Final Report

Reducing wastage and increasing shelf life of root vegetables during washing, packing and retailing

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1. PREFACE

Rotting of potatoes after washing and packing can be a significant cause of consumer complaints, and as a result has economic consequences for the fresh potato supply chain.

The pathogens responsible for causing soft rotting are pectolytic bacteria, principally *Pectobacterium* species (previously known as *Erwinia carotovora* subsp. *atroseptica* and *Erwinia carotovora* subsp. *carotovora* and now referred to as *Pectobacterium atrosepticum* and *Pectobacterium carotovorum*, respectively).

Currently there isn't a method available to rapidly and reliably identify stocks at risk of soft rotting. This short feasibility study was commissioned to determine if techniques currently used in other sectors, such as the medical and veterinary industries, could be of value in the potato industry. In particular, the researchers were asked to establish whether the techniques could be used by staff in packhouses to rapidly quantify the numbers of soft rotting bacteria on tubers at intake and/or after washing. Data on the numbers of bacteria present on a sample of tubers and the extent of soft rotting in the same stock were recorded with the aim of developing soft rot risk categories, with high risk stocks being those that are likely to show significant soft rot breakdown problems under commercial conditions.

At the start of the project several techniques, with potential for use in packhouses were identified and these were evaluated in parallel to determine which offered the greatest potential. The techniques were:

Serological Lateral Flow Device tests (LFD).
Based on the use of antibodies. The approach would use a sample of macerated tissue (eg tuber peel). The work involved testing the suitability of currently available antibodies for use in a LFD.

Linear After The Exponential Polymerase Chain Reaction (LATE-PCR).
Based on the detection of specific DNA sequences. The technique was originally developed by researchers at Brandeis University, USA. It has benefits over conventional quantitative polymerase chain reaction (PCR) techniques (such as TaqMan real-time PCR) in that it allows the numbers of many different species of pathogen in a sample to be quantified at the same time. Smiths Detection have an exclusive license for LATE-PCR from Brandeis University and have developed instrumentation for deployment of LATE-PCR in field conditions. Their technology was initially evaluated however, the company subsequently ceased to be involved in the project consortium and as result greater emphasis was given to evaluating other techniques.

Loop mediated isothermal Amplification (LAMP).
Based on the detection of specific DNA sequences. Unlike conventional quantitative PCR techniques, it does not require equipment to produce temperature changes to allow the underlying molecular reactions to progress.

The numbers of several different species of pathogen in a sample can be quantified at the same time. Portable equipment is required to detect and display the results from the underlying reactions. The Optigene Genie II instrument was used in this study.

The BioSeeq instrument (Smiths Detection), designed for use with LATE-PCR, would allow integrated DNA extraction and amplification. For the other technology (LAMP), DNA extraction would be performed as a separate step prior to carrying LAMP on the Optigene instrument. DNA extraction would normally be carried out under laboratory conditions, where precautions can be taken to minimise cross contamination of samples. As a result, work was carried out to develop and compare methods for DNA extraction that could be used on-site in packhouses.

The project was jointly funded by HortLink, Potato Council and HDC, with contributions from several vegetable packing companies and companies specialising in the development of diagnostic technologies. Given the range of crops of interest to these funders, work on other pathogens of carrots and parsnips was also included in the study. *Dickeya* species (previously referred to as *Erwinia chrysanthemi*) are also pectolytic bacteria able to cause soft rotting and were also included as target pathogens in the study.

Conclusions

DNA extraction

A simple DNA extraction technique, based on the use of ChargeSwitch® purification technology, was developed. It can be used on-site without requiring complex equipment or precise manipulations.

LATE PCR

The design of the molecular tools that would allow many different pathogens to be detected at one time was considered to be complex and it was not possible to produce an assay combining all the pathogens by the end of the project. The technique also involved long reaction times (greater than 2 hours) and required complex instrumentation and consequently it was concluded that LATE-PCR is not currently suitable for on-site deployment in packhouses for plant pathogen detection.

LAMP

Separate assays for the detection of pectolytic bacteria (*Pectobacterium* and *Dickeya* spp.) and *Pythium ultimum* have been developed and showed good potential for application in on-site tests. Further development will be needed before a suitable assay for *Sclerotinia sclerotiorum* is available.

Risk categories

Using the LAMP technique it was possible to relate rotting of potato tubers (under optimum conditions for pathogen multiplication) to the initial numbers of *Pectobacterium* spp. present on the tubers. Three rotting categories were tested using commercial stocks of potatoes: 0-10^3 colony forming units (cfu)/tuber (low risk); 10^3 to 10^5 cfu/tuber (medium risk); 10^5 cfu/tuber and

above (high risk). All tested stocks identified as being in the high risk category showed significant soft rot breakdown problems under conducive conditions.

Originally it was anticipated that this short feasibility study would be used as the basis to develop a full HortLink research proposal. This would have provided a route to carry out further development of the diagnostic technologies. However, by the end of the feasibility study, the HortLink funding stream was no longer considering new proposals. Therefore, although it has been possible to demonstrate the potential of packhouse diagnostics, further development work, with the aim of reducing the time required to analyse samples, has not been pursued.

Potato Council, 2012

2. SUMMARY

Diagnostic assays have been developed at Fera to key pathogens that cause rotting of potato, carrot and parsnip. The assays have been validated under laboratory conditions using pure cultures of target pathogens and related organisms as well as infected potato and carrot samples. Serological lateral flow device (LFD) tests were too specific for detection of the full diversity of pathogens commonly associated with rotting vegetables. LATE-PCR assays designed for detection of pectolytic soft rot bacteria (*Pectobacterium* and *Dickeya* spp.) and the fungal pathogens *Pythium violae* and *Sclerotinia sclerotiorum* were less effective than existing TaqMan real-time PCR methods in terms of specificity and sensitivity. Loop mediated isothermal amplification (LAMP) assays, designed to detect the same range of pathogens, were more reliable than LATE-PCR under laboratory conditions, although the LAMP assay developed for *Sclerotinia sclerotiorum* was not found to be reliable during subsequent laboratory validation.

For the soft rot bacteria, quantification of inoculum loading on potato tubers was related to risk of rotting by testing artificially-inoculated potato samples in the laboratory and washed potatoes at different stages during commercial packing. Inoculum loadings were successfully identified which lead to medium and high rotting potential when samples from different stocks were placed under conducive controlled environment conditions or under actual commercial washing, packing and retailing conditions. Conversely, stocks identified with low inoculum loadings had low rotting potential.

An assessment of rotting potential of stocks was based on the categories: 0-10^3 colony forming units (cfu)/tuber (low risk); 10^3 to 10^5 cfu/tuber (medium risk); 10^5 cfu/tuber and above (high risk). These studies used routine laboratory methods (isolation on CVP medium) to quantify inoculum loading. However, the three risk categories could also be identified using LAMP assays performed on the same stocks indicating the potential of LAMP to determine the risk of rotting in a commercial environment. All tested stocks which had shown significant soft rot breakdown problems under commercial conditions were identified as being in the high risk category.

Methods for simple DNA extraction from vegetable material using lateral flow device (LFD) membranes or magnetic beads were compared with crude extraction methods for use with the LAMP assays. Protocols for on-site detection were optimised. The feasibility of using the protocols for semi-quantitative on-site testing was investigated using the Genie II automated diagnostic platform from Optigene. Successful detection of soft rotting bacteria at inoculum loadings relating to high risk of rotting after washing was demonstrated to quality control staff under commercial washing and packing conditions. Application of the technology to monitor pathogen loading on potato, carrot and parsnip, in relation to risk of rotting prior to or after washing and packing, was concluded to be fully feasible for on-site use. Further commercial-scale optimisation and validation is now needed.

3. INTRODUCTION

In reaching current supermarket quality requirements, a significant proportion of parsnip, carrot and potato production is rejected during commercial washing and packing operations. Reduction of carrot and parsnip waste is listed as a priority in the HDC R&D strategy following estimates from the British Carrot Growers Association that *circa* 40% of production is wasted from harvest to final packing. Potato Council estimate annual losses of 10-20% of potato production. Losses have increased in recent years due to wetter growing and harvesting conditions which increase inoculum loading of fungal and bacterial pathogens on harvested crops. Furthermore, these vegetables are listed by WRAP as major contributors to the 6.7 million tonnes of avoidable household food waste produced each year in the UK of which more than 10% is attributed to parsnips, carrots and potatoes. This costs around £1 billion in wasted food and equates to around 1.8 million tonnes of carbon dioxide emitted as methane when the waste goes to landfill and including the energy spent in production, storage, packing and transportation.

The vegetable packing industry currently lacks the means to identify high risk crops on arrival and to accurately determine and control the effect of washing and packing procedures on shelf-life of the packed product before and after dispatch to the retailer. New molecular diagnostics technologies currently used in hospital and veterinary screening programmes have potential for adaptation for rapid on-site detection and quantification of key plant pathogens without the need for specialist handling. Quantitative diagnostic methods suitable for field operation include Linear After The Exponential polymerase chain reaction (LATE-PCR) using the Bioseeq platform from Smiths Detection Ltd. and Loop mediated isothermal AMPlification (LAMP) using the Genie II platform from OptiGene Ltd. Field test kits, based on lateral flow device (LFD) technology from Forsite Diagnostics, also have potential for development as quantitative on-site antibody- and LAMP-based assays as well as for rapid *in situ* DNA extraction.

This one-year feasibility study aimed to demonstrate the potential of these novel diagnostic platforms (LATE-PCR, LAMP and LFD and associated portable instrumentation) for multiplex detection and quantification of key

rotting pathogens affecting washed and packed vegetables. Moreover, it aimed to evaluate the practicality and cost-efficiency of accommodating the diagnostic technologies within commercial vegetable washing and packing operations.

The study included an assessment of the value of rapid detection and quantification of the key pathogens in assessing the risk of post washing breakdown and predicting shelf-life of the stored product. Target pathogens mainly included *Pectobacterium* and *Dickeya* spp. involved in bacterial soft rots of potato, carrots and parsnips as well as *Sclerotinia sclerotiorum* and *Pythium violae* which cause rotting of carrots and parsnips after washing and cavity spot of field-stored carrots, respectively. Monitoring of pathogen population dynamics at critical points throughout storage, washing, packing and retailing processes aimed to identify critical parameters (e.g. operating temperatures, packing materials, disinfection measures etc.) which could be manipulated to suppress pathogen build up and increase the shelf life of packed vegetables.

Specific objectives were:

1. Adapt and optimise novel diagnostic platforms (LATE-PCR, LAMP and LFD) for rapid on-site detection of key parsnip, carrot and potato pathogens.
2. Prove that diagnostics are suitable and cost-efficient for entry stage quality control and monitoring pathogen population changes in commercial washing and packing environments.
3. Demonstrate the effect of pathogen loading on shelf-life of packed vegetables.
4. Accurately quantify current losses and expected shelf life of packed vegetables.
5. Prepare full HortLink proposal with enlarged consortium representing all stages of the supply chain.

4. MATERIALS AND METHODS

4.1. Suitability of antibodies for potential use in LFD assays

The specificity of a number of available antibodies was assessed against a panel of isolates of *Pectobacterium carotovorum* and *Dickeya* spp. using a standard ELISA format.

4.2. Development and laboratory validation of LATE PCR assays

The advantages of LATE-PCR for on-site testing are predominantly due to the use of this technology on the Smiths Detection BioSeeq instrument for integrated DNA extraction and amplification. After the departure of Smiths Detection from the Consortium, work continued on development of LATE-PCR assays to assess the value of LATE-PCR for improved multiplex detection in

comparison with methods based on conventional symmetrical PCR. The production of single-stranded amplification products in LATE-PCR is reported to improve multiplexing capability by allowing probes with different melting temperatures (T_m) to be used in addition to probes with different fluorescent reporters (Sanchez et al, 2004). In a standard multiplex reaction, multiple probes can be used in the same reaction if they are labelled with different fluorescent labels; in this approach, discrimination of labels with similar wavelengths is a limiting factor. In the LATE-PCR approach, probes with the same reporter but different T_ms can be used in the same reaction by performing fluorescence monitoring at multiple temperatures, since no complementary strand of the amplification product is present to compete with the low T_m probe. LATE-PCR primers were designed for pectolytic bacteria, *S. sclerotiorum* and *P. violae*, followed by design of probes with different reporters and T_ms.

4.2.1. Pectolytic bacteria (*Pectobacterium* and *Dickeya* spp.)

For efficient generation of single-stranded amplification product, LATE-PCR uses primers at an unequal ratio (approx. 20:1). In order to compensate for the change in melting temperature caused by a change in concentration, the length of the limiting primer (present at a low concentration) must be increased relative to that of the excess primer. LATE-PCR primers for detection of pectolytic bacteria (*P. carotovorum* subsp. *atrosepticum, P. carotovorum* subsp. *betavasculorum, P. carotovorum* supsp. *odifera, P. carotovorum* subsp. *wasabiae, P. carotovorum* subsp. *brasiliensis, P. atrosepticum*, and *Dickeya* spp. (formerly *Erwinia chrysanthemi*)) were adapted from the currently used PEC TaqMan real-time PCR primers by increasing the length of the forward primer (the limiting primer in LATE-PCR) to give a T_m^L ≥5°C above that of the reverse (excess) primer. The reverse primer was also modified by the addition of a base at the 5′ end to give a T_m^X closer to the T_m of the amplicon, as recommended by Pierce et al (2005).

Primers were assessed by performing real-time PCR in the presence of EvaGreen intercalating dye to monitor generation of double-stranded amplification products in the initial stage of LATE-PCR. Each 25 µl reaction contained 0.625 units AmpliTaq Gold DNA polymerase, 1 x Buffer A, 5.5 mM MgCl2, 200 µM each dNTP, 1 µM excess primer, 50 nM limiting primer, and 0.5 x EvaGreen. PCR was carried out on an ABI 7900HT instrument using the following cycling condition: 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, 60 seconds at 60°C and 30 seconds at T_{mon} (variable fluorescence monitoring step). For comparison with TaqMan real-time PCR, the PEC TaqMan assay was used with the same conditions, except that the forward and reverse primers were both used at a final concentration of 300 nM and instead of EvaGreen the TaqMan probe was added at a final concentration of 100 nM. For TaqMan real-time PCR the same cycling conditions were used with the omission of the fluorescence monitoring step (fluorescence monitoring was performed during the 60°C annealing and extension step).

4.2.2. *Sclerotinia sclerotiorum*

LATE-PCR primers for *S. sclerotiorum* were designed by modification of the conventional primers developed by Rogers et al (2009). In order to obtain the required difference in melting temperatures, the reverse primer of the original assay was adjusted by 1 base (excess primer in LATE-PCR) and a limiting primer was designed to give the required melting temperature without introducing secondary structure. Primers were assessed using the conditions described above.

4.2.3. *Pythium violae*

LATE-PCR primers for *P. violae* were designed based on an alignment of COX II sequences for target and non-target species (see section 4.3.3). A region specific for *P. violae* was identified and used as the template for automated primer design using Primer Express 3 software. Primers identified by the design software were then modified to give the required melting temperatures, as described above. Primers were assessed as described above.

LATE-PCR primers described in sections 4.1 are shown in Table 1.

TABLE 1. PRIMER SEQUENCES FOR LATE-PCR.

Primer name	Target	Sequence (5′-3′)
Pec ex 1	Pectolytic bacteria	CCTCTACAAGACTCTAGCCTGTCAGTTTT
Pec lim 1b		CGGTAATACGGAGGGTGCAAGCGTTAATCGGAATG
Scler ex	*S. sclerotiorum*	CTTGTTTTTAGGGACAGGCTTAATGC
Scler lim		GTCAGAAGATGATCGAAAGAGAAATGGATTATCGCGGA
Pv ex v2	*P. violae*	TGTTGTACATGGTGCAACTATTG
Pv lim v2		CCATTGAATATAATAAAGCAAATGAAGGTAC**G**GCTACA

Molecular beacon probes were designed for the three primer pairs described above with the characteristics shown in Table 2. Reporters were selected for use on the Cepheid SmartCycler.

TABLE 2. PROPERTIES OF MOLECULAR BEACON PROBES DESIGNED FOR USE WITH LATE-PCR PRIMERS.

Target	Tm (°C)	5′ Reporter	3′ Quencher
Pectolytic bacteria	55.6 (high)	FAM	Dabcyl
S. sclerotiorum	53.5 (high)	Texas Red	BHQ2
S. sclerotiorum	53.5 (high)	Cy3	BHQ1
P. violae	55.9 (high)	JOE	BHQ1
P. violae	44.6 (low)	FAM	BHQ1

4.3. Development and laboratory validation of LAMP assays

Loop-mediated isothermal amplification (LAMP) is performed using 6 primers designed to target the species of interest, comprising external primers (F3 and B3), internal primers (FIP and BIP) and loop primers (F-loop and B-loop). LAMP was performed using conventional reagents and also using a novel Isothermal Master Mix which reduces run times and allows real-time detection using the Genie II instrument.

4.3.1. Pectolytic bacteria (*Pectobacterium* and *Dickeya* spp.)

Two sets of LAMP primers were designed to detect the following pectolytic bacteria: *P. carotovorum* subsp. *atrosepticum, P. carotovorum* subsp. *betavasculorum, P. carotovorum* supsp. *odifera, P. carotovorum* subsp. *wasabiae, P. carotovorum* subsp. *brasiliensis, P. atrosepticum*, and *Dickeya* spp. (formerly *Erwinia chrysanthemi*). Primers were designed based on an alignment of 16S rRNA sequences of target and non-target species used by Toth *et al* (1999) for development of a PCR-based assay. Assay 1 was designed in the same region as the currently used PEC TaqMan real-time PCR assay which was based on the conventional PCR assay of Toth *et al*; assay 2 was designed to target a different region of the 16S sequence. Due to differences in the sequences of the different target species, primers in both assays contained degenerate bases and/or mismatched bases. Initial testing indicated superior performance for assay 1, so this primer set was used for all further testing. Primers were tested using DNA extracted from cultures of target and non-target species.

LAMP was carried out in 25 µl reactions containing 0.32 U/µl *Bst* DNA polymerase (New England Biolabs, Ipswich, MA), 1 x Thermopol buffer, 1.4 mM each dNTP, 6 mM $MgSO_4$ (including 2 mM in the Thermopol buffer), 1.2 M betaine, 200 nM each external primer (F3 and B3), 2 µM each internal primer (FIP and BIP), and 1 µM each loop primer (F-loop and B-loop). Reactions were incubated at 65°C for 60 minutes, then at 80°C for 5 minutes to denature the polymerase. Amplification products were analysed by agarose gel electrophoresis with ethidium bromide staining.

Real-time LAMP was carried out on the Optigene Genie II instrument which performs concurrent temperature control and fluorescence monitoring. Each 25 µl reaction contained 1x Isothermal Master Mix (includes EvaGreen intercalating dye) and primers using the concentrations given above. Reactions were incubated on the Genie II at 65°C for 40-60 minutes followed by analysis of the amplification products by cooling from 95°C to 75°C with a slow ramp rate to generate an annealing profile (analogous to a melt profile).
Real-time LAMP results were analysed in terms of time to positive (earlier amplification indicates a higher target DNA concentration) and melt peak (which is characteristic of the specific product for each assay).

All LAMP primers are shown in Table 3.

TABLE 3. PROPERTIES OF LAMP PRIMERS

Primer name	Target	Sequence (5′-3′)
Pec1 F3	Pectolytic bacteria (16S)	CGTTACYCGCAGAAGAAGCA
Pec1 B3		CCGCTACACCTGGAATTCTACC
Pec1 FIP		CATTCCGATTAACGCTTGCACGCTAACTCCGTGCC AGCA
Pec1 BIP		TGGATGTGAAATCCCCGGCAAGACTCTAGCCTGT CAGTTTTG
Pec1 F-loop		CTCCGTATTACCGCGGC
Pec1 B-loop		GCTTAACCTGGGAACTGCATT
Sc v2 F3	*S. sclerotiorum* (mt small subunit rRNA intron and ORF1)	GAAGATGATCGAAAGAGAAATGGA
Sc v2 B3		CAGTCAAAAATCAAGGATTGATATTCT
Sc v2 FIP		CTTGTTTTTAGGGACAGGCTTAATGCGGATCTAAA TCAGTAATACCCCAA
Sc v2 BIP		TGTGTTTAAGGTGTACTCTAATGGGCTACGTAAAT ATTTGAAGTAGAATAGAACCT
Sc v2 F-loop		CTACCGTCTACTCGTTGCTC
Sc v2 B-loop		GAAAGAAGTTATCAAATCAGAATCCT
Pv v2 F3	*P. violae* (ITS)	GATCGCGTGCAGTTGTCG
Pv v2 B3		GTCCTCTACCCACAACAAAATG
Pv v2 FIP		CACGCAACATTGTACAGCCGAAGCTCGGCGTGAA CATATGGAG
Pv v2 BIP		TGTGCCTTGAGGTGTACTGACGGCGTCGCTGTAC ATCACACAA
Pv v2 F-loop		AACATACCGCGAATCGAGGC
Pv v2 B-loop		GTTGTGTGCTTGAACTGGAG

4.3.2. *Sclerotinia sclerotiorum*

LAMP primers were designed based on an alignment of the mitochondrial small subunit rRNA intron and ORF1 (Rogers *et al.,* 2009) and related non-target sequences identified by BLAST analysis. Primers were designed to the same region as the conventional PCR assay of Rogers *et al*. Primers were tested using DNA extracted from cultures of target and non-target species. LAMP was carried out as described above, primers are shown in Table 3.

4.3.3. *Pythium violae*

LAMP primers were designed based on alignments of COX II sequences and ITS sequences for target and non-target *Pythium* species, as used by Villa et al (2006) for construction of phylogenies of the genus. The ITS assay was designed close to the region targeted by Klemsdal *et al* (2008). Primers were designed to exploit sequence differences between *P. violae* and the closely related species *P. irregulare*, *P. sylvaticum*, *P. ultimum* and *P. spinosum* (COX II alignment) and *P. heterothallicum* (ITS alignment). Primers were tested using DNA extracted from target and non-target species. LAMP was carried out as described above, primers are shown in Table 3.

4.4. Optimisation of DNA extraction methods for on-site assays

For development and optimisation of DNA extraction from potato, samples were either healthy tubers, healthy tubers spiked with bacterial suspensions,

or symptomatic or asymptomatic infected tubers. To assess DNA extraction from healthy tubers, extracts were tested for potato DNA using a LAMP assay for detection of the plant cytochrome oxidase gene (Tomlinson, *et al.,* 2010).

4.4.1. Crude DNA extraction and LFD format extraction

Crude extracts were prepared by maceration of approx. 300 mg sample material (e.g. potato or carrot peel) in 5 ml LFD Buffer C (Forsite Diagnostics) either by shaking in a bottle containing the buffer and 5 stainless steel ball bearings for up to 1 minute or by crushing in a bag containing buffer with a small hammer. Crude extract was added directly to amplification reactions. Alternatively, 70 µl homogenised sample was applied to the release pad of a 2-minute DNA LFD (a lateral flow device without antibodies in a dipstick format) and left to dry for several minutes. A small section (approx 2 mm x 1.5 mm) was excised from the membrane and added directly to the amplification reaction.

4.4.2. ChargeSwitch extraction

DNA was extracted using the ChargeSwitch Plant kit with some modifications. Briefly, approx 150 mg potato peel was roughly homogenised using a small hammer as described above in a bag containing 2.5 ml ChargeSwitch Lysis Buffer. Approx. 1 ml homogenate was transferred to a tube containing 100 µl SDS. The sample was incubated at room temperature for 2-5 minutes, then 400 µl Precipitation Buffer was added and the sample was centrifuged at approx. 11,000 x g for 5 minutes. Approx. 1 ml of the resulting supernatant was transferred to a clean tube with 40 µl ChargeSwitch Beads and 100 µl Detergent. A PickPen device was used to transfer the magnetic beads and bound DNA through 2 wash steps in 1 ml Wash Buffer and into 150 µl Elution Buffer from which the beads were removed and discarded. DNA (1 µl was added to amplification reactions. For on-site use, the centrifugation step is replaced by filtration of the sample through a 5 µm syringe filter, and the number of wash steps was varied between 0 and 2. To further simplify the method for on-site use, all reagents were pre-aliquotted, such that the only pipetting step required is for transfer of DNA to the amplification tube.

4.5. Relationship between pathogen loading and bacterial soft rot potential

Potato seed tubers cv. Maris Piper that were free from *Pectobacterium* spp. were selected for the inoculation experiment. Two replicate sets of 5 tubers were artificially inoculated by vacuum infiltration at -0.9 bar in serially diluted aqueous suspensions containing *Pectobacterium carotovorum* strain P3414. Control tubers were infiltrated with sterile distilled water. Soft rot incidence was recorded after 3 days anaerobic incubation of the wet tubers in plastic bags at 28°C.

In addition, inoculum loading on potato stocks demonstrating problems of soft rotting during commercial washing and packing were also determined and compared with stocks in which no evidence of soft rot was identified.

Inoculum loading was determined by routine laboratory procedures as follows: samples of 100 randomly chosen and apparently healthy tubers were tested in sub-samples of 20. Strips of peel from around each tuber and including the point of stolon attachment were removed from each tuber and ground in phosphate buffer (pH 7.0) containing sodium pyrophosphate antioxidant. Serial dilutions of the extract were then spread on modified crystal violate pectate (CVP) medium and pectolytic colonies were enumerated after 48 hr incubation at 27°C.

4.6. Quantitative detection of pathogen populations in infected potatoes

Potato seed tubers were vacuum infiltrated as described in Section 4.5. The tubers were air dried then immediately processed for DNA extractions using the ChargeSwitch extraction method described in Section 4.4.2 for subsequent testing by real-time PCR and LAMP. Testing by LAMP was done using the Isothermal Master Mix for pectolytic bacteria and the Genie II instrument, as described in section 4.3.1. Testing by real-time PCR was done using the methods described in section 4.2.1.

4.7. On-site application of LAMP assays for detection of soft rotting bacteria

Samples were tested on-site at a potato packhouse using the ChargeSwitch extraction method described in section 4.4.2 and LAMP for pectolytic bacteria using Isothermal Master Mix and the Genie II instrument, as described in section 4.3.1.

4.8. Quantification of losses due to rotting

Estimates of losses incurred during the year as a direct result of soft rot during commercial potato washing and packing were made by the industrial partners. This information was based on a number of criteria including:

- The incidence of rotting identified during intake QC checks,
- The amount of breakdown observed in samples during routine hot box and shelf-life analyses,
- Additional staff costs required for QC,
- Additional staff costs required for inspection and removal of rotting tubers,
- Internal rejection of a rotting stock after packing but before dispatch (including, transfer costs, loss of downgraded/discarded product, unpacking costs, wasted packaging and lost energy and staff costs),
- Rejection at depot prior to transfer to retailer (including inspection cost, lost packaging, haulage return, replacement haulage, loss of downgraded product, unpacking product, lost energy, lost sales),
- Fines imposed by major retailers as a result of customer complaints following breakdown at retailer or after purchase.

5. RESULTS

5.1. Suitability of antibodies for LFD assays

All available antibodies were too specific to allow detection of the full range of pectolytic bacteria which cause soft rot of potato tubers and carrots. Furthermore, the sensitivity of detection was considered insufficient to allow reliable detection of rotting pathogens at populations usually present prior to the development of rotting symptoms. Further development of new LFD assays or the utilisation of existing commercially-available LFD assays (e.g. for *Pythium* detection), were not considered valid options for assessing rotting potential.

5.2. Development and laboratory validation of LATE-PCR assays

5.2.1. Pectolytic bacteria (*Pectobacterium* and *Dickeya* spp.)

The LATE-PCR primers designed for detection of pectolytic bacteria were found to have similar performance to the TaqMan real-time PCR assay on which they are based (Table 4).

TABLE 4. LATE-PCR AND TAQMAN REAL-TIME PCR RESULTS FOR DETECTION OF PECTOLYTIC BACTERIA AND NON-TARGET BACTERIAL SPECIES. (VALUES SHOWN ARE MEAN CT VALUES FOR DUPLICATE REACTIONS.)

	LATE-PCR	**TaqMan**
P. carotovorum subsp. *atroseptica*	19.37	20.16
P. carotovorum subsp. *betavasculorum*	27.47	29.49
P. carotovorum subsp. *odifera*	18.53	19.06
P. carotovorum subsp. *wasabiae*	34.61	33.53
P. carotovorum subsp. *carotovora*	34.52	37.20
Erwinia chrysanthemi (Dickeya)	17.13	18.72

Preliminary optimisation of primer ratios was carried out for this assay (Fig. 1). The LATE-PCR primers were used in a ratio of 20:1 with a molecular beacon probe; optimal amplification plots were observed using the molecular beacon at 200 nM, with a fluorescence monitoring step at 50°C (Fig. 2). All molecular beacon probes (see Table 2) were subjected to melt analysis to investigate the differences in fluorescence at high and low temperatures (in the open and closed conformation, respectively) (Fig. 3). The Pec molecular beacon was also subjected to melt analysis in the presence of the LATE-PCR amplicon (Fig. 3) to demonstrate the reduced quenching in the presence of the target strand (maintaining the open conformation at low temperature).

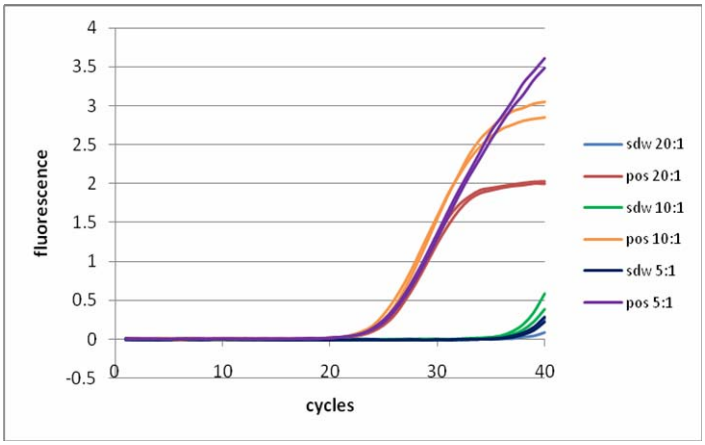

FIGURE 1. AMPLIFICATION PLOTS FOR LATE-PCR AMPLIFICATION OF PECTOLYTIC BACTERIA USING DIFFERENT PRIMER RATIOS. THE INTERCALATING DYE DETECTS DOUBLE-STRANDED AMPLIFICATION PRODUCTS, THE GENERATION OF WHICH REACHES A PLATEAU AT A LOWER LEVEL IF A MORE SYMMETRICAL PRIMER RATIO IS USED.

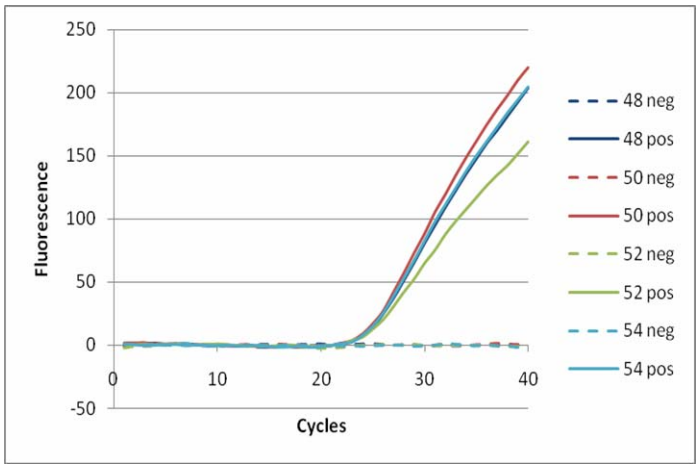

FIGURE 2. AMPLIFICATION PLOTS FOR LATE-PCR AMPLIFICATION OF PECTOLYTIC BACTERIA USING DIFFERENT FLUORESCENCE MONITORING TEMPERATURES RANGING FROM 48°C TO 54°C.

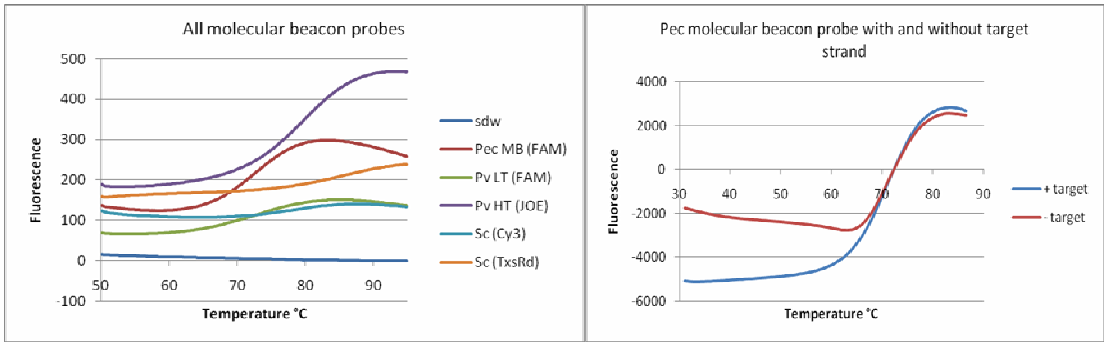

FIGURE 3. MELT ANALYSIS OF MOLECULAR BEACONS IN THE ABSENCE OF TARGET STRAND (LEFT: ALL PROBES) AND IN THE PRESENCE AND ABSENCE OF TARGET STRAND (RIGHT: PEC PROBE ONLY). (DIFFERENCES IN SCALE IN THE TWO PANELS ARE DUE TO THE USE OF DIFFERENT INSTRUMENTS FOR FLUORESCENCE MEASUREMENT.)

5.2.2. *Sclerotinia sclerotiorum*

The LATE-PCR primers designed for detection of *S. sclerotiorum* were found to amplify *S. sclerotiorum* but not the non-target species *Botrytis cinerea* when used with EvaGreen for detection of double-stranded amplification products in the exponential phase of LATE-PCR. However, very poor signals were obtained using the same primers with the molecular beacon probes described in Table 2. Low signals (difference in fluorescence for open vs closed conformations) were observed for molecular beacons labelled with both Texas Red and Cy3 (Fig. 3). The signal strength for the Texas Red probe was improved by reducing the Mg^{2+} concentrations, but this was not found to improve detection of amplification products using this probe. This may be attributable to inadequate labelling of the probe or flaws in its design, or could reflect the fact that the sequence used for design of the LATE-PCR and LAMP assays for *S. sclerotiorum* may be unreliable or unrepresentative.

5.2.3. *Pythium violae*

Testing of the COX II LATE-PCR primers for detection of *P. violae* indicated poor detection of the target species, and a lack of specificity (see Table 5). Initial results had indicated better detection of *P. violae*, despite cross-reactivity with non-target species, so molecular beacon probes were designed for use with these primers (Table 2), with the intention of improving specificity. Subsequent ITS sequencing indicated that the isolate of *P. violae* used initially had been misidentified or contaminated, and isolates positively identified as *P. violae* by ITS sequencing gave poor results (Table 5). Subsequent LAMP results indicated that ITS would be a better target for assay design for this species (see section 5.3.3), but this was not possible within the timescale of the project.

TABLE 5. LATE-PCR RESULTS FOR DETECTION OF PECTOLYTIC BACTERIA AND NON-TARGET BACTERIAL SPECIES. (VALUES SHOWN ARE MEAN CT VALUES FOR DUPLICATE REACTIONS.)

	Pv LATE-PCR v1	**Pv LATE-PCR v2**
No template control	-	37.34
Pythium violae	39.17	35.29
P. violae	-	36.98
P. cryptoirregulare	26.77	33.45
P. irregulare	26.16	32.73
P. debaryanum	18.73	16.54

5.3. Development and laboratory validation of LAMP assays

5.3.1. Pectolytic bacteria (*Pectobacterium* and *Dickeya* spp.)

Table 6 shows the results of testing target and non-target species using the pectolytic bacteria LAMP assay. These results suggest that the LAMP assay will produce broadly equivalent results to the real-time PCR assay on which it is based. Weak positives were observed for neat extracts of two of the non-

target species tested, although this may reflect a low level of contamination with *Pectobacterium* DNA in these extracts, as sequence analysis does not indicate a greater likelihood of cross-reactivity with the these species compared to the other non-target species tested. Non-target bacteria found to cross react in the LAMP assay are not expected to be present at detectable levels on potato, carrot or parsnip.

The pectolytic bacteria LAMP assay was used for testing extracts during optimisation of DNA extraction: further results are shown in section 5.4.1.

TABLE 6. COMPARISON OF TAQMAN REAL-TIME PCR AND LAMP (USING BST POLYMERASE AND GEL ELECTROPHORESIS) FOR TESTING DNA EXTRACTED FROM CULTURES OF TARGET PECTOLYTIC BACTERIA (TOP) AND NON-TARGET BACTERIA (BOTTOM).

		LAMP	
		neat	10^{-1}
P. carotovorum subsp. *atroseptica*	7017	+	+
P. c subsp. *betavasculorum*	4617	+	+
P. c subsp. *odifera*	3255	+	+
P. c subsp. *wasabiae*	3254	+	+
P. c subsp. *carotovora*	3024	+	+
Erwinia chrysanthemi (Dickeya)	BLEU 1A	+	+
E. nigrifluens	4410	+	+
E. salicis	1387	-	-
E. cacticida	2405	-	-
E. amylovora	36	(+)	-
E. rhapontici	378	-	-
E. persicinus	6112	-	-
Enterobacter dissolvens	2623	(+)	-
Enterobacter cangerogenus	6138	-	-
P. cypripedii	4419	-	-
Escherichia coli	5020	-	-
Pantoea agglomerans	6149	-	-

5.3.2. *Sclerotinia sclerotiorum*

Detection of *S. sclerotiorum* using LAMP was unsuccessful using two assays designed based on the mitochondrial small subunit rRNA intron and ORF1 (Rogers *et al.,* 2009). For this region, however, only one sequence has been published for *S. sclerotiorum*, so failure of the LAMP assays may be due to differences between isolates. The same region was used successfully as a target for PCR-based assays (PCR primers of Roger et al and the LATE-PCR primers described here) but these assays have a shorter amplicon than the LAMP assay, which has primers distributed across entire amplicon. To develop a successful LAMP assay for *S. sclerotiorum* it would be necessary to target a different region (eg ITS, the SCAR marker identified by Yin et al, 2009 or the laccase2 gene used by Hirschhäuser & Fröhlich, 2007), and this may also require the generation of new sequence data for multiple isolates. While the distribution of LAMP primers across the 200-300 bp amplicon has the potential to increase specificity in comparison with PCR, which is a crucial requirement for detection of specific targets in complex matrices, the same feature exacerbates the need for accurate sequence data for multiple isolates to ensure robust primer design.

5.3.3. *Pythium violae*

Detection of *P. violae* by LAMP was unsuccessful using primers designed to COX II sequence (similar results were obtained for LATE-PCR primers designed in the same region, but was successful using primers targeting ITS sequence (Fig. 4)).

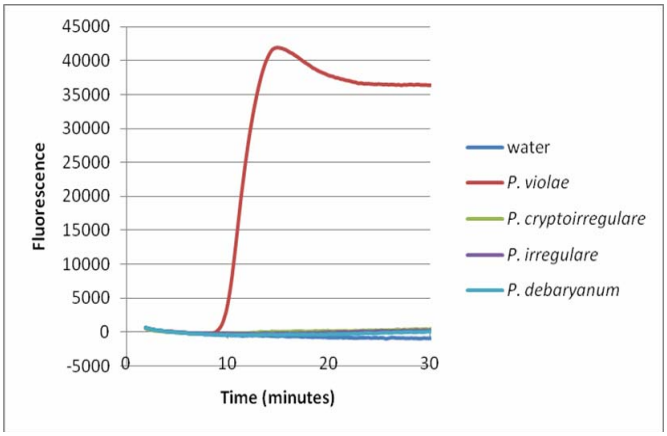

FIGURE 4. PYTHIUM VIOLAE REAL-TIME LAMP ON THE GENIE II. FIGURE SHOWS AMPLIFICATION PLOTS FOR DNA EXTRACTED FROM CULTURES OF TARGET AND NON-TARGET SPECIES.

Use of the ITS-based *P. violae* LAMP assay for detection of *P. violae* in soil samples from affected fields was demonstrated; furthermore, no cross reactivity was observed with DNA extracted from healthy carrot (Fig. 5). Further work would be required to determine sensitivity of this assay for detection of *P. violae* in carrot and soil samples, and to investigate whether the level of sensitivity is of value in the prediction of disease.

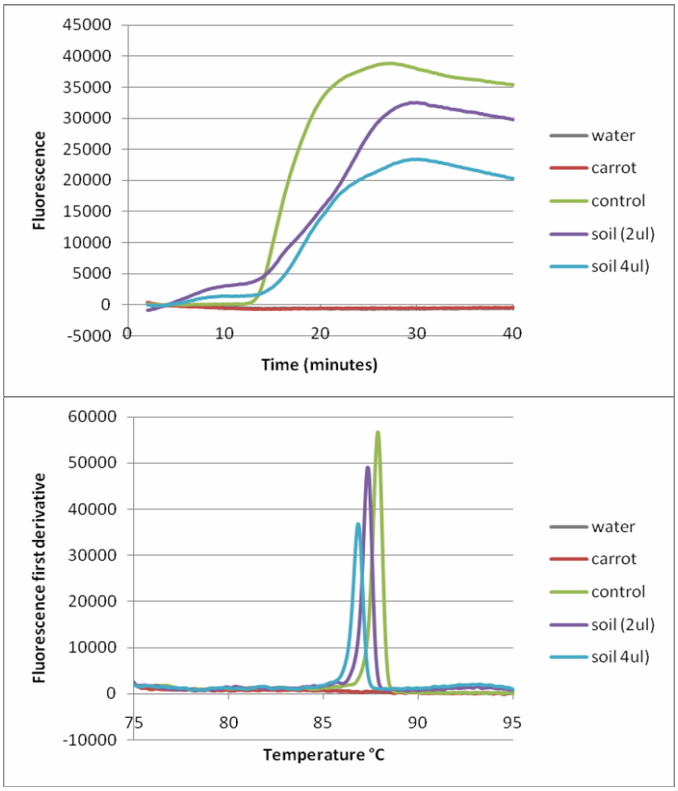

FIGURE 5. AMPLIFICATION PLOTS (TOP) AND MELT ANALYSIS (BOTTOM) FOR REAL-TIME LAMP DETECTION OF P. VIOLAE ON THE GENIE II.

5.4. Optimisation of DNA extraction methods for on-site assays

5.4.1. Crude DNA extraction and LFD format extraction

Initial experiments were carried out to assess extraction of DNA using the LFD method by extracting from healthy potato and carrot and testing the extracts by TaqMan real-time PCR for plant DNA (cytochrome oxidase). Fig. 6 shows TaqMan results for LFD extraction using different shaking times. Lower Ct values (indicating more DNA) were observed for carrot than potato, and DNA yield from potato was not improved by longer shaking.

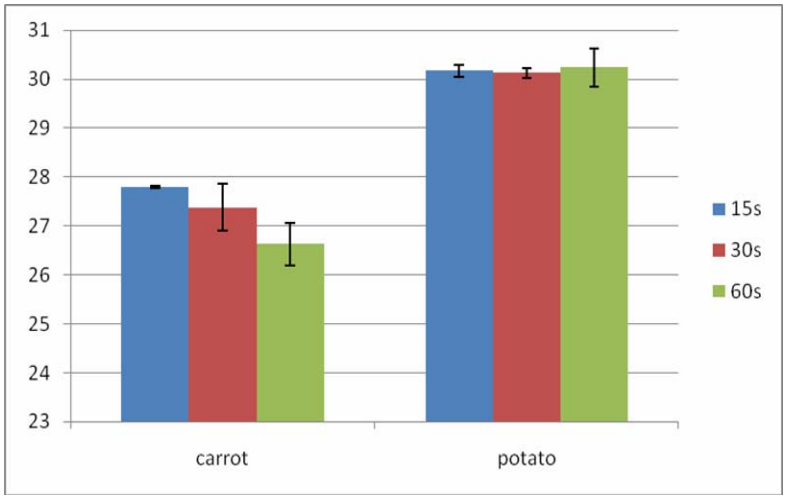

FIGURE 6. TAQMAN REAL-TIME PCR RESULTS FOR LFD DNA EXTRACTION FROM CARROT AND POTATO WITH DIFFERENT SHAKING TIMES. FIGURE SHOWS MEAN CT VALUES FOR DUPLICATE REACTIONS.

In order to investigate whether the relatively high Ct values for LFD extraction from potato were attributable to low DNA yield or the presence of inhibitory substances, sections of LFD membrane were added to LAMP and TaqMan reactions for pectolytic bacteria detection that were spiked with known amounts of *Pectobacterium* DNA. No adverse effect on amplification by LAMP or TaqMan was observed, even at the limit of detection, indicating the absence of inhibitors in LFD-extracted DNA from potato.

These results suggest that the low DNA yields from potato using LFD extraction may compromise sensitivity of detection of pectolytic bacteria. In order to increase efficiency of DNA extraction, experiments were carried out to investigate the use of a modified ChargeSwitch extraction method for extraction from potato. In a direct comparison, plant DNA (cytochrome oxidase) TaqMan real-time PCR Ct values of 30.5 and 24.8 were observed for LFD and ChargeSwitch extraction, respectively, indicating a difference in DNA yield of approximately 50- to 100-fold. DNA was also extracted using the LFD and ChargeSwitch methods from potatoes which had been vacuum infiltrated with pectolytic bacteria at different concentrations (10^3, 10^5 and 10^7), and tested by TaqMan real-time PCR and LAMP for pectolytic bacteria and plant DNA (cytochrome oxidase). As shown in Table 7, for LFD-extracted DNA, detection of pectolytic bacteria was only possible at the highest concentration of bacteria tested, and only by TaqMan real-time PCR. For ChargeSwitch-extracted DNA, bacteria were detectable by both TaqMan and LAMP in samples containing 100-fold less bacteria than was detectable using LFD extraction.

TABLE 7. TAQMAN REAL-TIME PCR AND LAMP RESULTS FOR TESTING DNA EXTRACTED FROM POTATO SAMPLES (VACUUM INFILTRATED WITH PECTOBACTERIUM AT THREE DIFFERENT CONCENTRATIONS) USING THE LFD AND CHARGESWITCH (C/S) METHODS.

	TaqMan		LAMP	
	Pec	**COX**	**Pec**	**COX**
No template control	-	-	-	-
control (Pec or plant)	15.94	25.11	+	+
LFD 1 (10^3)	-	27.90	+	+
LFD 2 (10^3)	-	27.02	-	+
LFD 1 (10^5)	-	27.18	-	+
LFD 2 (10^5)	-	26.62	-	+
LFD 1 (10^7)	38.11	27.31	-	+
LFD 2 (10^7)	37.23	26.77	-	+
C/S 1 (10^3)	-	23.76	-	+
C/S 2 (10^3)	-	23.50	-	+
C/S 1 (10^5)	33.68	23.51	+	+
C/S 2 (10^5)	34.40	23.00	+	+
C/S 1 (10^7)	31.00	23.58	+	+
C/S 2 (10^7)	29.78	23.21	+	+

Further development of the ChargeSwitch extraction method is described in section 5.4.2. In addition, experiments were carried out to investigate the possibility of direct addition of crude extracts to amplification reactions (without application to an LFD and excision of the membrane). This method has been found to be effective for detection of pathogens present at high levels (i.e. symptomatic infections) in plant matrices including potato tubers. For detection of high titre targets, very small samples can be taken from symptomatic material; however, for this application, much larger samples must be taken. Initial results indicated that while crude extraction (direct addition of crude extract to amplification reaction) may offer a small sensitivity advantage over LFD extraction, non-specific amplification artifacts were often observed in negative LAMP reactions when this method was used. While the non-specific amplification products can be distinguished from specific LAMP products by melt analysis, this makes interpretation of results more difficult.

5.4.2. Charge-switch extraction

Results showing the comparison of the ChargeSwitch and LFD extraction methods are shown in section 5.4.1; ChargeSwitch extraction was observed to result in higher DNA yields than LFD extraction. Modifications to the manufacturer's protocol (Precipitation Buffer used at room temperature, syringe filtration instead of centrifugation, reduction of wash steps to 1 or 0) were not observed to adversely affect DNA extraction from potato, as assessed by TaqMan real-time PCR and LAMP for plant DNA and pectolytic bacteria. Example results are shown in Table 8.

TABLE 8. REAL-TIME COX (PLANT DNA) LAMP RESULTS FOR DNA EXTRACTED FROM POTATO USING THE CHARGESWITCH METHOD WITH DIFFERENT NUMBERS OF WASH STEPS.

Number of wash steps	Time to positive (min:sec)	Melt peak (°C)
0	14:15	85.36
1	14:30	85.45
2	14:30	85.50

The modified ChargeSwitch method optimised for on-site use is as follows:

- Place sample (approx 150 mg) into grinding bag, add contents of Lysis Buffer tube.
- Roughly homogenise sample by manual disruption (e.g. using small hammer).
- Tip contents of bag into Precipitation Buffer tube to level indicated.
- Pass sample through 5 µm syringe filter into Bead tube.
- Use PickPen to transfer beads into Wash tube, mix then transfer beads to Elution tube.
- Transfer 1 µl eluted DNA to LAMP reaction tube for testing on Genie II.

5.5. Relationship between pathogen loading and bacterial soft rot potential

Rotting of potato tubers under optimum conditions for pathogen multiplication was directly related to the initial inoculum concentration. Some rotting generally occurred at each inoculum level but significantly more rotting was observed when tubers were inoculated at 10^7 colony-forming units (cfu) per ml than at 10^5 or 10^3 cfu per ml (Table 9). All tubers eventually rotted at the threshold inoculum level of 10^7 cfu per ml.

TABLE 9: ROT INCIDENCE IN ARTIFICIALLY INOCULATED TUBERS

Inoculum concentration	**No. tubers rotting after 3 days**			**No. tubers rotting after 6 days**		
(cfu per ml of inoculum)	**Rep A**	**Rep B**	**Mean**	**Rep A**	**Rep B**	**Mean**
10^3	0	1	0.5	1	1	1
10^5	1	2	1.5	3	3	3
10^7	5	4	4.5	5	5	5

Testing of 25 stocks sampled during commercial washing and packing in 2009 and 2010 indicated a good correlation between inoculum loading of *Pectobacterium* detected on samples of apparently healthy tubers selected from stocks identified as healthy or as having soft rot problems either in store or after retailing (Table 10). All stocks without rotting tubers were identified as low risk or at the lowest end of medium risk. All stocks with soft rotting tubers were identified as high risk.

TABLE 10: RELATIONSHIP BETWEEN PECTOBACTERIUM LOADING AND ROT INCIDENCE DURING COMMERCIAL PACKING AND RETAILING.

Packer	Stock	Mean cfu per tuber	Rot potential*	Observed rotting in stock
A	1	$2.1x10^2$	Low	Healthy
	2	$2.1x10^2$	Low	Healthy
	3	$2.5x10^8$	High	Customer complaints, stock recalled due to soft rot
	4	$2.4x10^6$	High	Customer complaints, stock recalled due to soft rot
	5	$3.7x10^7$	High	Rotting in store, stock withdrawn internally
	6	$5.6x10^7$	High	Rotting in store, stock withdrawn internally
	7	$2.8x10^5$	High	Rotting in store, rotted tubers graded out
	8	$2.7x10^5$	High	Rotting in store, rotted tubers graded out
B	1	$1.7x10^1$	Low	Healthy
	2	0	Low	Healthy
	3	0	Low	Healthy
	4	0	Low	Healthy
	5	$1.1x10^1$	Low	Healthy
	6	0	Low	Healthy
	7	$1.0x10^3$	Medium	Healthy
	8	$1.2x10^3$	Medium	Healthy
	9	$3.1x10^3$	Medium	Healthy
	10	$7.0x10^3$	Medium	Healthy
	11	$4.9x10^3$	Medium	Healthy
	12	$4.8x10^6$	High	Rotting in store
	13	$3.4x10^6$	High	Rotting in store
	14	$9.2x10^6$	High	Rotting in store
	15	$1.2x10^7$	High	Rotting in store
	16	$1.2x10^8$	High	Rotting in store
	17	$2.1x10^6$	High	Rotting in store

* Low = 0-10^3 cfu per tuber; Medium = >10^3<10^5 cfu per tuber; High = 10^5 cfu per tuber and above.

5.6. Quantitative detection of pathogen populations in infected vegetables

Potato seed tubers that had been vacuum-infiltrated with *Pectobacterium carotovorum* (Section 5.5) were tested by real-time PCR and LAMP. There was generally an increase in the amount of target DNA detected with increasing *P. carotocorum* inoculum (Table 11). Both the real-time PCR and LAMP tests failed to detect *P. carotovorum* on tubers at 10^3 cells/ml inoculum but both methods detected the medium and high inoculum loads. Correlation between the quantitative real time PCR and semi-quantitative LAMP results indicated the potential to use LAMP to predict the risk of rotting in potato stocks in commercial washing and packing environments.

TABLE 11. THE RELATIONSHIP BETWEEN BACTERIAL LOADING OF PECTOBACTERIUM CAROTOVORUM ON SEED TUBERS, ROT RISK AND LEVELS OF PATHOGEN DETECTED BY REAL-TIME PCR (OR QPCR) AND LAMP

***P. carotovorum* loading (cfu per ml of inoculum)**	**QPCR (Ct)**	**LAMP (Tp)**	**Rot risk**
10^3	ND	ND	Low
10^5	34.0	38:11	Medium
10^7	30.4	28:42	High

ND = not detected
Ct = cycle threshold. The value decreases with increasing target DNA
Tp = time to positive. The value decreases with increasing target DNA

5.7. On-site application of LAMP assays for detection of soft rotting bacteria

The modified ChargeSwitch extraction method and real-time LAMP using the Genie II instrument were trialled at a potato packhouse on the 11th May, 2011 . Positive results were obtained for ChargeSwitch extracts prepared and tested on-site (Table 12).

TABLE 12. RESULTS OBTAINED BY CHARGESWITCH EXTRACTION FOLLOWED BY REAL-TIME LAMP DURING DEMONSTRATION ON 11TH MAY 2011.

	Pectolytic bacteria		**Plant DNA (COX)**	
	time to positive (min:sec)	melt peak (°C)	time to positive (min:sec)	melt peak (°C)
C/S extract 1	16:00	89.44	11:15	85.14
positive control	17:30	89.36	13:00	84.72
no template control	-	-	-	-

Results and feedback obtained during this visit indicated that the on-site deployment of the methods developed in this project is feasible. Further work is required to scale-up the extraction method to allow testing of bulked samples (the method used for the demonstration extracted from peel samples from individual tubers). Initial homogenisation in water or phosphate buffer would be required to allow the method to be scaled up without significantly increasing the cost. On-site testing using the LAMP method took approximately 10 minutes for preparation of each sample and around 30 minutes to run up to 14 prepared samples through the Genie II. The cost of consumables for each sample was around £2-3 per sample.

A positive result was also obtained for a sample of wash water during the visit and this preliminary result indicates the potential for this method to be used for testing water in addition to tubers.

5.8. Quantification of losses due to rotting

Industrial partners involved with commercial potato washing and packing have estimated a 12 month average of 0.42% loss of product as a result of rotted potatoes removed during inspections. Where rotting of individual loads or stocks leads to rejection of whole packed consignments, losses are considerably higher due to the need for re-grading, re-packing, downgrading and disposal of the rotting portion or even the whole consignment. Losses increase the further down the process the rotting is detected.

6. DISCUSSION

6.1. Selection of diagnostic platforms for on-site application

The approach of using LATE-PCR was initially favoured due to the advantages conferred by the Bioseeq instrument which integrates automated DNA extraction and detection by LATE-PCR, despite the high per-sample cost for testing using the Bioseeq, the high cost of the instrument, and the long reaction times required for LATE-PCR (> 2 hrs). Without the availability of a Bioseeq instrument, we continued with preliminary development of LATE-PCR in order to investigate the potential advantages for multiplex detection that result from the unique characteristics of this amplification method. For this reason, in addition to an assay for detection of pectolytic bacteria, assays were also designed to detect two further pathogens (*S. sclerotiorum* and *P. violae*) with the specific intention of investigating multiplexing. In order to leverage the multiplex capabilities of LATE-PCR it is necessary to design fluorescent probes with specific characteristics. While LATE-PCR primers were successfully developed for two out of the three intended targets, development of probes suitable for multiplexing was not possible within the timescale of the project, although a probe was developed for pectolytic bacteria. Complexity of design of probes suitable for exploiting LATE-PCR's multiplexing potential, combined with long reaction times and the requirement for complex instrumentation, indicated that LATE-PCR is not currently suitable for on-site deployment for plant pathogen detection. In contrast, LAMP offers advantages of shorter reaction times and less expensive instrumentation (Genie II). Results for design of LAMP assays for the different target pathogens indicated the importance of reliable sequence information for assay design.Validation of assays designed for detection of the pectolytic bacteria (*Pectobacterium* and *Dickeya* spp.) and *Pythium ultimum* showed good potential for application in on-site tests. Further development will be needed before a suitable assay for *Sclerotinia sclerotiorum* can be validated.

Results indicated that while DNA can be extracted from carrot and potato using the LFD extraction method, this method is likely to be insufficiently efficient for testing asymptomatic material as required for this application. Addition of crude extract directly to LAMP reactions is possible for small samples containing high levels of pathogen (i.e. symptomatic material), but the sample:buffer ratios required to test large samples of asymptomatic

material resulted in an increased rate of both false negatives (failed reactions) and false positives (non-specific amplification artifacts). A simplified ChargeSwitch extraction was developed which increases sensitivity of detection, but with an associated increase in cost. The method is designed to be simple to perform on-site without requiring complex equipment or precise manipulations. An important area for further work will be modifying the initial sample processing steps to allow bulked samples to be testing without significantly increasing the per-test cost.

6.2. Feasibility of on-site diagnostics in commercial QC environment

The potential for use of on-site diagnostics prior to, or during, commercial vegetable washing and packing successfully was demonstrated during this project. The results represent the first application of any on-site diagnostic method to measure inoculum loading for assessment of the risk of rotting in vegetables undergoing washing and packing. A key factor in the success of this was the correlations observed between (a) the results of testing with the on-site diagnostic (LAMP) and established laboratory (PCR and isolation) methods, and (b) the inoculum loading quantified on asymptomatic samples and the incidence of rotting observed under conditions favouring multiplication of soft rot bacteria. Another important factor in the feasibility of application is the cost per test. Even at the prototype stage it was possible to conduct testing for under £3 per test with good prospects to reduce this cost with further development. In terms of time, it was possible to test up to 12 samples or subsamples per hour and to fit the testing into current routine practices.

6.3. Further R&D

The HortLink Programme was discontinued during the feasibility stage, so a full HortLink proposal to continue this work was not prepared as originally planned. At present, rotting potential is assessed according to the experience and intuition of growers, fieldsmen and technical managers. The technology coming from this project will now enable direct measurement of pathogen loading which can then be related to incidence and severity of rotting during washing, packing and transport as well as to the level of customer complaints.

Future R&D will need to establish the critical points along the production and packing chain at which crops should be analysed. Sampling should be statistically valid so as to best indicate the health status of the whole crop or consignment. Furthermore, the diagnostic methods should be validated on large samples in order to ensure that bulked samples can be accurately assessed. Ideally, a traffic light system able to distinguish crops with high, medium and low risk of rotting would be possible, allowing upgrading of equipment software (e.g. Genie II) to improve ease interpretation of results.

Finally, the results of repeated on-site testing should be related to the actual losses recorded during storage and packing and to the shelf life of the packed product. Repeated use of the technology would aim to fine tune storage, washing and packing conditions to minimise multiplication of soft rot bacteria.

The most suitable incubation conditions for QC shelf-life testing could also be identified.

7. CONCLUSIONS

- LATE-PCR and LAMP assays have been developed for rapid detection of key parsnip, carrot and potato pathogens.

- Pathogen DNA extraction methods have been developed and validated for use with LAMP assays under laboratory conditions and on-site during commercial washing and packing.

- Automated real-time LAMP analysis with the Genie II platform allowed semi-quantification of low, medium and high risk pathogen levels.

- Inoculum loading on potato samples has been related to risk of bacterial soft rot under laboratory and commercial washing and packing conditions.

- Current losses due to bacterial soft rot were quantified and justify use of on-site diagnostics for entry stage quality control and monitoring pathogen population changes in commercial washing and packing environments.

- Application of the technology for on-site monitoring of pathogen loading during commercial vegetable packing was demonstrated to be fully feasible.

- Further commercial-scale optimisation and validation is now needed.

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