populations A-E) 51% of encysted eggs remained unhatched after 8 weeks of exposure to PRD. The density and viability of PCN population described by hatching in PRD assay in inaccurate and should not be performed independently as an indication for the selection of management strategy. However, it can be successfully used to provide accurate information about the hatching dynamics of PCN populations for research purposes. Testing *G. pallida* populations by staining in Meldola's blue dye showed that, on average, 38% heat-killed eggs remained unstained. The overestimation of viable egg in heat treated samples, when examined by hatching in PRD followed by Meldola's blue staining assay, reached 70%.This result was entirely from secondary staining by Meldola's blue. The necessity for visual assessment, even if performed by trained staff, leaves a margin of uncertainty due to personal interpretation of partially stained eggs. This variability, across different laboratories, will not be welcomed by customers. Both the hatching bioassay and the trehalose assay provided convincing estimates from

heat-treated samples, with viability estimated as<1%. In light of this research, the trehalose assay should be recognised as a good alternative to existing less accurate methods of viability assessment. However, it is necessary to test the trehalose assay on a wider range of field populations and further investigate the value of ΔA viable egg^{-1} to consider its development as a commercial test.

One approach to managing PCN infestations is the use of potato varieties with resistance derived from members of the *Solanum* genus. Development of genotypes with the H_1 resistance gene (*S. tuberosum* ssp. *andigena* CPC1673) have proven to be highly effective in controlling *G. rostochiensis* but at the same time increased the risk of selection of *G. pallida* which is more challenging to manage. Distinct pathotypes of *G. pallida*, which vary in their ability to overcome different sources of resistance, are presented in GB. There is some evidence that mixed populations occur together and may become more virulent over successive generations. The European and Mediterranean Plant Protection Organization (EPPO, 2017) concluded that when using Kort *et al*. (1977) scheme many populations cannot conclusively be assigned to pathotypes. It is partly because the complexity of virulence found in PCN field populations is not well defined by methodology used in Kort *et al*. (1977) which includes a limited selection of resistance genes and lack an important genotype currently used in potato breeding programs. The risk of overcoming the resistance, which is often associated with long-term use of resistant varieties, should be minimized and monitored (EPPO, 2017). It was proposed that multiplication ability of populations with new or unusual pathogenicity should be tested on a set of different potato genotypes and described by their resistance score. The effectiveness of the Kort and EPPO methods in the assessment of virulence of GB field populations of *G. pallida* with potato genotypes possessing different types of resistance has not been investigated previously.

Assessment of selected *G. pallida* field populations and control populations of known pathotype showed that genotypes with resistance derived from *S. vernei* ('Innovator') performed better, in controlling reproduction, over genotypes derived from S*. tuberosum* spp. *andigena* CPC2802 ('Vales Everest') (Chapter 4). Regardless of the differences, both genotypes, with resistance derived from sources being used in the UK's breeding programs, were mainly characterised with the top two resistance scores, which was then confirmed by a multiplication ratio below one. Also genotype 'P55/7' showed partial resistance to all of the field populations tested. This indicates that using these genotypes in the field is likely to be highly effective in reducing *G. pallida* populations in the short-term at least. In the future, the durability of the resistance of these genotypes should be monitored. Additionally, breeding programmes should seek to develop broad-spectrum resistance through combining different sources of resistance. In general, the multiplication ratio (Kort *et al*., 1977) of *G. pallida* field and control populations was supported by resistance score (EPPO, 2006), based on four different potato genotypes ('Desiree', 'Innovator', 'P55/7' and 'Vales Everest'). *Globodera pallida* populations used in this study mostly, but not always, showed similar level of virulence on pathotype with the same source of resistance (Chapter 4). Notably, population HAU167 had much lower resistance score, on all three partially resistant genotypes, then other populations, including these also collected in East Midlands. Interestingly, this population was characterised by lowest hatchability (%). These results proved earlier discussed variability between virulence of PCN populations. Frequent testing virulence of the field population will allow quick detection of any changes *e.g*. new introduction to GB or appearance of *G. rostochiensis* population overcoming resistance.

Mitotyping provides an efficient way to assess the complexity of *G. pallida* populations, by describing population introductions, and identifying populations that may be of concern. This novel method (Eves-van den Akker *et al*., 2015) allowed to study the diversity of field populations by investigating occurrence of partial sequence of the mitochondrial gene, cytochrome *b* (cyt-*b*) descriptive of the three main introductions into Europe (Plantard *et al*., 2008). *Globodera pallida* field populations from England tested by next generation sequencing were found to be mitotype 1 (2%), mitotype 3 (59%) or a mixture of mitotypes 1 and 3 (39%). The lack of detection of mitotype 2 in populations originating from England, while all three mitotypes were found in Scotland, may be due to limited distribution or influenced by environmental conditions and/or management practices.

5.3. *Importance and relevance to the industry*

Changing customers preferences by bring attention to the potato varieties with the characteristics *e.g*. texture similar to those favoured in varieties like ‘Maris Piper’, but furnished with high resistance to *G. pallida* pathotypes, will be beneficial. Further research for genotypes with higher or full resistance to pathotypes Pa1 and Pa2/3 is extremely important but, if not selected by customers and by that, through potato market, by the growers, they will not serve desired purpose. Marketing of varieties like ‘Innovator’ or ‘Vales Everest’, which showed promising level of resistance to control *G. pallida* multiplication, should be considered by AHDB as an aim for upcoming years.

The development of dependable, quick, simple and economic viability test has many benefits for crop agronomy where it would aid the selection of appropriate pest management strategies. The trehalose viability assay may provide a more sensitive and reliable method for determining soil population densities of PCN. The

test will enable Plant Clinics to accurately assess populations after management inputs to determine their performance. In addition, the test will help growers make a more accurate assessment of PCN soil densities prior to planting and thus make informed decisions on the application of synthetic nematicides. In addition, it is also needed in research where a robust technique is required as a tool to assess the efficacy of developing control methods such as biofumigation.

Knowledge presented here might aid plant breeding for resistance programs with new resistance sources from wild *Solanum* spp. The frequently observed limited genetic diversity of European populations supports the hypothesis that only a fraction of virulence existing in South America is represented by European pathotypes (Hockland *et al*., 2012). To avoid fields contamination measures *e.g*. phytosanitary regulations, which are already in place, should be supported by more intensive screening test. Reliable pathotyping scheme will serve as a tool for quicker detection of populations with unknown virulence so the spread can be prevented.

The biological significance of mitotypes needs to be fully evaluated, but it may have potential to aid in the selection of appropriate potato cultivars for specific field populations leading to a more strategic pest management.

5.4. *Proposed future research*

Following the observations made during this project, the following areas for future work in the study of ‘Characterisation of potato cyst nematode populations in Great Britain for sustainable crop management’ are recommended.

Understanding the species composition and factors affecting species variability is highly beneficial for the development and preservation of resistant varieties. Future investigations should consider the relationship between the previously grown potato variety and the PCN species present. Due to lack of

information provided by growers, it was not possible to evaluate this relationship in this study. To investigate the changes and adaptation of populations to the management strategies to full extend it is recommend that soil samples for next national PCN survey should be also collected from fields investigated in this study. This approach will provide better information about population's response to specific control methods. If fields reported with mixed population in previous survey could be tested in this study, suggested selection of *G. pallida* over G*. rostochiensis* in mixed populations in field regularly planted with varieties susceptible towards *G. pallida* but resistant towards G*. rostochiensis*, could be directly confirmed. Due to the data protection regulations samples collected for purpose of this research could not be collected from fields tested by Minnis *et al*. (2002).

As mentioned earlier the trehalose assay showed the potential to serve as a commercial test for the viability assessment on the samples originating from field. So far no similarities were found between sample background noises from populations originating from fields with the same characteristics *e.g*. soil texture. Additional field populations should be tested and compared against each other to determine if the signal can be uniformed. As showed here increase in the reaction volume was beneficial to the assay and should be investigated further as a part of process to increasing the sensitivity of the assay. Based on the experiments conducted in this study, further research should be conducted on samples with a known proportion of heated and non-heated eggs from field populations. The results of this research can be then tested against glasshouse populations. Including a glasshouse population of high (near 100%) viability will be beneficial but comparisons must be treated carefully. Critical differences between field and glasshouse populations were earlier reported by Kroese *et al*. (2011). When

development of the trehalose assay to commercial test will be will be completed its cost effectiveness should be investigated.

To prevent biased results, soil samples taken for the survey were collected from fields of unknown infestation status and population density. The wide geographic distribution of survey sites and time limitations did not allow sampling of a large volume of soil in individual fields and consequently the final number of cysts collected was not sufficient to allow testing on a wider range of differential genotypes for pathotype identification. Additionally, glasshouse space limitation and the ability to process samples in the time dedicated for this part of the project prevented further virulence studies on a larger number of PCN survey populations and differential genotypes, which should be considered as future work.

The lack of detection of mitotype 2 in populations originating from England cannot be decisively confirmed due to limited number (n=93) of *G. pallida* populations examined for mitotype composition. Further tests are required on a larger scale to investigate the observations reported in this study.

Chapter 6

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Chapter 7

Appendices.

Appendix 7.1. Letter and questionnaire sent to growers, independent agronomists and agronomy companies as an invitation to contribute to the PCN survey.

Dear Sir or Madam,

I am a PhD student at Harper Adams University sponsored by Potato Council. For my project, I am planning to develop a new PCN species distribution map for England and Wales to update previous work conducted by Harper Adams University between 1998 and 1999. Below you can see the example of the map produced as a result of last survey.

To achieve this challenging task we are seeking help from growers, agronomists and crop specialists for the collection of soil samples. In order to make our work as representative as possible we are looking to obtain soil samples from potato growing land (ware crop) across the country.

For each field site, it is important that I also collect background cropping/agronomic information to help with the overall interpretation of the data. Therefore, I would appreciate your assistance with completing the attached questionnaire.

The University is very grateful for your help and would like to assure you that all data will be kept strictly confidential by following research ethics procedures.

For further information or if you have any questions please do not hesitate to contact me.

Thank you very much.

Yours sincerely,

Kasia Dybal

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Sites where PCN cysts were found between 1998-1999 (Minnis *et al*., 2002).

Appendix 7.1. Letter and questionnaire sent to growers, independent agronomists and agronomy companies as an invitation to contribute to the PCN survey (Continuation).

SURVEY QUESTIONNAIRE FOR PCN DISTRIBUTION MAP DEVELOPMENT

Ref. (for HAU use only):
_____

Name:
_____

Address/Contact details:
_____

County/Postcode:
_____

Sampling date:
_____

Field name:
_____

Soil type/Field size:
_____

Total area of potatoes grown:
_____

Current crop:
_____

Previous crop:
_____

Years since potatoes grown:
_____

History of PCN (Yes/No):
_____

Species presented (if known):
_____

Field grid reference:
_____

Comments:
_____

Thank you.

Appendix 7.2. Agronomic factors and results of potato cyst nematodes detection and species identification for survey samples.

HAU Reference	Geographic origin	Soil texture	Previous crop	Rotation	History of	Soil used for cysts	PCN presence	*G. pallida*	*G. rostochiensis*
1	East of England	Loamy	Cereal	7	no	566	no	no	no
2	East of England	Loamy	Cereal	7	no	566	yes	yes	no
4	East of England	Loamy	Cereal	7	no	554	no	no	no
6	East of England	Loamy	Cereal	7	no	544	no	no	no
7	East of England	Loamy	Cereal	7	no	531	yes	yes	no
8	East of England	Loamy	Cereal	7	no	557	no	no	no
9	East of England	Loamy	Cereal	7	no	544	no	no	no
10	East of England	Loamy	Non cereal		no	550	no	no	no
11	East of England	Loamy				533	yes	yes	no
12	East of England	Loamy				503	yes	yes	no
13	East of England	Loamy				506	yes	yes	no
14	East of England	Loamy				519	no	no	no
15	East of England	Loamy				543	yes	yes	no
16	East of England	Loamy				533	no	no	no
17	East of England	Loamy				534	no	no	no
18	East of England	Loamy				508	yes	yes	no
19	East of England	Loamy				550	yes	yes	no
20	East of England	Loamy				595	yes	yes	yes
21	East of England	Loamy				554	yes	yes	yes
22	East of England	Loamy				501	no	no	no
23	East of England	Loamy				506	yes	yes	yes
25	East of England	Loamy				518	yes	yes	no
26	East of England	Loamy				522	no	no	no
29	East of England	Loamy				521	yes	yes	yes
31	East of England	Loamy				520	yes	yes	no
32	East of England	Loamy				547	yes	yes	no
33	East of England	Loamy				532	yes	yes	no
34	East of England	Loamy				526	no	no	no
35	East of England	Loamy				519	no	no	no
36	East of England	Loamy and clayey				516	yes	yes	no
37	East of England	Loamy and clayey				525	no	no	no
38	East of England	Loamy and clayey				546	yes	yes	no

Appendix 7.2. Agronomic factors and results of potato cyst nematodes detection and species identification for survey samples (Continuation).

39	East of England	Loamy and clayey				520	yes	yes	no
40	East of England	Loamy and clayey				521	yes	yes	no
41	East of England	Loamy and clayey				522	yes	yes	no
42	East of England	Loamy and clayey				524	yes	yes	no
43	East of England	Loamy and clayey				517	yes	yes	no
44	East of England	Loamy and clayey				532	yes	yes	no
45	East of England	Loamy	Cereal	6	no	502	no	no	no
47	East of England	Loamy				543	yes	yes	no
48	East of England	Loamy				507	yes	yes	no
49	East of England	Loamy				530	yes	yes	no
50	East of England	Loamy				524	yes	yes	no
51	East of England	Loamy				519	yes	yes	no
52	East of England	Loamy				538	no	no	no
53	East of England	Loamy				528	no	no	no
54	East of England	Loamy				526	no	no	no
55	East of England	Loamy				518	yes	yes	no
56	East of England	Loamy				521	yes	yes	no
57	East of England	Loamy				520	yes	yes	no
58	East of England	Loamy				508	yes	yes	no
59	East of England	Loamy				540	no	no	no
60	East of England	Loamy				524	no	no	no
61	East of England	Loamy				556	no	no	no
62	East of England	Loamy				545	yes	yes	no
63	East of England	Loamy				520	yes	yes	no
64	East of England	Loamy				538	no	no	no
65	East of England	Peaty	Cereal	7	yes	523	yes	yes	no
66	East of England	Peaty	Cereal	7	yes	535	yes	yes	no
67	East of England	Peaty	Non cereal		no	521	yes	yes	no
68	East of England	Peaty	Cereal	6	unknown	512	yes	yes	no
69	East of England	Peaty	Cereal	6	no	493	yes	yes	no
70	East Midlands	Loamy and clayey				531	yes	yes	no
71	East of England	Loamy				527	no	no	no
72	East of England	Loamy				514	yes	yes	no
73	East of England	Loamy				519	no	no	no

Appendix 7.2. Agronomic factors and results of potato cyst nematodes detection and species identification for survey samples (Continuation).

74	East of England	Loamy				535	no	no	no
75	East of England	Loamy				521	no	no	no
77	East of England	Chalky				512	yes	yes	no
78	East of England	Chalky				523	no	no	no
80	East of England	Chalky				513	yes	yes	no
82	East of England	Chalky				518	yes	yes	no
83	East of England	Chalky				517	yes	yes	no
84	East of England	Chalky				517	no	no	no
85	East Midlands	Loamy and clayey	Cereal	6	yes	543	yes	yes	no
86	East Midlands	Loamy and clayey	Non cereal	5	yes	535	yes	yes	no
87	East Midlands	Loamy and clayey	Cereal	13	no	519	yes	yes	no
88	East Midlands	Loamy and clayey	Non cereal	13	no	525	no	no	no
89	East Midlands	Loamy and clayey	Non cereal	13	no	515	no	no	no
90	East Midlands	Loamy and clayey	Non cereal	13	no	544	no	no	no
91	East Midlands	Loamy and clayey	Non cereal	7	yes	536	no	no	no
92	East Midlands	Loamy and clayey	Non cereal	6	yes	525	no	no	no
93	East Midlands	Loamy and clayey	Non cereal	8	yes	514	yes	yes	yes
94	East of England	Loamy and clayey				513	no	no	no
95	East of England	Loamy and clayey				703	no	no	no
96	East of England	Loamy and clayey				548	no	no	no
97	East of England	Loamy and clayey				527	no	no	no
98	East of England	Loamy and clayey				546	no	no	no
99	East of England	Loamy and clayey				553	no	no	no
100	East of England	Loamy and clayey				510	no	no	no
101	East of England	Loamy				566	no	no	no
102	East of England	Loamy				526	no	no	no
103	East of England	Loamy				515	no	no	no
104	East of England	Loamy	Cereal	8	no	516	no	no	no
105	East of England	Loamy	Non cereal	7	no	504	no	no	no
106	East of England	Loamy	Non cereal	6	yes	511	no	no	no
108	East of England	Loamy	Cereal	8	yes	502	no	no	no
109	East of England	Loamy	Non cereal	7	yes	524	no	no	no
110	East Midlands	Loamy and clayey				534	yes	yes	no

Appendix 7.2. Agronomic factors and results of potato cyst nematodes detection and species identification for survey samples (Continuation).

111	East Midlands	Loamy and clayey				538	yes	yes	no
112	East Midlands	Loamy and clayey				521	yes	yes	no
113	East Midlands	Loamy and clayey				530	yes	yes	no
114	East Midlands	Loamy and clayey				534	yes	yes	no
115	East Midlands	Loamy and clayey				549	yes	yes	no
116	East Midlands	Loamy and clayey				569	yes	yes	no
117	East of England	Sandy				536	no	no	no
118	East of England	Sandy				561	no	no	no
119	East of England	Sandy				510	no	no	no
120	East of England	Sandy				515	no	no	no
121	East of England	Sandy				510	yes	yes	no
122	East of England	Sandy				562	no	no	no
123	East of England	Sandy				523	no	no	no
124	East of England	Sandy				561	no	no	no
125	East of England	Sandy				524	no	no	no
126	East of England	Loamy				548	yes	yes	no
127	East of England	Loamy				579	no	no	no
129	East of England	Loamy				551	no	no	no
130	East of England	Loamy				552	no	no	no
131	East of England	Loamy				508	yes	yes	yes
132	East of England	Loamy				543	no	no	no
133	East of England	Loamy				513	yes	yes	no
135	East of England	Loamy				540	yes	yes	no
137	East of England	Loamy				539	no	no	no
138	East of England	Loamy				625	no	no	no
139	East of England	Loamy				556	yes	yes	yes
140	East Midlands	Loamy and clayey				763	yes	yes	no
142	East of England	Sandy	Cereal	5	yes	509	yes	no	yes
143	East of England	Loamy and clayey	Potato	6	yes	528	no	no	no
144	East of England	Sandy	Cereal	5	yes	550	yes	no	yes
145	East of England	Sandy	Non cereal	5	yes	555	yes	no	yes
146	East of England	Loamy and clayey	Non cereal	5	yes	513	yes	no	yes
147	East of England	Loamy	Non cereal	5	yes	531	no	no	no
148	East of England	Sandy	Non cereal	5	yes	550	no	no	no

Appendix 7.2. Agronomic factors and results of potato cyst nematodes detection and species identification for survey samples (Continuation).

149	East of England	Loamy	Non cereal	5	yes	548	no	no	no
150	East of England	Sandy	Non cereal	5	yes	506	yes	no	yes
152	East of England	Sandy	Non cereal	6	yes	N/A - GrTo	yes	yes	no
153	East Midlands	Loamy and clayey				521	yes	yes	no
154	East Midlands	Loamy and clayey				528	yes	yes	no
155	East Midlands	Loamy and clayey				532	yes	yes	no
156	East of England	Clayey				521	yes	yes	no
157	East Midlands	Loamy				530	yes	yes	no
158	East Midlands	Loamy and clayey				550	yes	yes	no
159	East Midlands	Peaty				539	yes	yes	no
160	East Midlands	Peaty				536	yes	yes	no
161	East of England	Clayey				516	yes	yes	no
162	East Midlands	Loamy and clayey				546	yes	yes	no
163	East Midlands	Loamy and clayey				548	yes	yes	no
164	West Midlands	Loamy and clayey	Cereal		yes	N/A -ToRi	yes	yes	no
165	West Midlands	Sandy	Non cereal		yes	N/A - MiBu	yes	yes	no
166	West Midlands	Loamy and clayey	Cereal	6	yes	N/A - RoBe	yes	yes	no
167	West Midlands	Sandy	Potato	4.00	yes	N/A - MaDa	yes	yes	no
168	North West	Sandy and loamy	Non cereal		yes	545	yes	yes	no
169	North West	Loamy and clayey	Potato			624	yes	yes	no
170	North West	Loamy and clayey	Potato			550	yes	yes	no
171	North West	Loamy and clayey	Potato			546	yes	yes	no
172	North West	Loamy and clayey	Potato			541	no	no	no
173	North West	Loamy and clayey	Potato			550	no	no	no
174	North West	Sandy	Cereal		yes	533	yes	yes	no
175	North West	Sandy	Cereal		yes	523	yes	yes	no
176	North West	Loamy and clayey	Non cereal		no	534	yes	yes	no
178	North West	Sandy	Cereal		yes	520	yes	yes	no
179	North West	Loamy and clayey	Cereal		no	547	yes	yes	no
180	North West	Sandy and loamy	Cereal	6	yes	537	yes	yes	no
182	Wales	Loamy	Potato	4		660	no	no	no
183	Wales	Loamy	Potato	4		679	yes	yes	no
184	Wales	Loamy	Potato	5		510	no	no	no
185	Wales	Loamy		5		507	no	no	no

Appendix 7.2. Agronomic factors and results of potato cyst nematodes detection and species identification for survey samples (Continuation).

186	Wales	Loamy		5		533	no	no	no
187	East of England	Loamy	Potato		no	534	no	no	no
188	East of England	Loamy	Potato		yes	512	yes	yes	no
189	East of England	Loamy	Cereal		no	513	yes	yes	yes
190	East of England	Loamy	Cereal	8	no	521	no	no	no
191	East of England	Loamy and clayey	Potato		yes	458	yes	yes	no
192	East of England	Loamy and clayey	Potato		yes	509	no	no	no
193	East of England	Loamy	Potato		yes	515	no	no	no
194	East of England	Loamy	Potato		no	518	no	no	no
195	East of England	Loamy	Potato		no	484	yes	yes	no
196	East of England	Loamy	Potato		no	526	no	no	no
197	South East	Loamy				521	no	no	no
198	South East	Loamy and clayey	Cereal	6	unknown	555	no	no	no
199	South East	Loamy and clayey	Non cereal	6	yes	473	yes	yes	no
200	South East	Loamy and clayey	Cereal	6	yes	482	no	no	no
201	South East	Loamy	Cereal	5	yes	513	yes	yes	no
203	South East	Loamy	Cereal		yes	520	yes	yes	yes
204	South East	Loamy	Cereal		yes	490	yes	no	yes
206	South East	Loamy	Potato		no	520	no	no	no
207	South West	Loamy	Potato	4	no	530	no	no	no
209	South West	Loamy	Cereal		unknown	696	no	no	no
210	South West	Loamy			unknown	514	no	no	no
211	South West	Loamy			unknown	479	yes	yes	no
212	South West	Loamy	Potato		no	479	no	no	no
213	South West	Loamy	Potato		no	552	no	no	no
214	South West	Loamy	Potato		no	529	no	no	no
215	South West	Loamy	Potato	10	no	525	no	no	no
216	South West	Loamy	Potato	10	no	544	no	no	no
217	South West	Loamy	Potato	6	no	505	no	no	no
218	South West	Loamy	Potato		no	529	yes	yes	no
219	South West	Loamy	Potato		no	525	no	no	no
220	South West	Loamy	Potato		yes	503	yes	yes	no
221	South West	Loamy	Non cereal	1	unknown	538	yes	yes	no
222	South West	Loamy	Potato		unknown	507	no	no	no

Appendix 7.2. Agronomic factors and results of potato cyst nematodes detection and species identification for survey samples (Continuation).

223	South West	Loamy	Non cereal		no	616	no	no	no
224	South West	Loamy	Non cereal	5	no	626	no	no	no
225	South West	Loamy		5	no	512	no	no	no
226	South West	Loamy	Cereal		no	512	no	no	no
228	South West	Loamy				521	no	no	no
229	West Midlands	Loamy	Potato	5	no	522	no	no	no
230	West Midlands	Loamy	Potato	5	no	519	no	no	no
231	West Midlands	Loamy	Cereal	6	unknown	533	yes	yes	no
232	West Midlands	Loamy	Cereal	6	unknown	529	no	no	no
233	West Midlands	Loamy	Cereal	4	no	512	no	no	no
234	West Midlands	Loamy	Cereal	4	no	527	yes	yes	no
235	West Midlands	Loamy	Potato		no	519	no	no	no
236	West Midlands	Loamy	Potato		no	512	no	no	no
237	West Midlands	Loamy				509	no	no	no
238	West Midlands	Loamy				488	no	no	no
239	West Midlands	Loamy				515	yes	yes	no
240	West Midlands	Loamy				519	no	no	no
241	West Midlands	Loamy	Potato	5	no	509	no	no	no
242	West Midlands	Loamy	Potato	5	no	513	no	no	no
243	West Midlands	Loamy	Potato		unknown	523	no	no	no
244	West Midlands	Loamy	Potato	5	no	516	no	no	no
245	West Midlands	Loamy	Potato	5	no	512	no	no	no
246	West Midlands	Loamy				509	no	no	no
247	West Midlands	Loamy				525	yes	yes	no
248	West Midlands	Loamy				516	no	no	no
249	West Midlands	Clayey	Cereal			516	yes	no	yes
250	West Midlands	Loamy	Non cereal			524	yes	yes	yes
251	West Midlands	Clayey	Non cereal			523	no	no	no
252	East Midlands	Loamy	Potato	3	yes	513	no	no	no
253	West Midlands	Loamy	Cereal			514	no	no	no
254	West Midlands	Loamy	Potato			529	no	no	no
255	West Midlands	Loamy	Cereal			510	no	no	no
256	West Midlands	Loamy			yes	516	yes	yes	no
257	West Midlands	Loamy	Cereal		yes	543	yes	yes	no

Appendix 7.2. Agronomic factors and results of potato cyst nematodes detection and species identification for survey samples (Continuation).

258	West Midlands	Loamy	Cereal		yes	559	no	no	no
259	West Midlands	Loamy	Cereal		yes	543	no	no	no
260	West Midlands	Sandy	Cereal		yes	531	yes	yes	no
262	West Midlands	Loamy	Cereal		yes	527	yes	yes	no
263	West Midlands	Loamy	Cereal		yes	529	yes	yes	no
264	West Midlands	Loamy	Cereal		yes	534	yes	yes	no
266	West Midlands	Loamy	Cereal		yes	533	yes	yes	no
268	West Midlands	Loamy and clayey	Cereal		yes	547	yes	yes	no
269	West Midlands	Loamy and clayey	Cereal		yes	536	yes	yes	no
270	West Midlands	Loamy and clayey	Cereal		yes	513	no	no	no
271	Yorkshire and the Humber	Loamy	Cereal		no	540	no	no	no
272	Yorkshire and the Humber	Loamy and clayey	Cereal		yes	519	no	no	no
273	Yorkshire and the Humber	Chalky	Potato		yes	528	no	no	no
274	Yorkshire and the Humber	Loamy	Cereal		yes	508	yes	yes	no
275	Yorkshire and the Humber	Loamy	Potato		yes	530	yes	yes	no
276	Yorkshire and the Humber	Chalky	Cereal	5	yes	516	yes	yes	no
277	Yorkshire and the Humber	Loamy	Potato		no	532	yes	yes	no
278	Yorkshire and the Humber	Loamy	Potato		yes	512	yes	yes	no
279	Yorkshire and the Humber	Loamy	Cereal	5	yes	616	no	no	no
280	Yorkshire and the Humber	Loamy	Potato		no	527	yes	yes	no
281	Yorkshire and the Humber	Loamy	Cereal		no	528	no	no	no
282	Yorkshire and the Humber	Chalky	Cereal		no	524	no	no	no
283	Yorkshire and the Humber	Chalky	Cereal		no	527	no	no	no
284	Yorkshire and the Humber	Chalky	Cereal		no	530	no	no	no
285	Yorkshire and the Humber	Chalky	Cereal		no	523	no	no	no
286	Yorkshire and the Humber	Chalky	Cereal		no	536	no	no	no
287	Yorkshire and the Humber	Loamy and clayey	Potato		yes	530	yes	yes	no
288	Yorkshire and the Humber	Loamy and clayey	Potato		yes	519	yes	yes	no
289	Yorkshire and the Humber	Loamy and clayey	Potato		yes	527	yes	yes	no
290	Yorkshire and the Humber	Loamy and clayey	Potato		yes	528	yes	yes	no
291	Yorkshire and the Humber	Loamy	Cereal	10	no	529	no	no	no
292	Yorkshire and the Humber	Loamy	Potato	11	no	511	no	no	no
293	Yorkshire and the Humber	Loamy	Cereal	10	no	519	no	no	no
294	Yorkshire and the Humber	Loamy	Cereal		no	519	no	no	no

Appendix 7.2. Agronomic factors and results of potato cyst nematodes detection and species identification for survey samples (Continuation).

295	Yorkshire and the Humber	Loamy	Cereal		no	517	no	no	no
296	Yorkshire and the Humber	Loamy	Potato		yes	518	yes	yes	no
297	Yorkshire and the Humber	Loamy	Cereal		yes	522	yes	yes	no
298	Yorkshire and the Humber	Loamy	Cereal		yes	521	yes	yes	no
299	Yorkshire and the Humber	Loamy	Potato		yes	521	yes	yes	no
300	Yorkshire and the Humber	Loamy	Cereal		yes	518	yes	yes	no
301	Yorkshire and the Humber	Loamy	Potato		yes	530	yes	yes	no
302	Yorkshire and the Humber	Chalky	Potato		yes	528	no	no	no
303	Yorkshire and the Humber	Loamy	Potato		no	508	no	no	no
304	Yorkshire and the Humber	Loamy	Potato		no	513	no	no	no
305	Yorkshire and the Humber	Loamy	Potato		no	509	no	no	no
306	Yorkshire and the Humber	Peaty	Cereal	11	no	513	no	no	no
307	Yorkshire and the Humber	Chalky	Potato		no	511	no	no	no
308	Yorkshire and the Humber	Chalky	Potato		no	510	no	no	no
309	Yorkshire and the Humber	Sandy and loamy				513	yes	yes	no
310	Yorkshire and the Humber	Sandy and loamy				509	yes	yes	no
311	Yorkshire and the Humber	Sandy				530	yes	yes	no
312	Yorkshire and the Humber	Sandy				514	yes	yes	no
313	Yorkshire and the Humber	Loamy				518	no	no	no
314	West Midlands	Sandy				517	yes	yes	no
315	West Midlands	Sandy				524	yes	yes	no
316	West Midlands	Sandy				507	yes	yes	no
317	East of England	Loamy	Potato		yes	509	yes	yes	no
318	East of England	Sandy	Potato		no	504	no	no	no
319	East of England	Sandy	Potato		yes	546	no	no	no
320	East of England	Loamy	Potato		yes	517	no	no	no
321	East of England	Sandy	Potato		yes	505	no	no	no
322	East of England	Loamy				520	no	no	no
323	East of England	Sandy				506	no	no	no
324	East of England	Loamy				523	yes	yes	no
325	East of England	Sandy				502	yes	no	yes
326	East of England	Loamy			yes	512	yes	yes	no
327	East of England	Loamy			yes	510	yes	yes	yes
328	East of England	Peaty			yes	498	yes	yes	no

Appendix 7.2. Agronomic factors and results of potato cyst nematodes detection and species identification for survey samples (Continuation).

329	East of England	Peaty				515	yes	yes	no
330	East of England	Peaty				526	yes	yes	no
331	East of England	Peaty				513	yes	yes	no
332	West Midlands	Loamy and clayey			yes	540	yes	yes	no
333	West Midlands	Loamy			yes	532	yes	yes	no
334	West Midlands	Loamy			yes	534	yes	yes	no
335	West Midlands	Loamy			yes	530	yes	yes	no
337	West Midlands	Loamy and clayey			no	519	no	no	no
338	West Midlands	Loamy			no	521	no	no	no
339	West Midlands	Loamy			no	521	no	no	no
340	West Midlands	Loamy			no	508	no	no	no
341	East of England	Peaty	Potato		yes	511	no	no	no
342	East of England	Peaty				512	yes	yes	no
343	East of England	Peaty				524	yes	yes	no
344	East of England	Peaty				520	no	no	no
345	East of England	Peaty	Potato			518	yes	yes	yes
346	East of England	Loamy	Potato		no	517	yes	yes	no
347	East of England	Peaty	Potato		no	504	no	no	no
348	East of England	Peaty	Potato		yes	503	no	no	no
349	East of England	Peaty	Potato		yes	522	yes	yes	no
350	East of England	Peaty	Potato		no	549	yes	yes	no
351	East Midlands	Sandy	Potato		yes	537	yes	yes	no
352	East Midlands	Sandy	Potato		yes	529	yes	yes	no
353	East Midlands	Sandy	Potato		yes	506	yes	yes	no
354	East Midlands	Sandy	Potato		yes	533	yes	yes	no
355	East Midlands	Sandy	Potato		yes	525	yes	yes	yes
356	North West	Peaty	Potato	8	yes	520	yes	yes	no
357	North West	Sandy and loamy	Potato	3	yes	503	yes	yes	no
358	North West	Sandy and loamy	Potato	5	yes	521	yes	yes	no
359	North West	Sandy and loamy	Potato	2	yes	515	yes	yes	no
360	North West	Sandy and loamy	Potato	2	yes	515	yes	yes	no
361	North West	Sandy and loamy	Potato	6	yes	509	yes	yes	no
362	South East	Loamy and clayey	Potato		yes	501	yes	yes	no
364	South East	Loamy	Cereal	3	no	507	yes	yes	no

Appendix 7.2. Agronomic factors and results of potato cyst nematodes detection and species identification for survey samples (Continuation).

365	South East	Loamy	Potato	10	no	504	no	no	no
366	South East	Loamy	Cereal			525	yes	yes	no
367	South East	Loamy	Cereal			521	no	no	no
368	South West	Loamy	Potato		no	493	no	no	no
369	South West	Loamy	Potato		no	504	no	no	no
370	South West	Loamy	Potato		no	492	no	no	no
371	South West	Loamy	Potato		no	498	no	no	no
372	South West	Loamy	Potato		no	499	no	no	no
373	South West	Loamy				527	no	no	no
374	East of England	Loamy and clayey	Potato		no	500	no	no	no
375	East of England	Loamy and clayey	Potato		yes	492	yes	yes	no
376	East of England	Loamy and clayey	Potato		no	543	no	no	no
377	East of England	Peaty	Potato		no	562	yes	yes	no
378	East of England	Loamy and clayey	Potato		no	529	no	no	no
379	East Midlands	Sandy and loamy	Potato	1	yes	539	no	no	no
380	East Midlands	Loamy and clayey	Cereal		no	512	yes	yes	no
381	East Midlands	Loamy and clayey	Cereal		no	540	yes	yes	no
382	East Midlands	Loamy and clayey	Potato		no	534	no	no	no
383	East Midlands	Loamy and clayey	Potato		no	582	yes	yes	no
384	East Midlands	Loamy and clayey	Potato		no	568	yes	yes	no
385	East Midlands					513	yes	yes	no
386	East Midlands					431	yes	yes	no
387	East Midlands					591	yes	yes	no
388	East Midlands					517	no	no	no
389	East Midlands					576	yes	yes	no
390	East Midlands					521	yes	yes	no
391	East Midlands					261	yes	yes	no
392	East Midlands					695	yes	yes	no
393	East Midlands					663	yes	yes	no
394	East Midlands					533	yes	yes	no
395	East Midlands					605	yes	yes	yes
396	East Midlands					618	yes	yes	no
397	East Midlands					538	yes	yes	no
398	East Midlands					330	no	no	no

Appendix 7.2. Agronomic factors and results of potato cyst nematodes detection and species identification for survey samples (Continuation).

399	East Midlands					352	yes	yes	no
400	East Midlands					295	yes	yes	no
401	East Midlands					307	no	no	no
402	East Midlands					409	yes	yes	no
403	East Midlands					356	yes	yes	no
404	East Midlands					332	no	no	no
405	East Midlands					398	no	no	no
406	East Midlands					441	no	no	no
407	East Midlands					500	no	no	no
408	East Midlands					623	yes	yes	no
409	East Midlands					524	yes	yes	no
410	East Midlands					542	yes	yes	no
411	East Midlands					524	yes	yes	no
412	Yorkshire and the Humber					552	no	no	no
413	Yorkshire and the Humber					792	no	no	no
414	Yorkshire and the Humber					503	yes	yes	no
415	Yorkshire and the Humber					631	yes	yes	no
416	East of England					549	no	no	no
417	East of England					626	no	no	no
418	East of England					663	yes	yes	yes
419	East of England					532	yes	no	yes
421	West Midlands					849	no	no	no
422	West Midlands					437	no	no	no
424	West Midlands					403	no	no	no
425	West Midlands	Sandy				530	yes	yes	no
426	West Midlands	Loamy				570	no	no	no
427	West Midlands	Loamy				555	no	no	no
428	West Midlands	Loamy				550	no	no	no
429	West Midlands	Loamy				538	no	no	no
430	West Midlands	Sandy			yes	575	yes	yes	no
431	West Midlands	Sandy			yes	561	yes	yes	no
432	West Midlands	Sandy			yes	548	yes	yes	no
433	West Midlands	Sandy			yes	540	yes	yes	no
434	West Midlands	Sandy				553	no	no	no

Appendix 7.2. Agronomic factors and results of potato cyst nematodes detection and species identification for survey samples (Continuation).

435	West Midlands	Sandy				560	yes	yes	no
436	West Midlands	Sandy				554	yes	no	yes
437	West Midlands	Sandy				561	yes	yes	no
438	West Midlands	Sandy				552	yes	yes	no
439	West Midlands	Sandy	Potato			530	yes	yes	no
440	West Midlands	Sandy	Potato			546	yes	yes	no
441	West Midlands	Loamy	Cereal		yes	572	yes	yes	no
442	West Midlands	Loamy	Cereal		yes	593	yes	yes	no
444	South West	Loamy				562	no	no	no
447	South West	Loamy				550	no	no	no
449	South West	Loamy				536	no	no	no
450	South West	Loamy				547	no	no	no
451	East Midlands					533	no	no	no
452	East Midlands					552	no	no	no
453	East Midlands					559	no	no	no
454	West Midlands	Loamy				576	no	no	no
455	West Midlands	Loamy				516	no	no	no
457	East Midlands	Sandy and loamy				510	yes	yes	no
458	East Midlands	Sandy and loamy				510	no	no	no
459	East Midlands	Sandy and loamy				518	no	no	no
461	Wales	Loamy			unknown	520	no	no	no
462	Wales	Loamy and clayey	Non cereal		unknown	505	no	no	no
463	East Midlands	Loamy	Potato		no	518	no	no	no
464	North East	Loamy			yes	522	yes	yes	no
465	North East	Loamy			yes	511	yes	yes	no
466	Wales	Loamy and clayey				510	yes	yes	no
467	South East	Loamy				N/A	no	no	no
468	South East	Loamy				N/A	no	no	no
469	South East	Loamy				N/A	no	no	no
470	South East	Chalky				N/A	no	no	no
471	East of England	Loamy				N/A	yes	no	yes
472	East of England	Loamy				N/A	no	no	no
473	East Midlands	Loamy and clayey				N/A	no	no	no
474	North East	Loamy and clayey				N/A	no	no	no

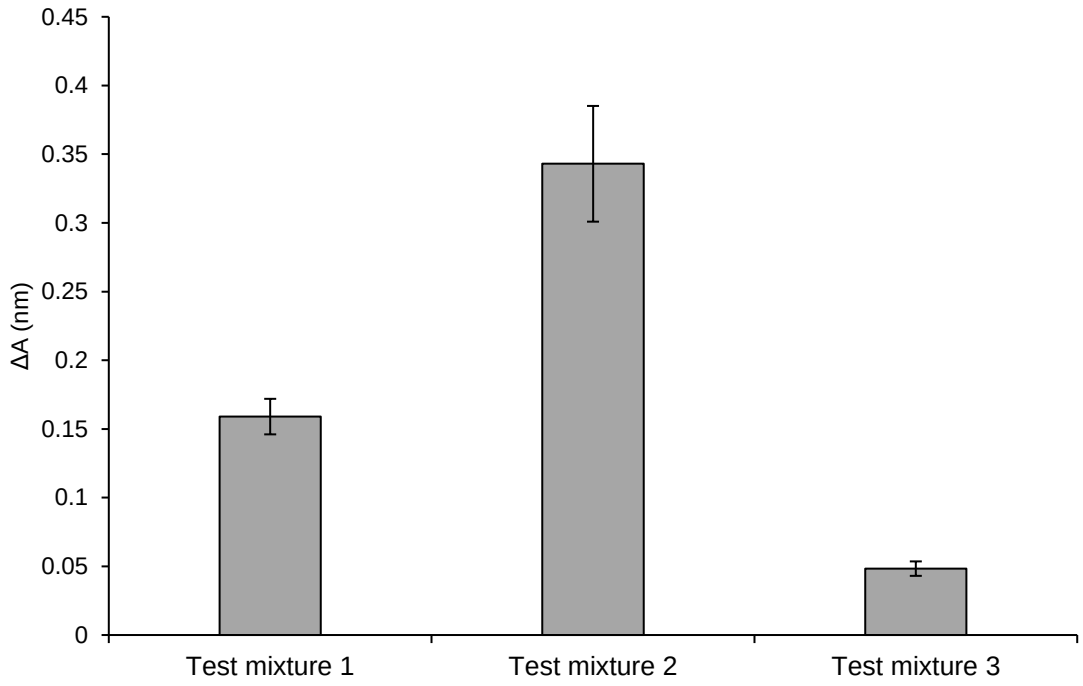
Appendix 7.2. Agronomic factors and results of potato cyst nematodes detection and species identification for survey samples (Continuation).

475	North East	Loamy and clayey				N/A	no	no	no
476	North East	Loamy and clayey				N/A	no	no	no
477	North West	Peaty				N/A	no	no	no
478	East of England	Loamy				N/A	yes	yes	no
479	East of England	Loamy				N/A	no	no	no
480	East of England	Loamy				N/A	no	no	no
481	East of England	Loamy				N/A	no	no	no
482	North West	Loamy and clayey				N/A	yes	yes	no
483	North West	Loamy and clayey				N/A	no	no	no
484	North West	Loamy and clayey				N/A	no	no	no
485	North West	Peaty				N/A	yes	yes	no
486	North West	Peaty				N/A	yes	yes	no
487	North West	Sandy and loamy				N/A	yes	yes	no
488	North West	Loamy and clayey				N/A	yes	yes	no
489	North West	Loamy and clayey				N/A	no	no	no
490	North West	Loamy and clayey				N/A	no	no	no
491	North West	Sandy and loamy				N/A	no	no	no
492	North West	Loamy and clayey				N/A	no	no	no
493	West Midlands	Loamy				N/A	no	no	no
494	West Midlands	Loamy				N/A	no	no	no
495	West Midlands	Loamy				N/A	no	no	no
496	West Midlands	Loamy				N/A	no	no	no
497	West Midlands	Loamy				N/A	no	no	no
498	East Midlands	Loamy and clayey				N/A	yes	yes	no
499	East Midlands	Chalky				N/A	no	no	no
500	East Midlands	Loamy and clayey				N/A	yes	yes	no
501	East Midlands	Sandy and loamy				N/A	yes	yes	no
502	East Midlands	Chalky				N/A	yes	no	yes
503	East Midlands	Loamy				N/A	no	no	no
504	East Midlands	Loamy and clayey				N/A	no	no	no
505	East Midlands	Loamy				N/A	no	no	no
506	East Midlands	Loamy				N/A	no	no	no
507	East Midlands	Chalky				N/A	no	no	no
508	East Midlands	Loamy				N/A	no	no	no

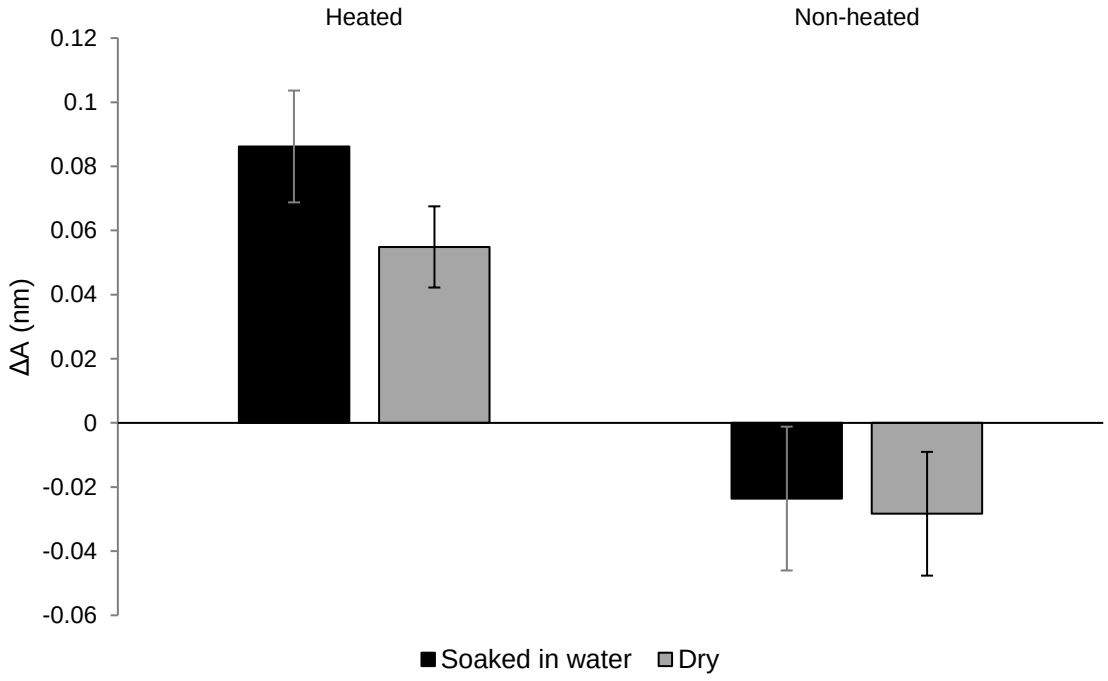
Appendix 7.2. Agronomic factors and results of potato cyst nematodes detection and species identification for survey samples (Continuation).

509	East Midlands	Loamy				N/A	no	no	no
510	East Midlands	Peaty				N/A	no	no	no
511	East Midlands	Loamy				N/A	no	no	no
512	East Midlands	Loamy				N/A	no	no	no
513	East Midlands	Peaty				N/A	no	no	no
514	East of England	Loamy				N/A	no	no	no
515	East of England	Clayey				N/A	no	no	no
516	East of England	Peaty				N/A	yes	yes	no
517	East of England	Loamy				N/A	no	no	no
518	East of England	Loamy				N/A	no	no	no
519	Yorkshire and the Humber	Loamy and clayey				N/A	yes	yes	no
520	Yorkshire and the Humber	Loamy and clayey				N/A	yes	yes	no
521	Yorkshire and the Humber	Loamy				N/A	no	no	no
522	Yorkshire and the Humber	Loamy				N/A	no	no	no
523	Yorkshire and the Humber	Loamy and clayey				N/A	no	no	no
524	Yorkshire and the Humber	Loamy				N/A	no	no	no
525	Yorkshire and the Humber	Chalky				N/A	no	no	no
526	Yorkshire and the Humber	Loamy				N/A	yes	yes	no
527	Yorkshire and the Humber	Loamy and clayey				N/A	no	no	no
528	Yorkshire and the Humber	Loamy				N/A	no	no	no
529	Yorkshire and the Humber	Chalky				N/A	no	no	no
530	Yorkshire and the Humber	Chalky				N/A	no	no	no
531	Yorkshire and the Humber	Chalky				N/A	no	no	no
532	Yorkshire and the Humber	Loamy				N/A	no	no	no
533	Yorkshire and the Humber	Loamy				N/A	no	no	no
534	Yorkshire and the Humber	Loamy and clayey				N/A	no	no	no
535	Yorkshire and the Humber	Loamy				N/A	no	no	no

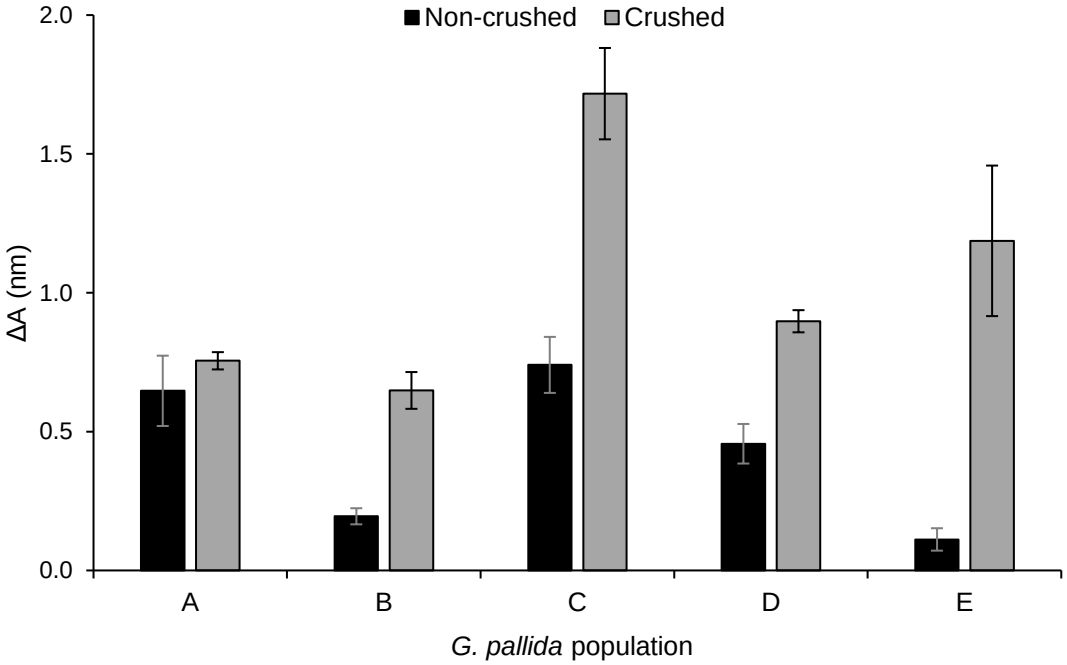
Appendix 7.3. The change of absorbance (ΔA) in samples with different reaction mixtures (Table 3.2.) tested to determine the optimal proportion of reagents required for the trehalose assay to assess the viability of *G. pallida* field populations.



Appendix 7.4. The change of absorbance (ΔA) in samples with cysts heated (30min, 99°C) or unheated and left dry or soaked in water 24 hours prior to the experiment tested to determine the optimal conditions required for the trehalose assay to assess the viability of *G. pallida* field populations.



Appendix 7.5. The change of absorbance (ΔA) in samples with cysts crushed and non-crushed prior to the experiment tested to determine the optimal conditions required for the trehalose assay to assess the viability of *G. pallida* field populations.



Appendix 7.6. Experimental design of glasshouse assay for virulence assessment of *G. pallida* field populations from England and Wales using different potato genotypes. Each *G. pallida* population/genotype combination was replicated four times and randomised.

Colour code:

PCN population/genotype	Ref. nr.
152 Desiree	1
152 Innovator	2
152 P55/7	3
152 Vales Everest	4
164 Desiree	5
164 Innovator	6
164 P55/7	7
164 Vales Everest	8
165 Desiree	9
165 Innovator	10
165 P55/7	11
165 Vales Everest	12
166 Desiree	13
166 Innovator	14
166 P55/7	15
166 Vales Everest	16
167 Desiree	17
167 Innovator	18
167 P55/7	19
167 Vales Everest	20
178 Desiree	21
178 Innovator	22
178 P55/7	23
178 Vales Everest	24
298 Desiree	25
298 Innovator	26
298 P55/7	27
298 Vales Everest	28
351 Desiree	29
351 Innovator	30
351 P55/7	31
351 Vales Everest	32
356 Desiree	33
356 Innovator	34
356 P55/7	35
356 Vales Everest	36
Luffness Desiree	37
Luffness Innovator	38
Luffness P55/7	39
Luffness Vales Everest	40
Pa1 Desiree	41
Pa1 Innovator	42
Pa1 P55/7	43
Pa1 Vales Everest	44

PCN population/genotype	Ref. nr.
Lindley Desiree	45
Lindley Innovator	46
Lindley P55/7	47
Lindley Vales Everest	48
blank	49
blank	50

Randomised design:

block 1 - Ref. nr.

8	39	2	13	38
43	31	19	7	15
42	5	11	47	3
17	44	27	45	48
29	30	36	46	32
10	12	26	4	24
6	25	16	23	1
20	34	22	33	14
49	18	40	28	35
9	21	50	41	37

block 2 - Ref. nr.

13	35	34	26	41
24	14	49	37	3
5	6	2	7	42
8	50	33	43	38
23	36	19	28	10
31	27	22	46	1
48	15	39	9	47
21	40	17	45	32
29	25	20	11	30
12	16	4	18	44

block 3 - Ref. nr.

32	48	29	50	1
21	12	47	13	22
7	8	37	3	6
20	16	41	45	19
43	28	9	44	2
11	30	38	46	25
10	27	36	49	33
31	35	40	18	5
26	17	42	34	4
39	15	23	14	24

block 4 - Ref. nr.

49	43	13	2	14
40	5	46	17	8
9	3	42	41	26
45	34	29	48	16
7	50	37	38	35
24	18	39	32	10
23	19	28	6	1
30	21	20	33	31
22	15	4	44	25
11	12	36	27	47

Appendix 7.6. Experimental design of glasshouse assay for virulence assessment of *G. pallida* field populations from England and Wales using different potato genotypes. Each *G. pallida* population/genotype combination was replicated four times and randomised (Continuation).

block 1

164 Vales Everest	Luffness P55/7	152 Innovator	166 Desiree	Luffness Innovator
Pa1 P55/7	351 P55/7	167 P55/7	164 P55/7	166 P55/7
Pa1 Innovator	164 Desiree	165 P55/7	Lindley P55/7	152 P55/7
167 Desiree	Pa1 Vales Everest	298 P55/7	Lindley Desiree	Lindley Vales Everest
351 Desiree	351 Innovator	356 Vales Everest	Lindley Innovator	351 Vales Everest
165 Innovator	165 Vales Everest	298 Innovator	152 Vales Everest	178 Vales Everest
164 Innovator	298 Desiree	166 Vales Everest	178 P55/7	152 Desiree
167 Vales Everest	356 Innovator	178 Innovator	356 Desiree	166 Innovator
blank	167 Innovator	Luffness Vales Everest	298 Vales Everest	356 P55/7
165 Desiree	178 Desiree	blank	Pa1 Desiree	Luffness Desiree

block 2

166 Desiree	356 P55/7	356 Innovator	298 Innovator	Pa1 Desiree
178 Vales Everest	166 Innovator	blank	Luffness Desiree	152 P55/7
164 Desiree	164 Innovator	152 Innovator	164 P55/7	Pa1 Innovator
164 Vales Everest	blank	356 Desiree	Pa1 P55/7	Luffness Innovator
178 P55/7	356 Vales Everest	167 P55/7	298 Vales Everest	165 Innovator
351 P55/7	298 P55/7	178 Innovator	Lindley Innovator	152 Desiree
Lindley Vales Everest	166 P55/7	Luffness P55/7	165 Desiree	Lindley P55/7
178 Desiree	Luffness Vales Everest	167 Desiree	Lindley Desiree	351 Vales Everest
351 Desiree	298 Desiree	167 Vales Everest	165 P55/7	351 Innovator
165 Vales Everest	166 Vales Everest	152 Vales Everest	167 Innovator	Pa1 Vales Everest

block 3

351 Vales Everest	Lindley Vales Everest	351 Desiree	blank	152 Desiree
178 Desiree	165 Vales Everest	Lindley P55/7	166 Desiree	178 Innovator
164 P55/7	164 Vales Everest	Luffness Desiree	152 P55/7	164 Innovator
167 Vales Everest	166 Vales Everest	Pa1 Desiree	Lindley Desiree	167 P55/7
Pa1 P55/7	298 Vales Everest	165 Desiree	Pa1 Vales Everest	152 Innovator
165 P55/7	351 Innovator	Luffness Innovator	Lindley Innovator	298 Desiree
165 Innovator	298 P55/7	356 Vales Everest	blank	356 Desiree
351 P55/7	356 P55/7	Luffness Vales Everest	167 Innovator	164 Desiree
298 Innovator	167 Desiree	Pa1 Innovator	356 Innovator	152 Vales Everest
Luffness P55/7	166 P55/7	178 P55/7	166 Innovator	178 Vales Everest

block 4

blank	Pa1 P55/7	166 Desiree	152 Innovator	166 Innovator
Luffness Vales Everest	164 Desiree	Lindley Innovator	167 Desiree	164 Vales Everest
165 Desiree	152 P55/7	Pa1 Innovator	Pa1 Desiree	298 Innovator
Lindley Desiree	356 Innovator	351 Desiree	Lindley Vales Everest	166 Vales Everest
164 P55/7	blank	Luffness Desiree	Luffness Innovator	356 P55/7
178 Vales Everest	167 Innovator	Luffness P55/7	351 Vales Everest	165 Innovator
178 P55/7	167 P55/7	298 Vales Everest	164 Innovator	152 Desiree
351 Innovator	178 Desiree	167 Vales Everest	356 Desiree	351 P55/7
178 Innovator	166 P55/7	152 Vales Everest	Pa1 Vales Everest	298 Desiree
165 P55/7	165 Vales Everest	356 Vales Everest	298 P55/7	Lindley P55/7

Appendix 7.7. The sequence, top three most replicated unique sequences, corresponds with 100% identity to the descriptive SNPs of each mitotype (described by plasmid Type 1, 2 and 3). Letter N describes the part of the sequence removed due to a sequencing error.

1st most replicated unique sequences = Type 3

AAGAAAATAAAGATGAAAAAAAACAAGAAAAACTAAAAAAAAAGGTAACAAAA
AATGCAAAGCAAAAAAAAACTTTAAGGAGTTCCCCCCAAAAATAAAAGCGCCT
CANATAAAATCAAGACCAAGATAAGGGACCACCCCCAAAAGCCTAGTGATTA
CAATCCCCGCTCAGAACGACATTTGAGACCAAACTATCACGTAACCTAAAAAA
GAGATAAGCATCAAAAAAACCAGAAGAATAACCCCGACCCTTCAAACC

2nd most replicated unique sequences = Type 2

AAGAAAATAAAGATGAAAAAAAACAAGAAAAACTAAAAAAAAAGGTAACAAAA
AATGCAAGGCAAAAAAAAACTTTAAGGAGTTCCCCCCAAAAATAAAAGCGCCT
CANATAAAATCAAGACCAAAATAAGGGACCACCCCCAAAAGACTAGTGATTAC
AATCTCCGCTCAGAACGACATTTGAGACCAAACTATCACGTAACCTAAAAAAG
AGACAAGCATCAAAAAAACCAGAAGAATAACCCCGACCCTTCAAACC

3rd most replicated unique sequences = Type 1

AAGAAAATAAAGATGAAAAAAAACAAGAAAAACTAAAAAAAAAGGTAACAAAA
AATGCAAGGCAAAAAAAAACTTTAAGGAGTTCCCCCCAAAAATAAAAGCGCCT
CANATAAAATCAAGACCAAAATAAGGGACCACCCCCAAAAGACTAGTGATTAC
AATCCCCGCTCAGAACGACATTTGAGACCAAACTATCACGTAACCTAAAAAAG
AGATAAGCATCAAAAAAACCAGAAGAATAACCCCGACCCTTCAAACC

Appendix 7.8. Initial number of cysts, total eggs and density (eggs cyst^{-1}) of *G. pallida* field and control populations used in the glasshouse experiment.

PCN population	Replicate	Cysts	Total eggs	Average total eggs	Eggs cyst^{-1}	Average eggs cyst^{-1}
HAU152	1	20	2244	2058	112	103
	2	20	1680		84	
	3	20	1766		88	
	4	20	2540		127	
HAU164	1	20	1125	1331	56	67
	2	20	1225		61	
	3	20	1250		63	
	4	20	1725		86	
HAU165	1	20	4575	4538	229	227
	2	20	3250		163	
	3	20	4750		238	
	4	20	5575		279	
HAU166	1	20	1275	1150	64	58
	2	20	1625		81	
	3	20	925		46	
	4	20	775		39	
HAU167	1	20	2250	1356	113	68
	2	20	850		43	
	3	20	1525		76	
	4	20	800		40	
HAU178	1	20	308	308	15	15
HAU298	1	20	959	1419	48	71
	2	20	926		46	
	3	20	2198		110	
	4	20	1592		80	
HAU351	1	20	610	747	31	37
	2	20	705		35	
	3	20	824		41	
	4	20	850		43	
HAU356	1	20	1727	1750	86	88
	2	20	1676		84	
	3	20	2008		100	
	4	20	1589		79	
Pa1	1	20	3072	3083	154	154
	2	20	3332		167	
	3	20	3048		152	
	4	20	2878		144	
Luffness	1	20	3894	3139	195	157
	2	20	2412		121	
	3	20	3270		164	
	4	20	2981		149	
Lindley	1	20	2065	3010	103	151
	2	20	2656		133	
	3	20	5493		275	
	4	20	1827		91	

Appendix 7.9. Final number of cysts, total eggs and density (eggs cyst^{-1}) of *G. pallida* field and control populations used in the glasshouse experiment.

PCN population	Genotype	Replicate	Cysts	Average cysts	Total eggs	Average total eggs	Eggs cyst^{-1}	Average eggs cyst^{-1}
HAU152	Desiree	1	471	335	163,437	101,291	347	286
		2	310		96,100		310	
		3	436		120,336		276	
		4	121		25,289		209	
	Innovator	1	5	7	887	1,321	177	253
		2	16		2,325		145	
		3	3		837		279	
		4	3		1,234		411	
	P55/7	1	18	66	3,025	11,083	168	177
		2	38		8,650		228	
		3	104		30,160		290	
		4	104		2,496		24	
	Vales Everest	1	16	9	2,825	1,534	177	155
		2	8		1,229		154	
		3	4		425		106	
		4	9		1,658		184	
HAU164	Desiree	1	193	157	82,990	59,407	430	386
		2	362		132,492		366	
		3	2		905		453	
		4	72		21,240		295	
	Innovator	1	2	2	79	128	40	46
		2	4		382		96	
		3	1		50		50	
		4	0		0		0	
	P55/7	1	20	21	4,400	4,570	220	206
		2	7		1,204		172	
		3	44		10,300		234	
		4	12		2,375		198	
	Vales Everest	1	0	3	0	439	0	101
		2	4		1,066		267	
		3	2		0		0	
		4	5		688		138	
HAU165	Desiree	1	500	458	134,500	152,162	269	325
		2	744		289,416		389	
		3	349		101,908		292	
		4	238		82,824		348	
	Innovator	1	5	4	1,550	849	310	194
		2	3		750		250	
		3	6		887		148	
		4	3		208		69	
	P55/7	1	18	58	4,775	14,842	265	280
		2	48		19,200		400	
		3	58		15,254		263	
		4	106		20,140		190	
	Vales Everest	1	7	6	860	792	123	90
		2	14		2,200		157	
		3	1		52		52	
		4	2		56		28	
HAU166	Desiree	1	260	250	63,700	76,120	245	317
		2	201		88,641		441	
		3	228		78,432		344	
		4	311		73,707		237	
	Innovator	1	9	7	1,900	1,731	211	262
		2	12		3,075		256	
		3	3		1,131		377	
		4	4		816		204	
	P55/7	1	46	42	8,100	8,621	176	214
		2	71		14,484		204	
		3	24		6,250		260	
		4	26		5,650		217	
	Vales Everest	1	3	3	309	460	103	146
		2	2		210		105	
		3	3		564		188	
		4	4		755		189	

Appendix 7.9. Final number of cysts, total eggs and density (eggs cyst^{-1}) of *G. pallida* field and control populations used in the glasshouse experiment (Continuation).

HAU167	Desiree	1	3	40	454	8,229	151	186
		2	18		3,200		178	
		3	56		11,200		200	
		4	84		18,060		215	
	Innovator	1	9	3	1,107	377	123	56
		2	0		0		0	
		3	4		402		101	
		4	0		0		0	
	P55/7	1	36	15	8,300	3,021	231	129
		2	15		2,800		187	
		3	10		983		98	
		4	0		0		0	
	Vales Everest	1	1	8	105	1,914	105	85
		2	0		0		0	
		3	32		7,550		236	
		4	0		0		0	
HAU178	Desiree	1	126	135	43,218	38,509	343	295
		2	39		11,800		303	
		3	200		39,000		195	
		4	176		60,016		341	
	Innovator	1	7	2	921	245	132	48
		2	0		0		0	
		3	0		0		0	
		4	1		59		59	
	P55/7	1	29	23	5,550	4,502	191	188
		2	38		8,400		221	
		3	12		1,650		138	
		4	12		2,408		201	
	Vales Everest	1	17	8	3,575	1,914	210	271
		2	5		1,000		200	
		3	4		1,120		280	
		4	5		1,960		392	
HAU298	Desiree	1	122	263	47,824	71,623	392	294
		2	552		138,000		250	
		3	201		54,471		271	
		4	177		46,197		261	
	Innovator	1	0	2	0	243	0	56
		2	1		0		0	
		3	5		936		187	
		4	1		36		36	
	P55/7	1	6	20	1,729	5,370	288	268
		2	25		7,400		296	
		3	26		7,800		300	
		4	24		4,550		190	
	Vales Everest	1	5	3	1,000	552	200	151
		2	0		0		0	
		3	3		508		169	
		4	3		700		233	
HAU351	Desiree	1	107	205	44,512	73,120	416	362
		2	126		41,454		329	
		3	453		160,362		354	
		4	133		46,151		347	
	Innovator	1	0	2	0	531	0	183
		2	2		803		402	
		3	4		1,321		330	
		4	0		0		0	
	P55/7	1	24	24	8,100	7,538	338	307
		2	25		6,925		277	
		3	27		10,050		372	
		4	21		5,075		242	
	Vales Everest	1	2	2	695	307	348	153
		2	0		0		0	
		3	2		480		240	
		4	2		52		26	

Appendix 7.9. Final number of cysts, total eggs and density (eggs cyst^{-1}) of *G. pallida* field and control populations used in the glasshouse experiment (Continuation).

HAU356	Desiree	1	225	282	71,100	89,046	316	316
		2	407		140,415		345	
		3	224		88,704		396	
		4	273		55,965		205	
	Innovator	1	3	5	633	590	211	178
		2	3		457		152	
		3	2		553		277	
		4	10		717		72	
	P55/7	1	57	104	13,395	35,769	235	317
		2	77		23,870		310	
		3	113		33,222		294	
		4	170		72,590		427	
	Vales Everest	1	3	9	225	1,177	75	115
		2	17		2,200		129	
		3	12		2,125		177	
		4	2		157		79	
Pa1	Desiree	1	734	370	241,486	120,452	329	334
		2	261		81,432		312	
		3	151		60,400		400	
		4	335		98,490		294	
	Innovator	1	0	0	0	0	0	0
		2	0		0		0	
		3	0		0		0	
		4	0		0		0	
	P55/7	1	1	4	185	444	185	128
		2	4		872		218	
		3	3		126		42	
		4	9		591		66	
	Vales Everest	1	2	1	663	277	332	159
		2	1		167		167	
		3	2		276		138	
		4	0		0		0	
Luffness	Desiree	1	359	276	115,598	91,409	322	342
		2	155		67,115		433	
		3	270		69,930		259	
		4	321		112,992		352	
	Innovator	1	3	2	66	129	22	68
		2	1		41		41	
		3	1		141		141	
		4	4		266		67	
	P55/7	1	62	81	12,028	22,247	194	291
		2	35		16,400		469	
		3	170		48,790		287	
		4	55		11,770		214	
	Vales Everest	1	11	8	1,125	1,042	102	134
		2	11		1,625		148	
		3	7		1,185		169	
		4	2		231		116	
Lindley	Desiree	1	738	465	261,252	176,490	354	377
		2	225		86,850		386	
		3	604		259,116		429	
		4	293		98,741		337	
	Innovator	1	1	7	474	1,809	474	302
		2	12		2,408		201	
		3	7		1,550		221	
		4	9		2,803		311	
	P55/7	1	18	42	3,150	12,902	175	199
		2	131		46,243		353	
		3	5		714		143	
		4	12		1,500		125	
	Vales Everest	1	21	27	3,900	3,713	186	139
		2	11		1,250		114	
		3	25		3,200		128	
		4	50		6,500		130	