Final Report

Investigating the Biology and Appropriate Control of *Dickeya* species Affecting GB Potatoes

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1. SUMMARY

Dickeya solani, within the genus *Dickeya* (previously known as *Erwinia chrysanthemi*), has emerged as a major threat to potato production in Europe and Israel. However, there is little practical information on its biology. This joint PCL-Scottish Government funded project (R437) follows on from a previous project on *D. solani* (SCR/927/09) to gain a greater understanding of the epidemiology of this pathogen and thus develop appropriate control strategies for implementation at the local and national level.

As part of the project, two new PCR-based diagnostics have been developed to detect and identify *D. solani*, one or both of which are now being used for statutory testing at SASA, Fera and NAK (Netherlands). They are also currently being evaluated for use throughout Europe as part of the Euphresco II project on soft rot and blackleg disease. Three further DNA-based methods for tracking different isolates of *D. solani* have been developed and their use investigated. However, as all isolates are highly similar these methods have some limitations in their ability to track outbreaks.

Independent data from both England/Wales and Scotland suggest that the movement of infected seed and not the environment is the principle route of spread of *D. solani*. No *Dickeya* infections have been found in Scotland since introducing legislation in 2010 and the numbers of non-Scottish origin seed is dwindling. Only very few waterways show contamination by the pathogen and, although in one case there is evidence of re-isolation over several years, the numbers isolated from water appear to be below that needed for disease to spread via irrigation. However, advice remains not to irrigate from such sources.

D. solani was able to spread from infected plants to neighbouring tubers at very low levels but there was no evidence that weeds were being contaminated, in contrast to findings in continental Europe where spread between potato plants and between potato and weeds is more efficient, perhaps due to differences in climatic conditions. Although no spread to or from weeds in the field / raised beds was observed under UK conditions, colonisation of some weeds and subsequent disease development in annual nettle was seen under glasshouse conditions.

There was little evidence for overwintering, although the pathogen was able to survive and in some cases increase slightly on stored tubers. The pathogen did not survive well on surfaces even in the presence of common materials, and standard disinfectants used at the correct concentration were able to control the pathogen on such surfaces. However, direct contact between healthy and rotting tubers was very likely to pass on contamination potentially leading to extensive disease development in the field.

Like *P. atrosepticum*, disease incidence caused by *D. solani* is related to the level of tuber contamination but seasonal conditions also have a major role. There was only slightly more disease initiated from 10 cell ml^{-1} for *D. solani* than for *P. atrosepticum* at 21°C (a temperature conducive to diseases development by both pathogens) and no statistically significant differences for more heavily contaminated tubers / stems.
D. solani caused up to 5 times, but typically 3 times, more tuber rotting at 27°C than at 21°C, with rotting at 21°C equivalent to that for *P. atrosepticum*. This would have major implications for disease in warmer seasons and could relate (although we have no data for it) to increased environmental spread in warmer countries.

Grower advice leaflets have been updated and information from this project disseminated on many occasions over the duration of the project. The project has also facilitated meetings of UK *Dickeya* researchers with others from across Europe and Israel with the aim of co-ordinating and maximising outputs from research across several other countries.

2. INTRODUCTION

A new species within the genus *Dickeya* (previously known as *Erwinia chrysanthemi*) has emerged as a major threat to potato production in Europe and Israel. The species is distinct from *Dickeya dianthicola* (*D. dianthicola*), which is known to have entered England and Wales on seed potatoes around 1990, and which has caused occasional losses under favourable environmental conditions since. Although the species name has yet to be formally accepted, *Dickeya solani* (*D. solani*) was proposed by researchers in the Netherlands and has been widely used (Tsror *et al*., 2009; Parkinson *et al*., 2009; **Note**: The formal description is currently 'in press' (van der Wolf *et al*., International Journal of Systematic and Evolutionary Microbiology doi:10.1099/ijs.0.052944-0) and the name will be validated in the near future). *D. solani* is highly aggressive on potato, especially under warm conditions, causing rapid wilting and blackleg-like symptoms when compared to *D. dianthicola* and the more common blackleg pathogen *Pectobacterium atrosepticum* (*P. atrosepticum*).

Direct losses to the Dutch seed potato industry, almost entirely due to *D. solani*, were reported to have reached €25m in 2007 due to downgrading and rejection of over 20% of stocks during certification. Losses in the Netherlands have since varied from season to season, being lower in cooler seasons. There are reports that *D. solani* has become the dominant cause of blackleg in the Netherlands (Czajkowski *et al.*, 2013). In addition to potato, *D. solani* has been isolated from field-grown flower bulbs (e.g. hyacinth), contributing to reported losses of €15m in 2007 to the Dutch bulb industry.

D. solani was first isolated on potato in England and Wales in 2007 and, in addition to the Netherlands, has also been found on potato crops in Belgium, Czech Republic, Finland, France, Georgia, Germany, Greece, Norway, Poland, Spain, Sweden and Switzerland. In 2009, all cases of *Dickeya* in ware potato crops sampled in England and Wales for testing at Fera were confirmed as *D. solani*. Over 20% wilting and rotting of progeny tubers before harvest were reported in some of these crops. All incidences were in crops originating from Dutch seed, some of which had been multiplied in England the season before. In Scotland in 2007, the pathogen was detected in irrigation water at one location in S.E. Scotland and has been detected from the same source every year since, with the exception of 2010. There have also been findings in commercial ware crops in Scotland in 2009 and 2010. In each case, seed had been multiplied once in England from seed of Dutch origin. In all cases the level of infection was low, never exceeding 0.1% of the crop, as determined by growing crop inspection. In addition, in 2009 *D. solani* was also detected at SASA on 3 potato varieties, grown from seed of Dutch and Belgian origin, during National List and independent variety trials. Since the last of these findings on non-Scottish origin ware and trial material in 2010, *D. solani* has not been found again on Scottish grown potatoes and to date, has never been found on Scottish-origin potatoes.

Following publication of the Potato Council review of *Dickeya* spp. affecting potatoes (Elphinstone and Toth, 2007), additional research on the status of *Dickeya* spp. in the UK, Europe and worldwide was funded by the Potato Council (project R290; 2007). In addition, previous Scottish Government-funded projects SCR/919/07 (*Dickeya*

dianthicola – a threat to Scottish seed potatoes; 2008-2011) and SCR/927/09 (Emerging threat to Scottish potato production posed by *Dickeya solani*; 2010-2011), involving the James Hutton Institute (then SCRI), SASA and Fera, sought to understand the biology, optimise testing methodologies and make recommendations on control measures for the genus. The latter 6 month project in particular, was restricted in its scope only focusing on 10 commercial crops in England and Wales and SASA's trial plots, in which infected National List candidate varieties from Belgium and the Netherlands were grown in 2009.

The preliminary research indicated that *D. solani* may be significantly more aggressive than *D. dianthicola* and *P. atrosepticum*. *D. solani* appears to be able to rapidly induce blackleg symptoms and also to rot developing progeny tubers, even when initial inoculum levels on the seed are low. Aggressiveness of *D. solani* appears to further increase at higher temperatures so there are implications for increased importance of this pathogen in response to global warming. However, at the start of this project (2010) there was little substantiated practical information on the biology of *D. solani* in relation to its host range, its ability to survive, establish and spread in the environment or its behaviour on stored potato tubers. As a result the objectives of this project were:

A. Refine, validate and apply diagnostic methods for specific detection and typing of *D. solani.*
B. Determine the extent of *D. solani* infection in the GB potato crop and evaluate the risks of spread to home-grown GB seed potatoes.
C. Assess the aggressiveness of a range of *D. solani* isolates in response to changes in temperature and humidity, and in comparison with earlier data obtained for *P. atrosepticum* and *D. dianthicola*.
D. Improve understanding of the epidemiology of *D. solani* infections and risks of pathogen establishment and spread following introduction of infected crops.
E. Refine specific recommendations for avoidance and control of this pathogen.

3. MATERIALS AND METHODS

Objective A: Refine, validate and apply diagnostic methods for specific detection and typing of *D. solani*.

3.1.1. Specific real-time PCR assay developed.

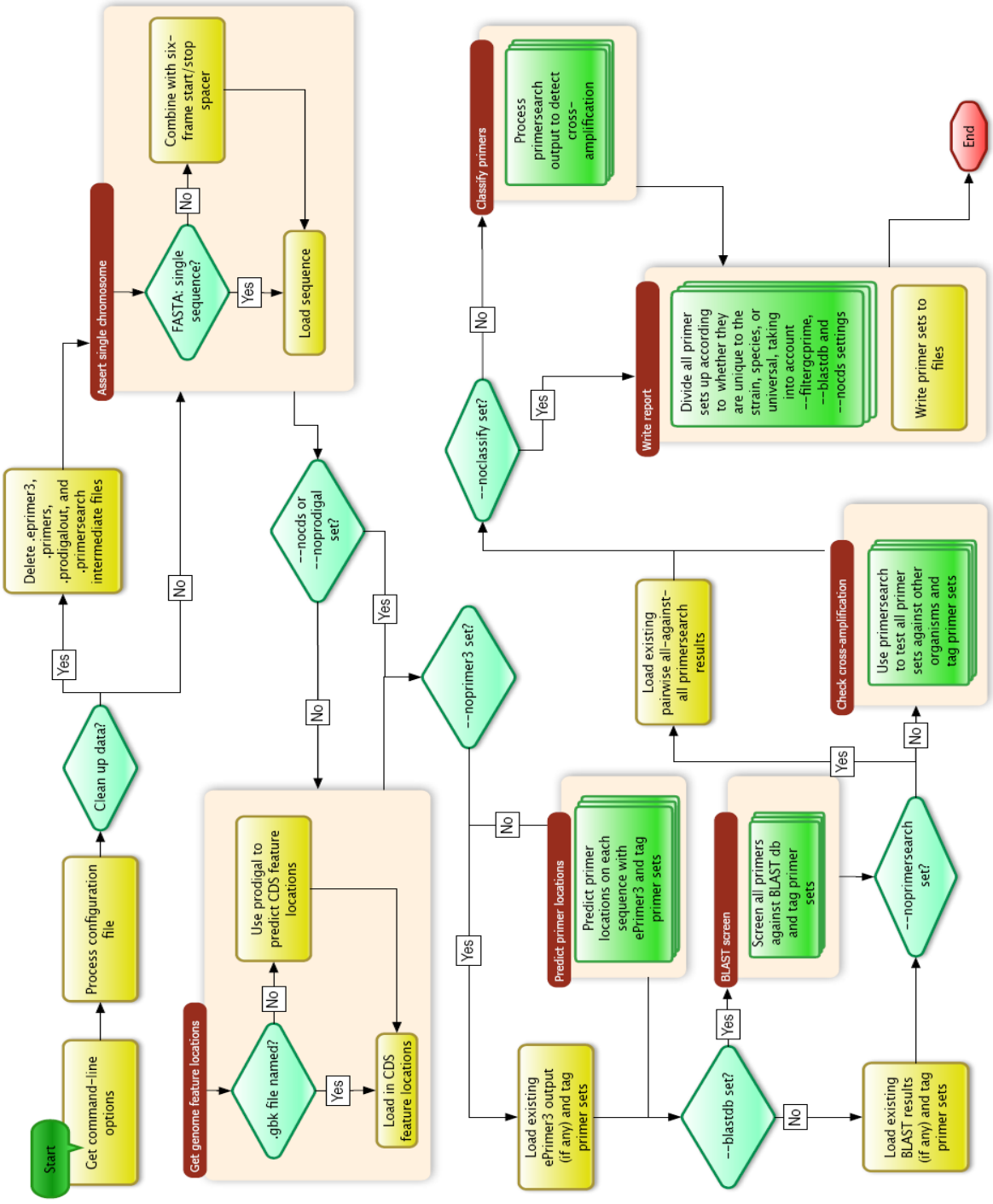
3.1.1.1. Design of D. solani species specific PCR primers using comparative genomics.

Considerable effort went into assembling pieces of sequence data for 4 *D. solani*, as well as 21 other *Dickeya* genomes at JHI. Once this information was available, a bioinformatics pipeline (Figure 1) was developed to identify 1000 PCR primer pairs and probes around the *D. solani* genome, before testing (computationally) the specificity of each primer and probe against each of the other genomes (Pritchard *et al.* 2013). Default parameters for real-time analysis were used for the pipeline (Figure 1), although such conditions can be altered according to desired testing protocols (Table 1). At the end of the analysis we were left with a list of primer pairs that matched sequences within the *D. solani* genome but were absent from all of the other genomes. These primers and probes were further validated in the laboratory at Fera by testing against a large panel of reference strains representing the known diversity of *Dickeya* spp. and related genera.

Table 1. Design parameters for flanking regions and internal probes of each primer set

Property	Amplification primers	Hybridisation probe
length	20bp (optimal)	13-30bp (range)
T_m	58-60°C (range) 59°C (optimal)	68-70°C (range)
GC%	30-80%	30-80%
Notes	No more than two G+C in last five nucleotides at 3` end	No G at 5` end
	Avoid runs of identical nucleotides	Avoid runs of identical nucleotides
	Fewer than four consecutive G bases	Fewer than four consecutive G bases
	Should not amplify any other enterobacteria	Avoid six or more consecutive A bases
		Avoid G at 3` end
		Avoid two or more CC dinucleotides in middle of probe
		Avoid G at position 2 at 5` end

Figure 1. Bioinformatic pipeline for primer predictions for *D. solani*.



3.1.1.2. Testing of selected real-time PCR primers and probes at Fera

From the comparative genomics study carried out at JHI, primer and probe sets were randomly selected from a total of 276 assays with predicted specificities to *D. solani* for amplification in real-time PCR using standard TaqMan conditions. Specificity was determined against an initial panel of 13 phylogenetically representative *Dickeya* strains. Selected assays demonstrating the required specificity were then further tested against a larger panel of 70 strains. A single selected assay for specific detection of *D. solani* (SOL-C) was then independently evaluated against a further panel of isolates and infected potato plants and tubers. Additional large scale validation has also been conducted independently using a large number of healthy and infected potato stocks at the NAK in the Netherlands.

3.1.1.3. Evaluating PCR assays for the detection of D. solani at SASA.

At SASA, an alternative approach to development of real-time PCR assays for specific detection of *D. solani* was followed. Primers and probes were designed by mining genes encompassed within a previously designed multi-locus sequence analysis (MLSA) system designed primarily to discriminate *D. dianthicola* strains amongst a panel of related species which also contained a number of *D. solani* isolates (Kowalewska *et al.*, 2010). An initial selection of gene targets was made by visually assessing each gene tree from the MLSA system and selecting genes that showed good separation of *D. solani* strains from all other *Dickeya* strains included in the study. In this way, the *fusA* gene was selected. The *fusA* sequences of 62 *Dickeya* and *Pectobacterium* strains were aligned using the ClustalW method and the MegAlign program in Lasergene v7.0.0 (DNASTAR Inc.). The TaqMan *D. solani* - specific probe DsolfusA-250T (TGAAAGCCATCAACTGGAATGATTC labelled with 6-FAM) was designed based on the 20 *D. solani* strains included in the original MLSA study. The flanking primer sequences are (5' to 3') DsolfusA-229F (GGT GTC GTT GAC CTG GTG AAA) and DsolfusA-300R (ATA GGT GAA GGT CAC ACC CTC ATC). Reaction mixtures were as described in Table 2. Master mix (24 µl) was added to each well of a 96-well plate (MicroAmp optical well plate with barcode) with an electronic pipette (Autorep) in a flow hood. 1µl of boiled cells (or 5µl of extracted DNA) was added to each well (1 µl of H_2O for negative controls). PCR was conducted on an Applied Biosystems 7900HT real time PCR machine using the conditions described in Table 3. The machine was run in standard mode, detecting FAM/TAMRA and using ROX as the passive reference.

Table 2. Components of PCR reaction mix used in the *fusA* real-time PCR assay.

Component	Volume
1x probe reaction mix	
Taqman master mix	12.5 µl
Forward primer (5 pmol)	1.5 µl
Reverse primer (5 pmol)	1.5 µl
Probe (5 pmol)	0.5 µl
Template	1 µl
H_2O (standard lab 18.2ΩM grade)	8 µl
TOTAL REACTION AMOUNT	25 µl

Table 3. Real-Time PCR protocol used in the *fusA* assay

Temp	Time	Cycles
95^0C	10 mins	x 1 cycle
95^0C	15 s	x 40 cycles
60^0C*	1 mins	

Data was taken at the extension (*) step only.

Two preliminary evaluation experiments were conducted using the *fusA* assay developed at SASA alongside SOL-C, the real-time assay designed at JHI and trialled at Fera, described above. In the first evaluation, a collection of 75 strains representing a wide diversity of the genus *Dickeya*, but with a strong emphasis of strains of *D. solani* was studied. The second evaluation was conducted with a European collection of strains, which are the core test set for the EUPHRESCO project.

3.1.1.4. Validation of specific real-time PCR assay at SASA

Using the primers and methodologies described above, an informal ring test was established with participating labs comprising: 1, AFBI, Belfast; 2, Fera, York; 3, JHI, Dundee; 4, NIAB, Cambridge; 5, SAC, Aberdeen and 6, Potato Council, Sutton Bridge. Details are given in Appendix 8.1. Blind spiked samples were sent out to all participants in the mini-ring test at the end of August 2011, along with the key reagents (primers, probes etc.) to perform the assays. To test the robustness of the new real-time PCR assays, participants used DNA extraction protocols, *Taq* polymerase enzyme, buffers etc. that were commonly used in each laboratory, rather than standardising reagents across all laboratories.

3.1.2. Variable number tandem repeat (VNTR) markers identified for typing of D. solani populations.

A draft genome sequence of *D. solani* strain IPO2222 (Pritchard *et al*., 2013) comprising 190 contigs, was used for identification of loci containing tandem repeat sequences using the DNAPrint programme at Fera, modified from 'mreps' (Kolpakov and Kucherov 2003). Five loci were selected and primers designed to flanking regions. Selected primer sequences, amplicon length, tandem repeat number and fluorochrome label are shown in Table 4. All loci were amplified using the following PCR conditions: Denaturation at 94^oC for 2 min, followed by 34 cycles of 94^oC for 30 sec, 60^oC for 30 sec and 72^oC for 30 sec and a final extension of 72^oC for 7 min. Amplicon sizes was determined by comparison with size standards (GeneScan™

350 ROX) separated using an ABI 3130 Genetic Analyser. Amplicons were diluted 1/60 and 1 µl was denatured in 8 µl formamide prior to loading onto the capillary column for size determination.

To investigate diversity within the 5 selected loci between isolates of *D. solani*, VNTR analysis was performed using the primers to amplify DNA purified from reference strains isolated from potato and hyacinth in Europe and Israel. VNTR profiles of each strain were then determined according to the relationship between their amplicon lengths and those from IPO2222.

Table 4. Tandom repeat sequences, flanking primers, amplicon lengths and number of repeats in *D. solani* IPO2222.

Locus 48	Repeated sequence	AATACA
	Number of repeats in IPO2222	9
	Amplicon length (bp) in IPO2222	124
	Forward primer	**00048FamF** GATGATTGTTACTTAGCATTCGACGAAGAATAG
	Reverse primer	**00048R** AAAGTCTTCACCAGAAACGAAGCTTATTC
Locus 82	Repeated sequence	GTTATAAATCGATA
	Number of repeats in IPO2222	5
	Amplicon length (bp) in IPO2222	141
	Forward primer	**00082FamF** CATACCCATTGTTGCGTCAGAGGAACG
	Reverse primer	**00082R** ATCGGTTTATAACCACAGGGTTATAACCATCG
Locus 94	Repeated sequence	CGCGAACCA
	Number of repeats in IPO2222	5
	Amplicon length (bp) in IPO2222	169
	Forward primer	**00094HexF** GAAACTATCGAAGCGGCATCCGATGAG
	Reverse primer	**00094R** GCTTATTACGGCGCTGTTCGTCACG
Locus 99	Repeated sequence	CGCTTTTGT
	Number of repeats in IPO2222	4
	Amplicon length (bp) in IPO2222	147
	Forward primer	**00099FamF** GCGTGTTCAGGCCGATATCCAACG
	Reverse primer	**00099R** CGAAGTTCGATCAGATCTACTACAGCTG
Locus 102	Repeated sequence	TTTCCTGTGATACATAG
	Number of repeats in IPO2222	6
	Amplicon length (bp) in IPO2222	200
	Forward primer	**00102HexF** ACTTCCAGCCTGACTTGCTGCGATAG
	Reverse primer	**00102R** GAGATTTATCACAGGAAACTATGCACCACAG

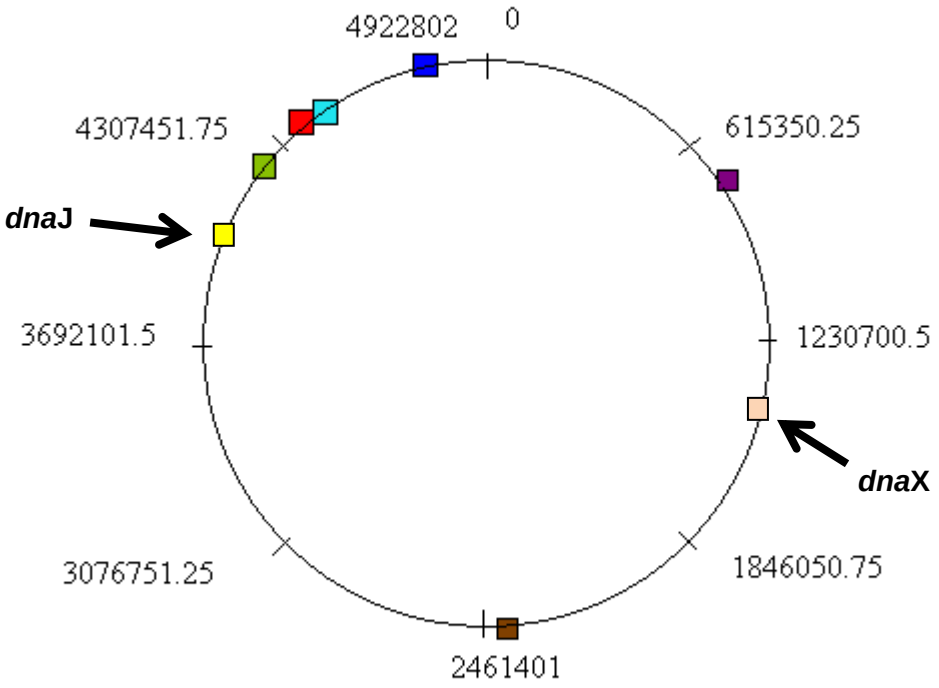
3.1.2.1. D. solani isolates fully typed by VNTR analysis*.*

Primers described above were used to amplify DNA purified from all *D. solani* isolates collected at Fera from potato and water survey samples during the 2010, 2011 and 2012 seasons. Amplicons were sized as described above. VNTR profiles of each strain were determined from the numbers of repeat sequences within each amplicon and compared with profiles obtained previously from the panel of reference strains.

3.1.3. MLSA system and web-based database.

The original MLSA system was based on 7 housekeeping genes, and this work was described previously in *Dickeya dianthicola* – A threat to Scottish Seed Potatoes Project Number: SCR/0919/07 (http://pubmlst.org/dickeya/). As part of the current project and to allow direct comparisons to be made with strains characterised in GB to those from other parts of Europe and the world, two additional genes have also been added to this system (*dna*J and *dna*X). Details of where these genes appear using the genome map of *D. dadantii* 3937 as a reference are given in Figure 2. Fragment sizes and primer sequences are given in Tables 5 & 6, respectively.

Figure 2. Location of marker genes used in the Dickeya MLSA scheme using the *Dickeya dadantii 3937* chromosome as a reference.



The r

The numbers of figure represent locations on chromosome (bp).

Table 5. Description of genes included in MLSA study

	Gene	Product	Left end	Right end	Gene Length (Fragment Length)
			bp		
		Genes Identified in previous study into *D. dianthicola* (SCR/927/09)			
	infB	translation initiation factor IF2	675726	678443	2718 (339)
	gapA	glyceraldehyde-3-phosphate dehydrogenase A	2315099	2316094	996 (399)
	purA	adenylosuccinate synthetase	4248437	4249735	1299 (336)
	rplB	LSU ribosomal protein L2p (L8e)	4389130	4389951	822 (336)
	fusA	protein chain elongation factor EF-G, GTP-binding	4394282	4396396	2115 (360)
	dnaN	DNA polymerase III, beta subunit	4849451	4850551	1101 (315)
	recA	Recombination protein *RecA*	3702951	3704027	1077 (525)
		Genes Identified in this Study			
	dnaJ	Chaperone protein *DnaJ*	4194484	4195617	1134 (258)
	dnaX	DNA polymerase III/DNA elongation factor III, tau and gamma subunits	1291469	1293487	2019 (276)

Table 6. Primers used for PCR reactions in the MLSA system

Gene	Forward primer	Reversed primer
dnaJ	AARAARGCKTAYAARGCKCTGGCGATGAA	CGGATCTCTTTVGTGACGCCRCG
dnaX	TATCAGGTYCTTGCCCGTAAGTGG	TCGACATCCARCGCYTTGAGATG

* - all primers shown in 5' – 3' orientation

Cultures were grown in Nutrient Broth overnight at 36˚C without shaking, and then 1ml was transferred into a sterile 1.5ml Eppendorf tube and centrifuged at 15,000g for 2 minutes. The supernatant was aspirated and discarded. DNA was extracted using a method adapted from Pastrik (2000) and the Invitrogen "Easy DNA" extraction kit.

Amplification of *dna*J and *dna*X was achieved using the reaction mixes and temperature programmes listed in Tables 7 – 10. Resultant, PCR products were purified by adding 0.5µl each of shrimp alkaline phosphatase (SAP) and exonuclease

I (EXO) to PCR reactions and incubating at 37°C for 45 min, followed by 80°C for 15 min then storing at 4°C, prior to sequencing.

Table 7. PCR reaction mix for *dna*X

Component	Volume per reaction (µl)
Sigma Jumpstart ReadyMix	10
Forward primer (10pmol/ µl)	1
Reverse primer (10pmol/ µl)	1
Sigma water	7
DNA	1
Total	**20**

Table 8. PCR temperature programme for *dna*X

Temperature	Time	
95˚C	5 min	35 cycles
94˚C	1 min	
59˚C	1 min	
72˚C	2 min	
72˚C	5 min	
4˚C	Cons.	

Table 9. PCR reaction mix for *dna*J

Ingredient	Volume per reaction (µl)
Sigma Jumpstart ReadyMix	12.5
Forward primer (10pmol/ µl)	0.5
Reverse primer (10pmol/ µl)	0.5
Sigma water	10.5
DNA	1
Total	**25**

Table 10. PCR temperature programme for *dna*J

Temperature	Time	
95˚C	5 min	
94˚C	30 sec	
60˚C	1 min	
72˚C	1 min	40 cycles
72˚C	7 min	
4˚C	Cons.	

Sequencing reactions were carried out either using the forward primer or the reverse primer, both in duplicate, using the reaction mix and temperature programme detailed in Tables 11 & 12. Sequence was generated using a ABI3130xl Genetic Analyzer (Applied Biosystems Inc; Foster City, CA, USA).

Table 11. Sequencing reaction mix

Component	Volume/reaction µl
ABI Big Dye 3.1 reaction mix	0.5
ABI Big Dye 5x buffer	1.75
Primer 10 µM (Forward OR reverse)	0.5
RNase Free Water	6.25
PCR product to be added	1
Final Volume	**10**

Table 12. Sequencing temperature programme

Temperature	Time	
96˚C	1 min	
96˚C	10 sec	
50˚C	5 sec	25 cycles
60˚C	4 min	
4˚C	Cons.	

The results were analyzed using 'Sequencing Analysis' (Applied Biosystems Inc; Foster City, CA, USA) software. The alignments were created using Lasergene-SeqMan Pro 11 (DNASTAR Inc., Madison, WI, USA), forward and reverse sequences were assembled and refined by eye to agree the consensus. The consensus sequences were saved as contig files and then copied to MEGA5

software (Centre for Evolutionary Functional Genomics, AZ, USA). The maximum likelihood trees for each gene were constructed via single linkage clustering, using 'tree function'. Bootstrap percentages were calculated with 500 replicates.

3.1.4. Single Nucleotide Polymorpism (SNPs)

In order to explore whether a higher level of resolution could be achieved in characterising *D. solani* strains a method based on detecting Single Nucleotide Polymorphism (SNPs) by pyrosequencing was explored. Individual SNP markers were identified by interrogating the genome of three *Dickeya* strains: MK10, MK16 and IPO2222 (described above), using the programme Mauve Multiple Genomic Alignment (Darling *et al.*, 2010). Twenty-one potential markers were identified and sequenced against the SASA isolates of MK10, MK16 and 2222 to rule out the possibility that sequencing errors from the original whole-genome analysis were the source of the possible SNPs detected by the Mauve analysis. From these 21 possible SNP sites, 8 markers were identified as 'true' SNPs and were trialled against a larger set of 11 *D. solani* isolates. Details of the primers used, designed through Pyromark Assay Design 2.0 (Qiagen) and the temperature programmes can be found in Tables 13 - 14. SNP reactions were performed on a Pyromark Q24 (Qiagen) following the manufacturer's protocols (Pyromark Q24 user manual, Qiagen).

Table 13. SNP PCR reaction mix

Component	Volume per reaction (µl)
Sigma Jumpstart ReadyMix	12.5
Forward primer (10pmol/ µl)	0.5
Reversed primer (10pmol/ µl)	0.5
Sigma water	9.5
DNA	2
Total	**25**

Table 14. SNP PCR cycles

Temperature	Time	
95˚C	15 min	45 cycles
94˚C	30 sec	
60˚C	30 sec	
72˚C	30 sec	
72˚C	10 min	
4˚C	Cons.	

Objective B: Determine the extent of *D. solani* infection in the GB potato crop and evaluate the risks of spread to home-grown GB seed potatoes.

3.1.5. England and Wales seed potato survey.

Surveys of approximately 800 seed potato stocks per year entered for classification in 2010, 2011 and 2012 were conducted by the Fera Plant Health and Seeds Inspectorate (PHSI) during growing crop inspections, and samples of infected plants were submitted to Fera for diagnosis from all stocks in which blackleg was observed. Soft rot bacteria were isolated on selective CVP-M medium and identified using specific PCR assays and according to fatty acid profile and partial *recA* gene sequence (Parkinson *et al*., 2009).

3.1.6. England and Wales river water survey.

River water samples (500 ml per sample) were collected by Fera PHSI during routine surveys for monitoring of the potato brown rot bacterium in September 2009, 2010, 2011 and 2012. A total of 162 samples in 2009, 287 samples in 2010, 200 samples in 2011 and 202 samples in 2012 were collected on up to 4 occasions per sampling point each year. Samples were tested for presence of soft rot bacteria by isolation onto CVP-M medium and isolates were identified as *Dickeya* using specific real-time PCR, fatty acid profiling and partial *recA* gene sequence.

3.1.7. Scotland growing crop survey.

The survey is designed to monitor growing crops of seed and ware in Scotland for the presence of *Dickeya* spp. It was established in support of new Scottish legislation launched in 2010, which established a “nil” tolerance for *Dickeya* infections in seed crops.

The survey is risk-based and targets crops which have the greatest risk of carrying or contracting the disease. As no crops grown from Scottish-origin seed have yet been found to be infected with *Dickeya* spp., the survey targets all seed and ware crops produced from non-Scottish origin seed (including farm saved seed from ware crops which had been grown from seed of non-Scottish origin), as well as potential close contact stocks - stocks grown on farms where there were positive findings in previous years. Also included were stocks grown near watercourses (previously) contaminated with *Dickeya* spp. All of these crops were inspected twice, as part of the routine Growing Crop Inspection, for plants showing a top-wilt of stems or classical blackleg symptoms. Stems of affected plants exhibiting symptoms were identified during field inspections, removed and wrapped in non-absorbent paper and delivered to SASA for testing. Diseased tissue was excised, suspended in Ringer’s Solution, allowed to stand for 10 min, then streaked onto crystal violet pectate modified (CVPM) agar and incubated at 36°C for 48 hours. *Dickeya* spp. were identified using PCR (Nassar *et al*., 1996), by selecting colonies that formed characteristic pitting in the media at 36°C, typical of *Dickeya* spp. The identity of *D. solani* and other *Dickeya* species was confirmed by *recA* sequencing (Parkinson *et al*., 2009; Kowalewska *et al.*, 2010).

In addition to the surveillance of the high risk stocks, a random survey of approximately 10% of Scottish-origin seed crops were also included in the study to ensure Scottish-origin seed remained free of the infection. These crops were selected from any SE or E crops which were being inspected as part of the routine

Growing Crop Inspection. Inspectors selected symptomatic stems as described above which were sent to SASA for testing.

3.1.8. Scottish post-harvest tuber survey.

This survey targeted all seed and ware produced from non-Scottish origin seed (including farm saved seed from ware of non-Scottish origin) each year, as well as potential close contact stocks - stocks grown on farms where there were positive findings in previous years. Representative tuber samples from the crop/stock were taken either from the field just prior to harvest or from store. Stocks were sampled at a rate of 200 or 3 x 200 tubers for Scottish and non-Scottish material respectively, where available. In cases of limited availability (i.e. some PB material) samples containing fewer tubers were submitted. Soft rot bacteria were isolated on CVP medium and identified using PCR followed by partial *recA* sequencing.

3.1.9. Scotland river water surveys.

All watercourses used to irrigate seed potato crops in the previous growing season were surveyed. Information on their location was obtained as part of the previous year's growing crop inspections. In addition, any watercourses that were found to be infested with *Dickeya* spp. in previous surveys were sampled each year. A watercourse in SE Scotland which was previously identified as being infested with *D. solani* was sampled multiple times each year, with sediment and representative weed samples also collected for testing. All water samples were collected in sterile bottles (250ml) from a range of sampling sites from each watercourse and delivered to SASA in cool boxes where they were processed within 24 hours. Samples were subdivided into aliquots of 40ml, clarified by centrifuging at a low speed (180g), and then 20ml of supernatant was mixed with an equal volume of Pectate Enrichment Medium (PEM; Meneley & Stanghellini, 1976) and incubated in an anaerobic chamber at 36^{o}C for 48 hours. Liquid cultures were then centrifuged at high speed (10,000*g*) to concentrate the bacterial fraction. Serial dilutions were prepared on the resuspended pellet prior to plating onto CVPM medium. All weed samples were first washed then crushed to expose the inner plant material and incubated in PEM for 48 hours at 36°C. The PEM was sieved and serial dilutions made down to 10^{-6}. One hundred microliters of each dilution was plated onto CVPM and incubated at 36°C for 48 hours. *Dickeya* spp. were identified using PCR (Nassar *et al*., 1996), by initially making a pre-selection of colonies that formed characteristic pitting in the media at 36°C, typical of *Dickeya* spp. The identity of *D. solani* and other *Dickeya* species was confirmed by *recA* sequencing (Kowalewska *et al.*, 2010).

Objective C: Assess the aggressiveness of a range of *D. solani* isolates in response to changes in temperature and humidity, and in comparison with earlier data obtained for *P. atrosepticum* and *D. dianthicola*.

3.1.10. Relative aggressiveness of D. solani determined in controlled environment studies.

3.1.10.1. Effect of inoculum level on tuber rotting

Bacterial strains were maintained on Luria Bertani (LB) agar (Miller 1972) and, when required, were grown overnight to log phase in 10 ml LB broth at 27°C with shaking. Bacterial cells were pelleted by centrifugation at 2880 x g for 10 min, washed in 10

mM $MgSO_4$ and resuspended in 10 ml of 10 mM $MgSO_4$. Whole potato tubers (cv. Maris Piper) were stab inoculated with *P. atrosepticum* SCRI1039 and *D. solani* IPO2222. The tubers were surface sterilised with a 5% sodium hypochlorite solution for 10 minutes, washed twice in SDW for 10 min and then left to air dry. Tubers were inoculated using a pipette tip with 10 µl of bacterial suspension at four different cell densities (equivalent to 10^4, 10^3, 10^2 or 10^1 CFU ml^{-1} per inoculation site). There were 20 inoculations for each bacterial strain at each cell density and the tubers were incubated in a damp box at 21°C for 6 days. Tubers inoculated with *D. solani* IPO2222 at the four cell densities were also incubated at 27°C for 4 days. *P. atrosepticum* SCRI1039 causes very little disease in tubers at a concentration of 10^4 CFU ml^{-1} when incubated at 27°C so was not included at this temperature. After incubation, disease was measured by weighing the tuber before and after scraping out the rot and the results expressed as 'weight of rot'.

Pathogenicity tests were also performed in potato stems (cv. *Estima*) by inoculating 20 replicate stems with *P. atrosepticum* SCRI1039 and *D. solani* IPO2222. Stems were inoculated with 10 µl of bacterial suspension at three different cell densities (equivalent to 10^3, 10^2 or 10^1 CFU ml^{-1} per inoculation site) at the lower junction of the stem and the second leaflet from the top of the plant. The inoculation site was wrapped with Nescofilm to seal the wound and the plants were well watered and kept in a controlled environment room at 21°C or 27°C for 14 days. Disease symptoms were assessed daily by measuring the length of blackleg lesions produced.

3.1.10.2. Effect of temperature on the amount of tuber rotting caused by different strains of D. solani

Tubers of cv. Maris Piper were inoculated as described above with *D. solani* IPO strains 2222, 2019, 2187, 3296, 3228, 2276, 3337, 3336, 3239, 3294, 3294, 3295 and 3494. Tubers were inoculated at a cell density of 10^4 CFU ml^{-1} per inoculation site and incubated at 27°C for 4 days and 21°C for 6 days. Initially, tubers were incubated at 21°C for 4 days. However, as little disease developed after this time period, tubers were incubated for a further 2 days to allow strain comparisons. Conversely, there would have been too much disease after 6 days at 27°C. While this made the different temperature difficult to compare in terms of absolute disease development, it clearly showed that the amount of disease caused was much higher for some strains at 27°C than at 21°C.

Objective D: Improve understanding of the epidemiology of *D. solani* infections and risks of pathogen establishment and spread following introduction of infected crops.

3.1.11. Potential of D. solani to establish on up to 10 weeds species determined in vitro*.*

3.1.11.1. Root binding

Ten plant species (a mixture of crops and common weeds) were tested for the ability of *D. solani* to bind to their roots (see below). Plants were removed from their pots and shaken gently to remove as much soil as possible. The roots were washed under running water to remove any remaining compost, rinsed in sterile distilled water and then detached from the plant and stored in 1 x phosphate buffered saline (PBS) until required. For each bacterium, 1g of roots was added to 10ml of a 10^8 CFU ml^{-1} bacterial suspension in a 50ml centrifuge tube. The roots were agitated gently using a flask shaker for 45 min and then rinsed 3 times in 20ml of fresh 1 x PBS by agitating vigorously for 5 min with a flask shaker. The roots were then ground to a fine slurry using a mortar and pestle with 2 ml PBS and a pinch of sterile sand and a serial dilution was prepared of the slurry down to 10^{-5} in PBS. From each of the 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions, 100µl was spread onto 2 crystal violet pectate (CVP) plates and incubated at 27°C for 48h. Any resulting colonies were counted.

Weeds and crops tested in experiment
Solanum lycopersicum (tomato)
Brassica oleracea (broccoli)
Viola arvesnsis (field pansy)
Solanum dulcamara (bittersweet)
Poa annua (annual meadow grass)
Capsicum annuum var. annuum (sweet pepper)
Solanum melongena (aubergine)
Sinapis alba (white mustard)
Spergula arvensis (corn spurry)
Chenopodium album (fat hen)

3.1.11.2. Root colonisation

Two weed species from the list above, bittersweet and meadow grass, were tested for the ability of *D. solani* to colonise their root systems for up to 28 days. Plants were left un-watered for 24hr to ensure the soil surrounding the roots was dry. The plants were removed from their pots and placed in a tray containing a *D. solani* suspension at a concentration of 10^7 CFU ml^{-1}, they were then left to soak up the inoculum for 1 hour before being replaced in their pots. The pots were placed on saucers and after 24hr were watered daily by pouring water into the saucers. After 1, 14 and 28 days the roots were tested for the presence of *D. solani* by removing the plants from their pots and shaking off any excess compost to leave only the adhering rhizosphere soil on the roots. The roots were then detached from the plant and 3 x 1.00g of roots was weighed out and ground to a fine slurry using a mortar and pestle with 2 ml PBS and a pinch of sterile sand. A serial dilution of the slurry was prepared down to 10^{-5} in PBS. From each of the 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions, 100µl was spread onto 2 CVP plates and incubated at 27°C for 48h. Any resulting colonies were counted.

3.1.12. Disease development and spread of D. solani in raised beds in Scotland.

Throughout these experiments *D. solani* MK13 (a strain recovered from an Israeli ware import into a Scottish packing plant) was used. The strain was grown from freezer beads stored at -80^{o}C onto Nutrient Agar (NA) and incubated at 36^{o}C for 48 hours. Cultures were re-isolated onto NA and incubated again at 36^{o}C for 24 hours. From plates (approximately 15 for each strain) colonies were added to Sigma water to produce an optical density at 600nm (OD_{600}) of 0.2 which equates to approximately 10^{8} CFU ml^{-1}. Suspensions were then diluted to a working concentration of 10^{5} CFU ml^{-1}. In this experiment the cultivar Nicola was selected as previous studies in Israel had shown that it was susceptible to infection by *D. solani* (Tsror *et al*., 2009). This experiment was repeated in each year of the project. However, in 2012 cv. Hermes was also included alongside cv. Nicola. Tubers were washed in tap water to remove excess dirt and allow access to the skin surface. These were then stored overnight in autoclave bags at a warm temperature (approximately 25^{o}C) to allow the lenticels to open. Following washing and drying overnight tubers were placed in net bags and vacuum infiltrated. Control tubers were processed first using distilled water. The tubers were exposed to water/bacterial suspension for 15 minutes at room temperature under vacuum then left overnight to dry. Tubers were planted by hand the following day as described below in separate raised beds in the quarantine testing facility at SASA (Figure 3). In the diagram 'I' denotes infected tubers and 'H' healthy. Infected Nicola potatoes were planted in close proximity to healthy Nicola potatoes to determine whether *D. solani can* be transmitted from the infected to the healthy tubers and also to test progeny tubers to determine whether the infection has passed from mother to daughter tubers.

These experiments were either conducted in beds of compost or in pots, to determine whether spread was by root-to-root contact or aerial splash, wind-blown etc. The experiment was carried out in 2010, 2011 and in 2012. In 2012 a separate bed was also included which contained cv. Hermes, using the same design as used with the cv. Nicola. All plants were watered heavily to ensure the growth media was kept moist throughout the growing season. Plants were harvested by hand and daughter tubers were tested individually from infected plants and daughter tubers were bulked from healthy plants.

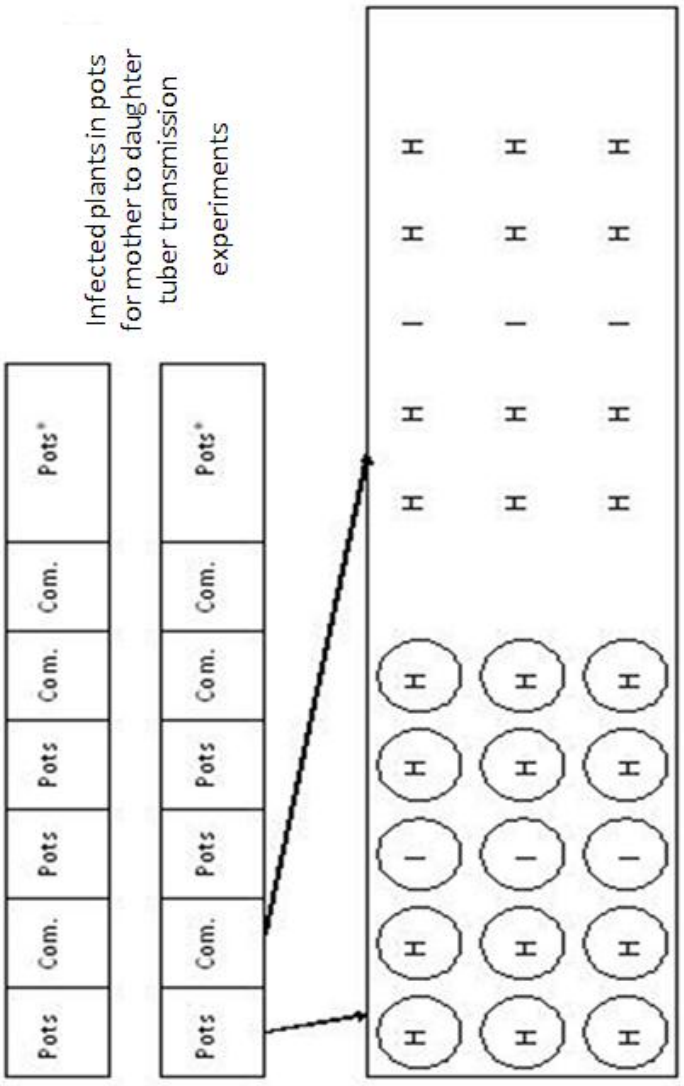


Figure 3. Infected plants in pots for mother to progeny tuber transmission experiments.

3.1.13. 3.14. Disease development and spread of D. solani in field observation plots in England.

3.1.13.1. Effect of seed tuber inoculum loading on disease incidence and potential for spread of D. solani.

Seed inoculated at SASA by vacuum infiltration in suspensions of *D. solani* MK13, as described above, was also provided to Fera for planting in field observation plots. An experiment was repeated in 2010, 2011 and 2012 to investigate the effect of inoculum concentration on disease incidence and potential for the pathogen to spread from inoculated to healthy plants in neighbouring rows. In each year, three replicated blocks of healthy potatoes (cv. Nicola) were planted in the first week of May. Within each block single rows were planted with seed inoculated in suspensions containing high (10^7 CFU ml^{-1}), medium (10^5 CFU ml^{-1}) and low (10^3 CFU ml^{-1}) populations of *D. solani*. Each inoculated row was bordered with double guard rows planted with uninoculated seed. In 2011 and 2012 supplementary overhead sprinkler irrigation was applied so as to provide weekly total inputs of 2.5

cm (including rainfall) in an attempt to maintain the soil moisture at seed tuber levels of at least 30%. No irrigation was applied in 2010. In 2012, the whole experiment was duplicated with a second potentially more susceptible variety (cv. Hermes). Blackleg plants were tested by real time PCR to determine the causal bacterium. The presence of *Dickeya* on progeny tubers harvested in the first week of September was also determined. DNA was purified from prepared potato peel samples taken from bulked samples of tubers from individual uninoculated plants and from individual tubers from inoculated plants. Pectolytic bacteria were enumerated using real-time PCR detection tests. These included a generic test for all pectolytic bacteria as well as specific tests for *D. solani* developed in this project.

3.1.13.2. Comparison of blackleg levels in different potato varieties after inoculation with D. solani or P. atrosepticum.

A second experiment was conducted in 2010 and 2011 to compare incidence of blackleg when different varieties were planted with seed inoculated either with *D. solani* or *P. atrosepticum*. Seed of the 10 most commonly grown potato cultivars was vacuum infiltrated in suspensions containing 10^5 CFU ml^{-1} of either *D. solani* MK13 or *P. atrosepticum* (NCPPB 549). Inoculated and uninoculated seed was planted in individual rows each of 6 plants. Supplementary irrigation was applied to achieve 2.5 cm total water per week to maintain soil moisture at or above 30% at the seed tuber level. Emergence and blackleg incidence were monitored weekly.

3.1.14. Evaluation of potential spread of D. solani from commercial potato crops grown in England.

In each of the 2010, 2011 and 2012 seasons, crops of ware or processing potatoes were identified in which over 20% blackleg symptoms caused by *D. solani* were detected during June of each year. Water from any field drains and nearby watercourses was sampled during the cropping season and after harvest in October/November and during the following spring. Replicated samples of 50 ml were sub-sampled and plated onto CVP medium before and after enrichment in double strength pectate medium for 48 hours at 27°C. Composite samples of predominant weeds and soil from 100 sites per field were also collected and tested as described above. In addition, potato tubers from the affected crops were sampled at harvest and after storage in the farm store and under controlled conditions at 8 °C at Fera. Tubers were tested in composite subsamples of 5 x 20 tubers after homogenising heel end vascular cores and strips of peel removed around the circumference of each tuber. DNA was extracted from the homogenate using the Promega Wizard Magnetic extraction kit for food and testing was performed using TaqMan real-time PCR tests.

3.1.15. Evaluation of weeds as a natural source of overwintering D. solani populations.

Naturally occurring weed populations were allowed to overwinter after harvest each year of the Fera observation plots in which potatoes inoculated with *D. solani* MK13 had been grown. Composite samples of the predominant weed species, each of 25 randomly selected plants, were then examined for *Dickeya* spp. in April. Stem base and rhizosphere samples were examined from three predominant annual weed species: speedwell (*Veronica* sp.), groundsel (*Senecio vulgaris*) and cleavers (*Galium aparine*). Rhizosphere soil (comprising root material shaken free of loosely

associated soil and homogenised in buffer) was inoculated into 10 ml of double strength pectate enrichment broth media and incubated for 48 h at 37°C after which aliquots were analysed using Taqman real time PCR tests. Stem-base samples (1 cm sections from 25 plants) were homogenised and plated onto CVP and PDA media.

3.1.16. Potential for survival of D. solani on artificially inoculated weeds and its capacity to contaminate potato plants and tubers.

3.1.16.1. Weed inoculation and root colonisation

Five replicate pots were set up with the following (25 pots total):

1. 4 week old seedlings from 6 weed species inoculated with *D. solani* IPO2222
2. Seeds from 6 weed species sown into pots inoculated with *D. solani* IPO2222
3. Soil only, no weeds or seeds inoculated with *D. solani* IPO2222
4. Control soil containing seeds from 6 weed species but not inoculated with *D. solani* IPO2222
5. Control soil containing 4 week old seedlings but not inoculated with *D. solani* IPO2222.

Seedlings were inoculated with *D. solani* IPO2222 as described previously. Soil was inoculated using 1 litre of a *D. solani* IPO2222 suspension at a concentration of 10^7 CFU ml^{-1}. Pots were placed at 19/10°C day time/night time temperatures in summer months with 16 hours daylight and 8 hours night with 80% RH (Scotland yearly average). All pots were kept water-logged for one week after inoculation with *D. solani* and were watered normally with water-logging once every 2-4 weeks as required. The day after inoculation, three weeds of each species were randomly sampled along with one core of soil from each pot with no weeds, to confirm whether *D. solani* had successfully colonised the roots and soil. Three weeks after inoculation a pathogen-free seed tuber was planted into the centre of all pots and the plants encouraged to grow and produce progeny tubers. All pots were sampled on 3 occasions during growth of the potatoes. At the end of the season the potato plants were observed for symptoms and once matured the progeny tubers were harvested and tested for the presence of *D. solani*.

Weeds used in experiment
Poa annua (annual meadow grass)
Avena fatua (wild oat)
Urtica urens (annual nettle)
Viola arvensis (field pansy)
Capsella bursa-pastoris (shepherd's purse)
Brassica napus (oil seed rape [commonly found as volunteers in fields])

To further investigate the capacity of *D. solani* on weeds to contaminate tubers and potato plants, a second experiment was set-up with 4 of the original weed species (annual nettle, field pansy, shepherd's purse and oil seed rape). Four week old seedlings of the 3 weed species were inoculated with *D. solani* IPO2222 as described above and the individual weed species planted in pots. There were five replicate pots for each weed species and 3 weeks after inoculation a pathogen-free seed tuber was planted into the centre of all pots. The experiment was carried out as before with the exception that only one weed species was present in any one experimental pot rather than having the weed species together in a single pot.

3.1.16.2. Sampling methods

Three random plants of each weed species were harvested 1, 2 and 3 months post inoculation during the growth of the potatoes. The roots were detached from the plant and 2 g of root was homogenised to a slurry with 1 x PBS. From each plant, 5 cm sections were cut from the stems just above (5 cm) ground level and homogenised to a slurry with 1 x PBS. A serial dilution of both the root and stem slurry was prepared down to 10^{-5} in PBS, and from each dilution, 100 µl was spread onto 2 CVP plates and incubated at 27°C for 48 h. Any resulting colonies were counted.

On three occasions a core of soil was removed from each of the five pots, pooled and homogenised to a slurry with 1 x PBS. A serial dilution of the slurry was prepared down to 10^{-5} in PBS and from dilution, 100 µl was spread onto 2 CVP plates and incubated at 27°C for 48 h. Any resulting colonies were counted.

From each potato plant, three 5 cm sections were cut from different stems just above (5 cm) ground level. The stem sections were homogenised in 1 x PBS and a serial dilution of the slurry was prepared down to 10^{-5} in PBS. From each dilution, 100 µl was spread onto 2 CVP plates and incubated at 27°C for 48 h, any resulting colonies were counted. In addition, 100 µl of slurry was added to 900 µl of single strength pectate enrichment medium (PEM) and incubated anaerobically 27°C for 2 days. Subsequently, a loopful of the PEM was streaked onto CVP plates and incubated at 27°C for 2 days. The pelleted slurry was kept for DNA extraction and testing with the *D. solani* qPCR primers developed within the project (see appendix 8.2 for details).

Resulting progeny tubers were peeled and the sap extracted from the peel using a Pollahne press. A serial dilution of the sap was prepared down to 10^{-3} in 1 x PBS, and from each dilution and the neat sap 100 µl was spread onto 2 CVP plates and incubated at 27°C for 48h. The sap was also enriched by adding 100 µl to 900 µl of single strength enrichment medium (PEM) and incubating anaerobically at 27°C for 2 days. Subsequently, a loopful of the PEM was streaked onto CVP plates and incubated at 27°C for 2 days. The sap was pelleted by centrifugation and kept for DNA extraction and testing with the *D. solani* qPCR primers developed within the project (see appendix 8.2 for details).

3.1.16.3. The effect of water-logging on the capacity of D. solani to spread from artificially inoculated weeds and to contaminate potato plants and tubers

The above experiment was repeated but on a smaller scale so that the pots could be submerged in water as in the previous experiments the size of the pots and the growth of the plants prevented the soil from being fully water-logged. Seedling roots of three weed species, *Urtica urens*, *Viola arvensis* and *Capsella bursa-pastoris* were inoculated with *D. solani* IPO2222 as described above and the individual weed species planted into smaller 10” pots. After 3 weeks, a pathogen free seed tuber was planted into the centre of all pots and once the potato plants had started to emerge the pots were submerged in boxes filled with water for 1 week. After a week of water-logging the pots were removed from the water for one week and this cycle continued for the duration of the experiment. Control pots were also set up containing un-inoculated weeds. Once matured the potato plants were sectioned into stem, roots, mother and progeny tubers and processed as described above.

3.1.16.4. The effect of inoculum level on root and systemic colonisation

Annual nettle (*Urtica urens*) and annual meadow grass (*Poa annua*) plants that had been left un-watered for 24hr were removed from their pots and placed in a tray containing a *D. solani* IPO2222 suspension at a concentration of 10^7 CFU ml^{-1}, 10^5 CFU ml^{-1} or 10^3 CFU ml^{-1}. Plants were then left to soak up the inoculum for 1 hour before being replaced in their pots. The pots were placed on saucers and after 24 h were watered daily by pouring water into the saucers. After 1 and 28 days the roots were tested for the presence of *D. solani* by removing the plants from their pots and shaking off any excess compost to leave only the adhering rhizosphere soil on the roots. The roots were then detached from the plant and 3 x 1 g of roots was weighed out and ground to a fine slurry using a mortar and pestle with 2 ml PBS and a pinch of sterile sand. A serial dilution of the slurry was prepared down to 10^{-5} in PBS. From each dilution, 100 µl was spread onto 2 CVP plates and incubated at 27°C for 48h. Any resulting colonies were counted. The sap was also pelleted by centrifugation and kept for DNA extraction and testing with the *D. solani* qPCR primers developed within the project. This experiment was repeated to verify the initial results.

3.1.16.5. Confocal microscopy of infected plants

Confocal microscopy was used to compare the invasion of annual nettle roots by bacteria with that of other weed species. In order to visualize the bacteria *in planta*, *D. solani* IPO2222 was transformed with a plasmid containing a green fluorescent protein reporter (pACYC-GFP). Seeds of annual nettle (*Urtica urens*) and meadow grass (P*oa annua*) were propagated on distilled water agar plates and left to grow for 3-4 weeks, after this time the seedlings were transplanted to hydroponic pots containing perlite and ½ x MS with no sucrose. The seedlings were left to grow for 4-5 weeks and then fluorescent dyes were added to the buffer in order to be taken up by the plants and stain the plant cells and mitochondria. The staining solution was removed after 24 hours and the plants were infected at the roots with *D. solani* IPO2222 + pACYC-GFP *(*10^7 CFU ml^{-1}) in fresh $MgSO_4$ buffer*.* The plants were incubated for 2 days with the bacteria and then images were taken of the intact roots.

3.1.16.6. Ability of rotting tubers/plants to contaminate weed species determined in glasshouse studies.

To investigate the ability of *D. solani* to spread from infected tubers/plants to contaminate weeds, tubers were infiltrated with a suspension of *D. solani* at 10^6 CFU ml^{-1}, left overnight to dry and then planted into large pots filled with compost. Tubers which had not been infiltrated were also planted into large pots as controls. To check the levels of *D. solani* infiltrated into the tubers, the peel from 5 tubers was removed, passed through a Polahne press and the resultant sap collected. The sap was diluted, plated onto CVP plates and the plates incubated at 27°C for 2 days. After this time, the colonies were counted to determine the level of *D.solani* in the peel of the infiltrated tubers. Once the potato plants had emerged (after approx 3 weeks), seeds from 4 weed species (*Urtica urens*, *Viola arvensis*, *Capsella bursa-pastoris* and *Brassica napus*) were sown in the pots. The pots were kept as wet as possible but without waterlogging and the weeds were tested for the presence of *D. solani* once a month.

3.1.16.7. Sampling methods

During growth of the potato plants, three random plants of each weed species were harvested 2, 3 and 4 months after sowing of the seeds. The roots were detached from the plant and 2 g of root was homogenised to a slurry with 1 x PBS. From each plant, 5 cm sections were cut from the stems just above (5 cm) ground level and homogenised to a slurry with 1 x PBS. A serial dilution of both the root and stem slurry was prepared down to 10^{-5} in PBS, and from dilution, 100 µl was spread onto 2 CVP plates and incubated at 27°C for 48 h. Any resulting colonies were counted. The sap was also pelleted by centrifugation and kept for DNA extraction and testing with the *D. solani* qPCR primers developed within the project (appendix 8.1).

3.1.17. The transmissibility and survival of D. solani on surfaces.

The aim of this experiment was to evaluate whether *D. solani* MK13, *D. dianthicola* 2260 (A5309) and *P. atrosepticum* NCPPB549 can survive on materials commonly used in potato grading and storage. Five materials (aluminium, hessian, rubber, steel & wood) were used to test whether the pathogens could survive.

These materials were cut to approximately 4 cm^2 and were autoclaved prior to use. A cell suspension of $1x10^8$ CFU ml^{-1} was made using sterile distilled water (SDW) and an overnight culture grown on nutrient agar. SDW was used as a negative control. The materials were incubated overnight in the cell suspension at 36°C (25°C for *P. atrosepticum*) and removed from the suspension and allowed to dry overnight. The materials were rinsed with 1ml SDW and incubated in pectate enrichment media (PEM) for 48 hours at 36°C (25°C for *P. atrosepticum*). The SDW that was used in the rinsing was also plated (100µl) onto CVPM and incubated for 48 hours at 36°C (25°C for *P. atrosepticum)*. After incubation, 100µl of the PEM suspension was plated onto CVPM and incubated at 36°C (25°C for *P. atrosepticum*) for 48 hours.

A revised method was also used with cell suspensions as previously described. However, PEM rather than SDW was used. After incubation for 48 hours at 36°C the materials were allowed to dry for 4 hours and then placed into PEM overnight at 36°C (or 25°C for *P. atrosepticum)*. The materials were not rinsed with SDW prior to incubation in PEM. 100µl of the PEM suspension was plated onto CVPM and incubated at 36°C (25°C for *P. atrosepticum)* for 48 hours. In addition, cell suspensions were also mixed into mashed-up potato material and then the paste was applied onto the materials and treated as described above.

3.1.18. Susceptibility of D. solani to common disinfectants.

Ten disinfectants (Fam30, GPC8, Halamid, Jet-5, Jeye's Fluid, Mikrozid AF, Sodium hypochlorite, V18, Vanoquat & Virkon S) commonly used in agriculture or the laboratory were tested for their effectiveness in controlling the growth of *D. solani* MK13, *D. dianthicola* 2260 (A5309) and *Pectobacterium atrosepticum* NCPPB549. A cell suspension of 10^8 cells ml^{-1} was made using an overnight culture. Various different concentrations were tested. Initially these were:

1. Lowest active range (LAR; i.e. recommended dilution for general use)
2. 50% dilution of LAR concentration
3. 25% dilution of LAR concentration

The disinfectants at the various dilutions were tested against the bacteria by adding 100 µl of suspension to 900 µl of the disinfectant solution. The mixture was vortexed

and left for the required incubation time (5, 10 or 30 minutes). Prior to the end of the contact time the suspension was vortexed again. The suspension was centrifuged at 13,000 rpm for one minute and the disinfectant (the supernatant) removed and 1 ml of sterile distilled water (SDW) added and vortexed to rinse the bacterial pellet. The solution was centrifuged again for 1 min at 13,000 rpm and the supernatant removed. The pellet was re-suspended in 1 ml of SDW and 100 µl of the sample plated out onto CVPM and incubated for 48 hours at 36^{o}C (25^{o}C for *P. atrosepticum*). Initial results showed no growth at the lowest active range (LAR), 50% LAR and 25% LAR. Further dilutions of 10% LAR, 5% LAR and 1% LAR also showed no growth. Dilutions of 0.5%, 0.4%, 0.3%, 0.2% and 0.1% LAR were chosen for comparison as some growth appeared at these concentrations. The disinfectants were also tested against *P. atrosepticum* and *D. dianthicola* at the lower concentrations.

4. RESULTS

Objective A: Refine, validate and apply diagnostic methods for specific detection and typing of *D. solani*

4.1.1. Specific real-time PCR assay developed.

4.1.1.1. Design of D. solani-specific PCR primers and probes using comparative genomics.

Following the comparative genomics primer design on three *D. solani* genome sequences, and comparing these primer pairs to 13 other *Dickeya* genome sequences, over 50 species-specific primer pairs were identified from each *D. solani* strain (Table 15). When these primer sequences were cross-checked against other members of this bacterial family (*Enterobacteriaceae* - which also contains *Pectobacterium*, *Escherichia coli* and *Salmonella*) this number reduced slightly to 36-41 primer pairs, suggesting that the original primer set contained a small number that would also amplify DNA from non-*Dickeya* strains. However, a large number of *D. solani* -specific primer pairs remained for laboratory testing and were sent to Fera for further analysis. No strain-specific primers were identified suggesting that the three *D. solani* strains studied were too similar to distinguish using this method.

Table 15. Primer pairs for both species- and strain-specific primer pairs for *D solani.* Figures in parentheses are primer pairs remaining after cross-checking with other closely related enterobacterial genomes.

Identifier	classification	Strain-specific	Species-specific
D. solani IPO2222	*solani*	0 (0)	57 (36)
D. solani MK16	*solani*	0 (0)	55 (41)
Dickeya unknown MK10	*solani*	0 (0)	57 (40)

4.1.1.2. Validation of selected real-time PCR primers and probes at Fera

Preliminary evaluation of randomly selected primer sets from a total of 276 assays with predicted specificities to different *Dickeya* species were used to identify candidates for real-time PCR development. Primers were selected that demonstrated

the required specificity and which generated a single amplicon in conventional PCR from template DNA purified from a small panel of 13 representative *Dickeya* strains. Predicted specificity to *D. solani* was confirmed in two (SOL-C and SOL-D) of five randomly selected primer sets. These were further evaluated under laboratory conditions against a larger panel of 70 *Dickeya* isolates selected as phylogenetically representative on the basis of *recA* sequence diversity (Table 16). Further independent evaluations were also made under laboratory conditions at SASA. Sensitivity and specificity of both assays to *D. solani* were confirmed with the exception of a single cross reaction with one isolate (NCPPB 3065) identified as *D. dadantii* according to *recA* sequence phylogeny. Specificity of SOL-C was independently confirmed at NAK in the Netherlands using a further 38 *Dickeya* and *Pectobacterium* isolates and 20 potato plants with blackleg symptoms and enriched extracts from 33 bulked tuber samples. Assays developed at PRI in the Netherlands lacked equivalent specificity in the same evaluation, leading to false positive results. The SOL-C assay will therefore be used in future routine testing in the Netherlands alongside other real-time PCR assays developed at Fera, including ECH with genus-specificity to *Dickeya* and PEC which detects all pectolytic *Dickeya* and *Pectobacterium* spp.

Table 16. Specificity of real time qPCR assays for *D. solani* (SOL_C and SOL-D) predicted using a bioinformatics pipeline tested on 70 *Dickeya* and related strains.

Test species	Isolates tested	*D. solani*-specific assay	
		SOL-C	SOL-D
D. dianthicola	7	0	0
D. solani (DUC-1)	16	16	16
DUC-2	5	0	0
DUC-3	1	0	0
D. dadantii	11	1	1
D. dieffenbachiae	6	0	0
D. chrysanthemi bv. *chrysanthemi*	7	0	0
D. chrysanthemi bv. *parthenii*	3	0	0
D. paradisiaca	1	0	0
D. zeae	11	0	0
New *Dickeya* species level clade (I)	1	0	0
New *Dickeya* species level clade (II)	1	0	0
Pectobacterium atrosepticum	1	0	0
P. carotovorum subsp. *carotovorum*	1	0	0
P. betavasculorum	1	0	0
P. carotovorum subsp. *odoriferum*	1	0	0
P. wasabiei	1	0	0
Pantoea agglomerans	1	0	0
Brenneria quercina	1	0	0
Erwinia amylovora	1	0	0

4.1.1.3. Evaluating additional PCR assays for the detection of D. solani at SASA.

A preliminary evaluation of the real-time PCR assays for the detection of *D. solani* was carried out. In all, 74 representative strains were tested against SASA-designed real-time PCR assays based on the *fusA* genes alongside the assay selected at Fera (SOL-C). The real-time assays (*fusA* & SOL-C) gave essentially similar results in that all *D. solani* strains studied (20/20) were detected. Only very few reference strains, MK2 & 1121 (2/54), from other *Dickeya* species gave false positive results (appendix 3, Table 17). The second evaluation of the EUPHRESCO strains showed that all *D. solani* strains studied (11/11) were detected, but only one, PRI 3328 (1/25) from other *Dickeya* species gave a false positive result (appendix 3, Table 18). These findings led to further evaluation of the assays involving a larger number of laboratories (see below).

4.1.1.4. Validation of specific real-time PCR assay completed.

Summaries of results from trialling the ECH, SOL-C and *fusA* real time PCR assays are shown in Tables 19-21 (see appendix 4), respectively. Results are presented in each case with and without enrichment prior to PCR. The ECH (and ADE) primers shown for some labs in Table 19 (in appendix 4) are designed to pick up all *Dickeya* strains whilst SOL-C and *fusA* are specific for *D. solani*. For the purposes of these experiments it was decided to set the cut-off for positive results for the qPCR at Ct 30; values below this reading we considered to be positive, above negative. In each case where unexpected results have occurred these are highlighted by prefixing the value with '***' (appendix 4, Tables 19, 20 & 21).

Looking at the results overall, there were 13 false positives from 760 assays. However, the number of false negatives was much higher at 168 overall. If all assays using the lowest cell concentrations (Rows 1 & 4 denoted by a 'L' in Tables 19, 20 & 21 - see appendix 4) were to be discounted this would account for 127 of all false negative results, leaving 41 false negatives in total. It is clear from these results that enrichment had minimal effect on boosting results, which may suggest that the concentration of cells was too low and could not be recovered sufficiently to facilitate an effective PCR reaction. It is also clear that boiling cells seems to give a more consistent set of results than extracting the DNA prior to PCR, though it should be stressed that DNA was not extracted by the same method in each laboratory so these methods cannot be universally discounted.

4.1.2. VNTR markers identified for typing of D. solani populations.

A highly discriminatory method was developed to type isolates of *D. solani* using variable number tandem repeat (VNTR) analysis in 5 selected loci. PCR amplification of these loci in a number of reference isolates of *D. solani* collected from around Europe was successfully performed using primers selected from the draft genome sequence of *D. solani* reference isolate IPO2222. The number of repeats per locus for each isolate was then determined from the amplicon lengths obtained after PCR in comparison with the expected amplicon lengths from IPO2222. The results (Table 22) indicated that all strains are highly similar with identical sequence in 4 out of the 5 selected loci. Slight sequence variation in one locus (48) identified 3 VNTR profiles amongst the *D. solani* reference isolates. The largest group (with profile 1) contained potato and hyacinth isolates from the Netherlands and potato isolates from England/Wales, France, Israel and Poland. Profile 2

contained potato isolates from Finland and Israel. Profile 3 was unique to the reference isolate IPO2222 from the Netherlands, currently proposed as the type strain of *D. solani*. Dutch researchers have requested further collaboration in this area so that the methods can be applied to a wider range of isolates from potato and ornamental hosts.

Table 22. Variable number tanden repeat (VNTR) analysis, indicating high levels of similarity within 3 profiles identified amongst reference isolates of *D. solani* from different sources around Europe and Israel.

Reference isolates	Date isolated	Origin	Host	Number of repeats per locus					VNTR profile
				48	99	82	94	102	
IPO 3337		France	potato	7	4	5	5	6	1
IPO 3239	2007	England/Wales	potato	7	4	5	5	6	1
IPO 2019		Netherlands	hyacinth	7	4	5	5	6	1
IPO 2187 (G87)	2006	Israel ex Netherlands	potato	7	4	5	5	6	1
IPO 2276	2005	Poland	potato	7	4	5	5	6	1
IPO 3228 (IMP J52)	2008 (d)	Israel ex Germany	potato	7	4	5	5	6	1
IPO 3296 (G298)	2008	Israel ex Germany	potato	8	4	5	5	6	2
IPO 3294		Finland	potato	8	4	5	5	6	2
IPO 3295		Finland	potato	8	4	5	5	6	2
IPO 2222	2007	Netherlands	potato	9	4	5	5	6	3

4.1.2.1. D. solani isolates fully typed by VNTR analysis.

All isolates of *D. solani* collected at Fera during 2010-2012 from blackleg plants submitted for commercial diagnosis or as part of the annual blackleg survey were typed by VNTR analysis (Table 23). Two isolates from surface water in England were also typed. All isolates were highly clonally related, falling into the same three VNTR profiles identified amongst the previously analysed reference isolates from around Europe and Israel. As previously, profile 3 was the least prevalent, being found only in a single potato sample in 2010. Most of the potato isolates types as either profile 1 or 2 and both isolates from river water typed as profile 2.

Table 23: VNTR profiles of *D. solani* isolates collected in England and Wales from infected potatoes or river water (2010-2012).

Year	Source of isolates	Total no. isolates collected	No. isolates VNTR profile 1	No. isolates VNTR profile 2	No. isolates VNTR profile 3
2010	Potato	14	9	4	1
	River water	1	0	1	0
2011	Potato	23	16	7	0
	River water	0	0	0	0
2012	Potato	5	1	4	0
	River water	1	0	1	0

4.1.3. MLSA system and web-based database.

The web-based MLST system has been established at (http://pubmlst.org/dickeya/), the original database being supplemented with sequence information from both *dnaJ* and *dnaX*. Although both genes have been used previously by other groups to characterise *Dickeya*, *Pectobacterium* or other members of the family, neither gene is entirely suitable for inclusion in an MLSA analysis as this form of characterisation works best when there are no gaps in sequence data. Unfortunately both genes show extensive evidence of insertion sequences when comparing strains making it difficult to identify a stretch which is found consistently in all strains. In the end the usable fragment sizes for both genes was relatively short with only 258 useable nucleotides available for *dnaJ* and 276 for *dnaX*. Both gene trees are shown in Figures 4 & 5, and the combined concatenated tree is in Figure 6. As with the previous analyses all *D. solani* strains were found to be identical and clearly distinct from other members of the genus.

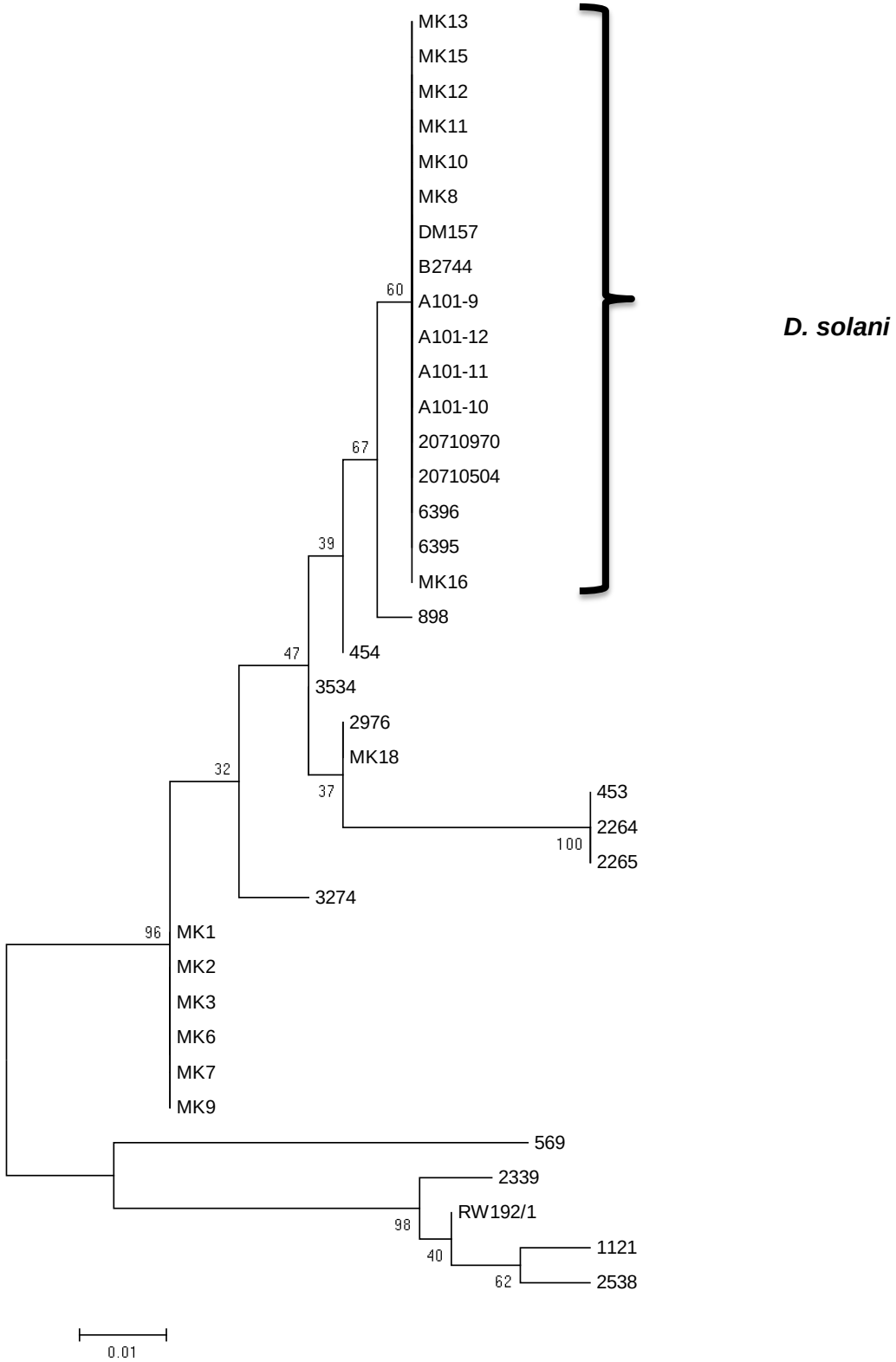


Figure 4. Maximum likelihood tree of *dnaJ* sequence data. Bootstrap analysis was conducted with 500 replications.

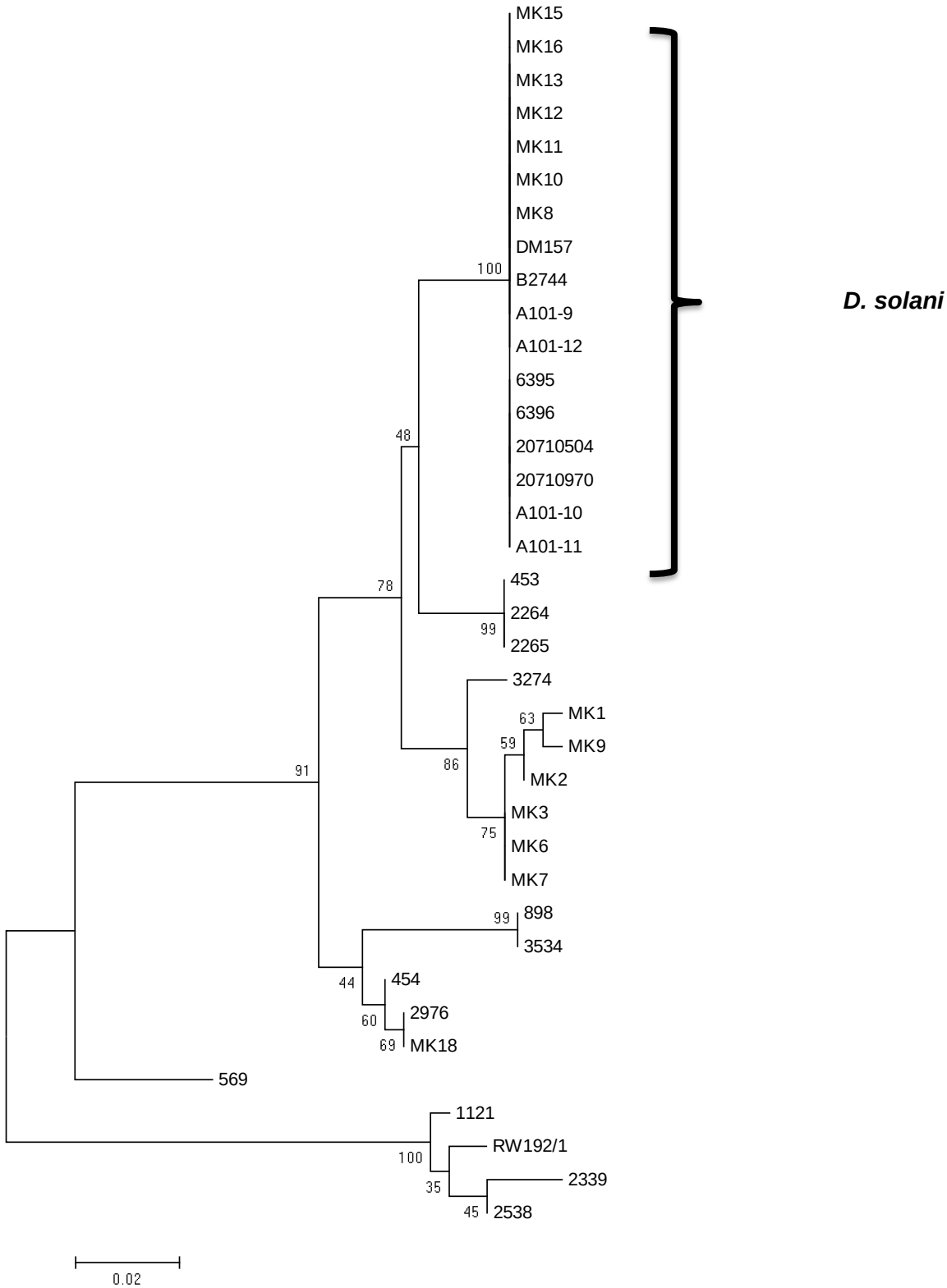


Figure 5. Maximum likelihood tree of *dnaX* sequence data. Bootstrap analysis was conducted with 500 replications.

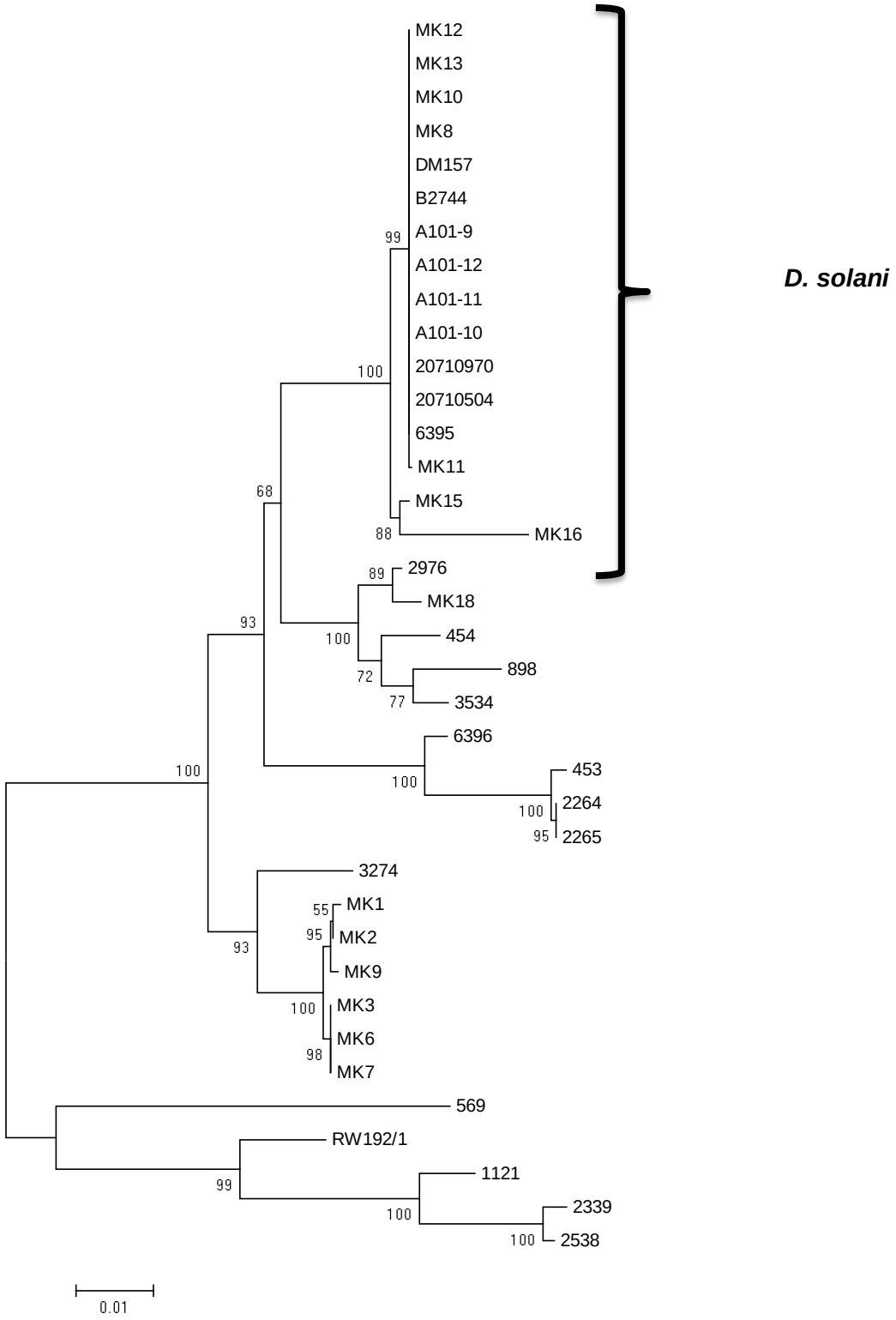


Figure 6. Maximum likelihood tree of concatenated sequence data (3144bp). Bootstrap analysis was conducted with 500 replications.

4.1.4. SNP Analysis

Eight SNP markers were used to characterise 14 strains of *D. solani*. In total 6 SNP profiles were recognised from these strains, the full results of which are presented in Table 24. The location of the polymorphisms is shown in the adjacent Table 25. The biggest grouping recovered, Group 1, was made up of MK11, MK14, MK15, MK16, A101-9, A101-10 and A101-11 all strains giving an identical profile. This profile group encompasses strains isolated from an Israeli ware import, three strains all isolated from the infested river in SE Scotland and a group of strains all isolated from Polish potatoes. The second largest group, Group 2, contains and B2745, DM157 and DM159, encompassed a group made up of an isolate recovered from a Belgian variety undergoing trialling at SASA and two isolates both of which were recovered from an infected ware crop of cv. Agria grown in Scotland, produced from once-grown English, Dutch-origin seed. The other strains studied here; IPO2222, MK10, B1 and B2744 all produced unique profiles and were recovered respectively from a Dutch potato (IPO2222 is the proposed type strain for the species), an Israeli ware import, a Spanish ware import and a Dutch variety undergoing trialling at SASA. It is encouraging to note that all strains isolated from the infested river in SE Scotland are identical possibly suggesting that the infestation may have arisen from a single contamination event.

Table 24. Presence of individual SNPs in *D. solani* isolates identified using Pyrosequencing

Strains/SNPs	117		118		172		311		446		523		799		834	
	a	b	a	b	A	b	a	b	a	b	a	b	a	b	a	b
MK10	1*	0	1	0	1	0	1	0	0	1	1	0	1	0	1	0
MK16	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1
2222	0	1	1	0	0	1	1	0	1	0	0	1	1?	0	0	1
MK11	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1
MK14	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1
MK15	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1
A101-9	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1
A101-10	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1
A101-11	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1
B1	0	1	0	1	1	0	0	1	0	1	1	0	0	1	0	1
B2744	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	1
B2745	0	1	0	1	0	1	1	0	1	0	0	1	1	0	0	1
DM157	0	1	0	1	0	1	1	0	1	0	0	1	1	0	0	1
DM159	0	1	0	1	0	1	1	0	1	0	0	1	1	0	0	1

Group 1 strains	
Group 2 strains	

*, 1 indicates SNP present; 0 indicates no SNP present.

Table 25. Identification of differences of individual SNPs

SNP	Sequence
117a	GTG**A**GCCTT
117b	GTG**G**GCCTT
118a	ATG**C**AAG
118b	ATG**A**AAG
172a	C**G**CGCAGT
172b	C**A**CGCAGT
311a	GATATT**G**TCT
311b	GATATT**A**TCT
446a	GGA**A**CGAG
446b	GGA**G**CGAG
523a	GGG**G**CCG
523b	GGG**A**CCG
799a	AAATGGAAG**C**CTA
799b	AAATGGAAG**T**CTA
834a	GCCGGATTT**A**C
834b	GCCGGATTT**G**C

Objective B: Determine the extent of *D. solani* infection in the GB potato crop and evaluate the risks of spread to home-grown GB seed potatoes.

4.1.5. England and Wales seed potato survey.

A summary of the results of an annual survey of seed potato stocks, conducted by the Fera Plant Health and Seeds Inspectorate is shown in Table 26. Approximately 800 seed stocks per year were each inspected twice for seed classification purposes. Blackleg was recorded in 257 (32.1%), 172 (21.5%) and 270 (33.8%) of these stocks in 2010, 2011 and 2012 respectively. *D. solani* was identified as causing blackleg in only 18 (2.3%), 4 (0.5%) and 5 (0.6%) of the total number of stocks inspected over the 3 years, compared with 193 (24.1%), 128 (16.0%) and 227 (28.4%) of the stocks in which blackleg caused by *Pectobacterium atrosepticum* was detected. *D. dianthicola* was also identified as causing blackleg in single stocks (0.1%) in 2010 and 2011 and in 5 stocks (0.6%) in 2012. In cases where the causal agent was not identified as *P. atrosepticum*, *D. solani* or *D. dianthicola*, it is not clear whether the original cause was not isolated or whether other *Pectobacterium* or *Dickeya* species may have been involved.

Table 26. Blackleg findings in seed potato stocks entered for classification in England and Wales (2010-2012).

	2010	**2011**	**2012**
% seed stocks with blackleg	32.1	21.5	33.8
% blackleg caused by *D. solani*	7.0	2.3	1.8
% blackleg caused by *D. dianthicola*	0.4	0.6	1.8
% blackleg caused by *P. atrosepticum*	75.2	74.4	84.1

All stocks in which *D. solani* was detected had been grown from seed of Netherlands origin (either directly or following multiplication in England and Wales in previous seasons) with the exception of one stock in 2012 which was third generation from seed of German origin. All *D. dianthicola* infected stocks had been grown from seed imported directly from the Netherlands. There were no findings of *D. solani* or *D. dianthicola* in seed stocks of GB origin in any of the three years. However, almost all blackleg caused by *Pectobacterium atrosepticum* was found in seed of GB origin.

The last 2 years of this survey were funded separately from this project, and additional details have been reported directly to the Potato Council in January 2012 and 2013 (http://potato.org.uk/sites/default/files/%5Bcurrentpage%3Aarg%3A%3F%5D/20123%20Dickeya%20Survey%20R454.pdf).

4.1.6. England and Wales river water survey.

Samples of river water collected in September each year by the Fera PHSI were tested for *Dickeya* spp. from 2009 to 2012. The results are summarised in Table 27.

Table 27: Detection of *Dickeya* spp. in samples of river water in England and Wales (2009-2012)

	2009	2010	2011	2012
No. samples tested	162	287	200	202
No. samples containing pectolytic bacteria	125	123	140	120
No. samples containing *Dickeya solani*	0	1	0	1
No. samples containing *Dickeya dianthicola*	0	2	0	3
No. samples containing *Dickeya zeae*	7	0	5	3
No. samples containing '*Dickeya aquatica*'	3	0	6	0

Although pectolytic bacteria were readily detected in between 42.1 and 77.9% of the samples each year, findings of *Dickeya* spp. were much less frequent. Nevertheless, *D. solani* was detected in single watercourses in 2010 and 2012, in the River Meese in Shropshire and in the River Ouse in Sussex respectively. *Dickeya dianthicola* was also detected in 2010 in Crown Lakes in Cambridgeshire and the River Avon in Devon and in 2012 also in the River Ouse in Sussex and Pilling Water in Lancashire. Although sampling mainly targeted different rivers each year, rivers in which *D. solani* was found were also intensively re-sampled both up- and down-stream in the following year. No further positive findings were found in the R. Meese in 2011, or in the River Ouse up until June of 2013. Further intensive sampling is planned for September 2013 in the River Ouse.

In addition to *D. solani* and *D. dianthicola*, a further two *Dickeya* species were identified in a small number of watercourses. According to *recA* sequence analysis (Parkinson *et al*., 2009) isolates of *D. zeae* belonged to *D. zeae* Phylotype II with the exception of a single isolate of *D. zeae* Phylotype I (Figure 7). The latter of these was the same genotype as found in river water in Scotland. The other *Dickeya* sp. was previously unknown and has been proposed as '*D. aquatica'*. All isolates of '*D. aquatica'* shared identical *recA* sequence homology which was also shared with similar isolates from river water in Finland. Isolates of both *D. zeae* and '*D aquatica*' were shown to readily rot potato tubers after injection into the cortex at 22 °C, but were unable to induce typical blackleg symptoms when inoculated directly into potato stems or when vacuum infiltrated into seed tubers before planting in pot experiments.

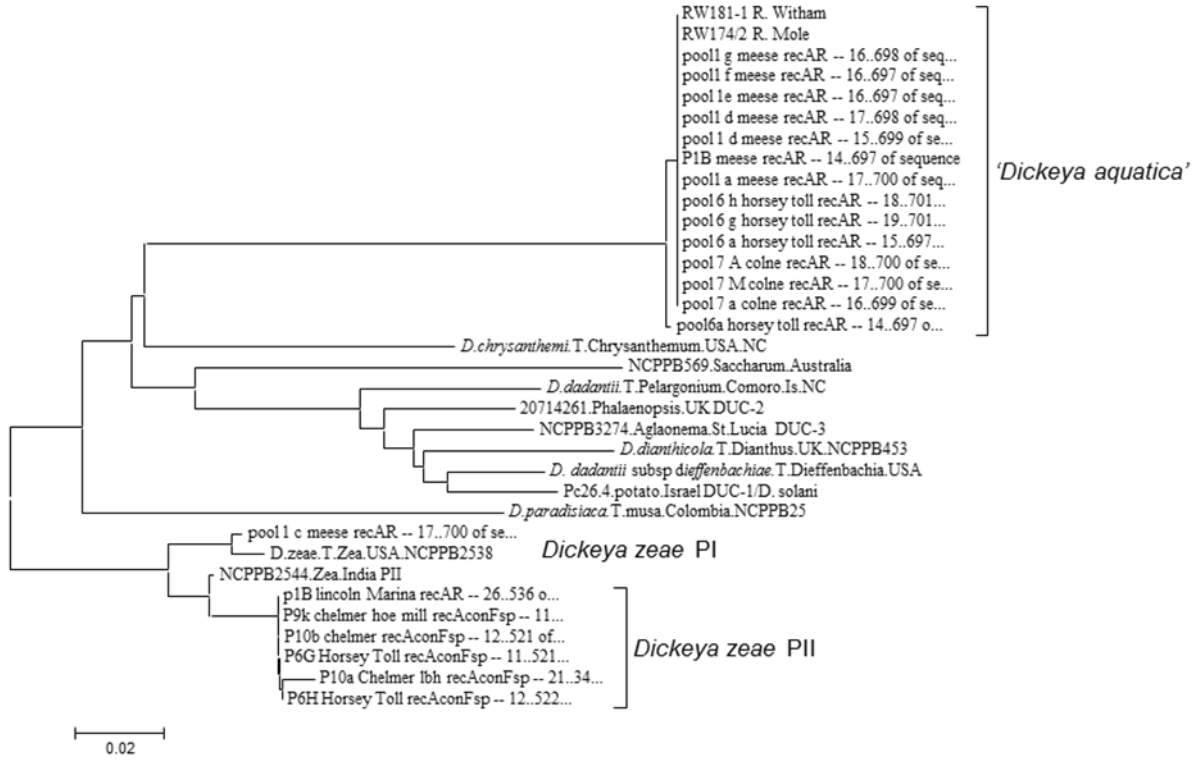


Figure 7. Phylogenetic tree based on *recA* sequence similarity showing identification of *Dickeya* isolates from river water as *D. zeae* and a new species proposed as '*D. aquatica*'. Reference type strains of *Dickeya* species are designated T.

4.1.7. Scotland growing crop survey.

The results from the *Dickeya* Growing Crop Inspection surveys for Scotland from 2010 – 2013 are shown in Table 28. In total 2,789 crops were sampled and tested, the only positive results obtained from this survey were found in 2010. That year, 9 ware crops all grown from Dutch origin seed were found in the survey encompassing 545 seed and ware crops (Table 28). All were subsequently identified as *D. solani* by *rec*A sequencing. Details of the geographic location of these crops, and the cultivars affected are given in Table 29. In all, three cultivars were affected, cvs Challenger, Innovator and Vivaldi. In subsequent years, the number of crops surveyed was 752, 821 and 671, respectively for 2011, 2012 and 2013. A number of samples of groundkeepers collected from fields which had previously been used to cultivate infected crops in 2009 and 2010 were also included. None of these samples tested positive for *Dickeya* spp.

Table 28. Number of crops sampled and positive findings from the Scottish growing Crop Inspection Survey 2010-2013

Year	Number of crops studied	Number of positive findings
2010	545	9
2011	752 +7 groundkeepers from 2009 + 2010	0 0
2012	821 +11 groundkeepers from 2009 + 2010	0 0
2013	671 +6 groundkeepers from 2009 + 2010	0 0

Table 29. Details of positive *Dickeya* findings in the 2010 *Dickeya* Growing Crop Inspection survey for Scotland survey.

SAMPLE NO	DATE SAMPLE TAKEN	DATE RECEIVED AT SASA	DATE PROCESSED	VARIETY	PARISH	PRELIMINARY POSITIVE RESULT	FINAL CONFIRMATORY RESULT	DATE NOTICE SERVED TO GROWER
DM573	04/08/10	20/08/10	20/08/10	Innovator	Fern	**+VE 24/08/10**	31/08/10	01/09/10
DM47	07/07/10	08/07/10	08/07/10*	Vivaldi	Carnbee	+ve 19/07/10	21/07/10	26/07/10
DM78†	08/07/10	09/07/10	09/07/10	Vivaldi	Eassie and Nevay	**+VE 15/07/10**	21/07/10	26/07/10
DM410	29/07/10	30/07/10	30/07/10	Challenger	Falkirk	+ve 11/08/10	31/08/10	01/09/10
DM84	08/07/10	09/07/10	09/07/10	Innovator	Meigle	+ve 15/07/12	21/07/10	26/07/10
DM87	08/07/10	09/07/10	09/07/10	Innovator	Meigle	+ve 21/07/10	23//07/10	26/07/10
DM46	07/07/10	08/07/10	12/07/10 *	Vivaldi	Scoonie	+ve 18/07/10	21/07/10	26/07/10
DM128	12/07/10	13/07/10	13/07/10	Innovator	St Martins	+ve 19/07/10	21/07/10	26/07/10
DM433	02/08/10	03/08/10	03/08/10	Innovator	Panbride	+ve 11/08/10	25/08/10	01/09/10

* SAMPLES UNABLE TO BE TESTED IMMEDIATELY – frozen
†, ALSO TESTED AS DM439 (SAMPLE TAKEN ON 04/08/10 AND PRELIMINARY POSITIVE RESULT ON 17/08/10, WITH FINAL CONFIRMATORY RESULT ON 25/08/10)

4.1.8. Scottish post-harvest tuber survey.

The *Dickeya* post-harvest tuber surveys for Scotland from 2010 – 2012 are shown in Table 30. In summary, a total of 1036 stocks of seed and ware were sampled and tested, including 201 stocks of non-Scottish origin. All were found free of infection.

Table 30. Number of stocks sampled and positive findings from the Scottish tuber Survey 2010-2013

Year	Number of crops studied (non-Scottish origin)	Number of positive findings
2010/2011	355 (71) (3 seed + 19 ware)	0
	+ 50 rotted tubers	0
2011/2012	321 (63) (1 seed + 4 ware)	0
	+ 51 rotted tubers	0
2012/2013	360 (67) (0 seed +0 ware)	0
	+7 rotted tubers	0

4.1.9. Scotland river water surveys.

Results from the *Dickeya* river survey for Scotland for 2010-2013 are shown in Table 31. A total of 314 separate watercourses were sampled over the three/four year period, encompassing 504 sampling sites. No new *Dickeya* infections were found during these surveys. However, a watercourse in SE of Scotland remained infested with *D. solani* during this period as did a river in NE Scotland which remains infested with *D. zeae*. A third river, previously identified as being infested with an unknown *Dickeya* sp. (DUC-3) in 2006 was found to be free from *Dickeya* throughout the survey period, possibly as a result of changes in the operation of a domestic sewage plant which emptied into the river and which was thought to be the source of the infestation. In both cases where a *Dickeya* infestation was detected local growers were advised either in writing or face-to-face that they should not use these sources for irrigating potato crops.

Table 31. Number of rivers*, sampling sites and positive findings from the Scottish river water survey 2010-2012

Year	Number of Watercourses* (sampling points)	Number of new positive rivers (identity and location)
2010	85 (170)	0
2011	81 (114)	0
2012	71 (107)	0
2013	77 (113)	0

*, Not including 3 rivers which were previously identified in Scotland as being infested with *Dickeya* ssp.

The river in SE Scotland was also intensively surveyed initially with the help of colleagues from the Centre for Ecology and Hydrology in 2010. From this it was evident that this small stretch of waterway had been heavily modified in the past and was now a straightened, over-deepened trapezoid channel, with steep banks and low riparian weed diversity. Weeds which were growing in or near the water were sampled multiple times. Species studied include; *Solanum dulcamara*, *Urtica dioica* (common nettle), *Impatiens glandulifera*

(Himalayan balsam) and *Epilobium* sp. (willow herb). No *Dickeya* species were ever isolated from these weeds.

Across the entire length of the watercourse there is little slope to the channel, resulting in low flow and high sedimentation rates. *Lemna minor (*Common duckweed), a non-rooting floating plant, was growing in the channel due to the near static flow. The low flow resulted in high levels of sediment retention. It was therefore considered possible that infected potato tissue, if washed into the channel, could form part of the organic rich sediments and remain in the channel for extended periods, even under high flows as flow refugia were present. No *Dickeya* species were found even after repeated sampling attempts.

Objective C: Assess the aggressiveness of a range of *D. solani* isolates in response to changes in temperature and humidity, and in comparison with earlier data obtained for *P. atrosepticum* and *D. dianthicola*.

4.1.10. Relative aggressiveness of D. solani determined in controlled environment studies.

4.1.10.1. Effect of inoculum level on tuber rotting

In tubers incubated at 21°C for 6 days, *D. solani* IPO2222 produced greater, but not significant, levels of disease than *P. atrosepticum* 1039 at 10^4 CFU ml^{-1}, approx. 4g and 5.5g of rot, respectively.. However, at 10^3 CFU ml^{-1} *D. solani* IPO2222 and *P. atrosepticum* SCRI1039 exhibited similar levels of disease with both strains producing just under 2g of rot (see Figure 8). At 10^1 and 10^2 CFU ml^{-1}, rotting was much reduced for both strains over the 6 day period. At 27°C, only *D. solani* IPO2222 was investigated at the four inoculum densities, where a large amount of rotting was observed at both 10^4 and 10^3 CFU ml^{-1} (approx. 14g and 7g respectively - see Figure 9).

In stems over 14 days at 21°C, *D. solani* IPO2222 and *P. atrosepticum* SCRI1039 showed similar levels of disease at 10^2 and 10^3 CFU ml^{-1} (Figure 10). However, at 10^1 CFU ml^{-1}, *D. solani* IPO2222 was significantly more aggressive than *P. atrosepticum* SCRI1039 ($P<0.05$). At 27°C, *D. solani* IPO2222, showed greater levels of disease than *P. atrosepticum* SCRI1039 at all 3 inoculum densities (Figure 11).

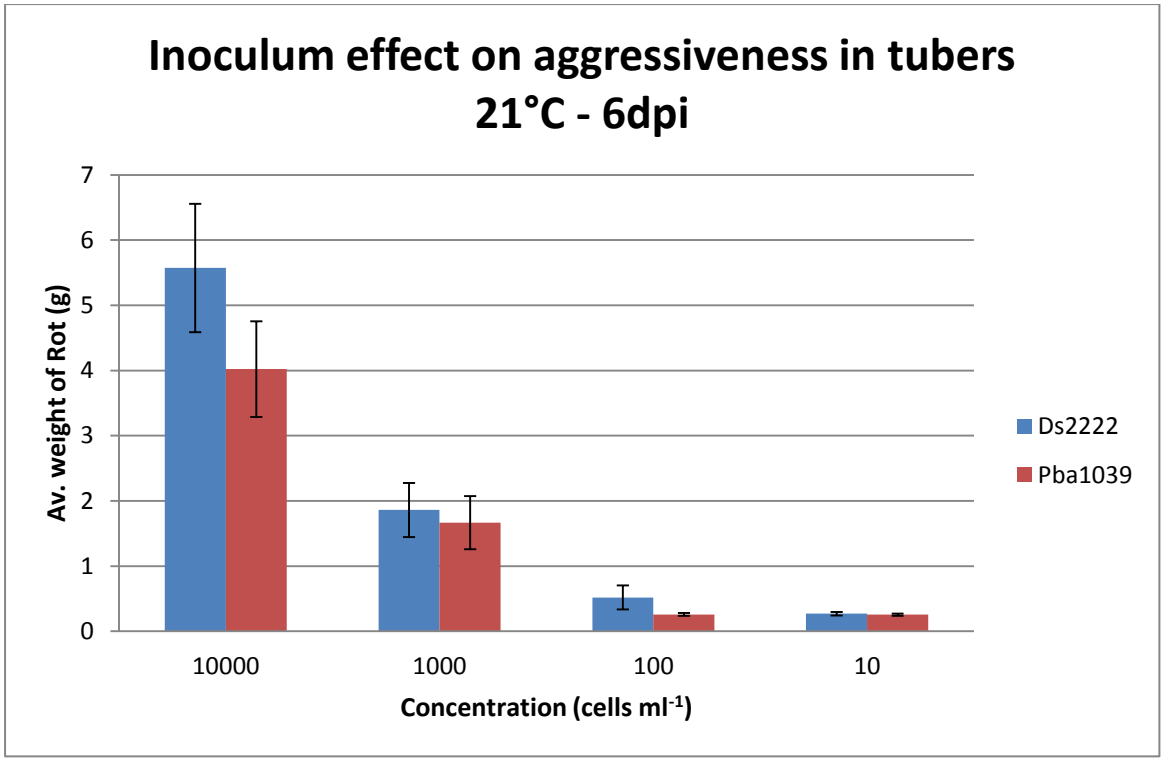


Figure 8. Effect of inoculum level on the aggressiveness of *P. atrosepticum* SCRI1039 and *D. solani* IPO2222 at 21°C in tubers. Results are expressed as average weight of rot from 20 inoculations after 6 days +/- SEM.

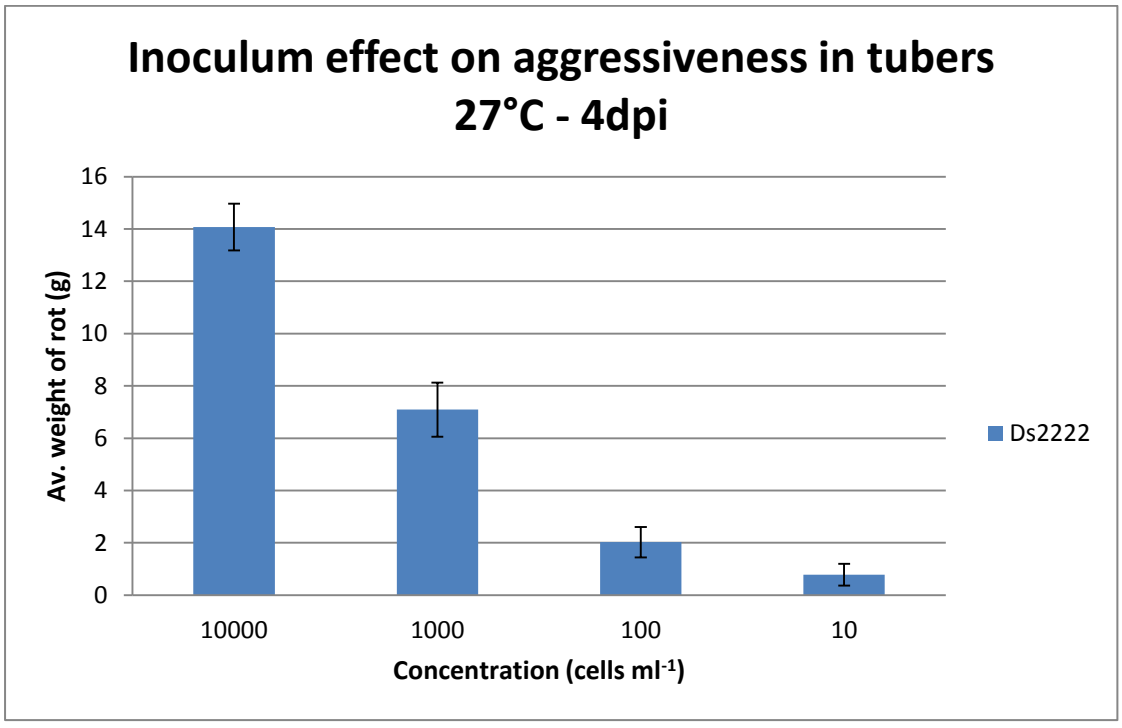


Figure 9. Effect of inoculum level on the aggressiveness of *D. solani* IPO2222 at 27°C in tubers. Results are expressed as average weight of rot from 20 inoculations after 4 days +/- SEM.

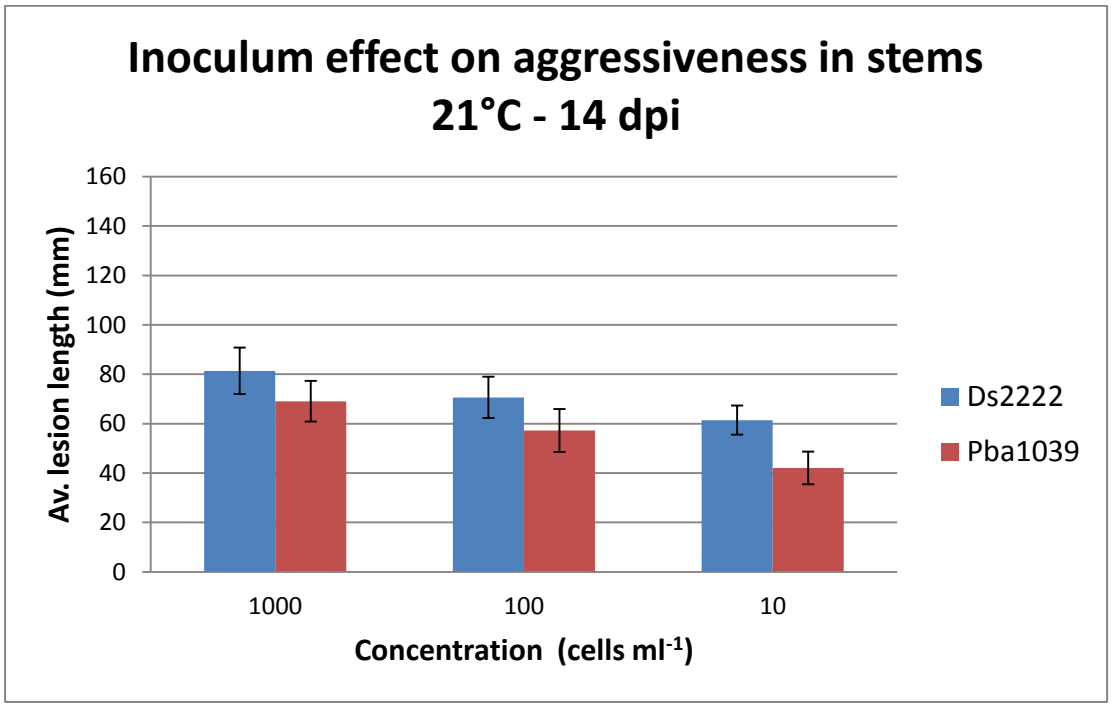


Figure 10. Effect of inoculum level on the aggressiveness of *P. atrosepticum* SCRI1039 and *D. solani* IPO2222 at 21°C in potato stems. Results are expressed as average lesion length from 20 inoculations after 14 days +/- SEM.

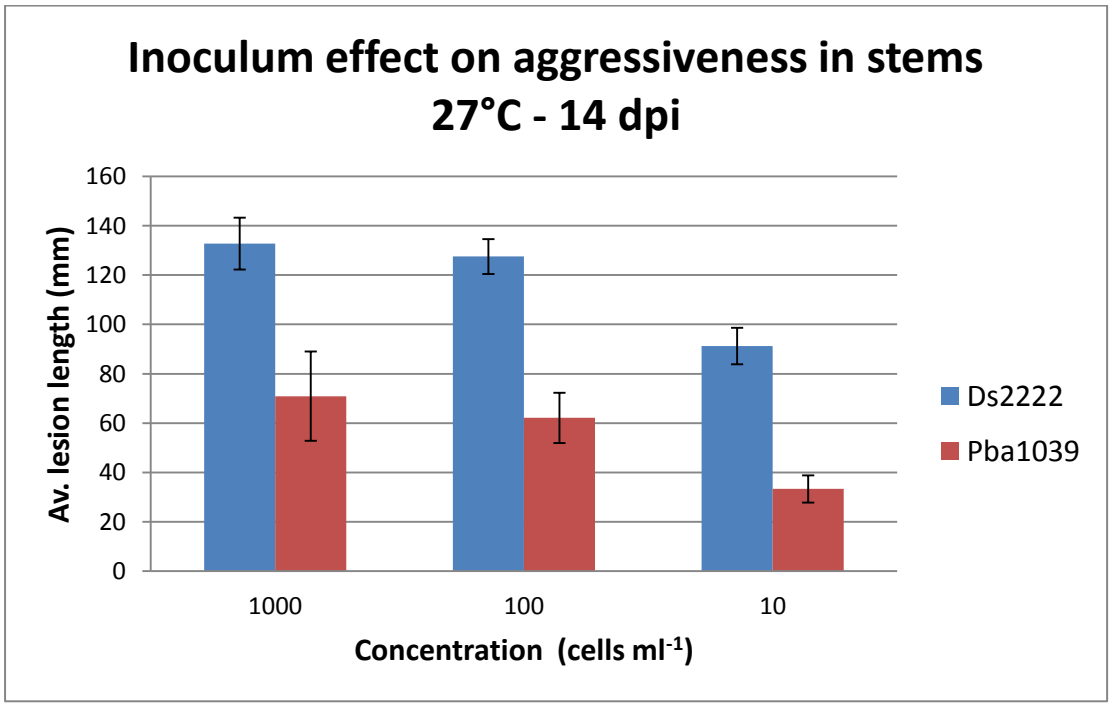


Figure 11. Effect of inoculum level on the aggressiveness of *P. atrosepticum* SCRI1039 and *D. solani* IPO2222 at 27°C in potato stems. Results are expressed as average lesion length from 20 inoculations after 14 days +/- SEM.

4.1.10.2. Effect of temperature on the amount of tuber rotting caused by different strains of D. solani

Ten of the twelve strains of *D. solani* tested were significantly more aggressive at 27°C than at 21° C (Figure 12), but were still capable of producing large amounts of rot at 21°C. The other two strains tested, *D. solani* IPO3296 and *D. solani* IPO3228 were considerably less aggressive at 27°C and produced very little disease at 21° C (Figure 12). The reasons for this difference in aggressiveness require further investigation. Typing techniques have been used to allocate some of the strains studied to 3 different VNTR profiles (see Table 22). There was no consistent trend for isolates with a particular VNTR profile to cause more or less disease at either temperature.

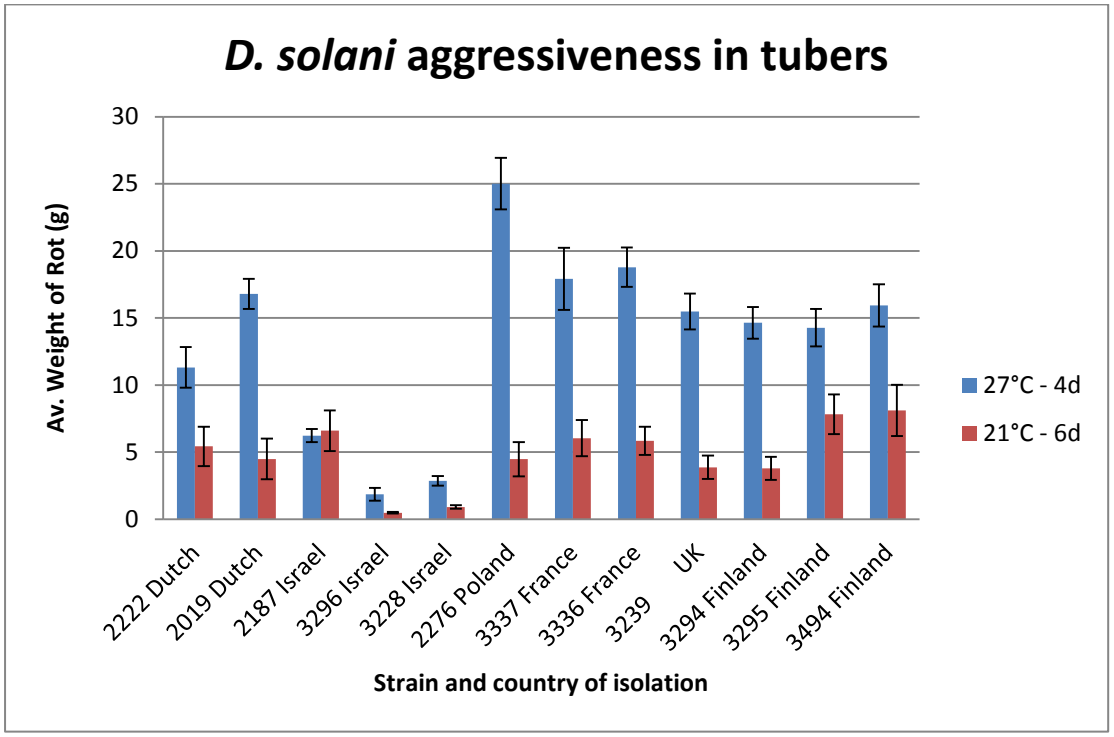


Figure 12. Comparison of the level of disease caused by various *D. solani* strains at 27°C for 4 days and 21°C for 6 days. Results are expressed as average weight of rot from 20 inoculations +/- SEM.

Objective D: Improve understanding of the epidemiology of *D. solani* infections and risks of pathogen establishment and spread following introduction of infected crops

4.1.11. Potential of D. solani to establish on up to 10 weeds species determined in vitro.

4.1.11.1. Root binding.

Ten plant species (a mixture of crops and common weeds) were tested for the ability of *D. solani* 2222 to bind to their roots using standard protocols developed at JHI. The results demonstrated that *D. solani* can bind at high levels to the roots of a wide variety of different plant species after 45 minutes of exposure to the pathogen (Figure 13).

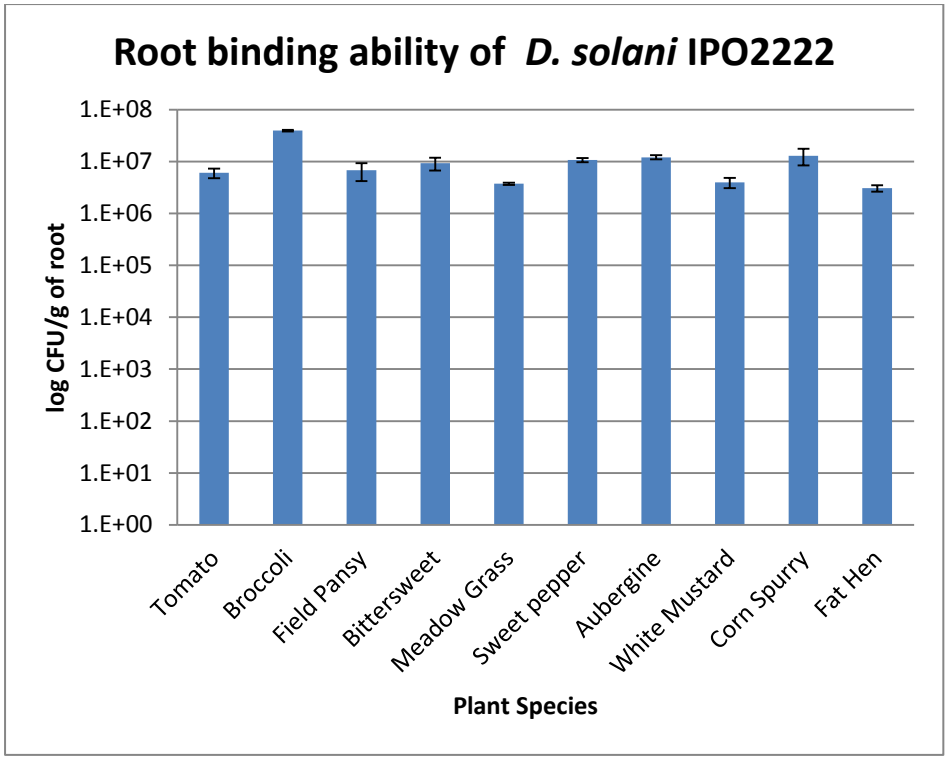


Figure 13. The ability of *D. solani* to bind to crops and weeds measured after 45 minutes exposure to 10^8 CFU/ml/g root.

4.1.11.2. Root colonisation

To investigate the potential for *D. solani* to establish on weed species further, we looked at the ability of the bacterium to colonise the root systems of two common weed species, bittersweet (*Solanum dulcamara)* and meadow grass (*Poa annua)*, for up to 28 days. The results revealed that after 14 days *D. solani* survived in the rhizosphere of both weed species at similar densities to the initial inoculum level (Figure 14). However, at day 28 we could only determine the numbers of *D. solani* cells colonising meadow grass, which were around 10^4 CFU ml^{-1} (Figure 14). This was due to the production of a bacterial film rather than discrete colonies produced on the agar medium of all the dilutions from bittersweet and on some of the dilution plates of meadow grass. This anomaly was also observed, but to a lesser extent, with some colonies recovered during the binding experiment. The reason for this film production is being investigated as it may be relevant to colonisation and/or aggressiveness in *D. solani*. These preliminary results suggest that *D. solani* may be capable of colonisation and survival on the roots of some weed species.

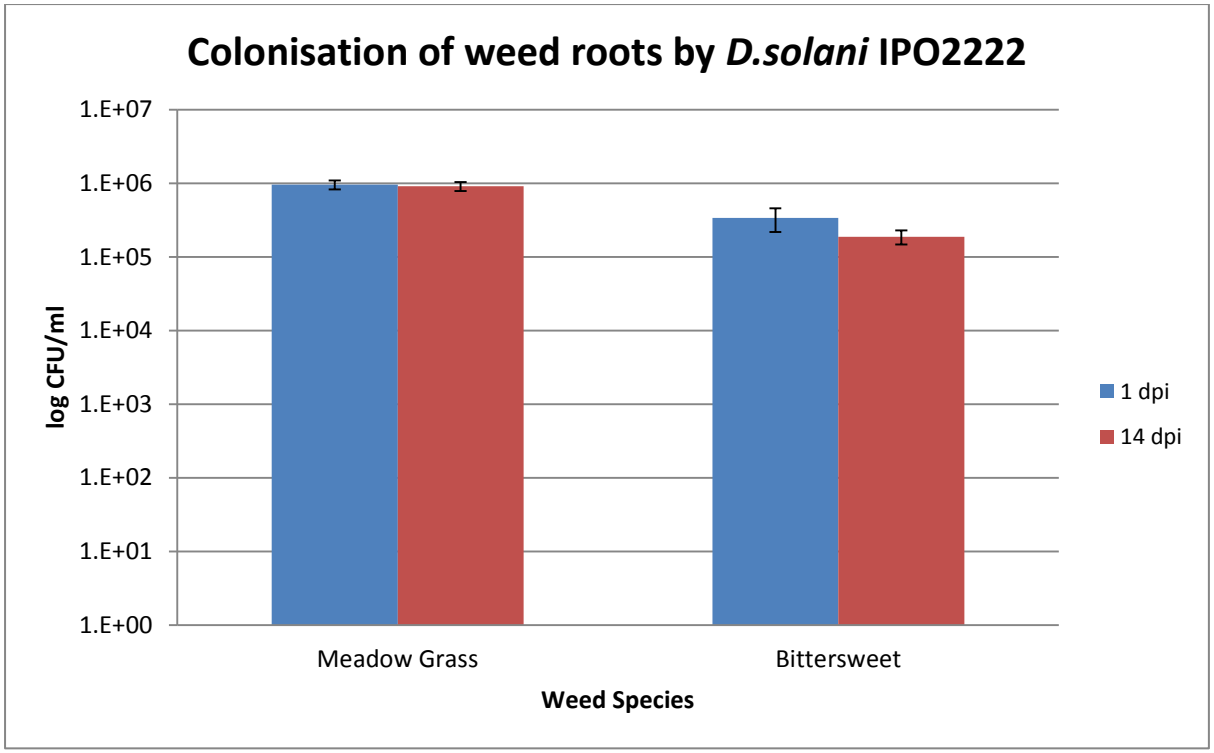


Figure 14. The ability of *D. solani* to colonise two weed species for up to one month. *Colonies could not be counted for bittersweet at 28 dpi due to the production of a bacterial film rather than discreet colonies although the presence of the pathogen was verified by PCR.

4.1.12. Disease development and spread of D. solani in raised beds in Scotland.

Post-harvest testing of plants produced from inoculated cv. Nicola seed tubers in the 2010, 2011 and 2012 experiments showed that only the minority of inoculated tubers went onto produce infected plants. Numbers are shown in Table 32 for 2010 (11/36), 2011 (17/36) and 2012 (2/36). Production of symptoms was also erratic with 4/11 infected plants producing symptoms in 2010, 8/17 in 2011 and 2/2 in 2012. Post-harvest testing of plants grown from uninoculated tubers adjacent to the infected plants showed that transmission was very low, with only 1 plant from 144 becoming infected in 2010, 4 from 144 in 2011 and no infection being found amongst the uninoculated plants in 2012. Only one infected plant, from the 2010 experiment, produced symptoms. In 2010, the infected plant produced from an uninoculated mother tuber was grown in compost directly adjacent to a plant grown from an inoculated mother tuber. In the 2011 experiment, all 4 infected plants produced from unioculated mother tubers were directly adjacent to plants grown form inoculated plants, 2 grown directly in compost beds and 2 grown in pots, embedded in peat.

It is difficult to draw definitive conclusions on the basis of this small data set, but there are clearly strong indications that under the prevailing Scottish conditions (at least those seen during the three years of the study), *D. solani* does not transmit readily from infected mother tubers to progeny tubers in the growing plant, although it was always seen in field plots at Fera (see below). It is also clear that when infection does occur in the plant it does not always lead to the expression of symptoms. Although rare, it is possible for the pathogen to be transmitted from an infected to a healthy plant growing in close proximity. On the basis of these limited data, it is impossible to determine how this transmission occurs as only 5 plants from the 432 grown from uninoculated mother tubers over this three-year experiment went

on to become infected; 3 of these were grown in compost directly adjacent to infected plants and 2 in pots. It cannot be discounted, however, that the pathogen can be transmitted through soil either by root-to-root contact or the movement of ground waters and that transmission may also be possible by foliar contact, the movement of wind and rain, etc. across the canopy. It is clear from these results, however, that whatever the number and nature of the infection routes available they are all highly inefficient under prevailing Scottish conditions and that the promulgation of an infection through a crop appears to be limited.

Taking the results from 2012 from the small study on cv. Hermes it would appear that this variety is more susceptible to *D. solani* than cv. Nicola as the majority of inoculated mother tubers went onto to produce infected plants with 10 from 18 plants showing infection. However, as was the case with the work on cv. Nicola, only the minority of infected plants produced symptoms (4/10). There was no evidence of any transmission from infected plants to adjacent plants grown from uninoculated plants grown in the vicinity.

Table 32. Results from the raised bed experiments carried out at SASA from 2010-2012. cv. Nicola was used throughout with cv. Hermes added to the experiment in the final year.

	cv. Nicola					
	No. of inoculated plants	Resultant no. of infected plants	Resultant no. of infected and symptomatic plants	No. of un-inoculated seed tubers	Resultant no. of infected plants	Resultant no. of infected and symptomatic plants
2010	36	11	4	144	1	1
2011	36	17	8	144	4	0
2012	36	2	2	144	0	0
	cv. Hermes					
2012	18	10	4	72	0	0

Figure 15. *Dickeya* symptoms in inoculated potato grown in irrigated field plots

4.1.13. Disease development and spread of D. solani in field observation plots in England.

4.1.13.1. Effect of seed tuber inoculation with D. solani on incidence of blackleg disease

Blackleg development increased with inoculum load of *D. solani* on the seed tubers in all three years (2010-2012) (Figure 16). No blackleg occurred in plants grown from uninoculated seed in 2010 or 2011. Blackleg observed in plants grown from uninoculated seed in 2012 was attributed to background levels of *Pectobacterium atrosepticum* present on the seed which was also isolated from the stems with blackleg symptoms. Interestingly, in the blackleg susceptible cultivar Hermes, the overall level of blackleg was lower in plants grown from seed inoculated with *D. solani* than in plants grown from the uninoculated seed. *D. solani* was only ever isolated from plants with blackleg symptoms when the seed of those plants had been inoculated with this species. Aerial blackleg, which appeared late in each season in non-inoculated rows was found to be caused by *P. carotovorum* which had infected through wind damaged stems and petioles in the aerial parts of the plant. Aerial blackleg was not included in the assessments shown in Figure 16. The period for evaluation of blackleg incidence was reduced in 2012 due to a high incidence of late blight in this exceptionally wet season.

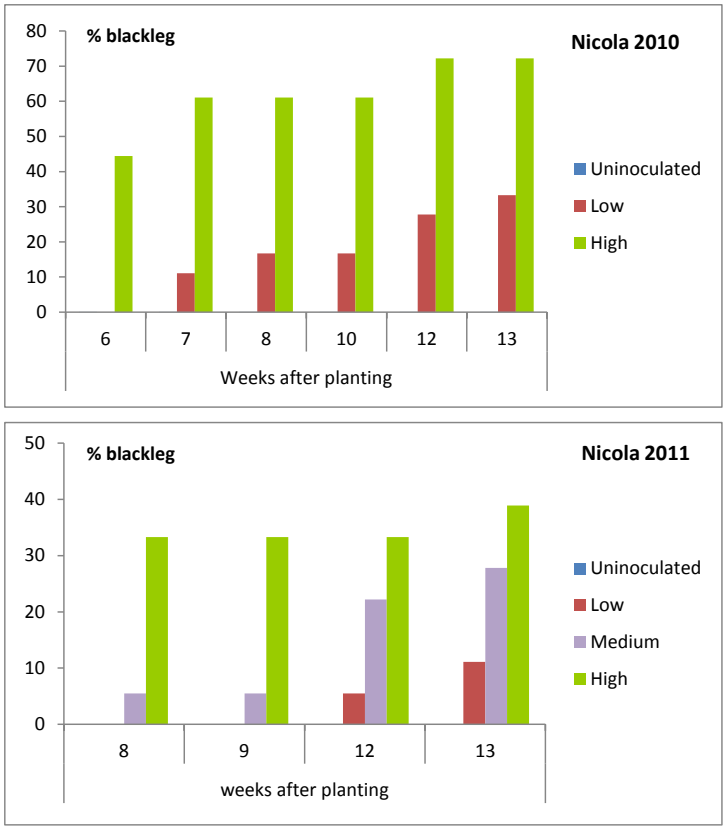


Figure 16. Mean incidence of blackleg in potatoes grown from seed vacuum infiltrated in high (10^7 CFU ml^{-1}), medium (10^5 CFU ml^{-1}) or low (10^3 CFU ml^{-1}) suspensions of *D. solani* strain MK13, compared with potatoes grown from un-inoculated seed

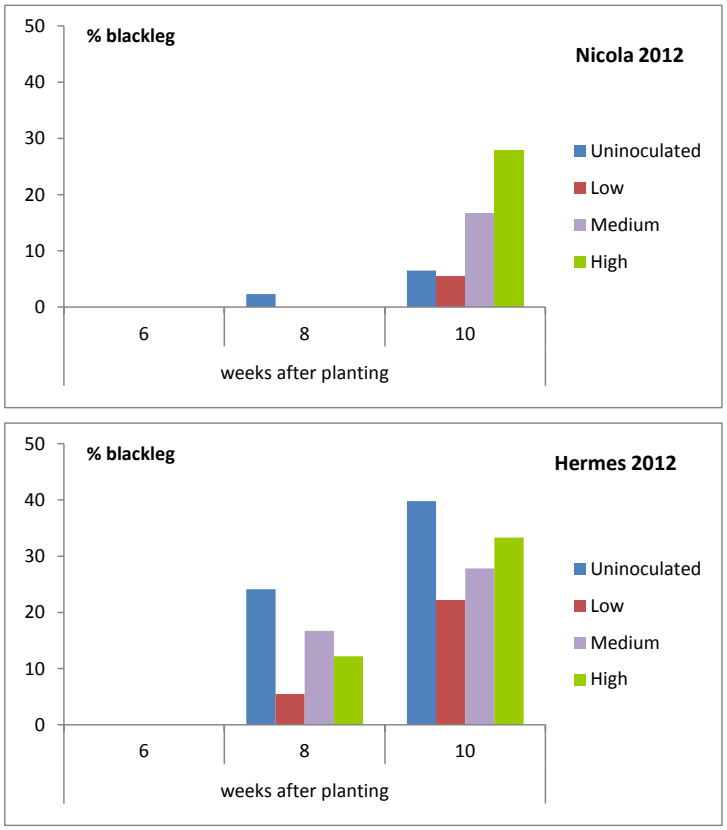


Figure 16 continued. Mean incidence of blackleg in potatoes grown from seed vacuum infiltrated in high (10^7 CFU ml^{-1}), medium (10^5 CFU ml^{-1}) or low (10^3 CFU ml^{-1}) suspensions of *D. solani* strain MK13, compared with potatoes grown from un-inoculated seed.

4.1.13.2. Effect of seed tuber inoculum loading on potential for spread of D. solani.

Spread of *D. solani* to progeny tubers produced from inoculated seed or from non-inoculated seed planted in adjacent guard rows was determined following harvest during the first week of September in each season. In all years Taqman tests for *D. solani* MK13 found the pathogen in progeny tubers harvested from all nine of the rows planted with vacuum infiltrated tubers at the beginning of the season indicating transmission of the pathogen directly to progeny tubers, regardless of the original inoculum level used (Figure 17). The mean C_T value for *D. solani* was 31.1, compared with a mean C_T value of 29.2 for total pectolytic bacteria in the same samples. In 2011 and 2012, *D. solani* was also detected at low level (mean C_T = 37.0) in progeny tubers harvested from some of the guard rows adjacent to the inoculated rows. In the drier conditions of 2010, when overhead sprinker irrigation was not used to maintain soil moisture, *D. solani* was not detected in progeny tubers harvested from any of the adjacent guard rows. Spread to progeny tubers in the adjacent guard rows was not related to the original level of inoculum used but appeared to occur in the least well drained areas of the field plots.

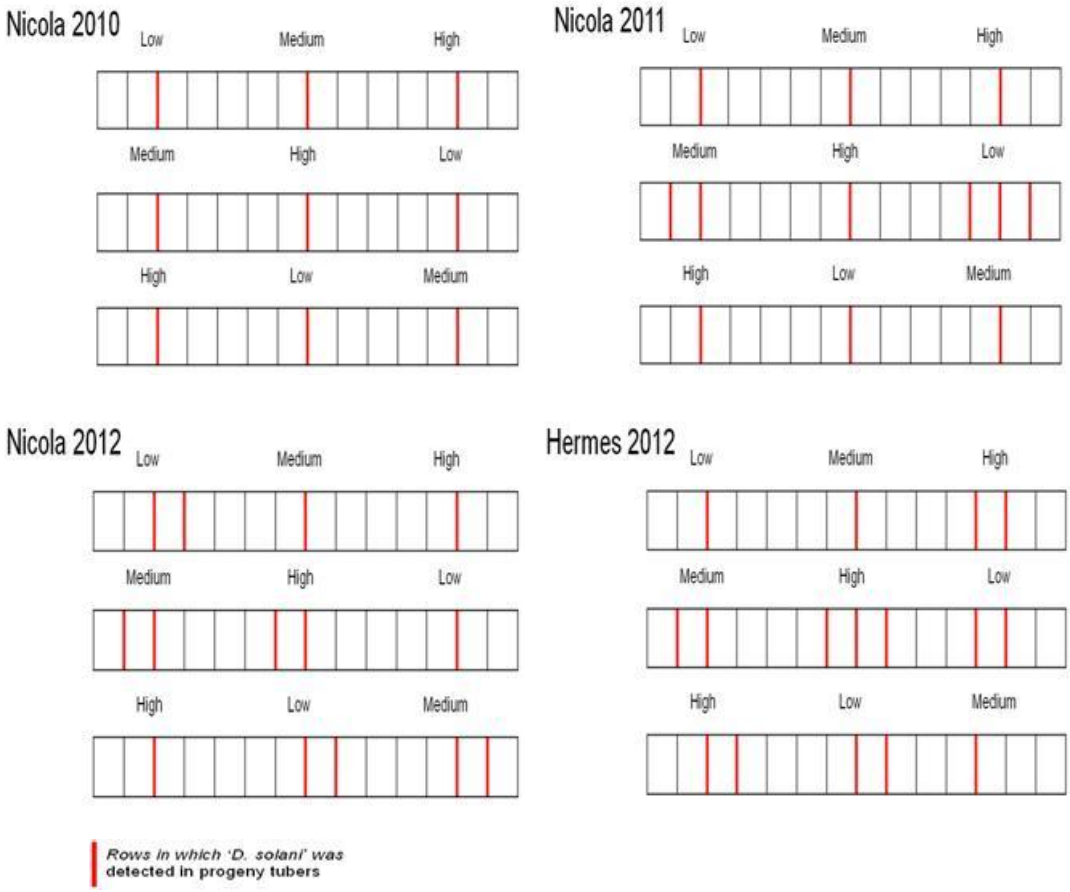


Figure 17. Detection of *D. solani* MK13 in progeny tubers harvested from rows originally planted with seed with varying inoculum loading and from neighbouring guard rows planted with un-inoculated seed.

Low = row planted with seed vacuum infiltrated in *D. solani* suspension containing 10^3 CFU ml^{-1}
Medium = row planted with seed vacuum infiltrated in *D. solani* suspension containing 10^5 CFU ml^{-1}
High = row planted with seed vacuum infiltrated in *D. solani* suspension containing 10^7 CFU ml^{-1}
All other rows planted with uninoculated seed.

4.1.13.3. Comparison of blackleg levels in different potato varieties after inoculation with D. solani or P. atrosepticum.

The incidence of blackleg recorded in field observation plots at Fera after planting seed inoculated at SASA by vacuum infiltration in suspensions containing 10^5 CFU ml^{-1} of *D. solani* or *Pectobacterium atrosepticum* is shown in Figure 18.

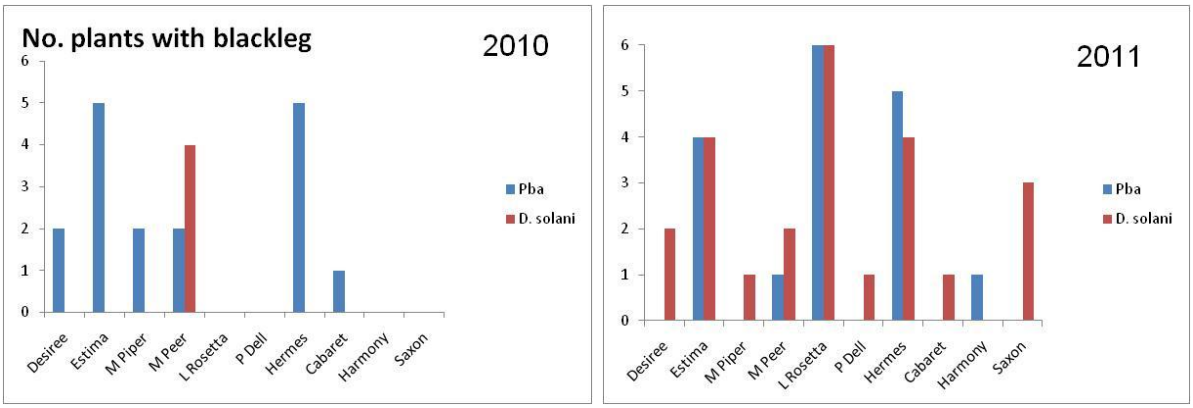


Figure 18. Blackleg incidence (mean number of plants with symptoms per row of 6) 13 weeks after planting inoculated seed in observation plots at Fera in 2010 and 2011.

Poor correlation in the incidence of blackleg across 10 cultivars was observed between the 2 years. Cooler early growing conditions in 2010 generally favoured development of blackleg caused by *P. atrosepticum* rather than by *D. solani* whereas both pathogens induced more blackleg in 2011. Whereas the cultivars Hermes and Estima tended to show more blackleg in both years, cvs Pentland Dell, Cabaret and Harmony were less affected. However, cv. Lady Rosetta was unaffected in 2010 but was the most affected by both pathogens in 2011, indicating that factors other than host genotype were influencing blackleg development.

4.1.14. Evaluation of potential spread of D. solani from commercial crops in England.

A number of commercial ware and processing potato crops with high incidence of blackleg caused by *D. solani* were identified during the growing seasons of 2010, 2011 and 2012. Twelve crops with >20% blackleg were further investigated in 2010. Of pectolytic bacteria isolated from soil samples collected 2 months after harvest and soil cultivation, 3-8% were identified as *D. solani* and 92-97% as *Pectobacterium carotovorum*. However, no *D. solani* could be detected in soil samples collected in the following spring from any of the 12 fields. *P. carotovorum* but not *D. solani* was detected in water samples collected from nearby drainage ditches and watercourses. *D. solani* was detected in tubers stored commercially for 2 months at 8-10 °C, where 1-3% of the pectolytic bacteria detected were identified as *D. solani* compared with 97-99% *P. carotovorum*. In 2011, 4 crops on 2 farms with >10% blackleg caused by *D. solani* were identified for further investigation, where growers estimated an approximate 13.5T per ha loss of yield due to the disease. As in the previous year, *P. carotovorum* but not *D. solani* was detected in composite soil samples and water samples collected from drainage ditches or nearby watercourses in the following spring. Detection by real-time PCR indicated that *D. solani* populations on harvested tubers remained constant during 6 months storage at constant 8°C in controlled environments at Fera but increased slightly when stored on-farm at temperatures between 8-12 °C (Table 33).

Table 33. Detection of *D. solani* in harvested tubers before and after storage by real-time PCR (data shown are C_T values obtained after testing DNA extracted from homogenized peel from subsamples of 20 randomly selected non-symptomatic tubers). Lower Ct values indicate that more *D. solani* was present than in samples with a higher Ct value.

Sub-sample	At store loading on farm	After 8°C storage at Fera	After 8-12 °C storage on farm
1	34.61	36	34.3
2	37.44	Not detected	33.5
3	36.71	39.5	30.1
4	Not detected	35.9	32.3
5	37.47	Not detected	35.6
Mean	37.2	37.9	33.2

In 2012, a further 3 crops with >30% blackleg caused by *D. solani* were identified for further investigation. *D. solani* was not detected in composite soil sample or in water samples collected from drainage ditches or nearby watercourses in the following April 2013. *D. solani* was again detected by real-time PCR in tubers harvested in the field from blackleg plants after 6 months storage in controlled environments at constant 8 °C. Furthermore, when samples of these asymptomatic tubers were planted under disease-conducive glasshouse conditions at 25 °C, blackleg developed from between 5 and 20% of the stored tubers (Table 34).

Table 34. Detection of *D. solani* in tubers harvested from blackleg plants after 6 months storage at 8 °C by real-time PCR (Taqman) and blackleg incidence following planting of the tested stored tubers in the greenhouse at 25 °C. The selected primers and probes detected *Pectobacterium atrosepticum* (ECA), total pectolytic bacteria (PEC) and *D.solani* (SOL).

Sub-sample	Taqman C_T			Blackleg incidence after planting
	PEC	ECA	SOL	
1	30	-	35	1/20
2	23	-	26	1/20
3	26	-	29	4/20

Real-time PCR data shown are C_T values obtained after testing DNA extracted from homogenized heel end cores from subsamples of 20 randomly selected non-symptomatic tubers.

To demonstrate the potential for spread of *D. solani* from a single soft-rotted tuber during handling, a tuber with soft rot caused by *D. solani* was shaken in a chitting tray with 100 healthy seed tubers from a stock which had tested free from *Dickeya*. After removal of the rotted tuber, the seed tubers were then planted under disease-conducive conditions in the glasshouse at 25 °C. Disease development was then recorded in the developing plants as

60% non-emergence and 14% blackleg with only the remaining 36% giving rise to healthy plants.

4.1.14.1. Evaluation of weeds as a source of surviving D. solani populations.

No evidence for survival of *D. solani* was found by testing (before and after enrichment in pectate broth) the stems or rhizospheres of composite samples of predominant weeds which had overwintered following the various blackleg-affected commercial crops described in 4.15, when sampled in April of 2011, 2012 or 2013. Furthermore, no evidence was found of *D. solani* surviving in association with weed species which grew after harvest of the field observation plots where potatoes artificially infected with *D. solani* had been grown (see 4.14). *Dickeya* spp. were not detected in stem base and rhizosphere samples (each of 25 randomly selected plants) from three predominant species: speedwell (*Veronica* sp.), groundsel (*Senecio vulgaris*) and cleavers (*Galium aparine*) when tested in April of 2011, 2012 or 2013.

4.1.15. Potential for survival of D. solani on weeds and its capacity to contaminate potato plants and tubers.

The levels of bacteria were checked on the seedling roots or in the soil one day after inoculation with *D. solani* IPO2222 and were all found to be just below the initial inoculum level of 10^7 CFU ml^{-1}. One month post inoculation, *D. solani* was still present on the roots of all six inoculated weed species (see Figure 19) with the highest levels being found on annual nettle (*Urtica urens*). However, after 2 months *D. solani* could only be detected on the roots of 3 weed species; annual nettle, field pansy (*Viola arvensis*) and shepherd's purse (*Capsella bursa-pastoris)* (Figure 19). In the pots where seeds were sown into inoculated compost, only 3 weed species, meadow grass (*Poa annua*), wild oat (*Avena fatua)* and oil seed rape (*Brassica napus*) had emerged after one month and from them *D. solani* could only be detected on the roots of oat grass. After 2 months, all six weed species had emerged but *D. solani* could not be found on the roots of any of these species. In the pots containing soil only, no weeds or seeds, *D. solani* survival was low, despite high initial inoculum levels (Figure 19), the bacteria could not be detected in the soil after one month. Lastly, *D. solani* was not found on the roots of any weeds growing in the inoculated control pots.

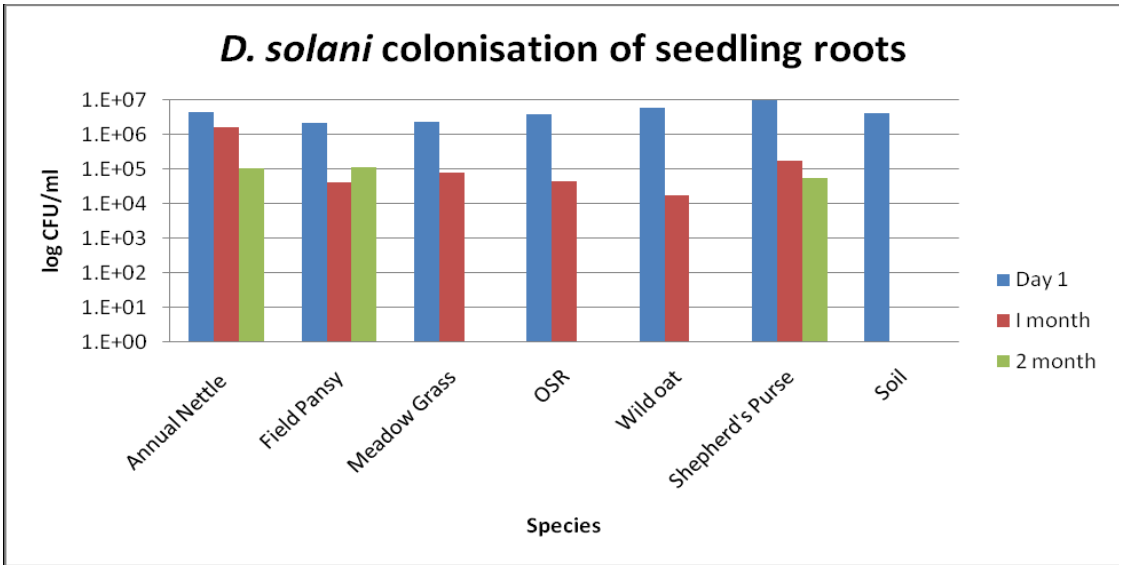


Figure 19. Survival of *D. solani* IPO2222 on the roots of weeds grown from inoculated seedlings. Pots contained groups of all 6 weed species.

During this experiment, all annual nettle plants that had been inoculated with *D. solani* IPO2222 as seedlings were systemically colonised by *D. solani* and showed signs of disease (Figure 20). Therefore, the stems of annual nettle plants were sampled and levels of *D. solani* in the plant were found to be almost identical to the numbers detected on the root one month post inoculation (Figure 21). After 2 months the annual nettle plants were beginning to show signs of recovery. However, the plants were still stunted in growth and while levels of *D. solani* were lower in the plant compared with levels on the root there was still a significant level of 4 x 10^3 CFU ml^{-1} present in the plant. Koch's postulates have so far not been tested. At 2 months post inoculation all other weeds were also tested for *D. solani* systemic colonisation and although no other weeds were showing signs of disease, *D. solani* was also detected in the stems of field pansy (Figure 21).

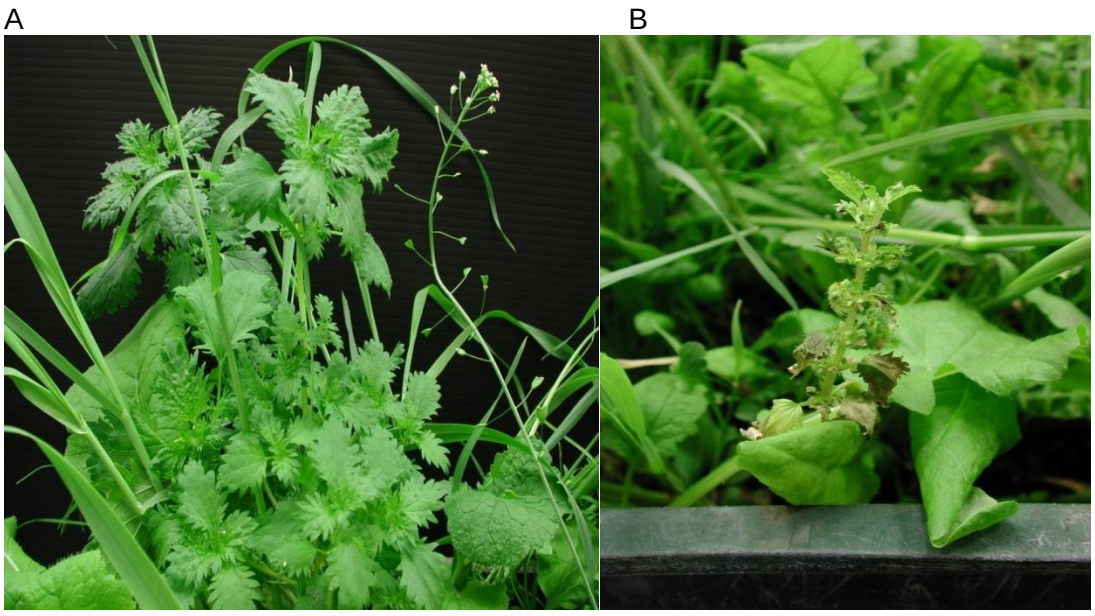


Figure 20. Comparison of annual nettle growing in un-inoculated control soil (A) and 4 weeks after inoculation in soil with *D. solani* IPO2222 (B).

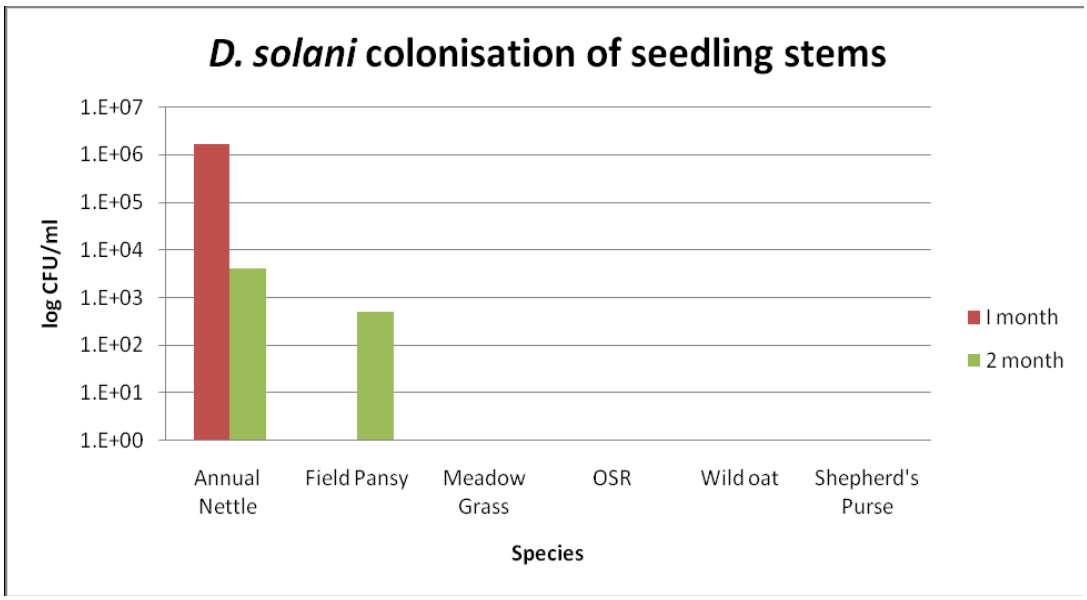


Figure 21. Systemic colonisation of weeds grown from inoculated seedlings. Pots contained groups of all 6 weed species.

Ten weeks post inoculation the weeds were so overgrown in the pots that they were preventing the potato plants from growing, and the weed roots had intertwined in the soil preventing individual sampling. Therefore, the decision was taken to cut back all the weeds and to sample the roots as a group rather than individual species. However, *D. solani* IPO2222 could not be detected, possibly due to the mass of roots preventing sufficient uptake of water for *D. solani* survival.

To investigate the capacity of *D. solani* on weeds to contaminate tubers and potato plants, a new experiment was set-up with 4 of the original weed species (annual nettle, field pansy, oil seed rape and shepherd's purse). The levels of bacteria were checked on the roots one day after inoculation and were all found to be just below the initial inoculum level of 10^7 CFU ml^{-1} (Figure 22). One month post inoculation, *D. solani* IPO2222 was detected on the roots of all four inoculated weed species with the highest levels being found on annual nettle (Figure 22). *D. solani* had also systemically colonised 3 of the 4 weed species, annual nettle, field pansy and shepherd's purse (Figure 23). Three months post inoculation with *D. solani* IPO2222, the pathogen was detected on the roots of 3 of the 4 weed species, annual nettle, field pansy and shepherd's purse but not on oilseed rape (Figure 23). *D. solani* IPO2222 was also found to have survived systemically in annual nettle and field pansy after 3 months (Figure 23). As before, annual nettle seedlings showed signs of disease in the first couple of months post inoculation and were stunted in growth, after this time the annual nettle plants began to show signs of recovery.

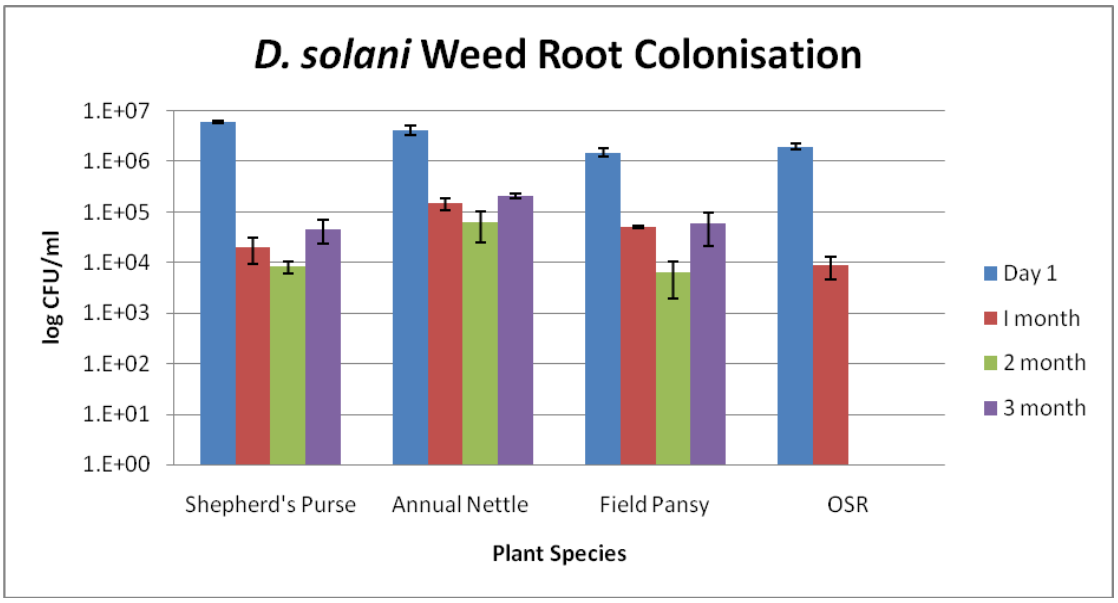


Figure 22. Survival of *D. solani* on the roots of weeds grown from inoculated seedlings. Pots contained individual weed species.

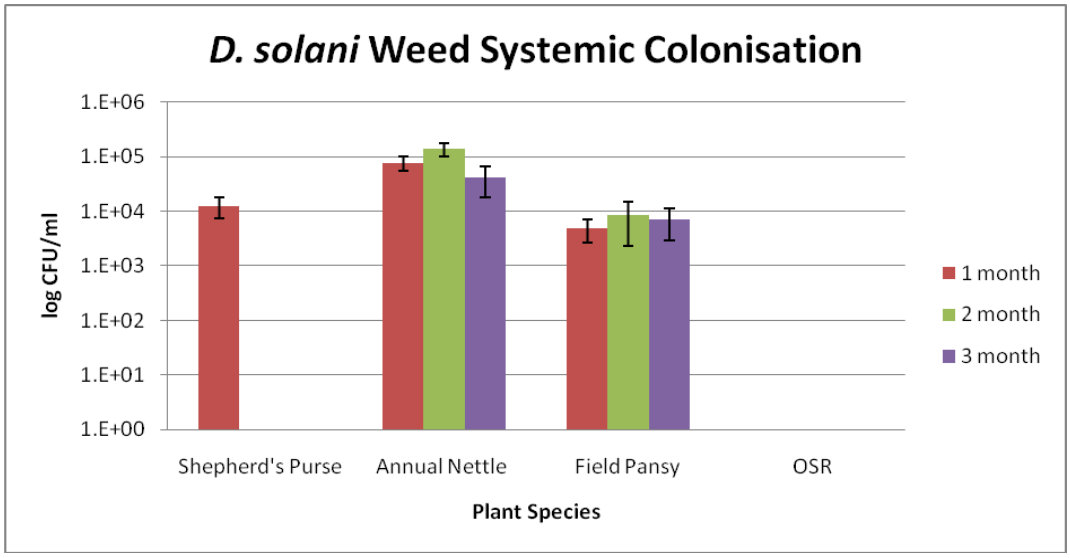


Figure 23. Systemic colonisation of weeds grown from inoculated seedlings. Pots contained individual weed species.

From the pathogen-free seed tubers planted into the centre of all weed containing pots, potato plants were grown and progeny tubers produced. However, no disease symptoms could be observed on the plants or tubers. From each potato plant, samples were taken from the stems, roots and peel of the progeny tubers. All samples were homogenised before being enriched in PEM media and streaked onto CVP plates. There were no positive *Dickeya* colonies produced on the CVP media, so DNA was extracted from all of the homogenised samples (see appendix 8.2) and analysed using the *D. solani* qPCR diagnostic primers, no *D. solani* was amplified from any of the samples.

4.1.15.1. The effect of water-logging on the capacity of D. solani to spread from artificially inoculated weeds and to contaminate potato plants and tubers

Following water logging, *D. solani* spread from the inoculated weeds (shepherd's purse, annual nettle and field pansy) to the roots and stems of potatoes grown together with all three weed species and, in the case of shepherds purse and field pansy, had also spread to the progeny tubers of the planted potatoes (see Figure 24). However, not all potato plants survived the water logging and there was only 1 replicate for both shepherd's purse and annual nettle and 2 replicates for field pansy. There were also no progeny tubers produced by the potato plant grown in the pots with infected annual nettle. Although the levels of *D. solani* detected on the potato plants were low, these preliminary results suggest that *D. solani* could have the potential to spread from inoculated weeds and contaminate potato plants and tubers.

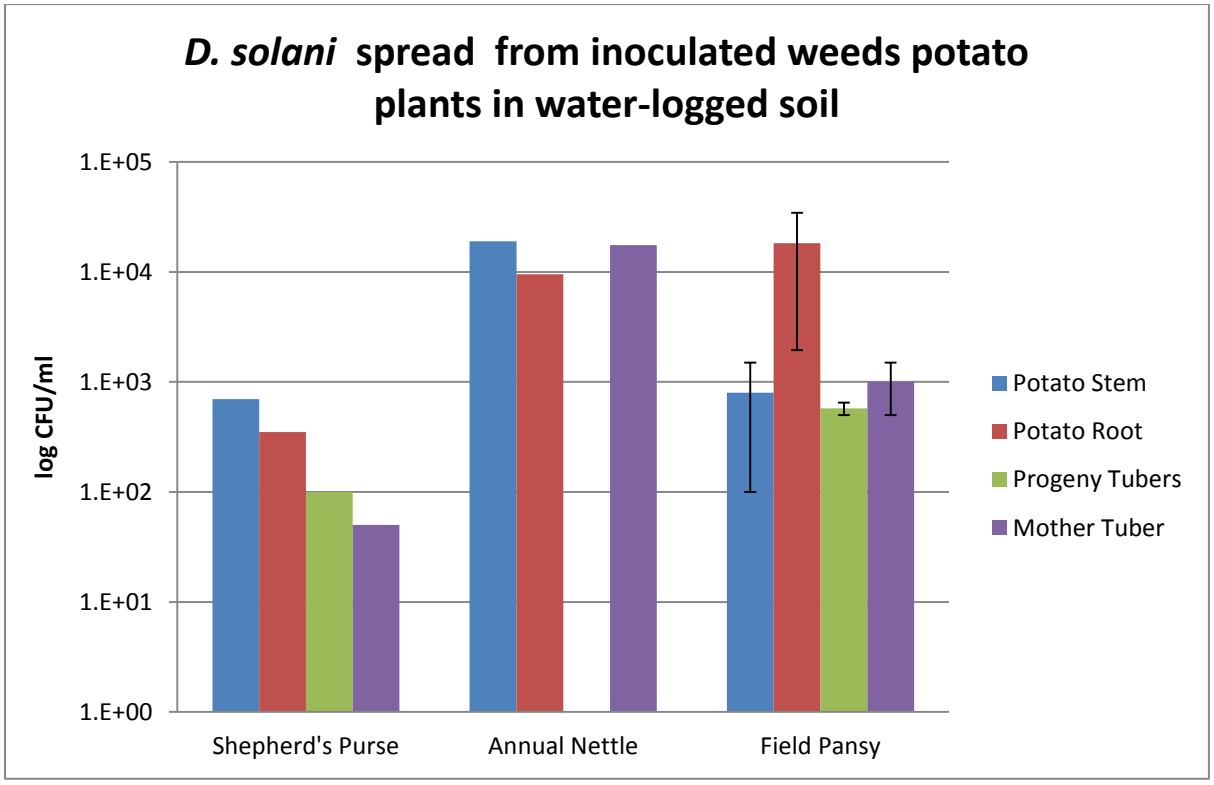


Figure 24. Spread of *D. solani* from artificially inoculated weeds to potato plants and tubers**.** There was only 1 replicate for annual nettle and shepherd's purse and the potato plant grown with infected annual nettle did not produce any progeny tubers (*).

4.1.16. The effect of inoculum level on root and systemic colonisation

To determine whether the level of *D. solani* IPO2222 on roots determines the extent of root invasion, roots of seedlings from 2 plant species (annual nettle and annual meadow grass, the latter was used as it had not shown systemic colonisation in previous experiments) were inoculated with 3 different bacterial concentrations (10^7, 10^5, 10^3 CFU ml^{-1}) of *P. atrosepticum* SCRI1039 and *D. solani* IPO2222. The levels of bacteria on the roots were determined one day after inoculation and all were found to be just below the initial inoculum levels of either 10^7,10^5 or 10^3 CFU ml^{-1}. After 2 months *P. atrosepticum* SCRI1039 and *D. solani* IPO2222 could only be detected on the roots and in the stem of annual nettle inoculated with 10^7 CFU ml^{-1} (Figure 25). This experiment was repeated to ensure consistency of results and again only infection at 10^7 CFU ml^{-1} resulted in colonisation and invasion of the stems of annual nettle. DNA was extracted (see appendix 8.2) from all the homogenised root and stem samples and amplified with the *D. solani* qPCR diagnostic primers to determine whether the bacteria were surviving at the lower concentrations but were below the detection level of dilution and CVP plating. However, there was no *D. solani* amplified from any of the 10^5 and 10^3 CFU ml^{-1} samples.

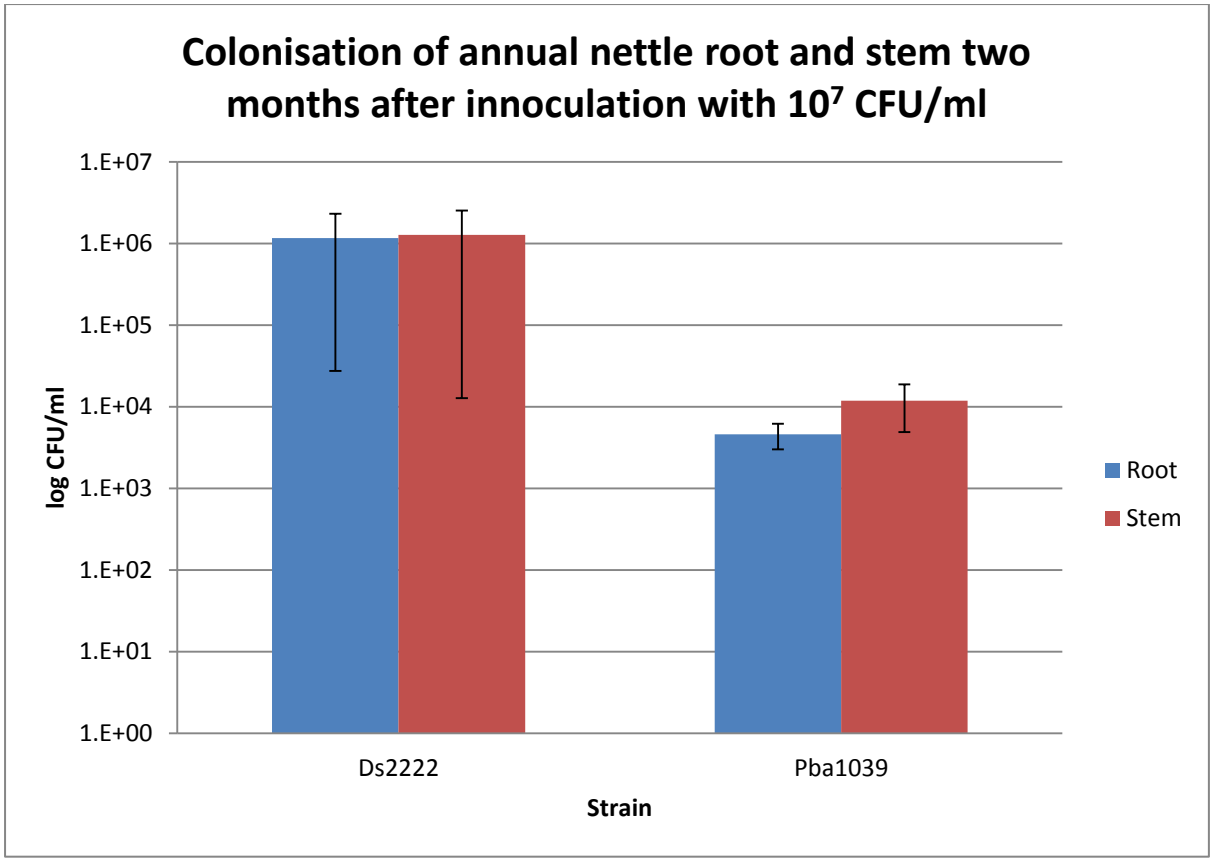


Figure 25. Colonisation of annual nettle plants by *D. solani* IPO2222 and *P. atrosepticum* SCRI1039 two months after infection with 10^7 CFU ml^{-1}

4.1.16.1. Confocal microscopy of infected plants

To compare the invasion of annual nettle roots by bacteria with that of other weed species, seedlings of annual nettle and meadow grass were inoculated with *D. solani* IPO2222. In nettle, *D. solani* was moving in and filing epidermal and cortex cells of the roots and were also moving into the apoplastic spaces between cells (Figure 26). It was not possible to tell from the images whether the bacteria were going into live cells or filling the space within dead cell. In meadow grass, the bacteria again were in the epidermal and cortex cells of the roots (Figure 27) and appeared to be in the space below the cortex cells and approaching the vascular tissue. However, due to the depth of the bacteria it was difficult to differentiate between autofluorescence and fluorescence from the bacteria. Unfortunately, after 2 days *D. solani* had killed the annual nettle plants and it was not possible to take images of the stem and leaves. The confocal imaging will require further work using lower concentrations of bacteria and rather than taking live *in planta* images the plants should be fixed and embed in wax and slices taken through the roots and stems to try and determine whether the bacteria are reaching the vascular tissue, where systemic colonisation is likely to begin.

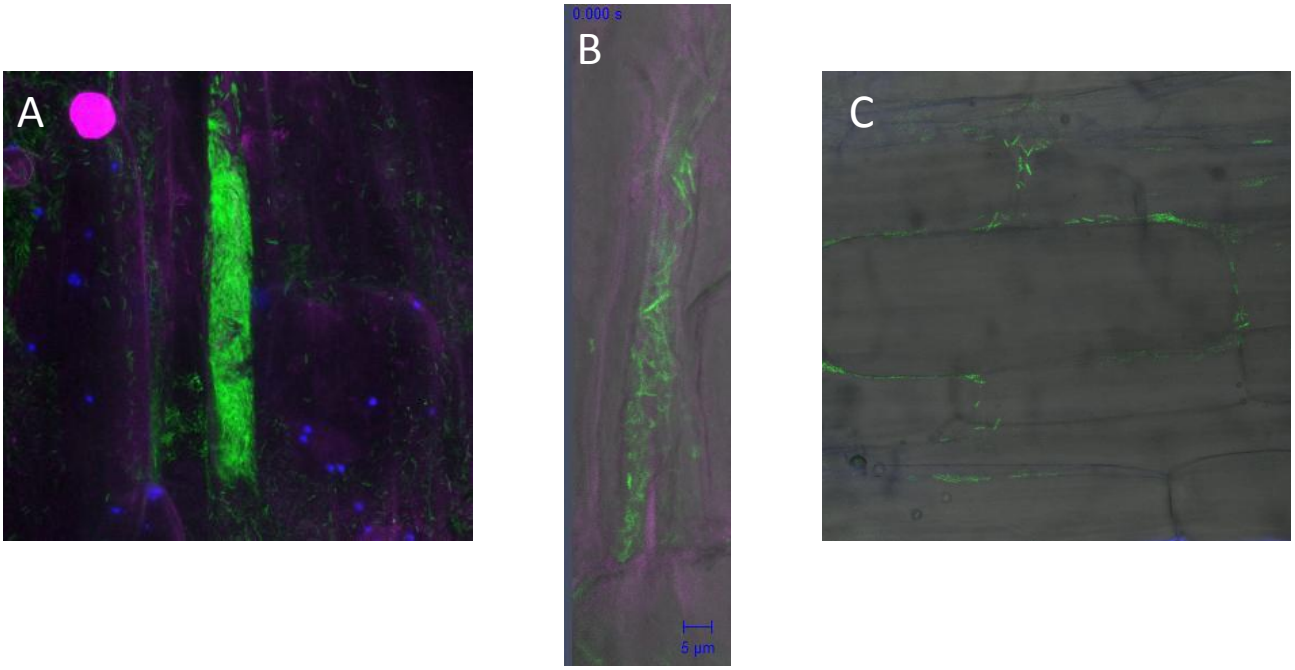


Figure 26. Confocal images of annual nettle roots showing *D. solani* 2222 in the cortex and epidermal cells of the roots (A and B) and in the space between cells (C).

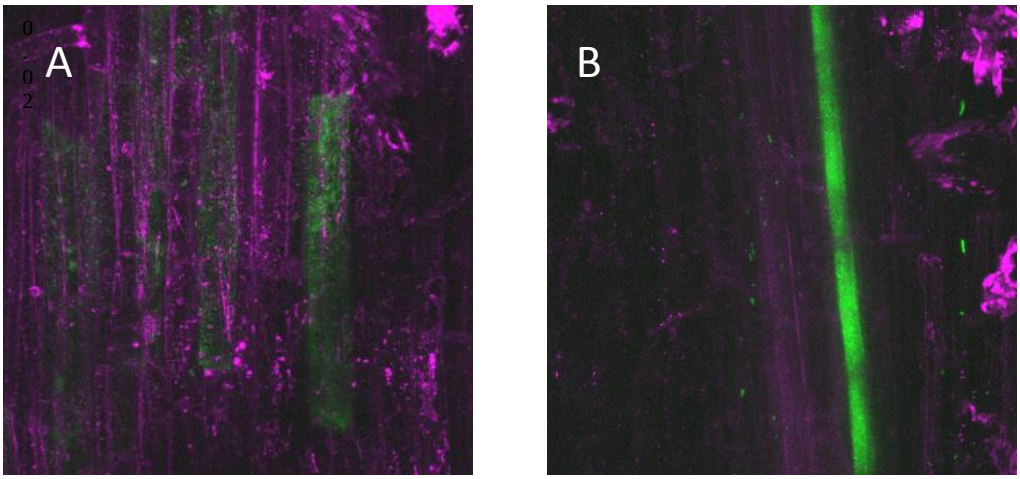


Figure 27. Confocal images of meadow grass roots showing *D. solani* 2222 in the cortex cells of the roots (A) and possibly at the vascular tissue (B).

4.1.16.2. Ability of rotting tubers/plants to contaminate weed species determined in glasshouse studies.

The level of *D. solani* IPO2222 was checked in the peel of the infiltrated tubers before planting and was found to be ~2 x 10^5 CFU ml^{-1} (just below the level of inoculation). In addition, the majority of potato plants developed blackleg during the experiment. Two months after sowing of the seeds no *D. solani* could be detected on the roots or in the stems of the four weed species. By 3 months, *D. solani* was detected at low levels on the roots of one plant from the 3 replicates for each weed species. However it was not possible to determine the exact numbers of *D. solani* colonising the weed species due to the production of a bacterial film rather than discrete colonies produced on the CVP medium. The bacterial film tested positive as *D. solani* when amplified with the *D. solani* qPCR primers. At the 4 month harvest there were no colonies on the CVP plates. However, *D. solani* was amplified from the roots of one field pansy plant using the *D. solani* qPCR primers. No *D. solani* was detected on the roots of the weeds grown in the pots with un-infiltrated tubers. These results suggest that *D. solani* could have the potential to spread to weeds surrounding infected potato plants/tuber. However, the duration of survival of *D. solani* on the roots and the levels of *D. solani* have not been determined.

4.1.17. The transmissibility and survival of D. solani on surfaces.

Initial results showed no growth from any of the storage materials and an apparent loss of the pathogens during the process so revisions were made to the experimental method. Using the revised method, in which bacteria were applied to surfaces in enrichment media rather than sterile distilled water all three pathogens were able to persist and be grown from all five storage materials. Subsequent experiments using mashed up potato material as an alternative media applied to all materials showed that *D. solani* did not survive on any material studied. Taken together these results indicate that *D. solani* does not survive well in the absence of the host and appears poorly adapted to surviving on surfaces for even relatively short periods of time.

4.1.18. Susceptibility of D. solani to common disinfectants.

It is clear from the results presented in Table 35 (see appendix 5) that all 10 disinfectants studied as part of this investigation were equally and highly effective at controlling *D. solani*, *D. dianthicola* and *P. atrosepticum* if applied at the concentration recommended by the manufacturer for general use. However, and probably more in keeping with the reality of how these products may be applied, the effectiveness of each disinfectant varied widely if diluted further down to 0.5, 0.4, 0.3, 0.2 and 0.1% of the manufacturer's recommended general use dilution. It is clear from these results that sodium hypochlorite and Vanoquat were the only disinfectants effective against all 3 pathogens even at 0.1% of the manufacturer's recommended general use dilution. Halamid also appears to be highly effective though it was evident that a small number of *D. solani* colonies were beginning to appear on the 0.2% and 0.1% dilutions when only 5 minutes of contact time was employed, indicating that at these dilutions and contact time the effective range of the product had been reached. It is encouraging to note that as the contact time increased to 10 minutes and beyond effective control was re-established. At the opposite end of the spectrum diluting Jeye's Fluid to 0.5% of the manufacturer's general use dilution was ineffective at controlling any of the 3 pathogens studied here, regardless of the contact time applied. Some of the disinfectants appeared more effective at controlling some species, but not all. This was particularly true for Jet 5, Mikrozid AF, V18 and Virkon S where *D. dianthicola* was highly sensitive to these products, in contrast to *D. solani* and *P. atrosepticum* that showed strong resistance. In the case of Jet 5 and Virkon S increasing the contact time produced greater control of the latter species, whilst increasing contact time was ineffective in the case of Mikrozid AF and V18.

The clear message from this work is that a range of products are available to control the spread of *D. solani* if used properly and adhering to the manufacturer's recommended concentration for general use. It is also clear that some disinfectants have a greater 'safety net' than others, and this is particularly true for sodium hypochlorite and Vanoquat. Finally care should be taken using products such as Jeye's Fluid as there is evidence that dilute solutions of the product have minimal effect on any of the pathogens tested here.

Prevention and control recommendations based on the available information from GB and overseas.

A major piece of work was intended to be a survey of 'mixed businesses'. However, after discussions with PCL it was decided that there was no easy way to identify such businesses, namely ware growers that grade their own crops and also use their premises to grade non-UK ware potatoes for customers in the retail or catering trade. It was decided that the best approach was to make contact with these businesses through blanket education rather than by contacting them through an industry-wide survey. Coincidental to this initiative, the Scottish Government brought forward a proposal to develop an information leaflet intended for all ware growers in Scotland entitled 'Defending your potato crop against disease'. The information leaflet highlights a range of plant health issues, including *Dickeya*, and the type of legislation and best practise available to counteract these threats. It gives brief information on the types of risks a mixed business runs. The information leaflet was sent out to over 786 Scottish ware growers on 29 March, 2011. The leaflet contains information which forms a major plank of the prevention control strategy for *D. solani* in Scotland as does the recently updated SASA website (see http://www.sasa.gov.uk/diagnostics/bacteriology/dickeya) and the linked information on the Scottish Government's (see http://www.scotland.gov.uk/Topics/farmingrural/Agriculture/plant/18273/PotatoHealthControls/PotatoQuarantineDiseases/Dickeya) which is regularly updated with news of surveys and advice to growers.

5. DISCUSSION

Objective A: Refine, validate and apply diagnostic methods for specific detection and typing of *D. solani.*

5.1.1. Development of PCR-based diagnostics

Two approaches were used to develop PCR-based diagnostics for *D. solani* – one using genome comparisons (SOL-C) and the other using the *fusA* housekeeping gene. Both sets of primers were validated against a wide range of *D. solani* and other strains and found to be *D. solani*-specific, with the exception of a single strain (classified as *D. dadantii*), which is currently being retested as it may have been misclassified. Both of our tests appear to work well, although some false negatives were seen during a ring test. However, this is more likely to do with the way the samples were prepared and tested rather than the primers themselves. The SOL-C primers were further validated by NAK in the Netherlands and have been chosen in favour of other primers developed in the Netherlands for further use. The SOL-C real-time PCR assay is currently being used in routine diagnostics at Fera and in the Netherlands. Both *fusA* and SOL-C primers have also been included in the list of preferred primers for ring testing within Europe though a EUPHRESCO *Dickeya* project, to standardise testing across Europe. The approach using whole genome comparisons and *in silico* primer selection is highly novel and has good potential for rapid diagnostics development for a range of future targets.

5.1.2. MLST, VNTR and SNP markers for tracking D. solani isolates

At the beginning of this project it was unclear as to the true identity of *D. solani*, how closely related it was to other members of the genus *Dickeya* and the level of diversity that existed between different strains of the pathogens recovered across Europe and Israel. As a consequence different approaches were applied, encompassing a range of sequencing and fingerprinting techniques, each able to resolve relationships at increasingly finer levels of resolution, starting with MLSA, then moving onto VNTR and SNP analyses. The major advantage of the sequencing based MLSA analysis is that it lends itself to the construction of an online database which other researchers can access to help them characterise new strains (see http://pubmlst.org/dickeya/). Although VNTR and SNP are able to distinguish between strains at higher levels of resolution and have the potential to characterise new outbreak strains it is vital that these techniques are only applied to organisms which are already known to be *D. solani*, hence the requirement to adopt a range of strategies in this work.

It is clear from the MLSA analysis that *D. solani* is an essentially clonal pathogen. Little variation can be found between strains, regardless of their country of origin or whether they have been recovered from infected potatoes or infested waters. The additional genes included as part of this study, *dnaJ* and *dnaX,* add the possibility of reading across from the diversity studies presented here to other studies conducted in Europe, in which *dnaX* is the marker of choice in strain characterisation. The addition of SNP analysis adds a further dimension in that this form of fingerprinting may have application in outbreak tracking. Studies are currently on-going to look at the diversity of the isolates recovered from the infested river in SE Scotland to determine whether this watercourse became infested as a result of a one-off event or if it is being repeatedly contaminated.

Primer sets were identified at Fera from the *D. solani* genome for amplification of a number of variable number tandem repeat (VNTR) sequences for use in population diversity and source tracing studies. VNTR profiles confirmed the clonal nature of all *D. solani* isolates collected across Europe and Israel, with minor variation detected in only one of the 5

selected loci. All 3 VNTR profiles identified were represented amongst isolates from England and Wales as well as those from the Netherlands, consistent with a common source of the infections. Confirmation of a shared VNTR profile between a single isolate from *Hyacinth* and other isolates from potato, also suggests movement of the same populations between the two hosts. Proposed collaboration with Dutch researchers working on Hyacinth and other ornamental hosts will allow analysis of a larger population of isolates and further tracing of the potential sources of infection.

Objective B: Determine the extent of *D. solani* infection in the GB potato crop and evaluate the risks of spread to home-grown GB seed potatoes.

5.1.3. Scotland surveys

With the introduction of legislation in Scotland in 2010 no new *Dickeya* infections have been found. Much credit must go to the industry for increasingly only sourcing Scottish-origin seed. The dwindling number of crops produced from non-Scottish origin seed is almost exclusively made up either by PBTC material brought in from Northern Ireland or as a result of small businesses spanning the border with England. All non-Scottish seed and ware crops were targeted in these surveys, alongside irrigated and close-contact crops, those grown on farms with previous *Dickeya* positives. It is therefore encouraging to note that the lack of any findings in these crops. It would suggest that infection does not spread readily through soil, weeds, ground keepers or through ground and irrigation waters. All indicators point to seed as the principal route for disease spread.

It should of course be noted that growers in the vicinity of infested water courses in Scotland are encouraged not to irrigate and, similarly, strict control programmes were imposed on affected growers of previous infections. It cannot be ruled out completely that irrigation and/or close contact are significant routes of infection to healthy crops/stocks, but clearly it would suggest that existing control measures and informed seed selection amongst Scottish growers appears to be working for now.

From previous river surveys conducted in Scotland since 2006, it is known that there were 3 rivers/water sources contaminated with *Dickeya* spp. One of these rivers in Central Scotland has been free of *Dickeya* (DUC-3; an as yet uncharacterised member of the genus that shows limited pathogenicity to potato and which is not a member of any of the currently known species) since 2010, probably as a result of changes to the operation of a domestic sewage plant that was likely source of the infection. Advisory notices to local growers suggesting they should not irrigate from this infested source of water were lifted in 2012, although monitoring of the waters has continued. The watercourse in SE Scotland is the biggest concern as, with the exception of 2010, *D. solani* has been regularly isolated from this source. Repeated attempts to identify an alternative host or refugia have met with failure, and discussions are currently underway with policy colleagues in Scotland with a view to scaling back our monitoring but putting in place a more permanent form of advisory notice for this watercourse. The river in NE Scotland contaminated with *D. zeae* maintains a consistent infestation, again similar discussions are underway to scale back monitoring but put in place a more permanent advisory notice.

It is encouraging that looking back over the last 3-4 year of river monitoring that only 3 rivers have been identified from the more than 300 sampled that are contaminated by *Dickeya* spp. in Scotland. Previous work conducted at SASA using suspensions of *Dickeya* spp. to water potato plants have suggested that concentration of approximately 10^3 CFU ml^{-1}or more are required to produce an infection. In all the cases where infested watercourse have been found we would estimate that rarely have we found much more than 1 CFU ml^{-1}. We

therefore conclude that the chance of establishing an infection through the use of infested irrigation water is low. However, it is felt that a precautionary approach should be applied in Scotland and it is likely that wherever and whenever an infestation is detected local growers will be advised that they should not use the affected watercourse as a source for irrigation water.

5.1.4. England and Wales surveys

Results from the seed potato surveys in England and Wales strongly suggested that the principal source of *D. solani* was infected seed of non-UK origin. In all but one finding of blackleg caused by *D. solani* the planted seed stock had either been directly imported from the Netherlands or previously multiplied in England and Wales from a seed stock originating in the Netherlands. Neither *D. solani* nor *D. dianthicola* were isolated from blackleg plants grown from seed or GB origin in any of the 3 years. It was therefore concluded that the source of '*Dickeya*' infecting potatoes in England and Wales was exclusively seed of non-UK origin and that there was no evidence for horizontal spread of *Dickeya* spp. to seed stocks of UK origin.

Monitoring of blackleg disease in England and Wales has found only a very low frequency (0.5-2.3%) of the seed stocks entered for classification to be affected by *D. solani*, restricted to stocks of Dutch or German origin. Similarly, *D. dianthicola* was also found infrequently (0.1-0.6% of stocks affected) and was associated only with stocks of Netherlands origin. The incidence of blackleg caused by *D. solani* was related to the inoculum level on the seed tuber, with lower incidence occurring later in the season with decreasing inoculum level. However, the incidence of blackleg caused by *D. solani* or *P. atrosepticum* was also highly yet independently affected by seasonal conditions.

River survey results in England and Wales identified the presence of *Dickeya* species in a small number of waterways in the UK. *D. solani* was found in only 2 river water samples from 2 unconnected rivers in different counties. Follow-up sampling in the same locations in the same year and in the following year failed to detect the pathogen, despite the use of sensitive enrichment methods and more intensive sampling both up- and down-stream from the original findings. Similarly, a low number of findings of *D. dianthicola* were confirmed in 4 unconnected watercourses and follow-up testing failed to demonstrate its survival or establishment.

Two other *Dickeya* species (*D. zeae* and a new species proposed as '*D. aquatica*') were found in a small number of rivers. Although both were pectolytic and caused soft rot of potato tubers, they did not induce typical blackleg when inoculated into potato stems. Interestingly, all isolates of '*D. aquatica*' were indistinguishable according to *recA* sequence analysis, suggesting the possibility of a common origin, despite their isolation from unconnected waterways. Most of the *D. zeae* Phylotype II isolates from water also showed *recA* sequence similarity, and were also similar to *D. zeae* isolated from potato blackleg and surface irrigation water in Australia (Parkinson *et al*, 2009). A single *D. zeae* Phylotype I isolate from river water was closely related to the isolate of *D. zeae* found in Scottish river water.

Whereas monitoring of *D. solani* or *D. dianthicola* in waterways in England and Wales has found no evidence of its colonisation in waterways, this is somewhat contradictory to repeated findings in consecutive years in the same waterway in Scotland. Further investigation of the reasons for this is needed. More evidence was found to support the hypothesis that *D. zeae* and '*D. aquatica*' may have become established in a small number of English rivers where they were found in subsequent years in the same watercourses.

Objective D: Improve understanding of the epidemiology of *D. solani* infections and risks of pathogen establishment and spread following introduction of infected crops.

5.1.5. Disease development and spread of D. solani in raised beds in Scotland

In raised bed observation plots at SASA in 2010, 2011 and 2012 from seed inoculated with *D. solani*, infection was detected in only the minority of plants. Of these infected plants symptoms were only observed in the minority of cases. Subsequent testing of harvested tubers from infected plants demonstrates that infection does move from mother to progeny tubers, albeit inefficiently, as the majority of progeny tubers from infected plants were found to have no detectable level of *D. solani*. Further there was limited evidence of spread in either the beds or pots from inoculated to un-inoculated plants. These findings are contrary to observations made in continental Europe, which indicate that spread is very effective between and within plants and may suggest that prevailing Scottish weather conditions are sub-optimal for disease spread.

5.1.6. Adherence of D. solani onto common materials

It is clear from the results presented here that *D. solani* is poorly equipped to persist on common surfaces such as wood, steel, rubber, hessian etc. Only when a nutrient source was available, in the form of pectate enrichment media, was it possible to detect the bacterium on these surfaces for any length of time. Experiments using macerated potato as a nutrient source surprisingly showed poor survival rates. It is therefore unlikely that surfaces carrying historic infections may pose much of a threat. It is clear, however, that experiments carried out at Fera and reported here suggest that tuber to tuber contact with rotting, infected tubers poses a major threat for disease spread.

5.1.7. Susceptibility of D. solani to common disinfectants

From the disinfectant results it is apparent that when using the recommended manufacturer's concentration all disinfectants tested are able to control *D. dianthicola*, *D. solani* and *P. atrosepticum*. Differences in efficiency were only evident at sub-optimal concentrations and only Sodium hypochlorite and Vanoquat appeared to be effective against all three species across all concentrations tested. These findings have implications for controlling disease spread by machinery and suggest that, if kept clean and the correct disinfectant is applied at the manufacturer's recommended concentration, the chance of onwards transmission from an infected crop to a healthy one could be greatly reduced.

5.1.8. Disease development and spread of D. solani in field plots in England

In field observation plot experiments, the incidence of blackleg was directly correlated with the population of *D. solani* present on vacuum infiltrated seed. Furthermore, blackleg caused by *D. solani* only developed in plants grown from the artificially inoculated seed. Plants in un-inoculated guard rows either remained disease free or, in some cases developed blackleg symptoms (as a result of infection by background levels of *P. atrosepticum* present on the seed before inoculation) or aerial stem soft rot (where airborne *P. carotovorum* entered damaged stems late in the season). These results strongly support the hypothesis that infected seed is the main source and pathway of spread of *D. solani*.

Spread of *D. solani* from inoculated seed to harvested progeny tubers produced on the same plants occurred at all levels of inoculation and was not necessarily related to the level of blackleg that developed. Spread to progeny tubers of un-inoculated plants in neighbouring rows occurred independently of the initial inoculum levels on the seed tubers but appeared to be related to the position of the infected plants in the field, spread occurring only in the wetter patches of the field. Spread to progeny tubers of un-inoculated plants was not observed at all in 2010 when soil moisture was not maintained by supplementary irrigation. Because of the unpredictability of disease development from inoculated seed, comparison of relative susceptibility of the 10 most popular potato varieties to *D. solani* was unreliable. Under disease conducive conditions, some varieties appeared equally susceptible to blackleg caused by both *P. atrosepticum* and *D. solani*.

5.1.9. Evaluation of potential spread of D. solani from commercial potato crops grown in England

Despite high levels (10-30%) of blackleg caused by *D. solani* in several commercial crops selected over a 3 year period, no evidence was found to support the hypotheses that the bacterium can survive overwinter in the soil or spread via drainage water to establish in nearby watercourses. *D. solani* was consistently found to survive in latently infected tubers over the storage period at 8°C and was even found to multiply slightly on tubers stored in commercial storage conditions (8-12°C) used for processing potatoes. Extensive spread of *D. solani*, from a single soft rotting tuber to a large number of healthy seed tubers during handling, was shown to result in high disease incidence when the contaminated tubers were immediately planted under conducive conditions. These results highlight the potential for large scale spread of *D. solani* during handling of an infected stock.

Objective C: Assess the aggressiveness of a range of *D. solani* isolates in response to changes in temperature and humidity, and in comparison with earlier data obtained for *P. atrosepticum* and *D. dianthicola*.

5.1.10. Relative aggressiveness of D. solani determined in controlled environment studies

In both tubers and stems, *D. solani* caused more disease at 27°C than at 21°C. However, there were still significant levels of disease at 21°C even at a concentration as low as 10^1 CFU ml^{-1} in the stems and 10^2 in tubers. Disease levels were similar to those of *P. atrosepticum* SCRI1039 at 21°C although *D. solani* produced more disease at 10^1 CFU ml^{-1} in stems than *P. atrosepticum*. Therefore, the results show that while *D. solani* prefers warmer temperatures, it is still able to cause disease under conditions seen in the UK. All strains of *D. solani* tested were highly aggressive at both 21°C and 27°C in tubers except for 2 isolates from Israel (one isolate originating from imported tubers and one isolate originating from tubers grown in Israel). The reason for these differences is not yet clear but might be due to length or condition of storage, and is currently being investigated.

5.1.11. Evaluation of weeds as a source of surviving D. solani populations

No evidence was found that *D. solani* is able to overwinter in soil following potato crops in England and Wales with high blackleg incidence or by establishing naturally in indigenous flora in potato fields or along associated waterways.

Root binding results at JHI demonstrate that *D. solani* is able to bind to and colonise different crop and weed species. Interestingly, unlike *P. atrosepticum*, after 30 days of colonisation the *D. solani* cells recovered from plant roots exhibited a mucoid consistency on agar plates typical of extracellular polysaccharide (EPS) production. The role of EPS is not known but may be linked to colonisation or pathogenicity by *D. solani*.

After 2 months *D. solani* was surviving on the roots of 3 weed species (of 6 tested) grown from inoculated seedlings; annual nettle (*Urtica urens*), field pansy (*Viola arvensis*) and shepherd's purse (*Capsella bursa-pastoris*). However, in pots where seeds were sown into inoculated compost, after 1 month *D. solani* could only be detected on the roots of oat grass, possibly due to the fact that oat grass was the fastest emerging weed and *D. solani* cannot survive in the soil for very long and certainly no longer than 1 month.

During the weed experiment it was observed that annual nettle seedlings were showing signs of disease, therefore the stems of weeds were tested for the presence of *D. solani*. Both annual nettle and field pansy had been systemically colonised by *D. solani* although the plants of field pansy were not showing signs of disease. This suggests that some weed species are more susceptible to colonisation and subsequent infection by *D. solani* than others and annual nettle is being investigated further.

In a follow up experiment using pots containing individual weed species three months post inoculation with *D. solani*, the pathogen was detected on the roots of 3 of the 4 weed species; annual nettle, field pansy and shepherd's purse but not on oil seed rape. *D. solani* was also found to have survived systemically in annual nettle and field pansy after 3 months confirming our previous results. The potential role of these weeds in the persistence and/or spread of *D. solani* in field conditions remains to be determined.

5.1.11.1. The effect of inoculum level on root and systemic colonisation

Lower concentrations of *D. solani* (10^5, 10^3 CFU ml^{-1}) did not result in colonisation of the roots or stems of annual nettle. This experiment was repeated to ensure consistency of results and again only infection at 10^7 CFU ml^{-1} resulted in colonisation and invasion of the stems of annual nettle. In addition, there was no *D. solani* amplified from any of the 10^5 and 10^3 CFU ml^{-1} samples using the *D. solani* qPCR primers.

5.1.12. Produce prevention and control recommendations and contingency plans based on the availability of information from GB and overseas

The following document has been updated based on the outcomes of this project:

Potato Council Growers' Advice – '*Dickeya* spp: What it is… and what you can do?' http://potato.org.uk/publications/dickeya-what-it-and-what-can-you-do

6. CONCLUSIONS

- Two new PCR-based diagnostics have been developed for *D. solani* and are now being used at Fera and at NAK in the Netherlands in their potato screening. The diagnostics are also now being trialled throughout the rest of Europe. This will ensure consistency of identification of *D. solani* amongst the countries using the assay.

- MLST, VNTR and SNP markers have been developed to track *D. solani* in the environment. While each of the methods show some degree of difference between isolates, the clonal nature of the pathogen means that only few groupings are apparent. While this could provide useful information it is also somewhat restrictive for tracking single strains / outbreaks.

- No *D. solani* infections have been found in Scotland following the introduction of legislation in 2010 and the numbers of non-Scottish origin seed is dwindling. This suggests that the movement of infected seed is the principle source of the pathogen and not the environment, at least in the case of Scotland. Seed surveys in England/Wales also suggest that the source of tuber infection is non-UK origin seed and there is no evidence of infection from the environmental.

- Although very few waterways in Scotland remain positive for *Dickeya* species, the levels of the pathogen appear to be below that needed for infection following irrigation. However, advice is not to irrigate from these sources and advisory notices have been issued regarding this. While a small number of unconnected rivers in England / Wales do show contamination *Dickeya* species, there is no evidence of long-term (>1 year) colonisation.

- Like *P. atrosepticum*, disease incidence caused by *D. solani* is related to the level of tuber contamination but seasonal conditions also have a major role. As with previous reports, which suggest that *Dickeya* species can initiate disease from contamination levels below that of *P. atrosepticum*, there was only slightly more disease initiated from 10 cell ml^{-1} for *D. solani* than for *P. atrosepticum* at 21°C (a temperature conducive to diseases development by both pathogens) and no statistically significant differences for more heavily contaminated tubers / stems.

- *D. solani* caused up to 5 times, but typically 3 times, more tuber rotting at 27°C than at 21°C, with rotting at 21°C equivalent to that for *P. atrosepticum*. This would have major implications for disease in warmer seasons and could relate (although we have no data for it) to increased environmental spread in warmer countries.

- Contamination of neighbouring tubers from infected plants did occur both in raised beds and field experiments but to low levels (and in wetter patches of the field). This may be due to the differences in prevailing climate or soil moisture at the experimental sites and European commercial conditions. It may also be due to higher incidence of undetected seed infections in the cases where this has been reported in continental Europe.

- *D. solani* does not survive well on common materials. However, tuber to tuber contamination during handling remains an important method of spread.

- All commonly used disinfectants are effective in killing *D. solani* but some work better than others at concentrations lower than the manufacturer's recommended doses. These include sodium hypochloride and Vanoquat.

- There was no evidence for overwintering of *D. solani* but it was able to maintain and even slightly increase in levels during commercial storage.

- Lab and glasshouse studies have demonstrated that *D. solani* is able to bind to the roots of some weeds and colonise some plants when it is present at high levels (10^7 CFU ml^{-1}). It can cause disease in annual nettle (*U. urtica*). The role of weeds in the spread of *D. solani* under field conditions in GB has not been quantified. Weeds may not be of major importance in the UK due to our climatic conditions. However, they may be an important source of spread to tubers in warmer countries. More recent data have shown that spread from weeds to tubers can take place in the glasshouse where waterlogging occurs but not where waterlogging is absent, albeit at high inoculum levels.

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8. APPENDICES

8.1.1. Appendix 1: Evaluation of real-time PCR assay for Dickeya solani

Overview

In order to begin the process of validating possible real-time assays for *D. solani* a simple evaluation exercise is planned using two potential specific assays and a small number of potato sap samples some of which have been spiked with different concentrations of *D. solani* and its relatives. You will be supplied with most of the materials needed to perform the assay but you will need to make some buffers and media, details contained in the Appendix, and you will need access to a real-time PCR machine. We suggest you assay the sap directly and after an enrichment step, we also propose that you should test the sap by plating out onto CVP to enumerate colonies and, if you can, testing some of the resultant colonies to determine whether they are *Dickeya* spp. or not. You can check whether colonies are *Dickeya* spp. by using the ECH real-time assay listed below and it would be helpful if you also use the conventional PCR assay based on the ADE primer set designed by Nassar *et al* 1996 (*Applied and Environmental Microbiology* 62; 2228–2235).

8.1.1. Protocol

1. From the 10 sap samples, streak out onto CVP agar plates and incubate for 48 hours at 37^{o}C. Suggest determining colony counts for each sample by spread plating onto CVP at the following dilutions: neat, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}, counting the number of colonies forming pits. Take a few random pit-forming colonies and check identity using ECH real-time assay and the conventional, 'Nassar assay'.
2. Test the 10 sap samples directly by taking 20µl and boiling at 100^{o}C for 5min. Use 1µl of the boiled cells in the PCR assays.
3. Extract DNA from the 10 sap samples using the method below. Use 5µl of extract in the PCR assays.
4. Take 250µl of each of the 10 sap samples and enrich in 250µl of Pectate Enrichment Broth and incubate for at least 48 hours at 37^{o}C.
5. Test the 10 enrichment samples directly by taking 20µl and boiling at 100^{o}C for 5min. Use 1µl of boiled cells in the PCR assays.
6. Extract DNA from the 10 enrichment samples using the method below. Use 5µl of extract in the PCR assays.

i. DNA extraction

DNA is extracted following a method adapted from Pastrik (2000) *European J Plant Pathol*. 106; 155-165 using Invitrogen "Easy DNA" extraction kit (See appendix for buffers). 220µl of lysis buffer was added to 100µl of plant tissue extract and heated for 10 minutes at 95^{o}C before being placed on ice for 5 minutes. 80µl lysozyme stock solution was then added and the samples incubated at 37^{o}C for 30 minutes. The samples were then mixed by vortexing with 220µl of Easy DNA solution A (Invitrogen) and incubated at 65^{o}C for 30 minutes. 100µl of Easy DNA solution B (Invitrogen) and 500µl of chloroform was added. The samples were centrifuged at 15000g for 20 minutes at 4^{o}C to separate the phases and form the interphase. The upper phase was retained, washed with 1ml of 100% ethanol (stored at -20^{o}C) and incubated on ice for 10 minutes. The ethanol was removed by centrifuging at 15000*g* for 20 minutes at 4^{o}C. The pellet was washed with 500µl 80% ethanol (stored at -20^{o}c) and was again centrifuged at 15000*g* and 4^{o}c but for 10 minutes. The ethanol was removed and the pellet allowed to air dry for at least 45 minutes. The pellet was resuspended in 100µl sterile molecular grade water and left at room temperature for at least 20 minutes before being stored at -20^{o}C until required for PCR. 5µl of the supernatant was used for the PCR and the

amount of sterile water used in the reaction was reduced accordingly to give a total reaction amount of 25µl.

ii. Real-Time PCR protocol

24 µl of master mix was added to 1µl of boiled cells (or 5µl of extracted DNA).
The cycle below was run on Applied Biosystems 7900HT real time PCR machine.

Table 35: real-time PCR cycle

95^0c	10 mins	x 1 cycle
95^0c	15 s	x 40 cycles
60^0c*	1 mins	

The machine was run in standard mode, detecting FAM/TAMRA and using ROX as the passive reference. Data was taken at the extension (*) step only.

Table 36: Primers for assays

Assay	Forward primer	Reverse primer	Probe
SOL-C	GCCTACACCATCAGGGCTAT	CACTACAGCGCGCATAAACT	CCAGGCCGTGCTCGAAATCC
fusA	GGTGTCGTTGACCTGGTGAAA	ATAGGTGAAGGTCACACCCTCATC	TGAAAGCCATCAACTGGAATGATTC
ECH	GAGTCAAAAGCGTCTTGCGAA	CCCTGTTACCGCCGTGAA	CTGACAAGTGATGTCCCCTTCGTCTAGAGG

Table 37: PCR reaction mixes

1x probe reaction mix	
Taqman master mix	12.5 µl
Forward primer (5 pmol)	1.5 µl
Reverse primer (5 pmol)	1.5 µl
Probe (5 pmol)	0.5 µl
Template	1 µl
H_2O (standard lab 18.2ΩM grade	8 µl
TOTAL REACTION AMOUNT	**25µl**

Table 38

Double strength pectate enrichment medium (D-PEM; Meneley & Stanghellini, 1976)

$MgSO_4$	0.64 g
$(NH_4)_2SO_4$	2.16 g
K_2HPO_4	2.16 g
Sodium polypectate (Slendid type 440; M. Bulger Enterprises)	3.4 g
Distilled water	to 1000 ml

1. Dissolve the first three salts separately in 300 ml distilled water. Heat if necessary.
2. Mix together in the order of the recipe (a precipitate may appear if this is not done) and make up to 1000 ml with distilled water.
3. Suspend 3.4 g of the polypectate in 5 ml of absolute ethanol and add to the salts solution, mixing well using a magnetic stirrer.
4. Steam until the polypectate is completely dissolved before adjusting the pH to 7.2, if necessary.
5. Prepare small aliquots (e.g. 50 ml) and sterilise by autoclaving at 120 ºC for 15 min. Store at 4 ºC. Once open, do not re-use to avoid contamination.

Solutions

TE: 10mM Tris-HCl, pH 8.0,
1mM EDTA

Lysis buffer: 100mM NaCl
10mM Tris-HCl (pH 8.0)
1mM EDTA (pH 8.0)

Lysozyme stock solution: 50mg lysozyme per ml in 10 mM Tris HCl pH 8.0

8.1.2. Appendix 2: DNA extraction from homogenised weed and potato samples

1. Homogenised samples were centrifuged for 10 minutes at 1000rpm at 4°C to remove large particulate material.
2. Supernatant was removed without disturbing the pellet and 2 x 5ml aliquots were dispensed into separate 15ml centrifuge tubes. If there was insufficient volume to create 2 x 5 ml aliquots, a single 5 ml aliquot was removed and any remaining supernatant was kept as a back-up.
3. The supernatant was centrifuged for 15 minutes at 5000rpm at 4^{o}C.
4. The supernatant was removed and the pellet stored at -20°C until required, at which point the pellet was re-suspend in 1ml of 1 x PBS buffer.
5. To each tube, 250µl of Buffer B (Promega Cat no. Z3191) and 750µl precipitation solution (Promega Cat no. Z3201) was added. The tubes were vortexed and then incubated at room temperature for 5 minutes.
6. Tubes were centrifuged at 5000rpm for 15 minutes at room temperature and then 750µl of supernatant was removed from each sample tube, whilst avoiding pellet, and pipetted into 2ml Eppendorf tubes.
7. To the Eppendorf tube, 750µl of isopropanol (stored at -20^{o}C) and 75µl of sodium acetate (3M) was added.
8. The tubes were inverted gently and incubated for 1 hour at room temperature.
9. The tubes were centrifuged at 13200rpm for 4 minutes and the supernatant removed.
10. To each tube 150µl of 70% ethanol was added and the tubes vortexed.

11. The tubes were centrifuged at 13200rpm for 2 minutes, then the ethanol removed and the pellet allowed to air dry for no longer than 10 minutes.
12. The pellet was re-suspended in 100µl TE buffer and the neat DNA stored at –20 °C until required.

8.1.3. Appendix 3: Screening of SASA and Fera D. solani real-time PCR assays

Table 17: Preliminary screening of SASA and Fera real-time PCR assays, using strains from SASA own collection

	SASA STOCK STRAINS											
	Strain	Attributed species	Host	Country	Year	CT values obtained in real-time qPCR assays						
						FusA	FusA	SOL-C	SOL-C	SOL-D	SOL-D	ADE
1	**MK1**	*Dickeya sp.*	*River 1*	Scotland	2007	39.1	32.0	Undetermined	17.4	Undetermined	Undetermined	+
2	**MK2***	*Dickeya sp.*	*River 1*	Scotland	2007	19.9	19.6	15.7	18.2	18.4	17.3	+
3	**MK3**	*Dickeya sp*	*River 1*	Scotland	2007	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	+
4	**MK4**	*Dickeya sp*	*River 1*	Scotland	2007	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	+
5	**MK5**	*Dickeya sp*	*River 1*	Scotland	2007	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	+
6	**MK6**	*Dickeya sp*	*River 1*	Scotland	2007	Undetermined	35.5	Undetermined	Undetermined	35.9	37.0	+
7	**MK7**	*Dickeya sp*	*River 1*	Scotland	2007	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	+
8	**MK8**	*Dickeya sp*	*River 2*	Scotland	2007	37.3	33.4	35.7	Undetermined	36.2	35.9	+
9	**MK9**	*Dickeya sp*	*River 1*	Scotland	2007	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	+
10	**MK10**	*Dickeya sp*	*Solanum tuberosum, rotten tuber.*	Israel	2006	17.8	17.3	15.3	15.5	17.4	15.2	+
11	**MK11**	*Dickeya sp*	*Solanum tuberosum, asymtomatic tuber*	import	2006	17.7	Undetermined	14.9	16.4	16.7	14.8	+
12	**MK12**	*Dickeya sp*	*Solanum tuberosum, asymtomatic tuber*	import	2006	18.3	17.1	14.3	16.7	17.7	15.9	+
13	**MK13**	*Dickeya sp*	*Solanum tuberosum, rotten tuber.*	Israel	2007	Undetermined	20.0	17.1	16.9	18.6	16.9	+
14	**MK14**	*Dickeya sp*	*River 2*	Scotland	2008	18.6	18.1	17.3	16.6	17.7	16.2	+
15	**MK15**	*Dickeya sp*	*River 2*	Scotland	2008	32.8	32.8	30.9	31.1	32.9	32.8	+
16	**MK16**	*Dickeya sp*	*River 2*	Scotland	2008	18.3	15.5	14.1	16.4	15.0	14.9	+
17	**MK17**	*Dickeya sp*	*Zea mays*	USA	1991	Undetermined	9.4	Undetermined	Undetermined	Undetermined	Undetermined	+
18	**MK18**	*Dickeya sp*	*Dieffenbactia picta*	Italy	1992	34.5	15.0	Undetermined	35.9	Undetermined	37.0	+
19	**MK19***	*Dickeya sp*	*River 3*	Scotland	2008	28.6	27.6	23.9	26.6	Undetermined	26.2	+
20	**MK20**	*Dickeya sp*	*River 3*	Scotland	2008	38.4	Undetermined	35.4	36.6	Undetermined	34.7	+
21	**A101-9**	*Dickeya sp*	*Solanum tuberosum*	Poland		18.4	17.7	15.9	16.7	16.2	15.9	+
22	**A101-10**	*Dickeya sp*	*Solanum tuberosum*	Poland		17.9	17.5	15.7	16.3	17.3	15.4	+
23	**A101-11**	*Dickeya sp*	*Solanum tuberosum*	Poland		19.4	18.8	18.0	17.3	19.2	17.6	+
24	**A101 12**	*Dickeya sp*	*Solanum tuberosum*	Poland		18.1	18.3	16.2	18.3	19.0	17.6	+
25	**B1(A)**	*Dickeya sp*	*Solanum tuberosum, tubers*	Germany	2009	Undetermined	7.6	Undetermined	Undetermined	35.6	33.2	+
26	**B1(B)**	*Dickeya sp*	*Solanum tuberosum, tubers*	Germany	2009	20.2	Undetermined	19.0	18.7	19.7	18.5	+
27	**B20714-261**	*Dickeya sp*	*Phalaenopsis*	England	2007	Undetermined	Undetermined	35.6	Undetermined	Undetermined	37.1	+
28	**B2744**	*Dickeya sp*	*Solanum tuberosum*	Scotland	2009	19.1	17.7	15.3	17.1	16.8	17.0	+
29	**B2745**	*Dickeya sp*	*Solanum tuberosum*	Scotland	2009	19.0	19.4	16.7	17.4	18.8	21.1	+
30	**DM157**	*Dickeya sp*	*Solanum tuberosum Agria*	England (Dutch seed)	2009	21.3	Undetermined	17.8	22.0	19.1	18.7	+
31	**DM159**	*Dickeya sp*	*Solanum tuberosum Agria*	England (Dutch seed)	2009	18.4	18.7	14.2	18.7	17.3	16.5	+
32	**RW192-1**	*Dickeya Zeae*	*River water*	England		Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	+
33	**RW240/1**	*New Dickeya sp.*	*River water*	England		37.2	26.6	36.7	25.2	Undetermined	37.3	+
34	**206216-74**	*Dickeya sp.*	*Hiacinth*	England	2007	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	+
35	**20708-100**	*Dickeya sp*	*Dahlia*	England	2007	28.9	28.6	28.5	24.5	32.9	29.7	+
36	**20710-504**	*Dickeya sp*	*Solanum tuberosum cv, Markies*	England	2007	16.9	17.7	14.6	16.1	16.0	14.7	+
37	**20714-521**	*Dickeya sp*	*Sedum*	England	2007	35.5	34.5	Undetermined	Undetermined	Undetermined	Undetermined	+
38	**312**	*P. carotovorum*	*Solanum tuberosum*	Denmark	1952	Undetermined	Undetermined	37.0	Undetermined	Undetermined	Undetermined	+
39	**402**	*D. chrysanthemi*	*Chrysanthemum morifolium*	USA	1958	36.1	35.2	33.2	Undetermined	Undetermined	37.4	+
40	**453**	*D. dianthicola*	*Dianthus caryophyllus*	England,	1956	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	+
41	**454**	*D. dadanti*	*Philodendron sp.*	USA	1957	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	-
42	**516**	*D. chrysanthemi*	*Chrysanthemum sp.*		1957	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	+
43	**549**	*P. artosepticum*	*Solanum tuberosum*	UK	1958	Undetermined	26.6	25.7	Undetermined	25.6	25.5	+
44	**569**	*Dickeya sp*				35.9	35.0	Undetermined	34.8	Undetermined	35.2	+
45	**898**	*D. dadanti*	*Pelargonium capitatum*	Comoros	1960	32.9	32.2	34.4	32.7	Undetermined	32.9	+

46	**1092**	*Erwinia chrysanthemi*				35.4	30.4	33.2	32.9	33.3	30.9	+
47	**1121**	*D. zeae*	*Ananas comosus*	Malaysia	1962	17.9	18.8	15.1	17.3	16.4	16.6	+
48	**1385**	*Dickeya sp*				35.1	36.9	34.2	33.6	Undetermined	33.4	+
49	**1578**	*P. rhapontici*	*Rheum Rhaponticum*	England	1963	37.5	32.1	Undetermined	Undetermined	Undetermined	35.6	+
50	**1861**	*D. chrysanthemi*	*Parthenium argentatum*	USA	1966	33.2	Undetermined	26.8	32.0	26.5	30.8	+
51	**1863**	*D. zeae*	*Zea mays*	USA	1966	Undetermined	Undetermined	35.8	Undetermined	Undetermined	35.9	+
52	**2260**	*Dickeya dianthicola*				36.4	36.3	35.1	35.7	Undetermined	33.5	+
53	**2264**	*Erwinia chrysanthemi*	*Solanum tuberosum*	England	1995	34.8	Undetermined	Undetermined	36.5	Undetermined	36.1	+
54	**2265**	*Erwinia chrysanthemi*				Undetermined	Undetermined	34.5	Undetermined	37.5	34.0	+
55	**2295**	*Erwinia stewartii*				Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	+
56	**2339**	*D. zeae*	*Chrysanthemum morifolium*	England	1970	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	+
57	**2511**	*Dickeya paradisiacal*				Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	+
58	**2538**	*D. zeae*	*Zea sp*	USA	1970	Undetermined	Undetermined	37.0	Undetermined	Undetermined	36.7	+
59	**2541**	*Dickeya zeae*				36.9	Undetermined	34.5	35.7	37.4	34.0	+
60	**2546**	*Dickeya zeae*				34.0	31.5	30.0	32.4	Undetermined	30.6	+
61	**2795**	*P. betavasculorum*	*Beta vulgaris*	USA	1975	34.1	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	+
62	**2971**	*Erwinia herbicola*				34.0	33.2	32.5	32.1	Undetermined	31.2	+
63	**2976**	*D. dieffenbachiae*	*Dieffenbachia sp.*	USA	1957	34.4	Undetermined	36.3	37.4	37.0	Undetermined	+
64	**3004**	*Erwinia cypripedii*				31.3	32.7	29.5	30.6	Undetermined	31.5	+
65	**3274**	*Dickeya sp*	*Agloanema sp. (DUC-3)*	St. Lucia	1983	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	+
66	**3531**	*Dickeya zeae phylotype II*	*Solanum tuberosum,*	Australia		35.3	17.0	36.1	36.3	Undetermined	37.1	-
67	**3532**	*Dickeya zeae phylotype I*	*Solanum tuberosum*	Australia	1980	Undetermined	34.4	33.4	35.3	Undetermined	Undetermined	-
68	**3533**	*Dickeya chrysanthemi*	*Solanum tuberosum*	USA	1987	38.0	Undetermined	37.1	35.3	Undetermined	Undetermined	-
69	**3534**	*Dickeya dianthicola*	*Solanum tuberosum*	Holand	1987	Undetermined	Undetermined	36.4	Undetermined	Undetermined	Undetermined	-
70	**3701**	*P. wasabiae*	*Eutrema wasabi*	Japan	1990	Undetermined	35.9	Undetermined	Undetermined	Undetermined	Undetermined	-
71	**3839**	*P. carotovorum subsp. odoriferum*	*Cichorium intybus*	France	1992	Undetermined	36.0	Undetermined	35.1	Undetermined	Undetermined	-
72	**6395**	*P. chrysanthemi*	*Solanum tuberosum*	England	2006	14.4	16.3	10.5	15.0	15.6	14.7	+
73	**6396**	*P. chrysanthemi*	*Solanum tuberosum*	England	2006	17.9	16.3	15.1	15.6	16.2	14.6	+
74	**6397**	*P. chrysanthemi*	*Solanum tuberosum*	England	2006	34.5	35.3	30.9	33.7	Undetermined	33.9	

Strains confirmed as '*D. solani*' by recA sequencing

Non-'*D.solani*' strains

Table 18: Screening of SASA and Fera real-time PCR assays, using Dutch collection of Dickeya strains

	DUTCH STRAINS							
	PRI nr. (NCPPB number)	Attributed species	Host	Country	Year	CT values obtained in real-time qPCR assays		
						FusA	SOL-C	SOL-D
1	**980**	*D. dianthicola*	*Solanum tuberosum*	Netherlands		35.3	Undetermined	Undetermined
2	**1259**	*D. dieffenbachiae*	*Solanum tuberosum*	Hungary		34.7	30.0	33.4
3	**2019**	*D. solani*	hyacinth			14.3	16.8	15.4
4	**2114**	*D. dianthicola*	*Dianthus caryophyllus*			Undetermined	Undetermined	Undetermined
5	**2115**	*D. dianthicola*	*Dahlia sp*			Undetermined	Undetermined	Undetermined
6	**2116**	*D. dianthicola*	*Solanum tuberosum*	France	1975	30.0	35.6	Undetermined
7	**2117**	*D. chrysanthemi*	*Parthenium argentatum*			Undetermined	Undetermined	Undetermined
8	**2118 (402)**	*D. chrysanthemi biovar chrysanthemi*	*Chrysanthemum morifolium*	USA	1956	Undetermined	35.7	Undetermined
9	**2119**	*D. chrysanthemi biovar chrysanthemi*	*Helianthus annuus*	France	1986	Undetermined	Undetermined	Undetermined
10	**2120**	*D. dadantii*	*Pelargonium capitatum*			Undetermined	36.9	Undetermined
11	**2121 (1121)**	*D. dadantii*	*Ananas comosus*	Malaysia	1961	Undetermined	Undetermined	Undetermined
12	**2122**	*D. dadantii*	*Ipomea batatas*	Cuba	1987	Undetermined	Undetermined	Undetermined
13	**2124**	*D. dieffenbachiae*	*Dieffenbachiae sp.*	France	1970	Undetermined	Undetermined	Undetermined
14	**2125 (2976)**	*D. dieffenbachiae*	*Dieffenbachiae sp.*	USA	1957	Undetermined	37	Undetermined
15	**2126**	*D. dieffenbachiae*	*Lycopersicon esculentum*	Cuba	1987	Undetermined	Undetermined	Undetermined
16	**2127**	*D. paradisiaca*	*Musa paradisiaca*	Colombia	1968	Undetermined	34.5	Undetermined
17	**2129 (2511)**	*D. paradisiaca*	*Musa paradisiaca var. dominico*	Colombia	1970	Undetermined	Undetermined	Undetermined
18	**2131 (2538)**	*D. zeae*	*Zea mays*	USA	1970	Undetermined	Undetermined	Undetermined
19	**2132 (2339)**	*D. zeae*	*Chrysanthemum morifolium*	UK	1970	Undetermined	Undetermined	Undetermined
20	**2133**	*D. zeae*	*Ananas comosus*	Martinique	1991	Undetermined	Undetermined	Undetermined
21	**2187**	*D. solani*	*Solanum tuberosum*	Israel	2006	13.2	14.7	14.2
22	**2222**	*D. solani*	*Solanum tuberosum*	Netherlands	2007	13.7	16.6	14.4
23	**2276**	*D. solani*	*Solanum tuberosum*	Poland	2005	13.6	16.3	14.3
24	**3228**	*D. solani*	*Solanum tuberosum*	Israel		15.1	14.9	14.2
25	**3239**	*D. solani*	*Solanum tuberosum*	UK		14.7	16.7	14.9
26	**3294**	*D. solani*	*Solanum tuberosum*	Finland		14.3	15.1	15.3
27	**3295**	*D. solani*	*Solanum tuberosum*	Finland		16.2	16.5	15.8
28	**3296**	*D. solani*	*Solanum tuberosum*	Israel		Undetermined	Undetermined	Undetermined
29	**3327 (3528)**	*D. dianthicola*	*Solanum tuberosum*	UK		30.8	37.6	Undetermined
30	**3328 (3530)***	*D. dianthicola*	*Solanum tuberosum*	UK		16.2	13.1	Undetermined
31	**3329 (3531)**	*D. zeae*	*Solanum tuberosum*	Australia		Undetermined	Undetermined	Undetermined
32	**3330 (3533)**	*D. chryanthemi*	*Solanum tuberosum*	USA		Undetermined	Undetermined	Undetermined
33	**3332 (3237)**	*D. dadantii*	*Solanum tuberosum*	Peru		Undetermined	Undetermined	Undetermined
34	**3334 (3344)**	*D. dianthicola*	*Solanum tuberosum*	France		Undetermined	36.6	Undetermined
35	**3336**	*D. solani*	*Solanum tuberosum*	France		15.8	15.8	15.5
36	**3337**	*D. solani*	*Solanum tuberosum*	France		17.6	16.6	15.0

Strains confirmed as '*D. solani*' by recA sequencing

Non-'*D.solani*' strains

8.1.4. Appendix 4: Real-time PCR results of Ring Test

Table 19: Real-time PCR results from all 6 laboratories using the ECH assay

Sample	Strain	Species	ECH												
			BOILED						DNA						
			1	2	3	4	5	6	1A	1B	2	3	4	5	6
1 unenriched	MK15 L	*D. solani* (River 2)	Not tested	***No Ct	***No Ct	***No Ct	***35.916	***No Ct	Not tested	Not tested	***No Ct	***34.5017	***36.6664	***No Ct	***39.29
2 unenriched	RW192/1	*D. zeae*	Not tested	27.7	28.2	23.8	18.0	16.9	Not tested	Not tested	17.8	14.1	22.0	***33.813	15.9
3 unenriched	Blank		Not tested	No Ct	39.3	30.9	No Ct	No Ct	Not tested	Not tested	No Ct	35.7	37.8	No Ct	39.9
4 unenriched	MK13 L	*D. solani* (Israel)	Not tested	***No Ct	***39.7946	***38.5540	***36.395	***38.64	Not tested	Not tested	***No Ct	***31.5934	***31.0893	***No Ct	***37.44
5 unenriched	MK15 H	*D. solani* (River 2)	Not tested	24.8	25.6	24.5	19.8	20.9	Not tested	Not tested	19.5	14.7	23.2	***35.809	17.9
6 unenriched	MK13 H	*D. solani* (Israel)	Not tested	27.0	26.4	27.2	17.4	22.8	Not tested	Not tested	19.0	17.1	24.8	28.2	18.6
7 unenriched	Blank		Not tested	No Ct	No Ct	37.5	No Ct	No Ct	Not tested	Not tested	No Ct	35.9	35.5	No Ct	36.2
8 unenriched	3327	*D. dianthicola*	Not tested	23.9	18.7	26.3	19.5	20.9	Not tested	Not tested	32.0	14.5	22.5	29.5	17.8
9 unenriched	549	*P. atrosepticum*	Not tested	Negative	33.7	32.2	No Ct	No Ct	Not tested	Not tested	No Ct	***28.8503	34.2	36.5	33.7
10 unenriched	20621674	*D. solani* (Hyacinth)	Not tested	24.9	27.3	27.0	12.3	22.6	Not tested	Not tested	18.3	17.0	24.0	***30.342	20.6
1 enriched	MK15 L	*D. solani* (River 2)	Not tested	***No Ct	24.4	24.0	19.5	22.1	Not tested	Not tested	***No Ct	17.0	25.6	***32.233	23.0
2 enriched	RW192/1	*D. zeae*	Not tested	29.7	18.8	24.2	17.6	16.4	Not tested	Not tested	26.7	13.5	21.7	29.0	14.6
3 enriched	Blank		Not tested	No Ct	No Ct	No Ct	No Ct	38.8	Not tested	Not tested	No Ct	32.6	35.2	No Ct	31.4
4 enriched	MK13 L	*D. solani* (Israel)	Not tested	***No Ct	***No Ct	22.3	***38.039	25.0	Not tested	Not tested	No Ct	***33.2861	23.7	28.5	***30.57
5 enriched	MK15 H	*D. solani* (River 2)	Not tested	21.0	19.3	23.5	19.2	19.6	Not tested	Not tested	24.4	13.6	22.9	***No Ct	16.7
6 enriched	MK13 H	*D. solani* (Israel)	Not tested	21.0	27.0	29.0	21.3	22.0	Not tested	Not tested	26.3	20.2	26.4	27.7	19.0
7 enriched	Blank		Not tested	No Ct	No Ct	No Ct	No Ct	No Ct	Not tested	Not tested	No Ct	***29.8979	34.0	No Ct	38.1
8 enriched	3327	*D. dianthicola*	Not tested	27.5	***31.8923	27.8	19.6	22.1	Not tested	Not tested	23.1	23.4	27.7	18.5	21.1
9 enriched	549	*P. atrosepticum*	Not tested	No Ct	No Ct	No Ct	***28.728	No Ct	Not tested	Not tested	No Ct	***29.5766	32.7	No Ct	33.6
10 enriched	20621674	*D. solani* (Hyacinth)	Not tested	18.9	27.1	24.8	21.8	19.6	Not tested	Not tested	26.4	20.8	26.9	***32.199	16.8

1A Easy DNA
1B Maxwell 16 Plant Kit

Institute 2 into enrichment broth 30/8/11
Institute 5 direct sample extraction 31 Aug/1 Sept

1. AFBI
2. Fera
3. JHI
4. NIAB
5. SAC
6. Sutton Bridge

D. solani

Blank or *Pectobacterium* spp.

Other *Dickeya* sp.

Table 20: Real-time PCR results from all 6 laboratories using the SOL-C assay

Sample	Strain	Species	SOL-C												
			BOILED						DNA						
			1	2	3	4	5	6	1A	1B	2	3	4	5	6
1 unenriched	**MK15 L**	***D. solani* (River 2)**	***No Ct	***No Ct	***No Ct	***36.4296	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***30.8423	***36.1721	***No Ct	***35.06
2 unenriched	**RW192/1**	***D. zeae***	No Ct	No Ct	36.6	No Ct	No Ct	36.7	No Ct	No Ct	No Ct	35.2	35.0	No Ct	No Ct
3 unenriched	**Blank**		No Ct	No Ct	35.6	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	34.5	No Ct	No Ct	No Ct
4 unenriched	**MK13 L**	***D. solani* (Israel)**	***No Ct	***No Ct	***36.1879	***33.3042	***37.922	***33.07	***No Ct	***30.91	***No Ct	27.2	***31.3797	***No Ct	***31.37
5 unenriched	**MK15 H**	***D. solani* (River 2)**	***No Ct	17.7	24.8	20.2	23.3	19.1	26.3	17.3	18.2	14.6	20.2	***37.822	17.7
6 unenriched	**MK13 H**	***D. solani* (Israel)**	***No Ct	***36.13	25.2	20.8	22.7	19.8	25.6	15.1	18.5	14.9	21.1	***31.767	16.8
7 unenriched	**Blank**		No Ct	No Ct	36.1	31.4	No Ct	No Ct	No Ct	No Ct	No Ct	33.0	33.0	No Ct	33.6
8 unenriched	**3327**	***D. dianthicola***	No Ct	***23.9	No Ct	30.9	No Ct	No Ct	No Ct	No Ct	***19.1	32.4	35.3	No Ct	31.7
9 unenriched	**549**	***P. atrosepticum***	No Ct	No Ct	37.1	34.7	No Ct	No Ct	No Ct	No Ct	No Ct	***27.8771	38.3	No Ct	***29
10 unenriched	**20621674**	***D. solani* (Hyacinth)**	***No Ct	18.2	24.8	19.7	19.6	21.1	26.1	15.0	17.9	13.6	20.9	***32.87	18.0
1 enriched	**MK15 L**	***D. solani* (River 2)**	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***34.7282	***36.6065	***No Ct	***No Ct
2 enriched	**RW192/1**	***D. zeae***	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	32.3	No Ct	No Ct	35.3
3 enriched	**Blank**		No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	33.1	39.3	No Ct	36.0
4 enriched	**MK13 L**	***D. solani* (Israel)**	***No Ct	***No Ct	***36.2361	***34.7736	***37.096	28.0	***No Ct	***No Ct	***No Ct	***34.2510	***36.6640	***34.583	25.8
5 enriched	**MK15 H**	***D. solani* (River 2)**	***No Ct	23.0	29.0	21.3	25.1	25.2	***32.51	***No Ct	24.4	22.3	24.2	***No Ct	21.4
6 enriched	**MK13 H**	***D. solani* (Israel)**	***No Ct	28.0	25.7	21.9	22.9	20.7	***No Ct	***No Ct	***32.07	18.4	23.2	***33.4	17.2
7 enriched	**Blank**		No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	32.3	34.5	No Ct	32.4
8 enriched	**3327**	***D. dianthicola***	No Ct	***24.02	37.6	No Ct	No Ct	No Ct	No Ct	No Ct	***24.99	32.1	33.7	No Ct	No Ct
9 enriched	**549**	***P. atrosepticum***	No Ct	No Ct	36.8	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	31.0	38.5	No Ct	34.5
10 enriched	**20621674**	***D. solani* (Hyacinth)**	***No Ct	19.5	25.8	19.6	22.0	18.6	***No Ct	***No Ct	20.3	20.5	22.8	***34.124	15.4

1A Easy DNA
1B Maxwell 16 Plant Kit

Institute 2 into enrichment broth 30/8/11
Institute 5 direct sample extraction 31 Aug/1 Sept

1. AFBI
2. Fera
3. JHI
4. NIAB
5. SAC
6. Sutton Bridge

D. solani

Blank or *Pectobacterium* spp.

Other *Dickeya* sp.

Table 21. Real-time PCR results from all 6 laboratories using the *fusA* assay and if performed the 'conventional' PCR assay using the ADE primers

Sample	Strain	Species	FusA													ADE				
			BOILED						DNA							1			2	
			1	2	3	4	5	6	1A	1B	2	3	4	5	6	Boiled	A	B	Boiled	DNA
1 unenriched	**MK15 L**	***D. solani* (River 2)**	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***36.6576	***38.8485	***No Ct	***37.91	Not tested	Not tested	Not tested	Negative	Negative
2 unenriched	**RW192/1**	***D. zeae***	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	39.2	No Ct	No Ct	Not tested	Not tested	Not tested	Positive	Positive
3 unenriched	**Blank**		No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	Not tested	Not tested	Not tested	Negative	Negative
4 unenriched	**MK13 L**	***D. solani* (Israel)**	***35.27	***No Ct	***No Ct	***37.1674	***No Ct	***36.32	***No Ct	***No Ct	***No Ct	***32.8273	***34.6805	***No Ct	***34.62	Not tested	Not tested	Not tested	Negative	Negative
5 unenriched	**MK15 H**	***D. solani* (River 2)**	28.9	21.4	28.4	25.2	25.5	22.7	28.4	***No Ct	***35.53	20.1	22.0	***No Ct	21.1	Not tested	Not tested	Not tested	Positive	Positive
6 unenriched	**MK13 H**	***D. solani* (Israel)**	28.1	24.7	28.5	26.0	22.5	23.8	26.8	***No Ct	20.0	19.8	22.8	***32.047	20.0	Not tested	Not tested	Not tested	Positive	Positive
7 unenriched	**Blank**		No Ct	No Ct	No Ct	37.5	No Ct	No Ct	No Ct	No Ct	No Ct	38.2	35.7	No Ct	35.5	Not tested	Not tested	Not tested	Negative	Negative
8 unenriched	**3327**	***D. dianthicola***	No Ct	***29.13	36.9	35.2	No Ct	35.2	No Ct	No Ct	31.2	35.4	36.3	No Ct	33.6	Not tested	Not tested	Not tested	Positive	Positive
9 unenriched	**549**	***P. atrosepticum***	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	32.8	No Ct	No Ct	33.9	Not tested	Not tested	Not tested	Negative	Negative
10 unenriched	**20621674**	***D. solani* (Hyacinth)**	27.6	18.2	28.2	24.9	22.6	24.8	27.1	***No Ct	21.0	19.5	22.3	***36.132	21.5	Not tested	Not tested	Not tested	Positive	Positive
1 enriched	**MK15 L**	***D. solani* (River 2)**	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***No Ct	***39.1293	***No Ct	***39.03	Not tested	Not tested	Not tested	Negative	Negative
2 enriched	**RW192/1**	***D. zeae***	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	37.8	38.9	No Ct	No Ct	Not tested	Not tested	Not tested	Positive	Positive
3 enriched	**Blank**		No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	37.1	***25.8973	No Ct	No Ct	Not tested	Not tested	Not tested	Negative	Negative
4 enriched	**MK13 L**	***D. solani* (Israel)**	***35.3	***No Ct	***No Ct	***38.1926	***No Ct	29.9	***35.24	***37.02	***No Ct	***36.1989	***37.8720	***37.968	29.9	Not tested	Not tested	Not tested	Negative	Negative
5 enriched	**MK15 H**	***D. solani* (River 2)**	25.8	***No Ct	***31.8878	26.4	26.9	27.2	***32.57	28.2	***34.59	27.2	26.5	***No Ct	24.9	Not tested	Not tested	Not tested	Positive	Positive
6 enriched	**MK13 H**	***D. solani* (Israel)**	24.7	25.0	28.4	26.0	25.2	22.3	***32.85	25.6	***31.31	22.9	24.9	***36.91	20.5	Not tested	Not tested	Not tested	Positive	Positive
7 enriched	**Blank**		No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	35.9	35.6	No Ct	35.7	Not tested	Not tested	Not tested	Negative	Negative
8 enriched	**3327**	***D. dianthicola***	No Ct	32.1	No Ct	No Ct	No Ct	34.5	No Ct	No Ct	31.2	36.1	36.4	No Ct	35.5	Not tested	Not tested	Not tested	Positive	Positive
9 enriched	**549**	***P. atrosepticum***	No Ct	No Ct	No Ct	39.5	No Ct	No Ct	No Ct	No Ct	No Ct	35.1	38.3	***17.719	38.6	Not tested	Not tested	Not tested	Negative	Negative
10 enriched	**20621674**	***D. solani* (Hyacinth)**	25.5	26.4	29.6	24.0	24.7	20.3	***30.05	25.3	29.6	23.2	24.7	***38.299	18.9	Not tested	Not tested	Not tested	Positive	Positive

1A Easy DNA
1B Maxwell 16 Plant Kit

Institute 2 into enrichment broth 30/8/11
Institute 5 direct sample extraction 31 Aug/1 Sept

1. AFBI
2. Fera
3. JHI
4. NIAB
5. SAC
6. Sutton Bridge

D. solani

Blank or *Pectobacterium* spp.

Other *Dickeya* sp.

8.1.5. Appendix 5: Susceptibility of D. solani to common disinfectants

Table 35. Susceptibility of '*D. solani*', *D. dianthicola* and *P. atrosepticum* to a range of disinfectants

Commercial Product (General use dilution)	Strains	Contact times (mins)	Colony Count					
			%					
			100	0.5	0.4	0.3	0.2	0.1
FAM 30 (1:180)	*D. solani*	5	0	0	0	0	510	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	TNTC	TNTC
	D. solani	10	0	0	0		104	TNTC
	D. dianthicola	10	0	0	0	0	0	1
	P. atrosepticum	10	0	0	0	TNTC	TNTC	TNTC
	D. solani	30	0	0	0	0	0	TNTC
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	TNTC	TNTC
GPC8 (1:35)	*D. solani*	5	0	0	2	1	TNTC	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	2	231
	D. solani	10	0	0	0	0	0	0
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	0	0	0	0	0
	D. solani	30	0	0	0	0	0	0
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	0	0
Halamid (0.3:100)	*D. solani*	5	0	1	2	0	1	3
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	0	0
	D. solani	10	0	0	0	0	0	0
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	0	0	0	0	0
	D. solani	30	0	0	0	0	0	0
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	0	0
Jet 5 (1:250)	*D. solani*	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	0	1
	D. solani	10	0	156	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	0	4	TNTC	TNTC	TNTC
	D. solani	30	0	0	0	0	70	TNTC
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	1	16	TNTC
Jeye's Fluid (5:100)	*D. solani*	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	30	0	TNTC	TNTC	TNTC	TNTC	TNTC

Mikrozid AF liquid	*D. solani*	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
Sodium hypochlorite (14:100)	*D. solani*	5	0	0	0	0	0	0
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	0	0
	D. solani	10	0	0	0	0	0	0
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	0	0	0	0	0
	D. solani	30	0	0	0	0	0	0
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	0	0
V18 (1:500)	*D. solani*	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
Vanoquat (1:300)	*D. solani*	5	0	0	0	0	0	0
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	0	0
	D. solani	10	0	0	0	0	0	0
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	0	0	0	0	0
	D. solani	30	0	0	0	0	0	0
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	0	0
Virkon S (1:100)	*D. solani*	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	0	0
	D. solani	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	0	0	0	0	0	1
	P. atrosepticum	10	0	0	0	0	0	0
	D. solani	30	0	0	0	0	0	0
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	0	0
-ve control (water)	*D. solani*	5	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	5	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	10	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	10	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	30	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	30	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	30	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC

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