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Development and Validation of Conventional and Quantitative Polymerase Chain Reaction Assays for the Detection of Storage Rot Potato Pathogens, *Phytophthora erythroseptica*, *Pythium ultimum* and *Phoma foveata*

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Abstract

The diseases pink rot, watery wound rot and gangrene are important storage rot diseases of potato associated predominantly with *Phytophthora erythroseptica* (*P.*), *Pythium ultimum* (*Py.*) and *Phoma exigua* (*Phoma*) var. *foveata* respectively. Reliable molecular-based diagnostic tests are required that will not only allow unequivocal identification of symptoms but will further advance epidemiological studies of these potato diseases to increase our understanding and contribute to more effective management and control strategies to the potato industry. Primers and probes were designed in specific regions of the internal transcribed spacer (ITS) regions to develop conventional and real-time quantitative polymerase chain reaction (PCR) assays able to detect all possible fungal and oomycete pathogens causing pink rot, watery wound rot and gangrene. The specificity of each diagnostic assay was rigorously tested with over 500 fungal/oomycete plant pathogen isolates from potato and reference culture collections, and both conventional and real-time PCR methods produced similar results. In terms of sensitivity, the detection limits for real-time PCR went below ag DNA levels compared with pg DNA levels with conventional PCR. The real-time PCR assays developed to detect *Phoma foveata* and *Py. ultimum* on tubers were suitable for the comparative C_t method ($\Delta\Delta$Ct) of quantification using the cytochrome oxidase gene of potato as a normalizer assay; an advantage as the need for a standard curve is eliminated. Each assay detected *Phoma* species (var. *foveata* or *exigua*) from naturally infected tubers showing symptoms of gangrene, and *P. erythroseptica* or *Py. ultimum* were also detected following inoculation of Russet Burbank tubers. Each diagnostic assay developed could reliably detect and distinguish between the pink rot, watery wound rot and gangrene-causing potato pathogens.

Introduction

The potato (*Solanum tuberosum*) is the world's fourth ranked food crop but both field and storage diseases can be limiting factors in sustainable and profitable potato production. Although potatoes can be stored for long periods, they are plagued by a range of storage rot disease problems (Secor and Gudmestad, 1999). The 'water rots' of potato, pink rot and watery wound rot (or leak), are predominantly caused by the soilborne oomycetes *Phytophthora erythroseptica* and *Pythium ultimum* respectively. The symptoms of both diseases are similar and cause a rapid dark discoloration and watery breakdown of tubers in storage. Pink rot is characterized by the pink colour that develops in infected tuber tissue when cut and exposed to air for 20–30 min, whereas watery wound rot is descriptive of the exudation of clear fluid from the soft infected tuber tissue. The main difference between these diseases is that *P. erythroseptica* infection can occur in the field prior to harvest through stolons, lenticels or eyes, whereas *Py. ultimum* infection only occurs in wounds and injuries that occur during harvest and generally rots out the entire central portion of the tuber.

Gangrene is another important storage rot disease primarily caused by the soilborne fungus *Phoma exigua* Desm. var. *foveata* (Foister) Boerema (often referred to as *Phoma foveata*), and tuber infection is predominantly through wounds made during harvest or subsequent handling. *Phoma exigua* Desm. var. *exigua* is another potato rot pathogen that can be associated with gangrene, and although morphologically similar, it is generally considered less aggressive than *Phoma foveata* (Aguelon and Dunez, 1984). Symptoms on tubers first appear as small dark depressions associated with wounds, which can enlarge to form 'thumb mark' or larger irregularly shaped areas.

Advanced gangrene also causes an internal rot and cavities beneath the superficial depressions. Gangrene lesion symptoms may be confused with dry rot disease symptoms produced by *Fusarium* species.

To guarantee the delivery of disease-free tubers, give assurances that tubers are free from pathogens likely to cause disease during storage, and to increase the understanding and potential to control such diseases, the potato industry requires rapid and reliable molecular-based diagnostic tests. The objectives of this research were to design both conventional and real-time polymerase chain reaction (PCR) assays and verify their reliability to detect and distinguish between the pink rot, watery wound rot, and gangrene-causing potato pathogens.

Materials and Methods

Isolation and identification of storage rot pathogens

Growers in the UK were requested to send samples of tubers potentially infected with storage rot pathogens. Diseased and symptomless tubers were obtained from 48 UK farms (three tubers per stock) during 1999–2000 so as to isolate storage rot pathogens. Tubers were washed under tap water to remove soil particles, surface-sterilized with 1% NaClO for 5 min, and again thoroughly washed under running water. An alcohol-sterilized blade was used to cut tubers into half slices avoiding infected areas, and tissue from internal regions of diseased lesions was inoculated in triplicate (per tuber) onto potato dextrose agar (PDA) (Difco, West Molesey, UK) media supplemented with 50 μg/ml each of three antibacterial antibiotics (chloramphenicol, neomycin and streptomycin). Plates were incubated at room temperature and potential storage rot fungi/oomycetes were subcultured onto the same isolation medium several times to obtain pure cultures. Cultures were identified by colony growth on PDA, and by microscopic examination of spore-bearing structures using classical keys.

Extraction of DNA from pure cultures and potato tubers

Fungal cultures were grown on PDA at room temperature, and mycelial DNA was extracted according to the method of Cullen et al. (2005) using a Mini-Bead-Beater-8 (Bio-Spec Products, Bartlesville, OK, USA). DNA was extracted from tuber peel sap after passage of peel strips through a roller press (Meuk, E. Pollähne, Wennisgen, Germany) following the methods described by Cullen et al. (2001).

Inoculation of potato tubers

Small pieces of mycelium were cut from the colony margin of agar cultures of *P. erythroseptica* and *Py. ultimum* and inserted separately into a wound cut in the middle of one side of Russet Burbank tubers with a sterile scalpel. Inoculated tubers were incubated in plastic bags (one tuber per bag) containing a wet paper towel to maintain high relative humidity, at room temperature in darkness until symptoms appeared (7–12 days). Peel was taken from areas adjacent to symptomatic areas (showing discoloration) and processed as described above.

Design of conventional and real-time PCR assays to detect pink rot, watery wound rot and gangrene-causing potato pathogens

Conventional and real-time PCR sets of primers and probes to detect *P. erythroseptica* (pink rot) and *Phoma exigua* varieties *foveata* and *exigua* (gangrene) were designed to the internal transcribed spacer (ITS) regions of the rDNA. The unpublished DNA sequences for ITS1 and ITS2 regions from 37 different *Phytophthora* spp. were supplied by D. E. L. Cooke [Scottish Crop Research Institute (SCRI)] and the ITS1 sequences from different *Phoma* spp., *Didymella* (the teleomorph of *Phoma* spp.), and other members of the family Pleosporaceae were retrieved from GenBank. Sequences were aligned using the CLUSTAL V package (Higgins et al., 1992) to identify regions of dissimilarity for the design of specific primers (Table 1). The published set of primers K1 and K3 designed (Kageyama et al., 1997) in the ITS1 and ITS2 regions to detect *Py. ultimum* (watery wound rot) were re-designed (at non-specific regions in K1) and new reverse primers were selected to reduce the amplicon size from 670 bp. In the design of all primers, annealing temperatures were standardized at 60°C to ensure operation under the same PCR conditions, and sets of real-time PCR primers were designed using PRIMER EXPRESS software (Applied Biosystems, Foster City, CA, USA); this software allows accurate T_m calculations to be made for sets of primers and probes such that the primers are within the range 58–60°C and probes are 10°C higher at 68–70°C, to improve specificity. In addition, the total length of each amplicon selected is below 150 bp to allow efficient amplification.

Conventional PCR conditions

Standard PCR amplification of all genomic and peel extract DNA samples was performed at an annealing temperature of 60°C and agarose gel electrophoresis analyses by the methods according to Cullen et al. (2001). To exclude false-negative results with each specific primer set, all template DNA samples were tested for PCR amplification using universal primers ITS5 and ITS4 for fungal 18S and 28S rDNA (White et al., 1990).

Real-time (TaqMan^{TM}) quantitative PCR conditions

Optimal conditions for real-time PCR were determined using a 3×3 matrix of 150, 300 and 900 nM of each primer concentration (Applied Biosystems) and a probe titration (100, 150 and 200 nM) using genomic DNA from appropriate species. Real-time PCR was performed in MicroAmp optical 96-well plates using either the automated ABI Prism 7900 HT or ABI 7700 sequence detectors (Applied Biosystems) following the recommended generic (three-stage) thermal cycle protocol (Applied Biosystems) and the procedures of

Table 1
Conventional and real-time polymerase chain reaction primers/probes designed for the specific detection of water rot and gangrene potato pathogens

Pathogen	ID	Primers and probe[a] sequences (5′–3′)	Product size (bp)
Conventional primers			
Phytophthora erythroseptica[b]	Pery2F1	TGGTGAACCGTAGCTGTGCTA	135
	Pery2R1	CGCCGAAGCGCACACAACG	
Pythium ultimum	Pu1F1	GACGAAGGTTGGTCTGTTG	307
	Pu2R1	CAGAAAAAGAAAGGCAAGTTTG	
Phoma foveata[c]	Phom1F2	AGGATCATTACCTAGAGTTGTA	130
	Phom1R1	TTTCAGACGCTGATTTCAATTA	
TaqMan primers/probes			
Phytophthora erythroseptica[b]	99F	TGTGCTAGGCTTGGCGTTT	78
	177R	CCTCGTCCACCCCAGCTTA	
	133T[a]	TGCGAAGTAGGGTGTCTGTTCCGGC	
Pythium ultimum	92F	TGTTTTCATTTTTGGACACTGGA	74
	166R	TCCATCATAACTTGCATTACAACAGA	
	116T[a]	CGGGAGTCAGCAGGACGAAGGTTG	
Phoma foveata[c]	72F	GCGTCATTTGTACCTTCAAGCTC	72
	144R	TTTAAGGCGAGTCTACACGCAA	
	97T[a]	CTTGGTGTTGGGTGTTTGTCTCGCC	
Cytochrome oxidase (COX) gene (Weller et al., 2000)	CoxF	CGTCGCATTCCAGATTATCCA	78
	CoxRW	CAACTACGGATATATAAGAGCCAAAACTG	
	Coxsol 151T[a]	AGGGCATTCCATCCAGCGTAAGCA	

[a]All probes were labelled at the 5′-end with FAM (6-carboxy-fluorescein), except for the COX gene assay in which the probe was labelled at the 5′-end with VIC (Applied Biosystems).
[b]Primers amplified species of *P. erythroseptica*, *P. cryptogea* and *P. drechsleri*.
[c]Primers amplified both *Phoma exigua* varieties *foveata* and *exigua*.

Cullen et al. (2005). Forward and reverse primers were included at 300 nM together with probe concentrations at 100 nM for all three optimized assays (Table 1). An assay to detect the cytochrome oxidase (COX) gene of potato was also included for use as an endogenous control and normalizer during quantification of tuber peel DNA extracts (Cullen et al., 2005). All fluorogenic probes were labelled at the 5′-end with the fluorescent reporter dye FAM (6-carboxy-fluorescein), and the 3′-end was modified with the quencher dye TAMRA (6-carboxy-tetramethylrhodamine).

Relative quantification, which describes the change in the concentration of the target relative to a reference sample (calibrator) was performed in this study. This is different from absolute quantification, which determines the input concentration of the target of interest using a standard curve (Applied Biosystems). The amplification efficiency of the assays developed for storage rot pathogens was examined such that a suitable quantification technique [standard curve or comparative threshold cycle ($\Delta\Delta$Ct) method] could be selected using the COX gene as the normalizing assay. The choice between the techniques used for relative quantification is based upon the efficiency of an assay compared with that of its normalizer assay. In the $\Delta\Delta$Ct method, Ct values obtained for the target (pathogen DNA), endogenous control (COX gene), and calibrator (reference sample) are used to calculate two parameters, $\Delta\mathrm{Ct} = \mathrm{Ct}_{\mathrm{target}} - \mathrm{Ct}_{\mathrm{endogenous\ control}}$ and $\Delta\Delta\mathrm{Ct} = \Delta\mathrm{Ct}_{\mathrm{target\ sample}} - \Delta\mathrm{Ct}_{\mathrm{calibrator}}$. The quantity of the target, normalized to the endogenous control and relative to the calibrator, is given by the amount of target $= 2^{-\Delta\Delta\mathrm{Ct}}$ (Applied Biosystems). To use the $\Delta\Delta$Ct method, the first step is to validate the $\Delta\Delta$Ct calculation by showing that both the efficiency of the target amplification and the endogenous control amplification are approximately equal. Validation experiments were carried out for each target assay on three separate occasions to calculate the efficiency and compare it over a range of genomic DNA dilutions (10^{-1}–10^{-5}) in triplicate. The dilutions of DNA were plotted on a log scale against the cycle threshold (Ct) value to provide a linear regression equation ($y = mx + b$) for each assay. If the efficiency of the target assay is comparable with that of the normalizer (i.e. the gradients of the two equations have a difference of < 0.1), then a comparative Ct method of quantification can be used. However, if the efficiencies of the reactions are different (> 0.1), a standard curve must be included for every plate.

Results

Isolation of storage rot pathogens

A total of 80 presumptive storage rot fungi/oomycetes were isolated from 120 isolations obtained from separate diseased tubers of UK potato stores. Fifty-four of these isolates were identified and confirmed as *Fusarium* spp. (Cullen et al., 2005), 12 isolates were identified as *Cylindocarpon* spp., and 14 isolates identified as *Phoma* spp. (Table 2). No strains of *P. erythroseptica* or *Py. ultimum* were isolated from any of the diseased or symptomless tubers during this survey (Table 2). In addition to these cultures, a wide range of other storage rot pathogens and related species, and other fungal/oomycete plant pathogens were obtained from culture collections at the SCRI, Central Science Laboratory (CSL) or from the Scottish Agricultural Science Agency (SASA) in order to test the specificity of each PCR assay (Tables 2 and 3). This involved testing over 500 European and North

Table 2
Numbers of plant pathogenic fungi/oomycetes used in this study

Source	*Phytophthora* spp. Pink rot	*Pythium* spp. Watery wound rot	*Phoma* spp. Gangrene
Isolated from diseased potato (1999–2000)	0	0	14
Culture collections[a]	78	19	169
Positive ID confirmation by			
Conventional PCR	22[b]	10	90[c]
Real-time PCR	22[b]	10	89[c]
Detection on infected tubers			
Conventional PCR	+[d]	+[d]	+
Real-time PCR	+[d]	+[d]	+

[a]Cultures were obtained from reference collections at the Scottish Crop Research Institute (Dundee, UK), Central Science Laboratory (York, UK) or from the Scottish Agricultural Science Agency (Edinburgh, UK).
[b]Both polymerase chain reaction (PCR) assays amplified DNA from all species of *P. erythroseptica*, *P. cryptogea* and *P. drechsleri*, and one strain of *P. primulae*.
[c]Conventional PCR amplified DNA from all *Phoma exigua* varieties *foveata* and *exigua*, as well as one culture of *P. complanata*, *P. glomerata*, *P. herbarum*, *P. medicaginis*, *P. telepii* and *P. exigua* var. *inoxydibilis*; real-time PCR produced signals for all *P. exigua* varieties *foveata* and *exigua*, as well as one culture of *P. complanata*, *P. glomerata*, *P. herbarum*, *P. lingam*, and *P. exigua* var. *inoxydibilis*.
[d]Positive signals were detected for tubers inoculated with cultures of *P. erythroseptica* and *Py. ultimum*; +, PCR products were detected from tuber peel DNA extracts.

Table 3
Plant pathogenic fungal and oomycete species used for testing the specificity of all water rot and gangrene-specific polymerase chain reaction assays

Phytophthora spp.	*Pythium* spp.	*Phoma* spp.
P. erythroseptica	*P. ultimum*	*P. exigua* var. *foveata*
P. cryptogea	*P. aphanidermatum*	*P. exigua* var. *exigua*
P. drechsleri	*P. catenulatum*	*P. betae*
P. arecae	*P. coloratum*	*P. clematidina*
P. botryosa	*P. dissotocum*	*P. complanata*
P. cactorum	*P. intermedium*	*P. epicoccina*
P. cajani	*P. pyrilobum*	*P. eupyrena*
P. cambivora	*P. sylvaticum*	*P. glomerata*
P. capsici	*P. torulosum*	*P. herbarum*
P. clandestina	*P. violae*	*P. heteromorphospora*
P. cinnamomi	*P. = Py.*	*P. leveillei*
P. citricola		*P. lingam*
P. citrophthora		*P. macrocapsa*
P. colocasiae		*P. medicagines*
P. fragariae var. *fragariae*		*P. multistrata*
P. gonapodyides		*P. nebulosa*
P. heveae		*P. pomorum*
P. humicola		*P. sorghina*
P. ilicis		*P. subboltshauseri*
P. inflata		*P. telephii*
P. infestans		*P. terricola*
P. iranica		*P. valerianellae*
P. katsurae		*P. exigua* var. *inoxydibilis*
P. lateralis		*P. = Phoma*
P. medicaginis		
P. megasperma		
P. melonis		
P. nicotianae		
P. palmivora		
P. porri		
P. primulae		
P. pseudotsugae		
P. sinensis		
P. sojae		
P. syringae		
P. tentaculata		
P. vignae		

DNA samples were extracted from cultures obtained from the reference collections at Scottish Crop Research Institute, Central Science Laboratory or Scottish Agricultural Science Agency.

American fungal/oomycete and bacterial plant pathogen isolates from potato and reference culture collections used in previous studies (Cullen et al., 2001, 2002, 2005) including several isolates of the potato pathogens, *Colletotrichum coccodes*, *Erwinia carotovora* ssp. *atroseptica*, *Erwinia carotovora* ssp. *carotovora*, *Fusarium avenaceum*, *Fusarium coeruleum*, *Fusarium culmorum*, *Fusarium sulphureum*, *Helminthosporium solani*, *Oospora lactis*, *Polyscytalum pustulans*, *Py. ultimum*, *Ralstonia solanacearum*, *Rhizoctonia solani* and *Spongospora subterranea*.

Specificity and sensitivity of PCR assays

Both conventional and real-time PCR assays designed for the detection of pink rot and watery wound rot causing oomycetes produced identical results during the testing of isolates (Table 2). Products were amplified from all 21 isolates of *P. erythroseptica*, *Phytophthora cryptogea* and *Phytophthora drechsleri* tested as expected, as these species have identical ITS1 and ITS2 regions. The DNA extracted from the remaining 34 different species of *Phytophthora* was not amplified by either PCR assay, except for one isolate of *Phytophthora primulae*. All 10 reference isolates of *Py. ultimum* were successfully confirmed by both conventional and real-time PCR assays, when no products were generated from isolates of *Pythium aphanidermatum*, *Pythium catenulatum*, *Pythium coloratum*, *Pythium dissotocum*, *Pythium intermedium*, *Pythium pyrilobum*, *Pythium sylvaticum*, *Pythium torulosum* and *Pythium violae* (Table 3).

Conventional and real-time PCR assays successfully confirmed the identity of all 83 isolates of *Phoma exigua* var. *foveata* and *Phoma exigua* var. *exigua* (Table 2). The PCR assays could not distinguish between the *Phoma exigua* varieties *foveata* and *exigua* as the ITS1 and ITS2 regions of these two species are identical based on multiple DNA alignments. All

presumptive gangrene-causing species isolated from infected tubers of the UK potato stores were also identified as either *Phoma exigua* var. *foveata* or *Phoma exigua* var. *exigua*. However, although the specificity of both assays was confirmed when testing 21 other *Phoma* species, products were also generated for reference isolates of some species including, *Phoma complanata*, *Phoma glomerata*, *Phoma herbarum*, *Phoma lingam*, *Phoma medicaginis*, *Phoma telepii* and *Phoma exigua* var. *inoxydibilis* (Tables 2 and 3).

The specificity of all PCR assays was further validated by testing a wide range of other fungal/oomycete and bacterial plant pathogens tested in our collection and used during the development of other specific PCR assays (Table 3; Cullen et al., 2001, 2002, 2005). The quality of all DNA preparations tested for amplification by PCR throughout this study was confirmed by the detection of product using the universal eucaryotic primers (ITS4 and ITS5). The sequence and size of PCR products amplified with each primer set was confirmed by sequencing products generated from three different isolates of each species following the procedures of Cullen et al. (2001).

The sensitivity of detection with agarose gel electrophoresis for each conventional PCR assay was set at 10 pg genomic DNA, whereas each real-time assay could reliably detect atto gram (10^{-18} g) levels of genomic DNA extracted from reference cultures of storage rot pathogens. The standard curves produced for each specific assay for real-time PCR showed high correlation coefficient (R^2) values (0.99), indicating a reproducible linear response in detection related to increasing DNA concentration.

Detection of storage rot pathogens on infected potato tubers

Phoma species (var. *foveata* or *exigua*) were successfully detected from naturally infected tubers of cultivars Spunta, Morene, Hermes, Desiree and Maris Piper with varying levels of gangrene symptoms using both conventional and real-time PCR (Table 2), following the method of tuber peel DNA extraction. *Phytophthora erythroseptica* and *Py. ultimum* were also detected by each specific PCR assay following inoculation of Russet Burbank tubers and the early development of the characteristic pink rot and watery wound rot symptoms during storage. No amplification of DNA was detected when clean and uninoculated tubers were tested by each specific PCR assay.

Validation of real-time PCR quantification methods

It was concluded that the real-time PCR assays developed to detect *Phoma foveata* and *Py. ultimum* were suitable for the comparative Ct method of quantification as the amplification efficiencies were comparable with those for the endogenous potato COX gene assay, i.e. the difference between the slope values of each standard curve was <0.1 (Table 4). In the case of *P. erythroseptica*, absolute quantification will have to be used as the efficiencies of the specific and endogenous control assays produced slope value differences >0.1.

Discussion

We have developed robust diagnostic conventional and real-time quantitative PCR assays to distinguish and identify the pink rot, watery wound rot and gangrene-causing complex of potato pathogens. Prior to the development of DNA-based molecular methods, identification of plant pathogens and differentiation of the many diseases was based solely on visual observation of symptoms and isolation of the causative organisms on selective media. Although the diseases pink rot and watery wound rot produce similar external symptoms on potato, each PCR assay was able to distinguish *P. erythroseptica* from *Py. ultimum* and vice versa, and can thus be used to differentiate symptoms of pink rot from those of watery wound rot. In addition, the assays designed to detect the water rot pathogens could distinguish *P. erythroseptica* and *Py. ultimum* from*Oospora lactis* (synonym of *Geotrichum candidum*), the pathogen responsible for causing rubbery rot of potato, a condition similar to pink rot which also produces a pinkish discolouration after tubers are cut. The same level of specificity using PCR diagnostics can be applied to distinguishing the potato diseases gangrene and *Fusarium* dry rot (Cullen et al., 2005); the primers designed to detect *Phoma exigua* varieties *foveata* and *exigua* did not cross-react with any of the *Fusarium* complex of potato pathogens and vice versa (Cullen et al., 2005).

All PCR assays were generally of a high specificity and allowed the detection of different water rot and gangrene-causing pathogens on naturally infected or inoculated potato tubers. A small number of symptomless tubers (25) collected from different UK stores were also tested for infection by each of the storage rot disease pathogens together with diseased tubers. Although the detection of symptomless infections was not demonstrated during this study, it is envisaged that

Table 4
Quantification method validation experiments for each real-time polymerase chain reaction (PCR) assay

Real-time PCR assay	Linear regression equation of standard curve ($y = mx + b$)[a]	Difference between gradient of target and COX assays	Suitability for comparative Ct method
COX gene assay	$y = -1.6461 \times +17.478$		
Phoma foveata	$y = -1.5869 \times +14.879$	0.0592	Yes
Pythium ultimum	$y = -1.6724 \times +14.92$	0.0263	Yes
Phytophthora erythroseptica	$y = -1.3768 \times +12.269$	0.2693	No

[a]y is the threshold cycle (Ct) value, m is the slope of standard curve line, x is the DNA dilution, $b = y$ – intercept of standard curve line.

the extraction and PCR assay protocols would allow this detection due to the successful demonstration using identical methods for other potato blemish and storage rot pathogens (Cullen et al., 2001, 2002, 2005) The reliability of the each diagnostic assay was rigorously tested with over 500 fungal/oomycete and bacterial plant pathogen isolates including those from potato and reference culture collections used in previous assay validation studies (Cullen et al., 2001, 2002, 2005), and both conventional and real-time PCR methods produced similar results. In terms of sensitivity, the detection limits for real-time PCR went below ag DNA levels compared with pg DNA levels with a single round of conventional PCR. Real-time PCR also has the crucial advantage of calculating the quantity of pathogen DNA in tuber samples. Other advantages over conventional PCR include no post-amplification processing, a reduced labour-intensity, and assays can be performed faster with an automated system greatly increasing throughput.

Real-time PCR assays incorporated primers/probe to detect the COX gene for use as a potato endogenous control during quantification. The COX gene assay can be used to normalize all the quantitative values for the target DNA by the amount of potato DNA present in the extract, and hence the level of target per amount of potato tissue sampled. This normalization process will account for differences in the amount of tissue sampled, the quantity of DNA extracted from each sample, and also any variance in the volumes of reagents and DNA aliquoted into each PCR reaction. In addition, the real-time PCR assays developed to detect *Phoma foveata* and *Py. ultimum* were suitable for the comparative Ct ($\Delta\Delta$Ct) method of quantification. The advantage of using the comparative Ct method is that the need for a standard curve is eliminated, which increases throughput, and also eliminates the adverse effect of any dilution errors made in creating the standard curve samples. The next stage of assay development would be to test the possibility that the singleplex real-time PCR assays could be incorporated into multiplex formats to include both target and normalizer primers/probes using different reporter fluorophore dyes to further increase throughput.

The PCR assays developed to detect the predominant pink rot (*P. erythroseptica*) and gangrene (*Phoma foveata*)-causing potato pathogens also cross-reacted with other species during specificity tests. It was no surprise that the assays designed to detect *P. erythroseptica* also amplified DNA from *P. cryptogea* and *P. drechsleri*, as all three species have identical ITS1 and ITS2 regions, all belong to Clade 8VI of the genus, and the latter two species have also been implicated in potato rot disease (Stamps, 1978a,b). The product was also amplified by both conventional and real-time PCR assays from one strain identified as *P. primulae*, which should not cause a problem in detection as the natural host of this pathogen is Primula species, and it has not been associated with potato. Tooley et al. (1997) previously designed a PCR assay to detect *P. erythroseptica* on tuber tissue based on a specific primer (PERY2) in the ITS2 region and a universal primer (ITS4). Although only nine different *Phytophthora* species and eight other potato pathogens were tested during the specificity testing of the assay, amplification was observed from DNA of *P. hibernalis*, in addition to that of *P. cryptogea* and *P. erythroseptica*. It is clear that the specificity of this *P. erythroseptica* assay should be further evaluated by testing DNA from a wider collection of related and other plant pathogenic oomycetes and fungi as used in this study.

Similarly, the gangrene disease-specific PCR assays developed amplified DNA from additional *Phoma* species other than *Phoma exigua* varieties *foveata* and *exigua*; products were generated from one strain from two or three isolates of *Phoma complanata*, *Phoma glomerata*, *Phoma herbarum*, *Phoma lingam*, *Phoma medicaginis*, *Phoma telepii* and *Phoma exigua* var. *inoxydibilis*. Although the alignment of the ITS1 and ITS2 sequences of all the different *Phoma* spp. revealed that some were highly similar, it was still possible to identify areas of sequence microheterogeneity in the ITS1 regions which should distinguish *P. exigua* varieties *foveata* and *exigua* from the other species. We believe that some of these isolates of *Phoma* that cross-amplified with the gangrene-specific PCR assays had been misidentified previously. In addition, none of the aforementioned *Phoma* species are pathogens of potatoes and therefore should not limit the usefulness of the assay. Macdonald et al. (2000) developed a PCR-restriction fragment length polymorphism (RFLP) marker assay to discriminate between *Phoma foveata* and *Phoma exigua* varieties from the sequence of a random-amplified polymorphic DNA (RAPD) product. Primers amplified a fragment (474 bp) for *Phoma foveata* and *Phoma exigua* varieties *exigua*, *diversispora*, *inoxydibilis* and *sambuci-nigrae*, and discrimination was based on the RFLP patterns generated from the PCR product using two enzymes. As well as being a more time consuming procedure, this PCR-RFLP assay was only validated by testing mycelial DNA and 11 different *Phoma* species. Although it is generally considered that *Phoma foveata* is predominantly responsible for causing gangrene of potatoes in northern temperate countries due to its tolerance of lower temperatures than *Phoma exigua* var. *exigua* (Boerema, 1967; Boyd, 1972), future work will investigate the possibility of designing specific real-time PCR assays based on sequence differences in the RAPD product described by Macdonald et al. (2000) to distinguish between these different *Phoma* species. The principal aim of this study was to validate the reliability of the DNA extraction and PCR assay procedures for the detection and discrimination of the gangrene-causing pathogens from the remaining storage rot pathogens. The occurrence of gangrene in seed stocks can also have an effect on the incidence of other diseases in the growing crop such as increasing the

susceptibility to the bacterial soft pathogen, *Erwinia carotovora* ssp. *atroseptica*, and therefore the detection of either *P. exigua* varieties *foveata* and *exigua* can have important implications.

The conventional and real-time PCR assays designed to detect *Py. ultimum* showed no cross-amplification with DNA from any other *Pythium* spp. or plant patho gens tested, indicating high specificity. Both PCR methods were based on the assay previously designed to detect *Py. ultimum* from damped-off seedlings by Kageyama et al. (1997). In the case of *Pythium* species, it is the ITS1 region that has interspecific differences whereas the ITS2 is not species specific (Levesque et al., 1994). Primer set K1 and K2 was designed in the ITS1 and ITS2 regions, respectively, and the specificity of the assay was validated by testing 20 other *Pythium* species (Kageyama et al., 1997). It was necessary to re-design the primers to detect *Py. ultimum* so as to ensure all primer sets operated under the same optimal conditions at an annealing temperature of 60°C, and to reduce the amplicon size of 670 bp down to 74 bp for real-time PCR requirements. In addition, the newly designed primer sets in this study were validated by testing a wide range of other potato pathogens.

The specific PCR assays and DNA extraction protocols developed in this study should allow rapid and sensitive detection of all potential water rot and gangrene-causing pathogens on infected potato tubers. The use of real-time PCR can provide high throughput and reproducible quantification, in addition to the accurate diagnosis of target plant pathogens in environmental samples, and will open new opportunities for research on the study of inoculum threshold levels, epidemiology and host–pathogen interactions. This should ultimately increase our understanding of these diseases and contribute to the implementation of effective management and control strategies to the potato industry.

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