Final Report

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

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CONTENTS

1. SUMMARY

- 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.*
- Highly significant differences (P<0.001) were found between the viability estimates (%) made 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 a trehalose quantification assay (assay IV).
- Highly significant differences (P<0.001) were found between the viability assessment for *G. pallida* field populations A-E.
- The differential potato genotypes used in the glasshouse have a highly significant effect on cyst production (P<0.001), fecundity of reproduced cysts (P<0.001) and Pf/Pi ratio (P<0.001).
- A highly significant difference was found between *G. pallida* populations for cyst reproduction (P<0.001), the fecundity of reproduced cysts (P<0.001) and on Pf/Pi ratio (P<0.001).
- The interaction between potato genotypes and *G. pallida* populations was significant for reproduction of cysts (P=0.03) and highly significant for the fecundity of reproduced cysts (P<0.001) and Pf/Pi ratio (P<0.001).

2. INTRODUCTION

Potato cyst nematodes (PCN) comprise two related species (*Globodera rostochiensis* and *G. pallida*) and are the most destructive pests of potato crops in the United Kingdom (UK) 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). Development of potato genotypes with the *H1* resistance gene have proven to be highly effective in controlling *G. rostochiensis* but at the same time increase the risk of selection of *G. pallida* which is more challenging to manage.

In the most recent survey *G. pallida* was identified as being the dominant species and present in approximately 92% of the infested potato fields (Minnis *et al*., 2002) - a shift in prevalence from *G. rostochiensis* to *G. pallida* which is often attributed to the growing of potato cultivars that are resistant to the former. The results indicate that the species distribution of potato cyst nematodes has been changing. This study aimed to characterise PCN populations of Great Britain by conducting a PCN survey in GB to provide an insight of the current potato cyst nematodes occurrence and distribution.

Distinct pathotypes of *G. pallida*, which vary in their ability to overcome different sources of resistance, are presented in the Great Britain (GB). There is some evidence that mixed populations of *G. pallida* occur together and may become more virulent over successive generations. To examine current virulence of *G. pallida* populations, glasshouse virulence tests were conducted on field populations using potato genotypes with a range of resistance. Additionally a metagenetics approach was undertaken, based on a region of mitochondrial DNA that was found to be descriptive of pathotypes, to determine the presence of *G. pallida* pathotypes in survey samples and thereby their distribution in GB.

Egg viability is not routinely estimated in PCN population density assessments as currently available viability tests are an additional expense for potato growers and for that reason not often requested for field samples. Causes for a decline in population vitality, as in-eggs mortality, can often leave intact, but not viable, eggs which can then be counted towards the population density. Determining the viability of eggs within the cysts is essential for assessing the effectiveness of control treatments and making decisions on inputs. The project explored and developed various techniques for estimating viability before testing the most promising methods on PCN field populations. The focus of this research was to directly compare their assessment of *Globodera* eggs viability under identical conditions and identify the most reliable assay.

Aims

(I) To estimate the proportion and distribution of GB potato fields infested with PCN, and to determine the distribution of the two species, *G. pallida* and *G. rostochiensis*.

(II) Amend and investigate the suitability of a trehalose quantification assay as a method to assess the viability of *G. pallida* field populations.

(III) Evaluate the comparative effectiveness of hatching in potato root diffusate (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 trehalose quantification assay (assay IV) to assess the natural viability of *G. pallida* field populations.

(IV) 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 trehalose quantification assay (assay IV) to assess the viability of *G. pallida* field populations on six designed viability levels.

(V) To assess the virulence of selected *G. pallid*a populations from PCN survey in England and Wales by testing their ability to reproduce on a range of the differential potato genotypes in the glasshouse.

(VI) To investigate genetic variability, as an aspect of virulence, of *G. pallida* DNA extracts obtained from PCN survey samples in a relation to potential sources of PCN introduction into Europe by use of a metagenetic approach to next-generation sequencing.

(VII) To validate novel next-generation sequencing as a virulence assessment method by investigating its reproducibility and comparison of the results obtained for *G. pallid*a populations with these obtained from the established virulence assessment method.

3. MATERIALS, METHODS AND KEY RESULTS

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

A stratified survey was conducted in England and Wales based on total ware potato growing area (ha) for 2013 provided by the Agriculture and Horticulture Development Board (AHDB) – Potato Data Centre (data obtained on the 10th of April 2014). 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 aim was to collect 500 soil samples to achieve range of the distribution presented in the most recent survey (Minnis, 2000). Required survey samples were systematically distributed across 34 counties (Table 1.).

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 soil sample was taken from each block using a "cheese-corer" style auger with 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

Table 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

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 appointed by growers and independent agronomists;
- 14% results were obtained from a national annual PCN survey conducted by Animal and Plant Health Agency (APHA).

Cysts 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). Thirty six samples, that were collected in the final stages of the project, were extracted at HAU using the Fenwick can, as originally 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 manually examined for PCN cysts under a binocular microscope at 30x magnification (Shepherd, 1986). When present, cysts were hand extracted from float material. The first 20 cysts, or less if sufficient number not present, were moved into Eppendorf tubes for PCN detection and species identification analysis.

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 PCN species determination performed using second assay of the deoxyribonucleic acid (DNA) based method by Reid *et al*. (2015). Soil samples collected after species testing at SASA, which contained PCN-like cysts, were subjected to DNA extraction by activated carbon (Sigma-Aldrich) method and PCN detection/species composition was investigated using a multiplex real-time PCR (Nakhla *et al*., 2010, modified).

Analysis of 500 survey samples from England and Wales showed that 241 samples (48%) were infested with PCN (Figure 1.)

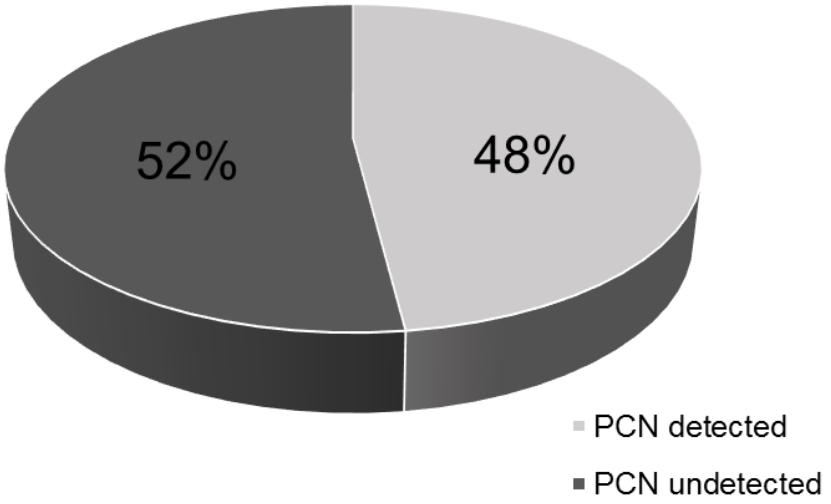


Figure 1. Survey samples undetected and detected as PCN infested from England and Wales presented as a percentage of the total survey samples.

The results for the 241 DNA extracts, where PCN identification was successful, showed that 15 samples were mixed populations (contained both species), 214 samples contained pure *G. pallida* and 12 samples contained pure *G. rostochiensis* populations (Figure 2.).

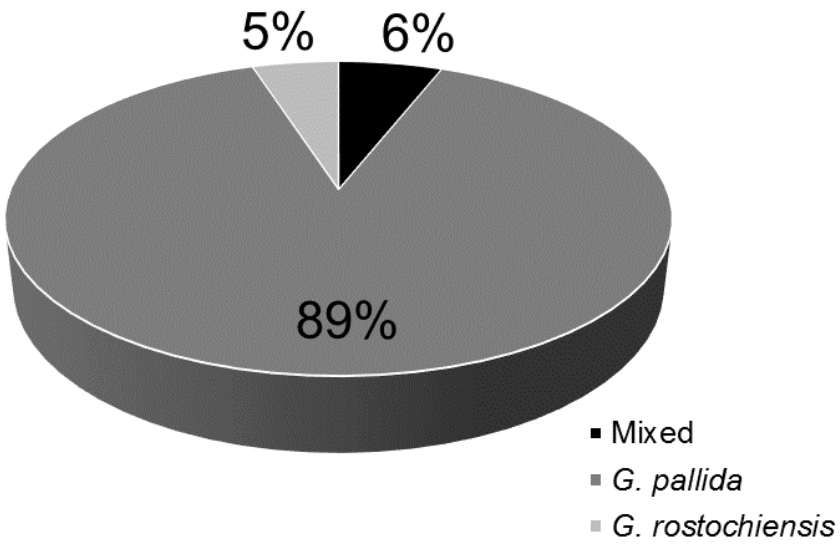
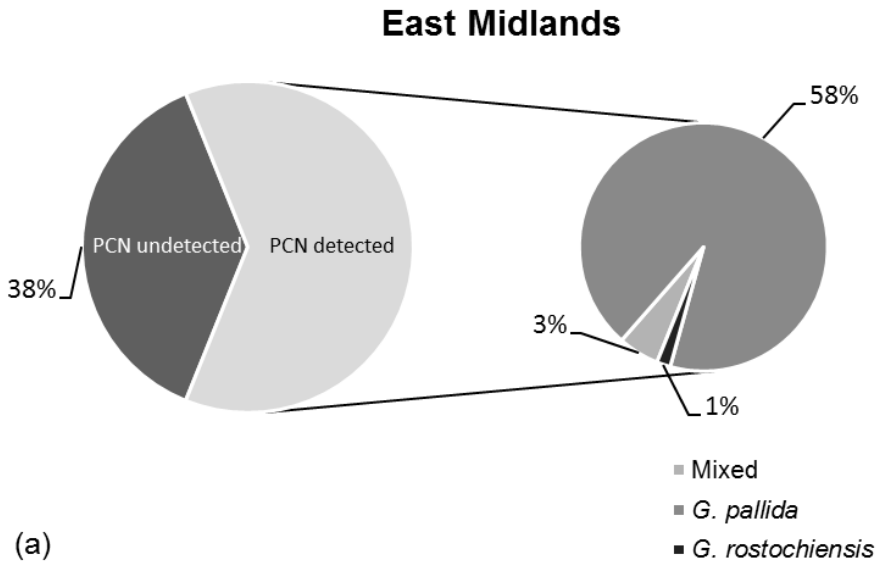
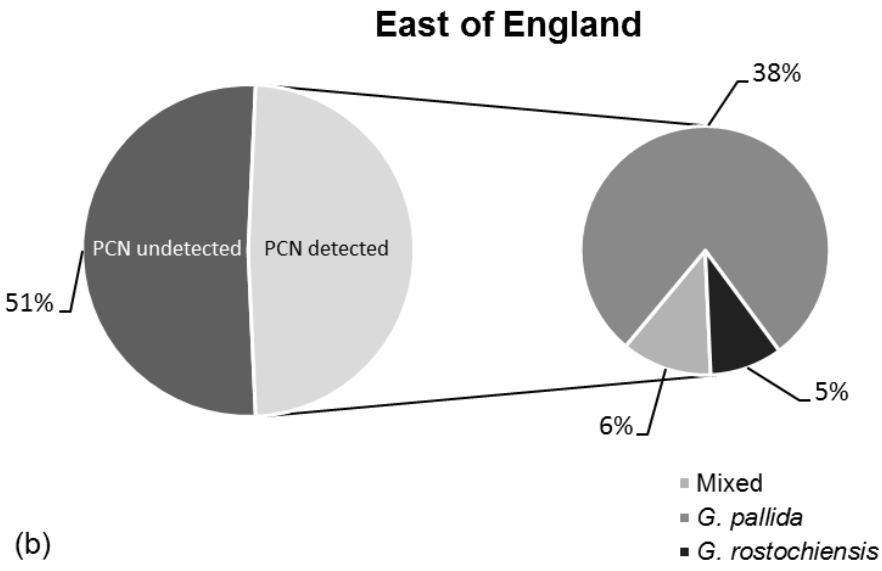


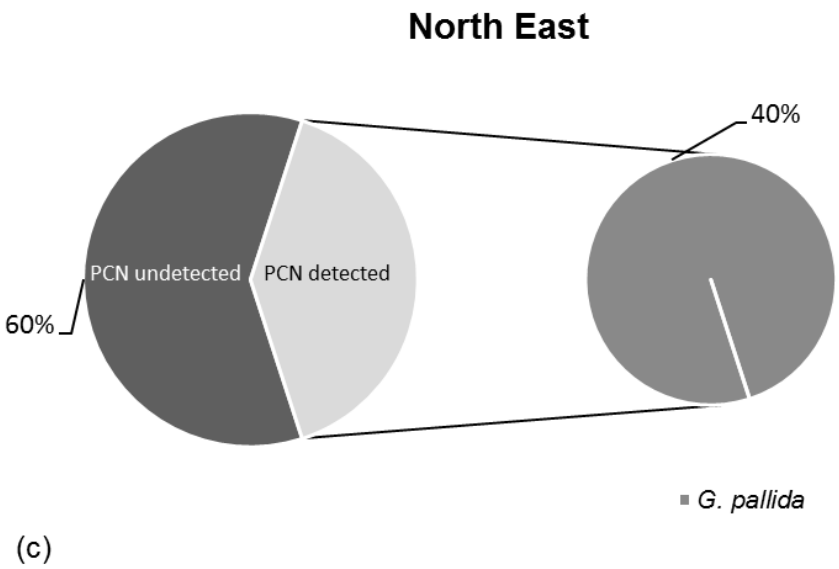
Figure 2. Survey samples containing both PCN species (mixed), pure *G. pallida* and pure *G. rostochiensis* populations from England and Wales presented as a percentage of the total PCN detected survey samples.

Success of the 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 detected species. Generally in the regions where *G. rostochiensis*, as pure or mixed populations, was detected (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 3.







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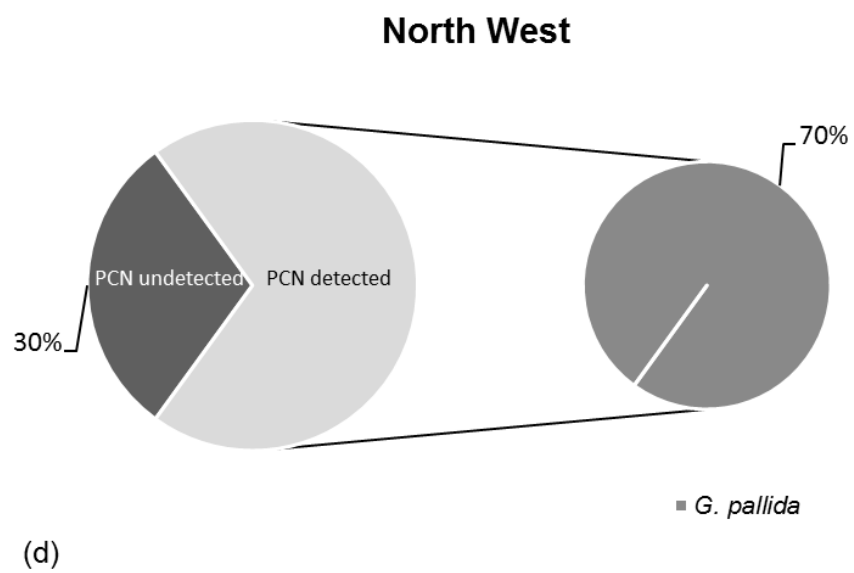

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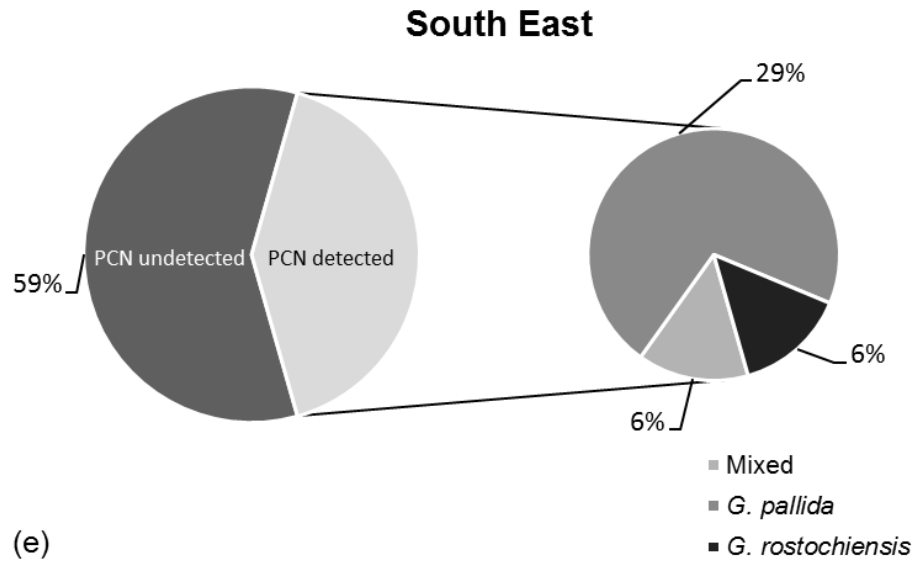

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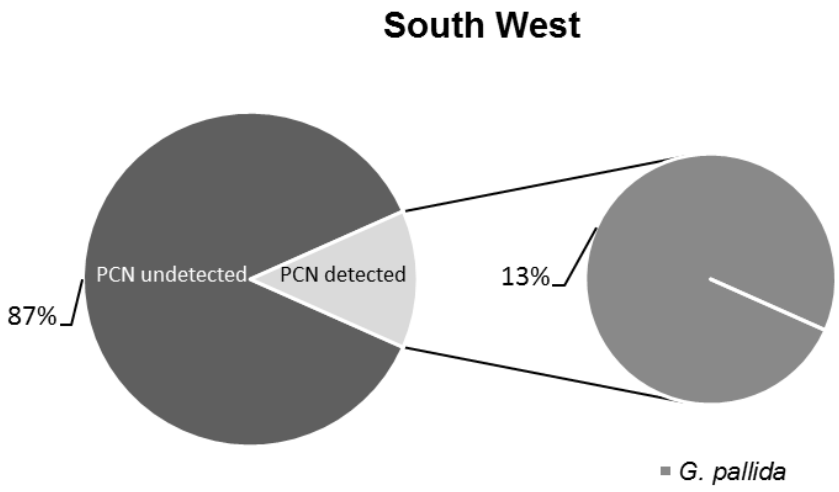

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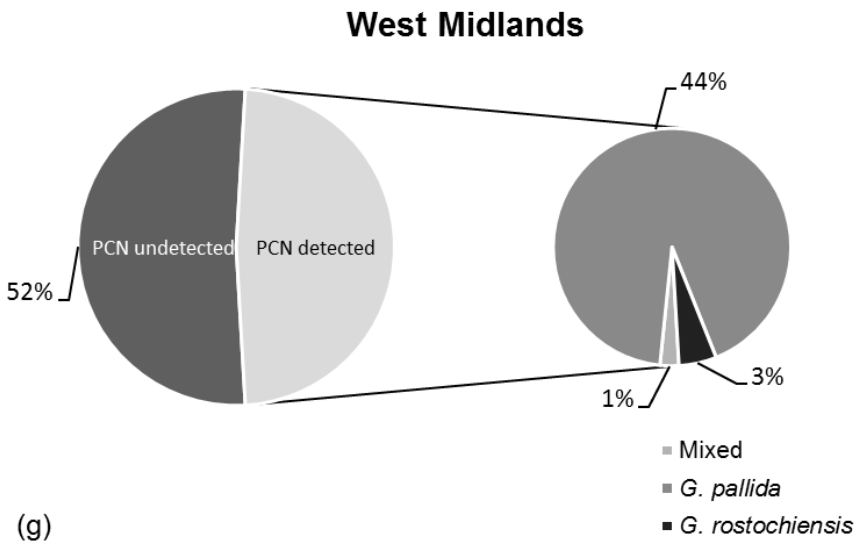

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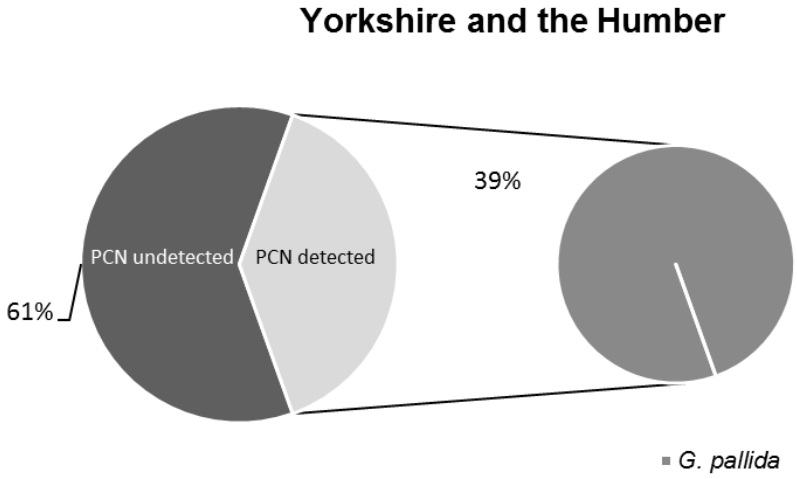

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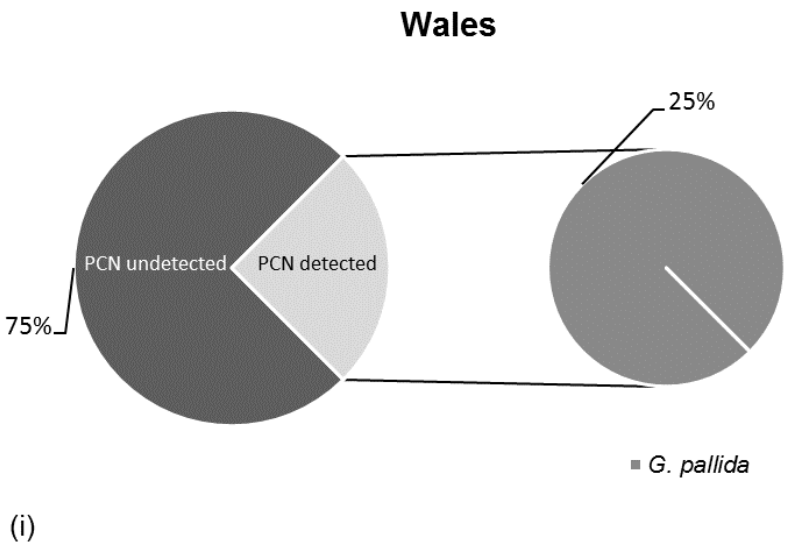


(i)

Figure 3. 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) and Wales (i) 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*.

From the 49 counties originally listed by the AHDB Potatoes - Grower Panel, 34 were investigated for PCN infestation and 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 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 of Worcestershire, were additionally confirmed to have fields infested with pure *G. pallida* populations. 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.

Figure 4. shows the distribution of the fields identified with pure *G. pallida* or containing *G. pallida* as a mixed population. Figure 5. shows the distribution of the fields with pure *G. rostochiensis* or containing *G. rostochiensis* as part of a mixed population. Figure 6. shows the distribution of the fields with mixed populations. Figures 4. – 6. serve as a graphical visualisation of the numerical results presented in Table 2. and discussed above.

Table 2. 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	Samples with PCN undetected	Samples with PCN undetected (% total	Samples with PCN detected	Samples with 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

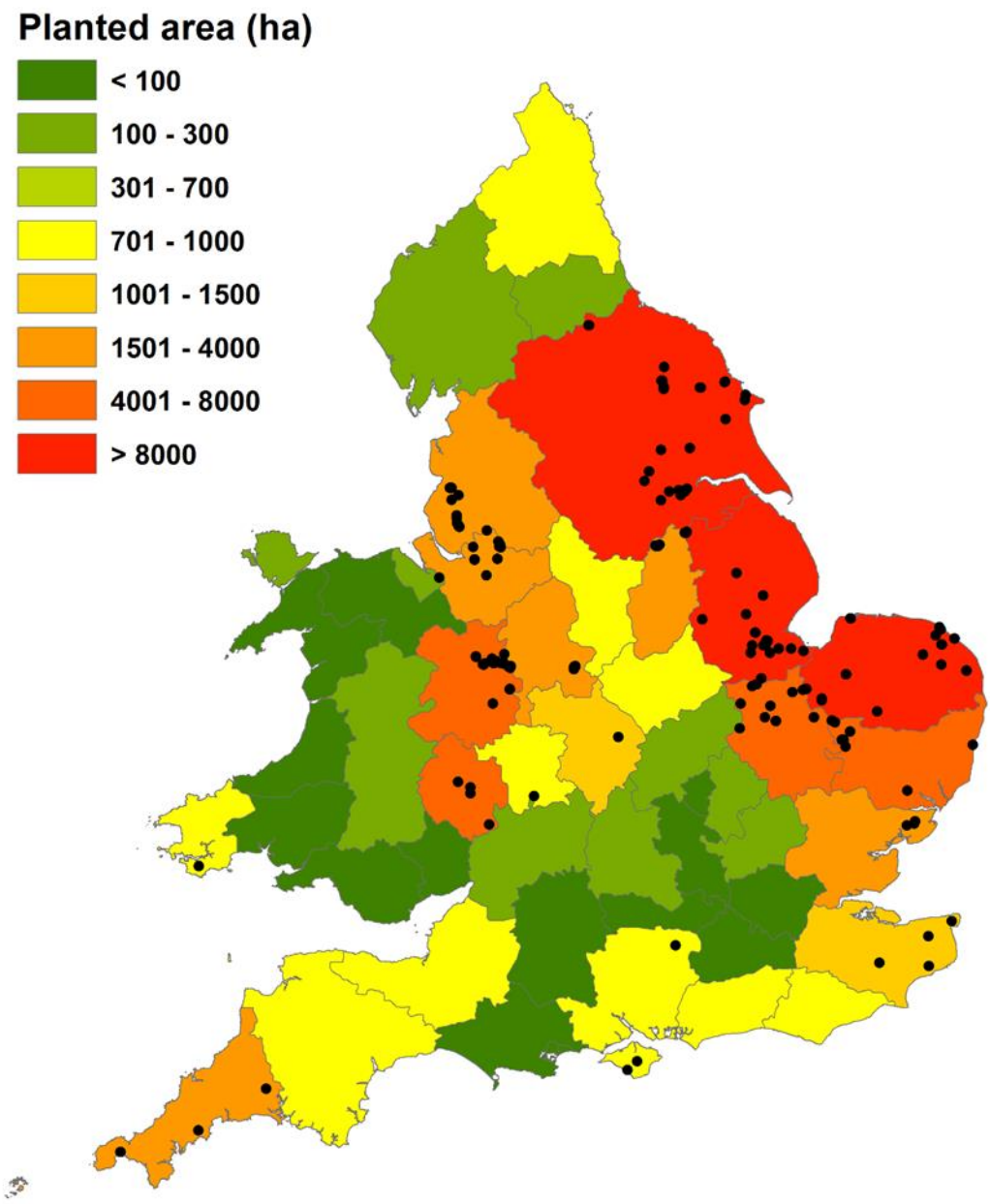


Figure 4. Distribution of the fields detected as PCN infested containing *G. pallida* (as a pure and mixed) populations in a stratified PCN survey (England and Wales) based on the total potato growing area (ha).

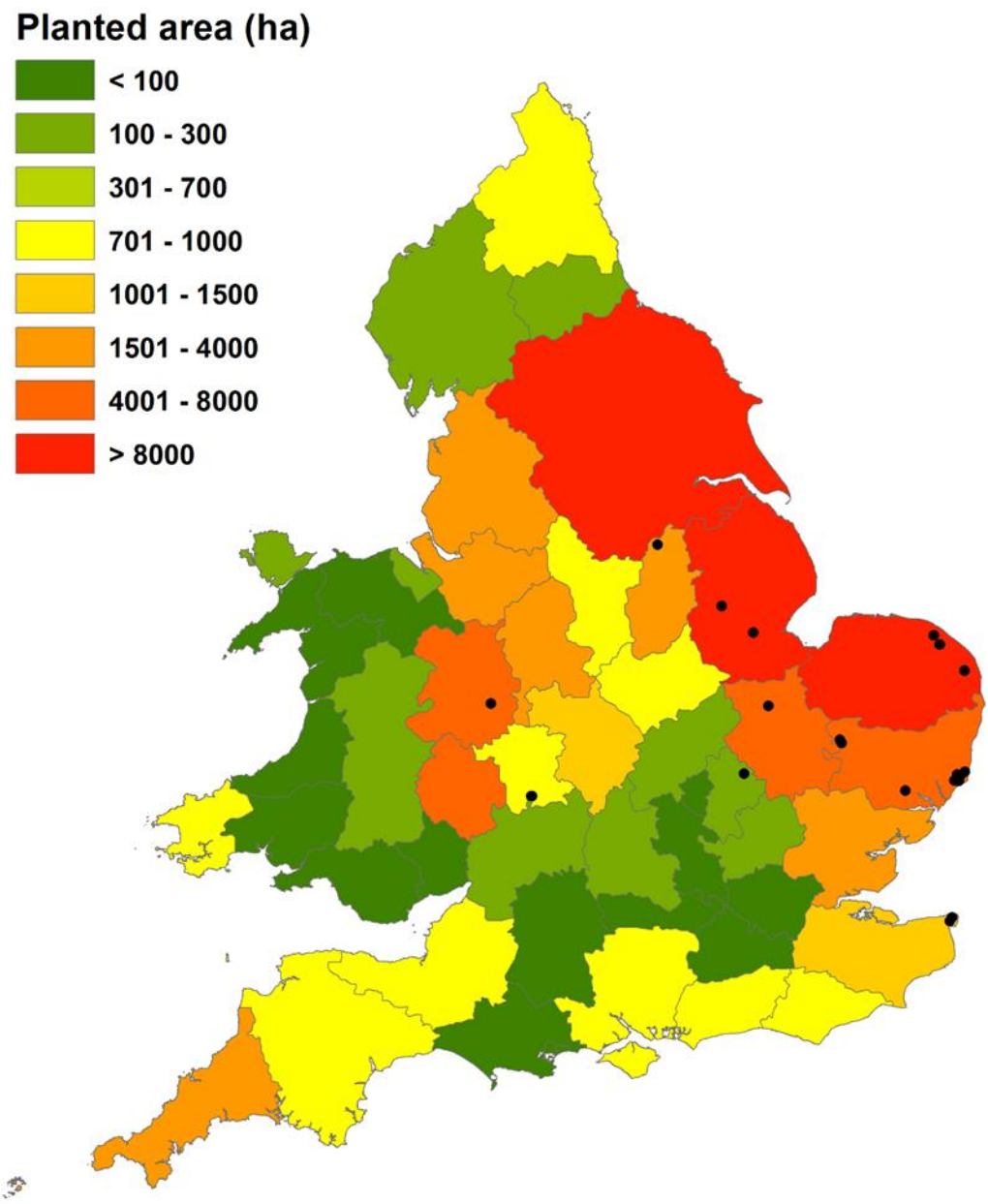


Figure 5. Distribution of the fields detected as PCN infested containing *G. rostochiensis* (as a pure and mixed) populations in a stratified PCN survey (England and Wales) based on the total potato growing area (ha).

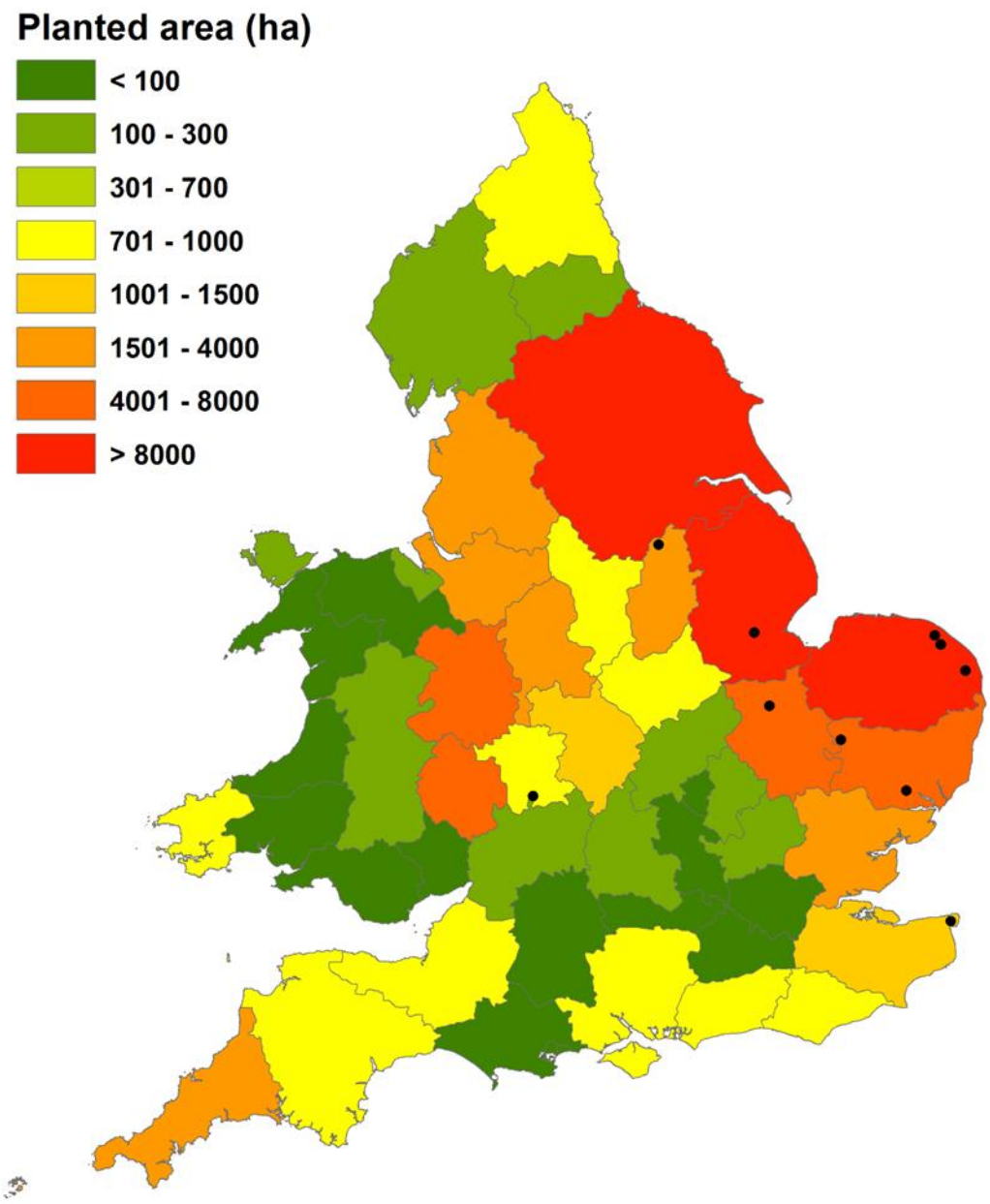


Figure 6. Distribution of the fields detected as PCN infested containing mixed (*G. pallida* and *G. rostochiensis*) populations in a stratified PCN survey (England and Wales) based on the total potato growing area (ha).

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. 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 7.). This observation, combined with increased occurrence of pure *G. pallida* populations, could indicate that by successful controlling of *G. rostochiensis* populations previously reported as mixed are now identified as pure *G. pallida*.

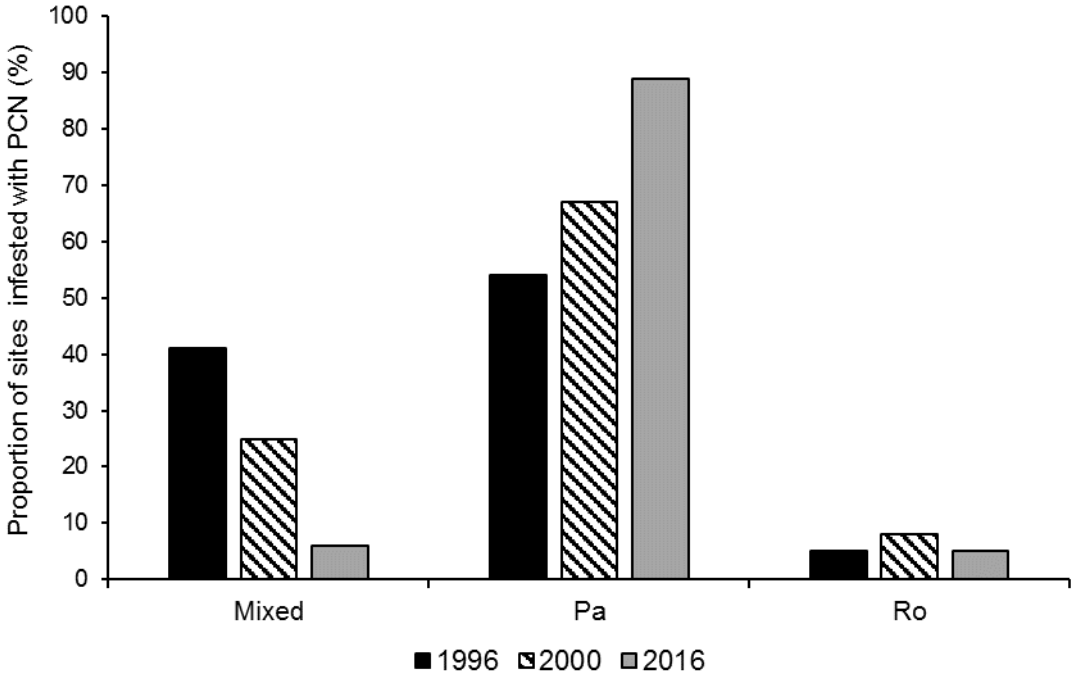


Figure 7. The proportion of PCN populations containing both species (mixed), pure *G. pallida* (Pa) and pure *G. rostochiensis* populations (Ro) as a percentage of the total survey samples confirmed as PCN infested in England and Wales for surveys conducted in 1996 (Hancock, 1996), 2000 (Minnis, 2000) and 2016.

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

Potato cyst nematode populations 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 – called further populations A–E). Viability of these populations was 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 a trehalose quantification assay (assay IV).

3.2.1. *Hatching in PRD assay - assay I*

The protocol was based on the plate assay method described by Ngala *et al*. (2015). The number of viable eggs cyst^{-1} was calculated using equation (1).

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

The percentage viability was calculated using equation (2).

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

3.2.2. *Hatching in PRD followed by Meldola's blue staining assay - assay II*

Cysts of five *G. pallida* populations were treated as described in Hatching in PRD assay - assay I until the last count of newly emerged J2 was recorded. After eight weeks of hatching in PRD the cysts with their unhatched eggs were stained as described in Meldola's blue staining assay - assay III to differentiate between non-hatched but viable and non-viable eggs. The number of viable eggs cyst^{-1} was calculated using equation (3).

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

The percentage viability was calculated using equation (4).

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

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

The viability of five *G. pallida* populations was assessed following the technique described by Shepherd (1986). 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).

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

3.2.4. *Trehalose quantification 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. Change of absorbance per viable egg was calculated using equation (7).

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

Eggs were described as viable by visual morphological determination using a binocular microscope at 60x magnification following EPPO guidance (EPPO, 2017). The number of viable eggs cyst^{-1} was calculated using equation (8).

$$\frac{\Delta\text{A}/\Delta\text{A viable egg}^{-1}}{\text{total cysts used}} = \text{viable eggs cyst}^{-1} \quad \text{(8)}$$

The percentage viability was calculated using equation (9).

$$\frac{\Delta A/\Delta A \text{ viable egg}^{-1}}{\text{total eggs}} = \text{viability (\%)} \quad (9)$$

3.2.5. *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 (exp.1, 2 and 3) to test the variability within the same technique.

3.2.6. *Experimental design – artificial viability*

A range of artificial viability levels were created by mixing cysts placed in distilled water and heated for 30 minutes at 99°C with unheated in various ratios (Table 3.). Supernatant from heated samples prepared for the trehalose quantification 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 (1-6) and each population (A-E).

Table 3. Artificial viability levels 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

The results of natural viability of *G. pallida* field populations (A-E) assessed by hatching in PRD assay (assay I) in exp.1, 2 and 3 are presented in Figure 8.

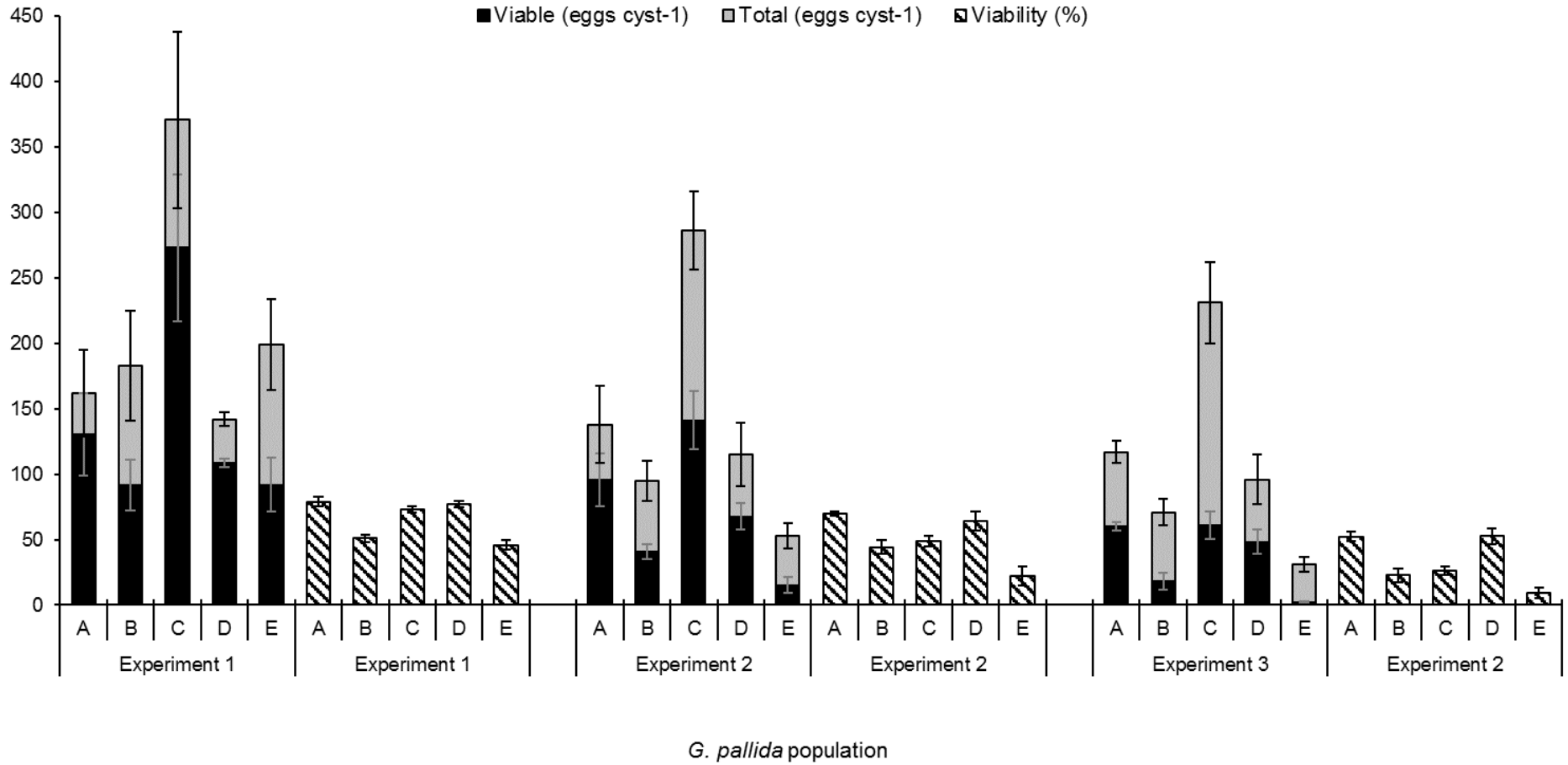


Figure 8. Viability of *G. pallida* field populations (A-E) assessed by hatching in PRD assay (assay I) reported as number of viable eggs of total eggs per cysts (viable/total eggs cyst^{-1}) and as a percentage viability (%). Error bars represent the standard error of the mean.

Natural viability assessed by assay I in exp.1 was 79,51,73,77 and 46% for populations A-E, respectively. The assessments of exp.2 were lower by comparison with exp.1 by 12, 13, 33, 17 and 52% for populations A-E, respectively. The assessments of exp.3 were again lower, by comparison with exp.1, for all *G. pallida* populations (A-E), by 34, 55, 64, 32 and 79%, respectively. The reduction was consistently greater across all populations in exp.3 than in exp.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.

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 exp.1, 2 and 3 are presented in Figure 9..

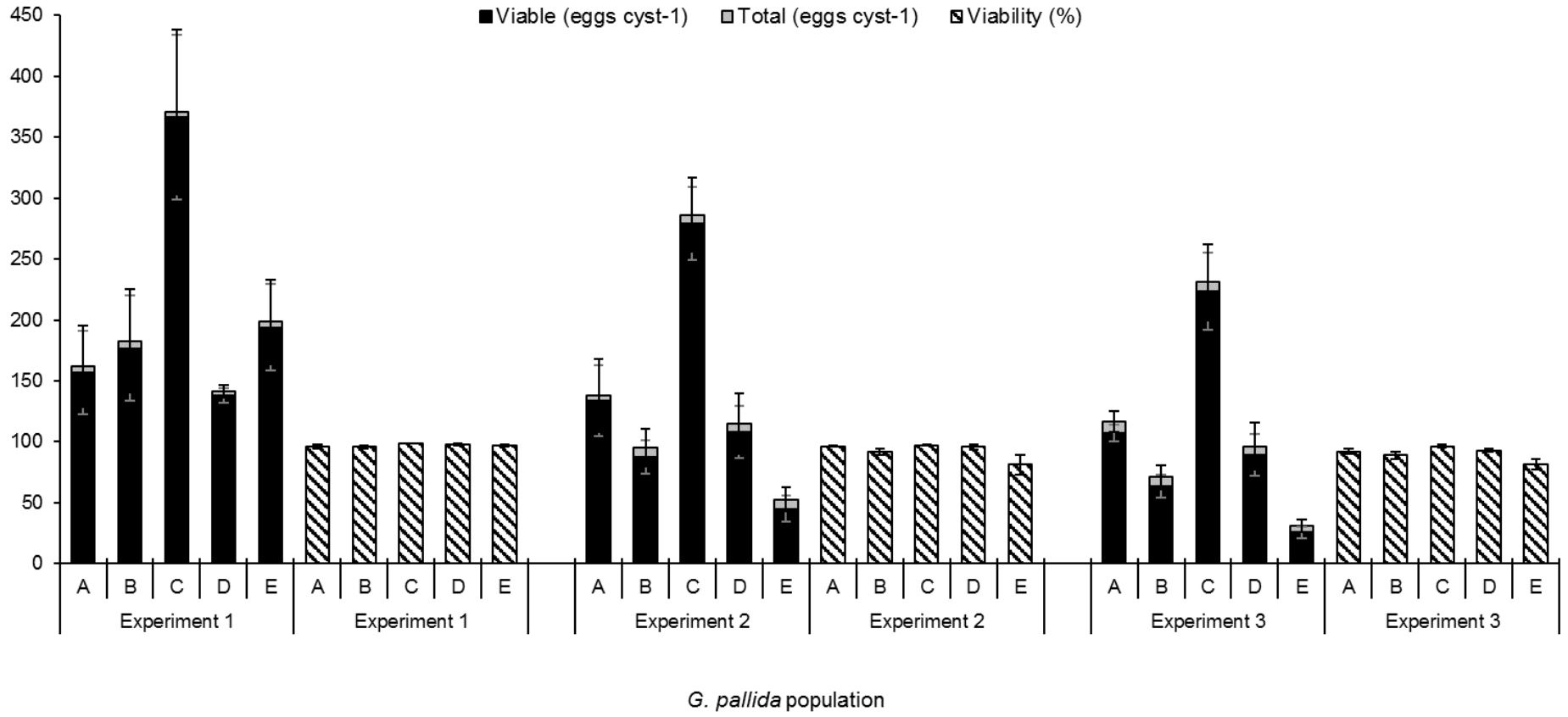


Figure 9. Viability of *G. pallida* field populations (A-E) assessed by hatching in PRD followed by Meldola's blue staining assay (assay II) reported as number of viable eggs of total eggs per cysts (viable/total eggs cyst^{-1}) and as a percentage viability (%). Error bars represent the standard error of the mean.

Natural viability assessed in exp.1 by assay II was 96, 96, 99, 97 and 97% for *G. pallida* field populations A-E, respectively. The assessments of exp.2 were lower, by comparison with exp.1, by 4, 1, 2 and 16% for *G. pallida* field populations B-E, respectively. Only population A had higher, by 1%, viability when assessed in exp.2. The assessments of exp.3 were lower for all *G. pallida* field populations (A-E), by comparison with exp.1, by 4, 7, 2, 5 and 16%, respectively. The reduction of viability was greater in exp.3 then in exp.2 when populations B, C and D were tested. Population E showed similar reduction in both experiments, by 16.2 and 15.8%, exp.1 and 2, respectively.

Secondary assessment by Meldola's blue staining after eight weeks (56 days) of stimulation by PRD showed high viability of the unhatched eggs (Figure 10.). Highest viability of unhatched eggs was detected for population C where 95%, in all experiments, was reported as alive. Population B and E showed high unhatched eggs viability, 92 and 94% respectively, in exp.1 which in following experiments was reduced to 86%, in both experiments, for popualtion B and to 78 and 79%, exp.2 and 3 respectively, for population E. Unhatched eggs of population D were assessed as viable by 89% in exp 1 and 2 and by 84% in exp.3. Population A had lowest, but still substantial, assessment by secondary Meldola's blue staining. Here 84, exp.1 and 3, and 88%, exp.2, of unhatched eggs were reported as viable.

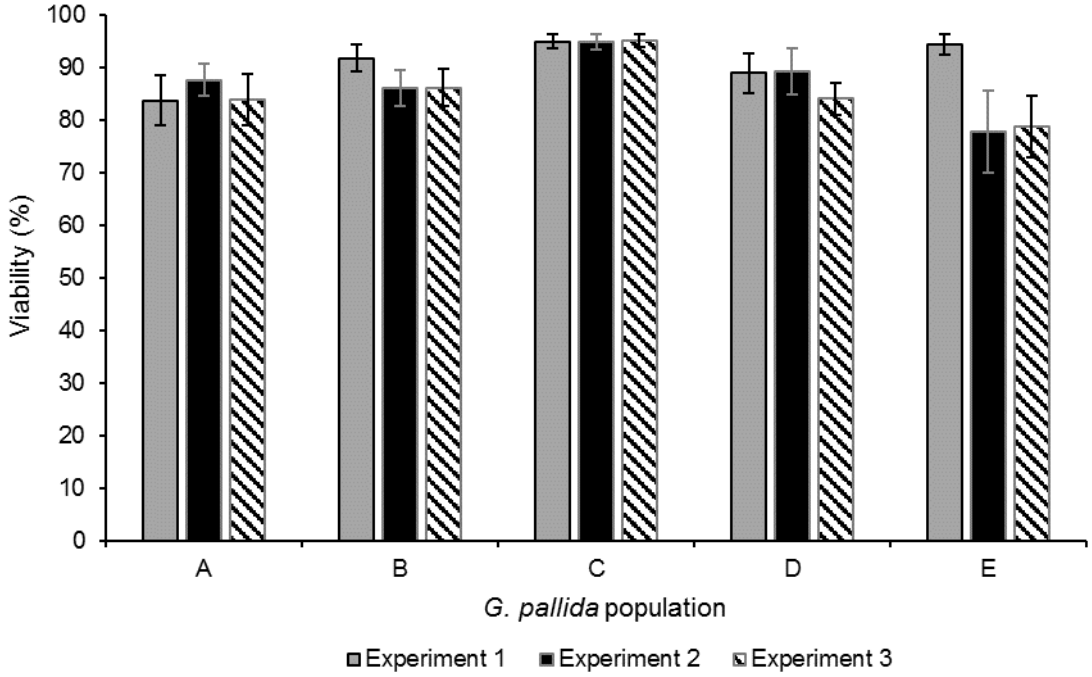


Figure 10. Viability (%) of the unhatched eggs, after eight week of hatching, assessed by Meldola's blue staining as a secondary viability assessment in hatching in PRD followed by Meldola's blue staining assay (assay II). Error bars represent the standard error of the mean.

The results of natural viability of *G. pallida* field populations A-E assessed by Meldola's blue staining assay (assay III) in exp.1, 2 and 3 are presented in Figure 11.

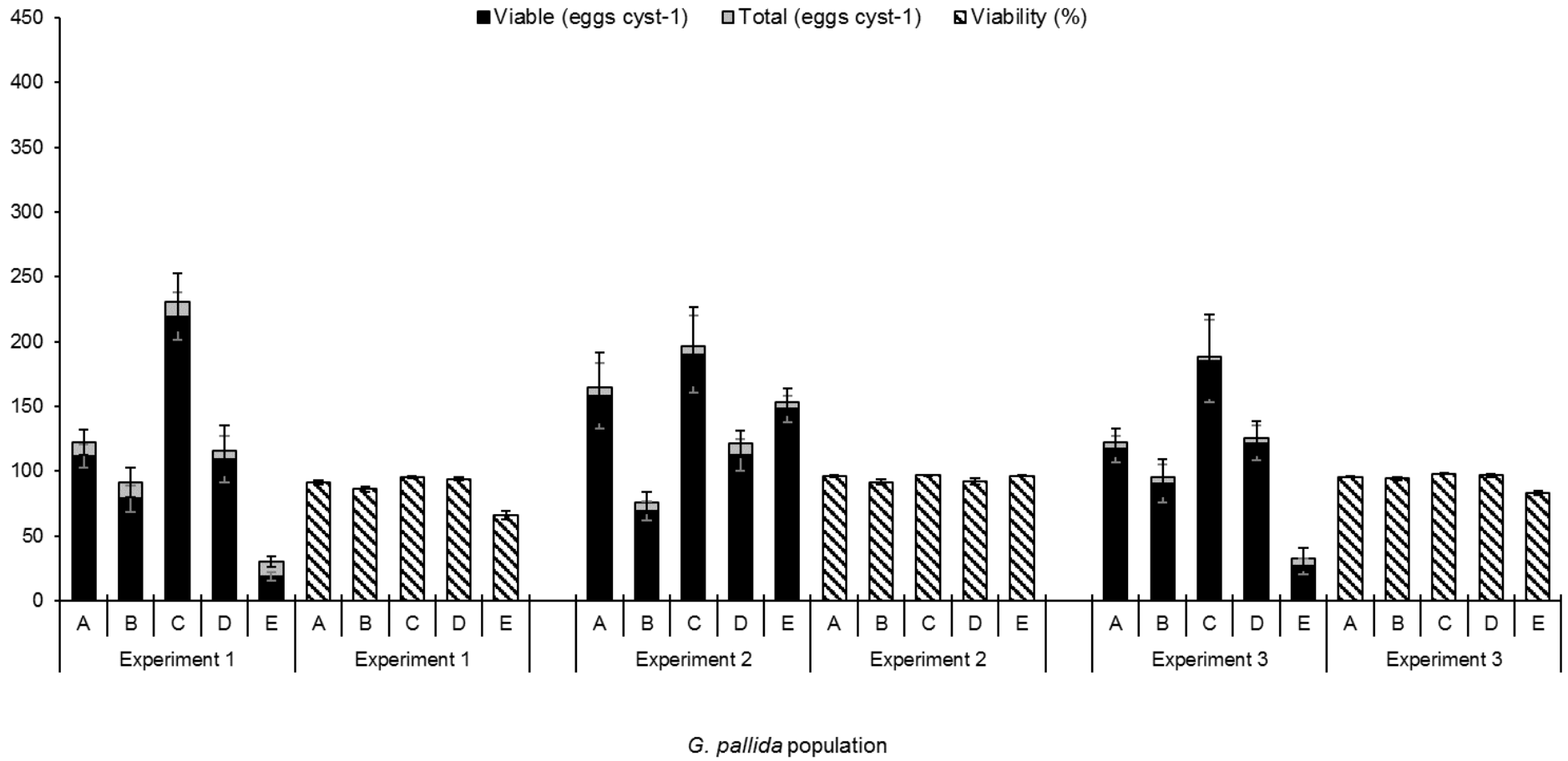


Figure 11. Viability of *G. pallida* field populations (A-E) assessed by Meldola's blue staining assay (assay III) reported as number of viable eggs of total eggs per cysts (viable/total eggs cyst^{-1}) and as a percentage viability (%). Error bars represent the standard error of the mean.

Natural viability assessed in exp.1 by assay III was 91, 86, 95, 94 and 66% for *G. pallida* field populations A-E, respectively. Comparison with exp.1 showed that assessments of exp.2 were lower, by 2%, only for population D. Remaining populations A, B, C and E showed an increase in viability by 5, 6, 1 and 46%, respectively. Experiment 3 assessed viability of all populations (A-E) higher than exp.1 by 5, 10, 3, 3 and 25%, respectively. The increase of viability was greater in exp.3 for populations B and C and greater in exp.2 for population E. Population A showed the same increase in both experiments.

The results of natural viability of *G. pallida* field populations A-E assessed by a trehalose quantification assay (assay IV) in exp.1, 2 and 3 are presented in Figure 12.

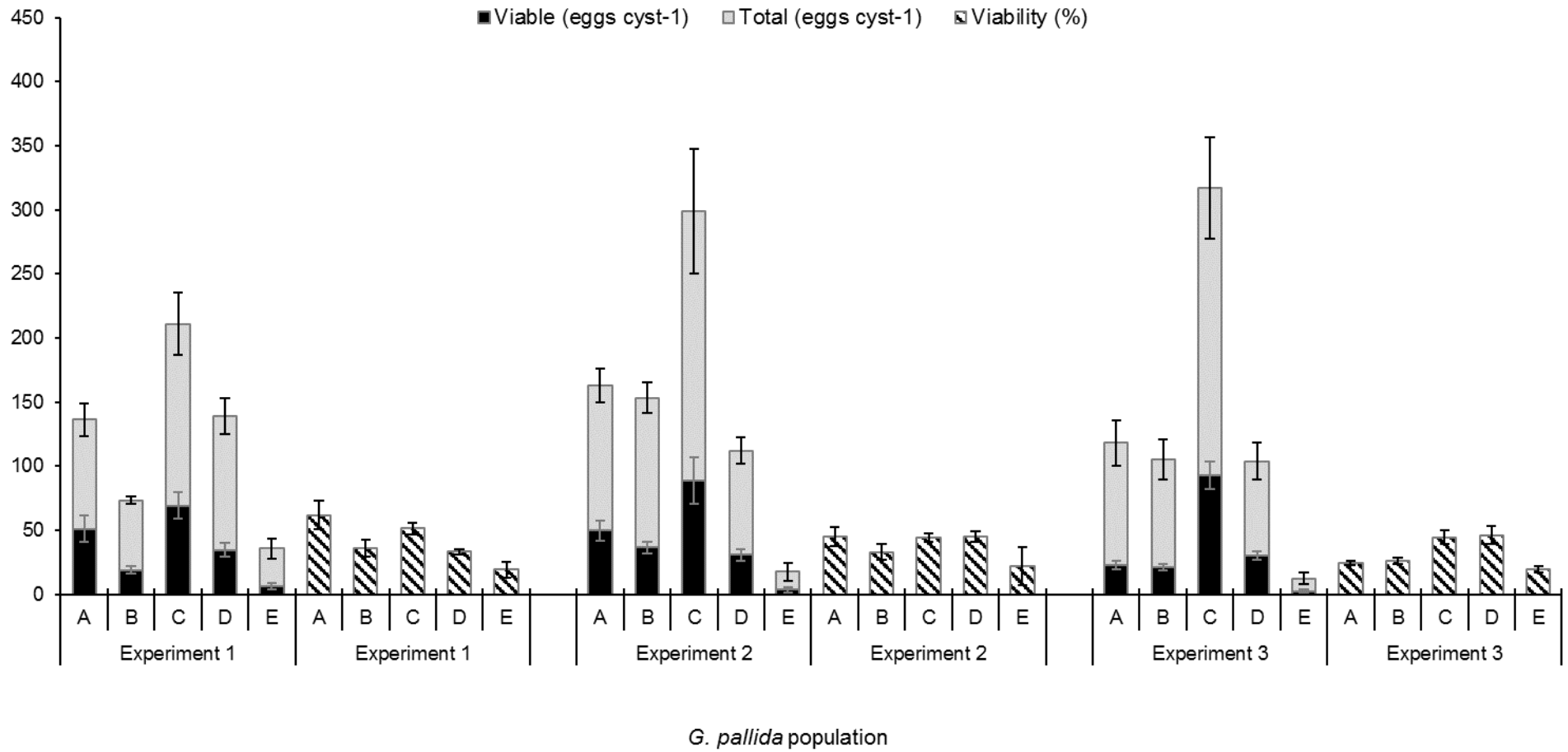


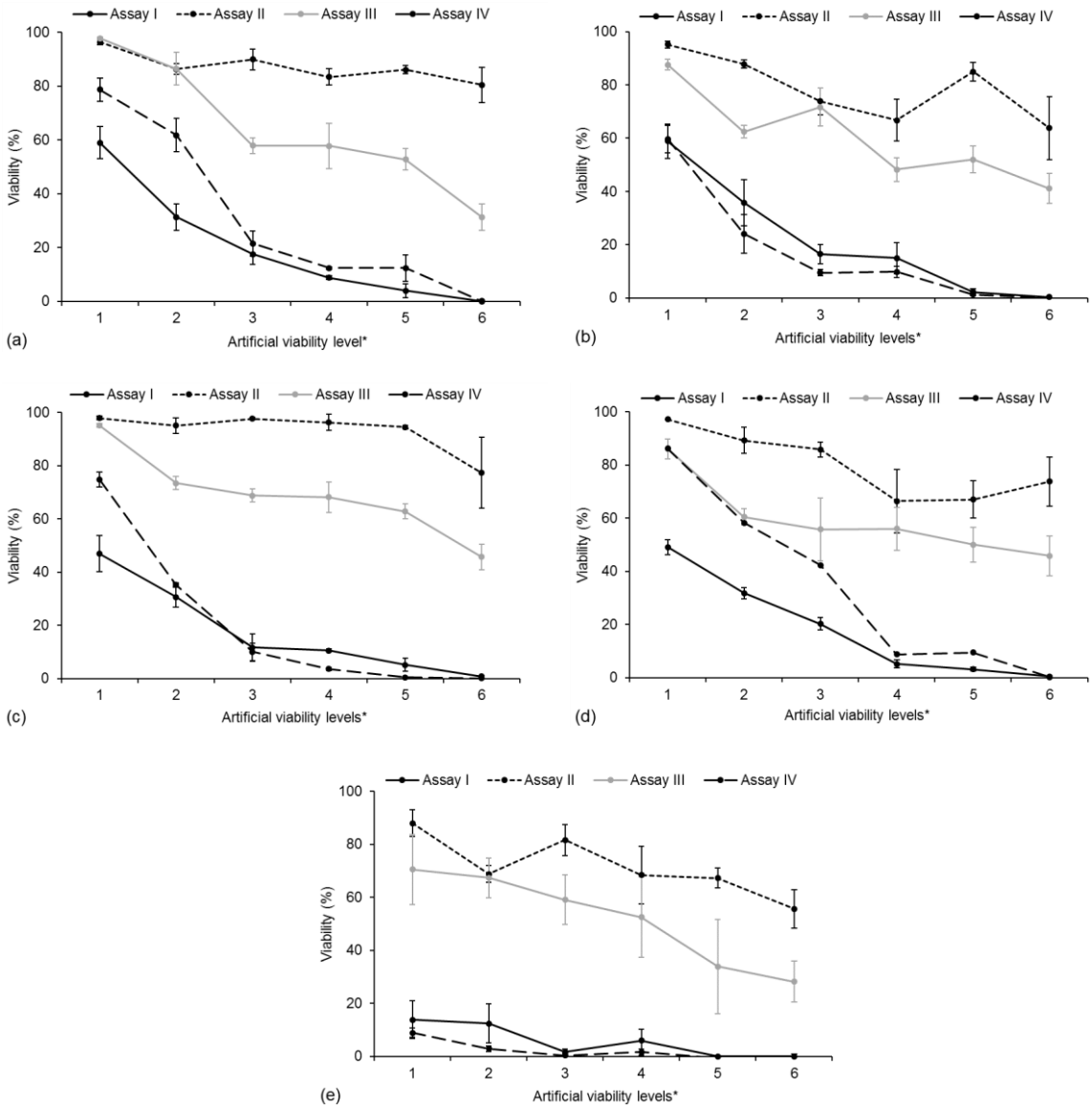
Figure 12. Viability of *G. pallida* field populations (A-E) assessed by trehalose quantification assay (assay IV) reported as number of viable eggs of total eggs per cysts (viable/total eggs cyst^{-1}) and as a percentage viability (%). Error bars represent the standard error of the mean.

Natural viability assessed in exp.1 by assay IV was 62, 36, 51, 33 and 19% for *G. pallida* field populations A-E, respectively. Lower estimation of viability, by comparison with exp.1, by 27 and 8% (exp.2) and by 60 and 27% (exp.3) was observed for populations A and B, respectively. Viability of populations D and E increased by 36 and 14% (exp.2) and by 39 and 1% (exp.3), respectively. Population C also showed reduction in viability assessment in exp.2 and 3 equally by 14% when compared against results from exp.1.

Highly significant differences ($P<0.001$), and accounted for 73, 81 and 79%, exp.1, 2 and 3 respectively, of the variance, were found between the estimates made by assays I, II, III and IV. Also highly significant differences ($P<0.001$), but accounted for only 10, 9 and again 10%, exp.1, 2 and 3 respectively, of the variance, were found between the viability assessment for populations A-E. Relationship between viability assessment methods (I-IV) and field populations (A-E) was found to be significantly different ($P<0.05$) and accounted for only 4 and 5% in exp.1 and 2, respectively, of the variance. In exp.3 a highly significant differences ($P<0.001$), accounted for 4% of the variance, were found when relationship between viability assessment methods (I-IV) and field populations (A-E) was tested.

Viability assessment on six levels of artificial viability assessed by assay I and IV expressed gradual reduction in viability in parallel with increasing number of heat-treated cysts in the samples across all field populations (A-E). Assessment by assay IV was higher than by assay I on levels 1-5 for population A and D and lower for population E. Higher assessment by assay IV was also observed on level 1 for population B and level 1 and 2 for population C. Remaining levels for these two populations had higher viability assessment by assay I with one exception, on level 6 (population B) both assays detected no viability (0%). Assay II and III assessed viability higher than assay I and IV on all levels for all populations. Comparison between assay II and III showed that, in case of 93% assessment (all levels/all populations), assay II detected higher number of

viable eggs. There was only one assessment when assay III returned viability 2% higher than assay II (level 1, population A) and one assessment when both assay agrees on the viability (level 2, population A) (Figure 13.).



* Artificial viability levels comprised of a mixture of unheated and heat-treated of *G. pallida* cysts (Table 3.)

Figure 13. Viability (%) distribution assesses 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 trehalose quantification assay (assay IV) for *G. pallida* field populations (a) A, (b) B, (c) C, (d) D and (e) E on six artificial viability levels. Error bars represent the standard error of the mean.

Differences between assays were clearly visible when only level 6 of artificial viability was observed. Assay I correctly detected non-viable nematodes in population

A, B, D and E and, in population C, where the viability was detected it was below 1%. Very similar outcome was seen for assay IV where populations A, B and C were correctly recognised as non-viable and only 1% viability, each, was detected for populations D and E. In contrast, assay II and III recognised viable eggs in heat-treated samples. Assay III detected viability ranging from 28 (population E) to 46% (population C and D). Population A and B were described by 31 and 41% viability, respectively. The assessment of viability on this artificial level was even higher when assess by assay II. Here detection of the viability ranged from 81 (population A) to 56% (population E). Viability assessment for population B, C and D was at 64, 77 and 74%, respectively.

3.3. Assessment of established and novel methods for the characterisation of the virulence of potato cysts nematodes populations from Great Britain

3.3.1. *Glasshouse experiment - virulence assessment*

Field populations, from the survey samples collected in GB, characterised for species composition and confirmed to be pure *G. pallida* populations were selected for pathotypes determination virulence tests. Additionally, three reference populations of *G. pallida* from the JHI collection were used (Table 4.).

Table 4. Tested (survey) and control (the JHI collection) *G. pallida* populations included in the glasshouse trial for pathotype assessment using the differential 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*	Pa2/3	JHI collection	Scotland	unknown	unknown	1
Lindley	*G. pallida*	Pa2/3	JHI collection	England	unknown	unknown	1

A range of potato genotypes, including a susceptible control and genotypes representing the main sources of resistance towards *G. pallida* pathotypes Pa1 and partial resistance towards Pa2/3, were used (Table 5.).

Table 5. The differential potato genotypes (susceptible and partially resistant to both *G. pallida* pathotypes) used in the glasshouse for pathotypes 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	Major gene (*H3*)
'P55/7'	*S. multidissectum* PH1366	High	Partial	Major gene (*H2*)
'Innovator'	*S. vernei*	High	High	Polygenes (*Gpa5*)

To examine multiplication ability of *G. pallida* populations a randomised glasshouse experiment was designed with four replicates per *G. pallida* population/variety combination (Figure 14.). Just before planting initial population (Pi) density was assessed for each *G. pallida* population. After 10 weeks of growth newly formed cysts were extracted and final population (Pf) density was recorded. An assessment of the reproduction (population multiplication ability), expressed as eggs cyst^{-1} was calculated using equation (10).

$$\frac{\text{Pi eggs } cyst^{-1}}{\text{Pf eggs } cyst^{-1}} = \text{Pf/Pi ratio} \quad \text{.....(10)}$$

Populations which produced more eggs cyst^{-1} than were used for inoculation and by that had Pf/Pi ratio larger (>) than 1 were characterised as susceptible. Analogously Pf/Pi ratio smaller or equal (≤) 1 indicated resistance (Kort *et al*., 1977).

Figure 14. Arrangement of the glasshouse for pathotypes assessment of *G. pallida* field populations from England and Wales (1 DAP).

Reduction in multiplication for Lindley and Luffness populations (Pa2/3) was strongest on 'Innovator' for which number of cysts pot^{-1} was 98 and 99%, respectively, lower than those produced on susceptible 'Desiree'. 'Innovator' was closely followed by 'Vales Everest' with reduction of 94 and 97% for Lindley and Luffness, respectively. 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 Lindley population had stronger (91%) reduction than Luffness (71%). Number of cyst pot^{-1} of population Pa1 (Pa1), post reproduction, was strongly reduced (99%) on both, 'P55/7' and 'Vales Everest'. No multiplication was recorded for population Pa1 (100% reduction) on 'Innovator' (Table 6.). Production (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 the susceptible genotype 'Desiree'. 'Vales Everest' achieved the reduction in cysts pot^{-1} between 80 and 99%. Populations HAU165, 298 and 351, exhibited the same reduction level on 'Innovator' and 'Vales Everest' with

multiplication 99% lower than ‘Desiree’. These three populations were also, alongside with HAU164, best controlled by ‘P55/7’, 87, 92, 88 and 87% reduction, respectively. Population HAU166 was the only one with the reduction in cyst multiplication weaker (97%) on ‘Innovator’ than on ‘Vales Everest’ (99%). Across all field populations the weakest reduction in cysts pot^{-1} was observed on ‘P55/7’ with range between 62 and 92% (Table 6.).

Table 6. Reduction in reproduction (%) of *G. pallida* field populations from England and Wales on the differential potato genotypes relative to reproduction on susceptible genotype ‘Desiree’ (cyst pot^{-1}).

Population	Potato genotype		
	'Innovator'	'P55/7'	'Vales Everest'
HAU 152	98	80	97
HAU 164	99	87	98
HAU 165	99	87	99
HAU 166	97	83	99
HAU 167	92	62	80
HAU 178	99	83	94
HAU 298	99	92	99
HAU 351	99	88	99
HAU 356	98	63	97
Pa 1	100	99	99
Luffness	99	71	97
Lindley	98	91	94

‘Innovator’ contains gene of high resistance to Pa1 and Pa2/3 which suggests that populations which low Pf/Pi ratio could be considered pathotype Pa2/3. ‘Vales Everest’ was also bred with resistance towards Pa2/3 which again suggests that, if Pf/Pi value is low, tested population could represent virulence characteristic of pathotype Pa2/3. ‘Vales Everest’ has high resistance to Pa1. Finally, ‘P55/7’ with its high resistance to Pa1 is described as partially resistance to Pa2/3 which suggest that, 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 are

expected to prevent the multiplication of pathotype Pa1. Consistency low Pf/Pi ratio (≤1) across differential genotypes indicates presence of *G. pallida* population with pathotype Pa1 characteristic of virulence. Lindley population (Pa2/3) had (Table 7.) the highest Pf/Pi ratio on 'Desiree' followed by 'Innovator', 'P55/7' and 'Vales Everest'. Luffness (Pa2/3) also produced the highest Pf/Pi ratio on 'Desiree' but the next highest ratio was produced on 'P55/7' followed by 'Vales Everest' and 'Innovator'. Population Pa1 (Pa1) had again highest Pf/Pi ratio on 'Desiree', which was followed by 'Vales Everest', 'P55/7' and 'Innovator'. None of the field populations showed the pattern of Pf/Pi ratios distribution expected for pathotype Pa1. Following recommendations by Kort *et al*. (1977) and focusing on Pf/Pi ratio only, pathotype Pa2/3 could be assigned to population HAU164, 165, 167 and 298. Additional interpretation based on comparison between results obtained for control and field populations could aid, with some dose of caution, the assessment of pathotypes. Distribution of multiplication ratios for Lindley population is also seen in population HAU152 and 166 which could suggest presence of pathotype Pa2/3. Pattern in distribution of Pf/Pi ratios for Luffness is also seen for population HAU351 and 356 which, even if actual values are higher than one, could suggest that these populations are pathotype Pa2/3. Population HAU178, with very high Pf/Pi ratios across all genotypes, could not be designated to *G. pallida* pathotype Pa1 or Pa2/3 (Table 7.).

Table 7. Reproduction ratios (Pf/Pi) on the differential potato genotypes and pathotype designation of field populations from England and Wales.

Population	Genotype				Pathotype designation
	'Desiree'	'Innovator'	'P55/7'	'Vales Everest'	
HAU 152	2.8	2.5	1.7	1.5	-
HAU 164	5.8	0.7	3.1	1.5	Pa2/3
HAU 165	1.4	0.9	1.2	0.4	Pa2/3
HAU 166	5.5	4.5	3.7	2.5	-
HAU 167	2.7	0.8	1.9	1.3	Pa2/3
HAU 178	19.7	3.2	12.5	18.0	-
HAU 298	4.1	0.8	3.8	2.1	Pa2/3
HAU 351	9.8	4.9	8.3	4.1	-
HAU 356	3.6	2.0	3.6	1.3	-
Pa 1	2.2	0.0	0.8	1.0	Pa1 control
Luffness	2.2	0.4	1.9	0.9	Pa2/3 control
Lindley	2.5	2.0	1.3	0.9	Pa2/3 control
Average	5.2	1.9	3.7	3.0	

The results showed that the differential potato genotypes used in the glasshouse have a highly significant effect ($P<0.001$) on reproduction of cysts, 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 for cyst reproduction ($P<0.001$) the fecundity of reproduced cysts ($P<0.001$) and on Pf/Pi ratio ($P<0.001$). The interaction between potato genotypes and *G. pallida* populations was significant for reproduction of cysts ($P=0.03$) and highly significant for the fecundity of reproduced cysts ($P<0.001$) and Pf/Pi ratio ($P<0.001$).

3.4. Next-generation sequencing - mitotype assessment

The mitotype diversity was investigated for 229 survey samples (experiment was conducted before completion of survey samples collection hence not all survey samples were tested) collected across England and Wales from individual fields previously used for ware potato production. Up to 20 cysts from each sample were moved into 2ml safe-lock tubes and sent to SASA for further analyses. To extract total DNA from cysts a commercial plant kit (BioSprint 96 DNA Plant Kit, Qiagen) was used as described by Reid *et al*. (2015). Samples were analysed to investigate the presence and composition of these mitotypes using SNPs as a molecular marker in sequencing methodology developed by Eves-van den Akker *et al*. (2015). They modified primers which amplify 310bp region of cyt-*b* by adding a distinctive 4bp barcode which was designed to allow the identification of individual survey samples during the data analysis. Additionally two 5' adenosines were added before barcode and actual forward or reverse primer sequences to act as a protecting buffer for the barcode in the samples metagenetic sequencing process. Sequencing of 310bp amplicon was carried out at Edinburgh Genomics (University of Edinburgh, Edinburgh, UK). Analyses were made in duplicates on survey samples (2x229), eight replicates of selected survey samples were included as internal control (8x5) and six replicates of mixes of ratios of plasmids containing known partial sequences of cyt-*b* as positive control (6x9). After sequencing, bioinformatic analyses and data analysis were performed.

Bioinformatic and data analysis on the data set obtained after next-generation sequencing was completed showed that the vast majority of the unique amplicons occurred only once for a given barcode pair and only frequency of mitotypes in the top 10 barcoded samples was higher than 10. Out of these 10 samples only nine could be, thanks to the unique character of the barcodes, identified and assigned to the actual samples included in the experiment. All of them proved to be a mixtures of plasmids, containing known partial sequences of cyt-*b*, used as positive controls. While many

barcode pairs could be assigned to survey samples, there was no detection of any mitotype. Several other approaches were exploited in an attempt to increase number of unique amplicons but none of them resulted in satisfactory improvement. Low occurrence of numerous unique amplicons in the data set obtained from next-generation sequencing and, when possible, detection of sequences corresponding with mitotype only in control samples thwarted further analysed and a conclusion (Eves-van den Akker *et al*., 2015) could not be drawn. Work is currently underway to run a repeat of the sequencing on 124 samples collected from the PCN survey. If successful, an updated version of this report will be made available via AHDB Potatoes.

4. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

- 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.*
- Trehalose quantification assay's upper threshold was reached at approximately 5,000 viable *G. pallida* eggs at ΔA just above 2nm.
- Trehalose quantification assay's lower threshold was reached at approximately 100 viable *G. pallida* eggs.
- Trehalose quantification assay's limitations should be set at approximately 100 for minimum and 5,000 for maximum number of *G. pallida* eggs. Strong relationship ($R^2 = 0.8$) was observed between ΔA and number of eggs in samples containing more than 100 and less than 5,000 eggs.
- Change of absorbance per viable egg need to be calculated individually for each population.

- Average ΔA trehalose quantification assay's background noise, from samples with no eggs, was -0.0002nm.
- Specimen's background noise need to be taken into account, individually for each *G. pallida* field population, when testing viability by trehalose quantification assay.
- Testing for variance in viability (%) between exp.1, 2 and 3 showed inequality between experiments for at least one comparison for each viability assay (I-IV).
- Highly significant differences ($P<0.001$) were found between the viability estimates (%) made by assays I, II, III and IV.
- Highly significant differences ($P<0.001$) were found between the viability assessment for populations A-E.
- Relationship between viability assessment methods (I-IV) and field populations (A-E) was found to be significantly different ($P<0.05$) in exp.1 and 2, and highly significantly different ($P<0.001$) in exp.3.
- Hatching in PRD and trehalose quantification assay mostly correctly detected samples containing non-viable nematodes. When viability was detected it was not higher than 1%.
- Hatching in PRD followed by Meldola's blue staining and Meldola's blue staining assay recognised viable eggs in heat-treated samples up to 46 and 81%, respectively.
- On average, partially resistant genotypes showed strong reduction in reproduction of cysts pot^{-1}, when compare with susceptible 'Desiree', by 98% for 'Innovator', 82% for 'P55/7' and 96% for 'Vales Everest'.
- When reported as number of eggs $cyst^{-1}$ the reproduction on 'Innovator', 'P55/7' and 'Vales Everest' was 48, 27 and 46%, respectively, lower than reproduction recorder on 'Desiree'.

- Reduction in multiplication for Lindley and Luffness populations (Pa2/3) was strongest on ‘Innovator’ closely followed by ‘Vales Everest’ and was also lower, relative to reproduction on ‘Desiree’, on partially resistant genotype ‘P55/7”.
- Population Pa1 (Pa1) reproduction was strongly reduced (99%) on both, ‘P55/7’ and ‘Vales Everest’ while no multiplication was recorded (100% reduction) on ‘Innovator’.
- Reproduction (cysts pot^{-1}) from the field populations was, with one exception, lowest on ‘Innovator’ closely followed by ‘Vales Everest’
- Across all field populations the weakest reduction in cysts pot^{-1} was observed on ‘P55/7’.
- Lindley population (Pa2/3) had the highest Pf/Pi ratio on ‘Desiree’ followed by ‘Innovator’, ‘P55/7’ and ‘Vales Everest’.
- Luffness (Pa2/3) also produced the highest Pf/Pi ratio on ‘Desiree’ but the next highest ratio was produced on ‘P55/7’ followed by ‘Vales Everest’ and ‘Innovator’.
- Population Pa1 (Pa1) had again highest Pf/Pi ratio on ‘Desiree’, which was followed by ‘Vales Everest’, ‘P55/7’ and ‘Innovator’.
- None of the field populations showed the pattern of Pf/Pi ratios distribution expected for pathotype Pa1.
- Based on Pf/Pi ratio only, pathotype Pa2/3 could be assigned to populations HAU164, 165, 167 and 298.
- Additional interpretation based on comparison to the results obtained for control populations suggested the pathotypes Pa2/3 could be also assigned to populations HAU152, 166, 351 and 356, although this interpretation should be treated with some dose of caution.
- Populations HAU178, with very high Pf/Pi ratios across all genotypes, could not be designated to *G. pallida* pathotype Pa1 or Pa2/3.

- The differential potato genotypes used in the glasshouse have a highly significant effect on reproduction of cysts ($P<0.001$), fecundity of reproduced cysts ($P<0.001$) and Pf/Pi ratio ($P<0.001$).
- A highly significant difference was found between *G. pallida* populations for cyst reproduction ($P<0.001$), the fecundity of reproduced cysts ($P<0.001$) and on Pf/Pi ratio ($P<0.001$).
- The interaction between potato genotypes and *G. pallida* populations was significant for reproduction of cysts ($P=0.03$) and highly significant for the fecundity of reproduced cysts ($P<0.001$) and Pf/Pi ratio ($P<0.001$).
- Low occurrence of numerous unique amplicons in the data set obtained from next-generation sequencing and, when possible, detection of sequences corresponding with mitotype only in control samples thwarted further analysed and the final conclusions could not be drawn.

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