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Christophe Lacomme *Editor*

Plant Pathology

Techniques and Protocols

Second Edition

Humana Press

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Edited by

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Preface

Pathogens pose a threat to plants in natural communities (i.e., forests, grasslands), horticultural commodities, or cultivated crops. Risks of pathogen spread have increased with increased human mobility and the globalization of trade. In addition, factors such as environmental changes (local or global climate fluctuations) and changes to pesticide legislation impact on whether pathogens and their vectors establish in different habitats and the selective pressures that will give rise to new pathotypes and pesticide- or antibiotic-resistant variants. Damages caused worldwide by either emerging, re-emerging or endemic pathogens are significantly important. The International Plant Protection Convention, Regional and National Plant Protection Organizations, have developed phytosanitary measures to prevent the spread of regulated pathogens (particularly quarantine pathogens) between countries in order to protect agricultural and natural plant systems.

Safeguarding plant biosecurity relies heavily on the early detection and diagnosis of the pathogen. Other than diagnoses based on morphological characteristics, diagnostic methods can be separated into three main categories: bioassay, serological and molecular methods, and sometimes a combination of these methods will be used. Since the late 1970s, the serological method of ELISA, using polyclonal and especially monoclonal antibodies, has been the method of choice for most diagnostic laboratories, due to its cost effectiveness and capacity to provide reliable detection and diagnosis for a large number of samples. However, over the past decade an increasing number of DNA/RNA-based assays, particularly PCR-based assays, are routinely used in diagnostic laboratories because of their increased sensitivity and specificity, the relative ease with which tests can be developed, their adaption to detect multiple targets, their requirement for minimal quantities of target, and their capacity to be automated for high-throughput testing. Moreover sequencing has contributed considerably to the increased knowledge of plant and microbial genomes and is now widely used either as stand-alone methods or in addition to other methods for diagnosis. Techniques such as end-point (conventional) PCR, real-time PCR, and diagnostic microarrays are versatile and can be used as either a generic or species-specific detection/diagnostic method. One of their drawbacks, however, is their reliance on prior knowledge of the genome of the target pathogen or pathogens. The rapid evolution of bioinformatics and computing technology to analyze very high numbers of complex datasets will make next-generation, high-throughput parallel sequencing platforms (also known as deep sequencing) accessible as a detection and diagnostic method. The application of these metagenomic approaches to diseased material offers the possibility to identify pathogens that have yet to be fully characterized or described. Importantly, recent advances in plant pathogen diagnoses have delivered field deployable portable diagnostic systems that do not require thermal cycling equipment. This allows rapid on-site identification of pathogenic agents, thereby passing the need for laboratory-based analysis. The development of any diagnostic assay requires thorough validation to ensure for example sensitivity, specificity, repeatability, and reproducibility and that the assay is fit for purpose.

This second edition of *Plant Pathology Techniques and Protocols* covers diagnostic methods that are currently used in laboratories for a broad range of plant species and matrixes. These include serological and molecular methods that have one or more of the

following characteristics: suitability for high-throughput testing, detection of a group of pathogens or of sometimes uncharacterized pathogens, detection and identification of specific pathogens, and high sensitivity. Qualitative and quantitative tests are described, as well as recently developed cutting-edge diagnostic methods. These chapters target an audience of plant pathologists and molecular biologists who will find information on how to perform the tests in their laboratories. Also provided is background information on many pathogens, which are endemic, nonendemic, or emerging and with different lifecycles that cause diseases of significant importance in a wide variety of hosts. Finally I would like to thank all authors that have contributed to this second edition of *Plant Pathology Techniques and Protocols.*

Edinburgh, UK ***Christophe Lacomme***

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Chapter 1

Detection of the Bacterial Potato Pathogens *Pectobacterium* and *Dickeya* spp. Using Conventional and Real-Time PCR

Sonia N. Humphris, Greig Cahill, John G. Elphinstone, Rachel Kelly, Neil M. Parkinson, Leighton Pritchard, Ian K. Toth, and Gerry S. Saddler

Abstract

Blackleg and soft rot of potato, caused by *Pectobacterium* and *Dickeya* spp., are major production constraints in many potato-growing regions of the world. Despite advances in our understanding of the causative organisms, disease epidemiology, and control, blackleg remains the principal cause of down-grading and rejection of potato seed in classification schemes across Northern Europe and many other parts of the world. Although symptom recognition is relatively straightforward and is applied universally in seed classification schemes, attributing disease to a specific organism is problematic and can only be achieved through the use of diagnostics. Similarly as disease spread is largely through the movement of asymptomatically infected seed tubers and, possibly in the case of *Dickeya* spp., irrigation waters, accurate and sensitive diagnostics are a prerequisite for detection. This chapter describes the diagnostic pathway that can be applied to identify the principal potato pathogens within the genera *Pectobacterium* and *Dickeya*.

Key words *Pectobacterium*, *Dickeya*, Real-time PCR, Blackleg, Soft rot

1 Introduction

Pectobacterium and *Dickeya* species (spp.) are plant pathogenic bacteria belonging to the family *Enterobacteriaceae*. They mainly consist of broad host range pathogens that cause wilts, rots, and blackleg disease on a wide range of plants and crops worldwide [1]. The major pathogenicity determinant of these bacteria is their copious production of plant cell wall-degrading enzymes (PCWDE) including pectinases, cellulases, and proteases, which macerate host tissue [2]. The genera were previously known collectively as the "soft rot erwinias" [3]. However, in 1998 the genus *Erwinia* underwent a major revision resulting in the soft rot erwinias being reassigned to the genus *Pectobacterium* [4], a name originally proposed by Waldee in 1945 [5]. Subsequent study of these taxa

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suggested further revision was required resulting in *Pectobacterium chrysanthemi* being reassigned to the newly established genus *Dickeya* [6]. *Dickeya* currently encompasses 7 species: *D. aquatica*, *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. paradisiaca*, *D. solani*, and *D. zeae* [6–9]. There are 6 species currently in the genus *Pectobacterium*, including *P. atrosepticum*, *P. betavasculorum*, *P. cacticidum*, *P. carotovorum*, *P. cypripedii* and *P. wasabiae* [10], with *P. carotovorum* being further subdivided into subsp. *carotovorum* and subsp. *odoriferum* [4]. A further subspecies "*P. carotovorum* subsp. *brasiliensis*" has recently been proposed [11] but has yet to be formally recognized.

A number of *Pectobacterium* and *Dickeya* species are known to cause blackleg, tuber soft rot, and stem wilt and rot in potato. These include *D. dadantii*, *D. dianthicola*, *D. solani*, *P. atrosepticum*, *P. wasabiae* and *P. carotovorum* subsp. *carotovorum* and *P. c.* subsp. *brasiliensis* [11–18]. World-wide, *Pectobacterium* is the main cause of blackleg and soft rot in potatoes, with *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum* the most significant production constraints. *P. carotovorum* subsp. *carotovorum* has a broad host range and a world-wide distribution in contrast to *P. atrosepticum* which is largely restricted to potato and principally only found in temperate regions [19]. Although originally described as affecting potatoes grown in Brazil [11] and New Zealand [14], respectively, "*P. carotovorum* subsp. *brasiliensis*" and *P. wasabiae* can now both be found in many of the potato-growing regions of the world. With regard to *Dickeya*, *D. dianthicola* and *D. solani* predominate in Northern Europe with the earliest report of a *Dickeya* spp. (*E. chrysanthemi*) causing disease on potato dating back to the 1970s [20]. Until 2005, all European potato isolates were likely to have been *D. dianthicola* [13, 21], but, more recently, several studies on potato strains isolated from diseased plants and tubers from a wide range of European countries and Israel have identified a new pathogen, subsequently named *D. solani*, as the principal *Dickeya* species affecting potato across the region [8, 12, 13, 22]. In other parts of the world, *D. dadantii* is known to cause blackleg and soft rot in potatoes, with a recent report highlighting its importance in Zimbabwe [23].

Disease spread is thought to be largely due to movement of latently infected potato seed tubers, which can lead to the introduction of highly pathogenic *Pectobacterium* and *Dickeya* spp. into different countries resulting in new disease outbreaks [19, 22, 24, 25]. In addition, *Dickeya* spp. have been detected in irrigation water in a number of potato-growing regions of Northern Europe, but the significance of these findings in relation to disease epidemiology remains unclear [9, 12, 26].

Blackleg, caused by *Pectobacterium* and *Dickeya* spp., is characterized by the production of a slimy, wet, black rot lesion spreading from the rotting mother tuber up the stems, especially under wet

Fig. 1 Symptoms of potato blackleg caused by *Pectobacterium atrosepticum* (**a**), *Dickeya solani* (**b**) and tuber soft rot caused by *Dickeya solani* (**c**)

conditions [19]. In dry conditions, symptoms tend to lead to stunting, yellowing, wilting, and desiccation of stems and leaves (Fig. 1). Under warm wet conditions, blackleg and soft rot symptoms in potato are similar whether caused by *Pectobacterium* or *Dickeya* spp., which makes it almost impossible to identify the causal agent by visual assessment alone. For this reason, it is important to be able to rapidly and reliably detect and identify the bacterial species or subspecies.

This chapter describes diagnostic methods for the isolation and differentiation of *Pectobacterium* and *Dickeya* spp. using dilution plating with conventional PCR or nucleic acid extraction with quantitative real-time PCR. The chapter provides methods for pathogen isolation from asymptomatic and symptomatic potato plants and tubers as well as irrigation waters and details diagnostic methods for the isolation and differentiation of *Pectobacterium* and *Dickeya* spp. using qualitative and quantitative PCR assays. The chapter covers pathogen isolation on selective crystal violet pectate (CVP) medium [27, 28] with conventional PCR and nucleic acid extraction with quantitative real-time PCR. While there are primers to identify most species and subspecies of *Pectobacterium* using conventional PCR, there is as yet only validated species-specific primers for *P. atrosepticum* and *P. wasabiae* using real-time PCR [29, 30]. The genera *Dickeya* can be detected as a group using conventional and real-time PCR. However, the only validated species-specific real-time PCR primers are currently available for *D. solani* and *D. dianthicola* [31]. It is expected that other *Dickeya* and *Pectobacterium* species-specific real-time PCR assays will be available in the near future.

2 Materials

2.1 Isolation of Bacteria from Plant Tissue

1. 70 % (aq., v/v) ethanol.
2. 10 % (aq., v/v) sodium hypochlorite.
3. Hand-held potato peeler or disposable scalpel.
4. Universal long Extraction bags (Bioreba).

5. 0.25 Strength Ringer's buffer: dissolve 1 tablet (Oxoid) in 500 mL of distilled water and sterilize by autoclaving.
6. Antioxidant: tetrasodium pyrophosphate or dithiothreitol.
7. Homex 6 (Bioreba) or mallet.

2.2 General and Selective Plating Media

1. Enrichment media: Double strength pectate enrichment medium (D-PEM; [32]):
   - Dissolve in order, the salts (0.64 g $MgSO_4$; 2.16 g $(NH_4)_2SO_4$; 2.16 g K_2HPO_4) in 300 mL distilled water.
   - Heat if required to dissolve salts then make up to 1,000 mL.
   - Suspend 3.4 g of sodium polypectate in 5 mL of absolute ethanol and add to the solution, mixing well with a magnetic stirrer.
   - Steam suspension until polypectate is completely dissolved, and adjust pH to 7.2 if necessary.
   - Dispense into small aliquots (50 mL) and sterilize by autoclaving at 120 °C for 15 min. Store at 4 °C until required.
2. Selective plating media:
   - Nutrient agar (NA) or Luria-Bertani agar (LBA).
   - Crystal violet pectate medium (CVP) can be successfully used as both a single or double layer media for the detection and isolation of *Pectobacterium* and *Dickeya* spp. [28]. Although the double layer is more time consuming and awkward to make, it can be more suited to samples with large numbers of bacteria due to its slower cavity development.
     - Single-layer CVP is prepared as two solutions, the crystal violet solution and the pectin solution. The two solutions should be prepared separately before being mixed together and autoclaved. Add the ingredients of both solutions sequentially in the order of the following recipes. Prepare the crystal violet solution in 500 mL distilled water and add 1.02 g $CaCl_2{\cdot}2H_2O$, 1 g tryptone, 5 g trisodium citrate, 2 g $NaNO_3$, 4 g agar and 1.5 mL crystal violet (1 % aqueous solution). Each ingredient of the crystal violet mix should be dissolved by stirring the medium before adding the following one. The pectin solution contains 2 mL NaOH (5 M) and 18 g pectin in 500 mL distilled water, and the pectin should be dissolved by stirring with a magnetic stir bar using heat if required. Mix the pectin solution with the crystal violet solution while stirring, and adjust the pH to 7.0 if required, before autoclaving. After autoclaving, mix the medium using a magnetic stir bar to avoid bubble formation and pour while still hot into Petri dishes in a laminar flow cabinet. Allow the medium

to set overnight and then store at 4 °C until required. Before use, dry plates in a laminar flow or drying oven to remove excess surface moisture.

– Double-layer CVP media is prepared in two steps. The first step is to prepare the basal layer mix in 1 L distilled water by sequentially adding the ingredients in the order of the following recipe: 5.5 g $CaCl_2 \cdot 2H_2O$, 1 g tryptone, 1.5 mL crystal violet (0.1 % solution), 1.6 g $NaNO_3$ and 15 g agar. After autoclaving for 15 min at 120 °C, cool the mix to 45–50 °C and pour ~15 mL into Petri dishes. Allow the basal layer to set and dry in a laminar flow to remove excess surface moisture. The second step is to prepare the over layer by adding 2 mL of 5.5 % EDTA (pH 8.0), 2 mL NaOH (5 M) and 20 g pectin in 800 mL distilled water. Dissolve the pectin by stirring with a magnetic stir bar using heat if required. Adjust the pH to 7.0 before autoclaving. Pour 7 mL of the over layer onto the dried basal layer.

2.3 Conventional PCR

1. PCR amplification.
   - Molecular grade water.
   - Oligonucleotide primers (Table 1).
   - Deoxyribonucleotides (dNTPs).
   - 5× PCR Reaction Buffer containing 7.5 mM $MgCl_2$ and 5 U/μL Taq DNA polymerase (Promega)-positive reference sample (*see* **Note 1**).
2. Gel electrophoresis.
   - Agarose.
   - SYBR safe (Life Technologies) or GelRed (10,000×; Thermo Scientific).
   - Loading buffer if required (*see* **Note 2**).
   - 100 bp or 1 kb ladder.
   - Thermal cycler.
   - Gel electrophoresis system.
   - 10× TBE electrophoresis buffer: 1 M Tris, 1 M boric acid and 20 mM EDTA (pH 8.3). Dilute to 1× for use.
   - UV gel documentation system.

2.4 Real-Time PCR

1. Nucleic acid extraction using kit.
   - Promega Wizard Magnetic DNA purification for Food (Thermo Labsystems).
   - Kingfisher 96 magnetic particle separator (Thermo Labsystems).

Table 1
Primers for conventional PCR

Target organism	Gene target	Primer name	Primer sequence (5′-3′)	Amplicon size (bp)	Reference
Pectobacterium and *Dickeya* spp.	16S rRNA	SR3F SR1cR	GGT GCA AGC GTT AAT CGG AAT G AGA CTC TAG CCT GTC AGT TTT	119	[33]
Pectobacterium atrosepticum	Genome	ECA1f ECA2r	CGG CAT CAT AAA AAC ACG GCA CAC TTC ATC CAG CGA	690	[34]
P. carotovorum subsp. *carotovorum*	Genome	EXPCCF EXPCCR	GAA CTT CGC ACC GCC GAC CTT CTA GCC GTA ATT GCC TAC CTG CTT AAG	550	[35]
P. wasabiae	YD protein gene	PW7011F PW7011R	CTATGACGCTCGCGGGT TGCTGTT CGGCGGCGTCGTAGT GGAAAGTC	140	[30]
"*P. carotovorum* subsp. *brasiliensis*"	16S-23S rRNA	BR1f L1r	GCG TGC CGG GTT TAT GCA CT CAA GGC ATC CAC CGT	322	[11]
Dickeya spp.	pectate lyase gene	ADE1 ADE2	GAT CAG AAA GCC CGC AGC CAG AT CTG TGG CCG ATC AGG ATG GTT TTG TCG TGC	420	[36]

2. Nucleic acid extraction using lab protocol.
   - Buffer B (Promega Cat No Z3201).
   - 750 µL precipitation solution (Promega Cat No Z3191).
   - Isopropanol.
   - Sodium acetate (3 M).
   - 0.25 Strength Ringer's buffer.
   - 70 % (aq., v/v) ethanol.
   - TE buffer: 10 mM Tris (pH 8) and 1 mM EDTA.
3. Quantitative detection and differentiation.
   - Molecular grade water.
   - Oligonucleotide primers and TaqMan® probes (Table 4).
   - TaqMan® Universal PCR MasterMix: the reaction mix is supplied at 2× concentration and contains AmpliTaq Gold® DNA Polymerase (Ultra-Pure), dNTPs and ROX™ as a passive reference.
   - Real-time PCR machine.

3 Methods

Bacteria can be extracted from the leaves and stem of potato plants and the peel and stolon end of tubers. The following methods are optimized for the detection from asymptomatic plants, but they can also be used for symptomatic plants (*see* **Note 3**). The methods describe (a) the isolation of *Pectobacterium* and *Dickeya* spp. from stem, tubers, and water samples, (b) the preparation of samples for molecular detection, (c) the qualitative detection and differentiation of *Pectobacterium* and *Dickeya* spp. using dilution plating on CVP and conventional PCR and (d) quantitative detection and differentiation using real-time PCR.

3.1 Isolation from Stems, Tubers, and Water Samples

Sampling and processing the peel and stolon end cores separately will determine whether the bacterial infection is systemic (found in the vascular tissue of stolon end) or can only be found externally as lenticel infection in tuber peel (*see* **Note 4**).

3.1.1 Sampling Potato Tubers

1. Wash tubers to remove excess soil.
2. Using a clean and disinfected hand-held potato peeler (*see* **Note 5**), remove one peel strip from each tuber in the sample, to include both the heel (stolon) and rose ends.
3. Rinse the tubers again, and then using a separate hand-held peeler or disposable scalpel, remove a small plug of tissue from the stolon end of each tuber in the sample (approximately 5–10 mm deep and wide) making sure not to take any peel.

3.1.2 Sampling Potato Plants

1. Rinse plants to remove any soil or debris.
2. Using a disposable scalpel, remove a 5 cm section of each stem just above ground level and a selection of leaves from all stems in the sample.

3.1.3 Processing of Plant and Tuber Samples

1. Place all plant and tuber samples into separate universal extraction bags (*see* **Note 6**) and weigh each bag.
2. Add 15 mL of 0.25 strength Ringer's buffer containing tetrasodium pyrophosphate (0.1 % final concentration) or dithiothreitol (final concentration 0.075 %) antioxidant (*see* **Note 7**) to each bag.
3. Pulverize the sample using a Homex 6 grinder or rubber mallet to give an oatmeal consistency.

3.1.4 Sampling and Processing Irrigation Water

1. Collect water samples in sterile bottles (250 mL) and transport to the laboratory in a cool box. Process within 24 h of collection.
2. Subdivide into aliquots of 40 mL and clarify by centrifugation at a low speed ($180 \times g$).

3. Remove 20 mL of supernatant and mix with an equal volume of D-PEM (*see* Subheading 2.2); incubate in an anaerobic chamber at 36 °C for 48 h.
4. Centrifuge at high speed (10,000 × *g*) to concentrate the bacterial fraction. Resuspend the pellet prior to serial dilution and plating onto CVP medium.

3.2 Qualitative Detection and Differentiation of Pectobacterium and Dickeya spp. Using Conventional PCR

Isolating *Pectobacterium* and *Dickeya* spp. can be problematic when secondary saprophytic microorganisms are present as they can outgrow the bacteria being isolated. Plating on the semi-selective media CVP (*see* Subheading 2.2) preferentially increases the pectolytic populations. The selectivity of the media is based on the addition of crystal violet, which inhibits the growth of Gram-positive bacteria, and on the use of pectin as the main carbon source. *Pectobacterium* and *Dickeya* spp. form characteristic deep cavities in the medium, due to their ability to break down and metabolize pectin. Although *Pectobacterium* and *Dickeya* spp. are both plated onto CVP, they should be incubated at 27 and 37 °C, respectively, for colony formation (*see* **Note 8**).

3.2.1 Selective Plating

1. Pipette off the extract from the homogenized sample and prepare a dilution series from 10^0 to 10^{-3} in 0.25 strength Ringer's buffer (*see* **Note 9**). This should ensure background saprophytes are diluted out and isolated *Pectobacterium* and *Dickeya* colonies are obtained.
2. Spread 100 μL of each dilution for each sample on to duplicate CVP plates previously dried to remove excess surface moisture. Incubate one plate at 27 °C and one plate at 37 °C for 48 h.
3. For use as a back-up stock, a 1 mL aliquot of the homogenate can be removed and added to 200 μL of 100 % sterile glycerol and stored at −20 °C or −80 °C for longer-term storage.
4. A dilution series of approximately 10^{-1}–10^{-4} CFU.mL^{-1} of a positive control for *Pectobacterium* and *Dickeya* spp. should also be prepared.
5. After 48 h select all colonies showing characteristic deep cavities and prepare a suspension of each colony by resuspending in molecular grade water.
6. This suspension will serve as a template for conventional PCR. Prior to PCR an aliquot of the sample should be transferred to a screw-top vial and incubated in a heat block at 96 °C for 5 min. Boiled samples can be frozen at −20 °C until required.

If any colonies require to be kept for reference stocks, then select, if possible, at least two well-spaced isolated colonies/cavities per CVP plate and re-streak the bacteria onto a fresh CVP plate.

Incubate at 27 °C or 37 °C (depending on genera) for 48 h. Once there are clean colonies showing characteristic cavities, select a colony and streak on to an NA or LBA plate previously dried in a laminar flow cabinet long enough to remove excess surface moisture. Incubate at 27 °C or 37 °C for about 1 week to ensure that only *Pectobacterium* or *Dickeya* spp. colonies are present. These bacteria form round convex creamy-translucent colonies on NA or LBA.

3.2.2 Conventional PCR

Set up PCR reactions in a contamination-free environment using primers specific for the species of interest (Table 1). There are multiple primer combinations for *Pectobacterium* and *Dickeya* spp., reaction conditions and PCR product electrophoresis and visualization methods routinely used by the authors that are described below.

1. Prepare the master mix as described in Table 2 (*see* **Note 10**). Prepare enough master mix for the number of samples to be tested and aliquot 24 µL into each PCR tube or each well of a PCR plate.
2. Add 1 µL of boiled cell sample for each 25 µL reaction. Samples should be amplified using both undiluted and diluted (1:10) boiled cell suspensions.
3. Negative controls should include the reaction mix without any template (i.e., no boiled cell suspension) and the reaction mix with 1 µL of any buffers used for sample processing. A positive control should also be included (*see* **Note 1**).
4. Using a thermal cycler, run the specific amplification protocol for the appropriate primers as described in Table 3.
5. After PCR amplification, the amplicon can be resolved by agarose gel electrophoresis on a 1.5 % gel in TBE buffer containing 0.01 % SYBR safe or GelRed.

Table 2
Master mix reagents for conventional PCR

Reagent	Working stock concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	–	16.38	–
Green GoTaq® reaction buffer with 7.5 mM $MgCl_2$	5×	5.0	1×
dNTP mix (dATP, dTTP, dCTP, dGTP)	10 mM of each	0.5	0.2 mM of each
Forward primer	10 µM	1.0	0.4 µM
Reverse primer	10 µM	1.0	0.4 µM
Taq DNA polymerase	5 U/µL	0.12	1.5 U
Template	–	1.0	–

Table 3
Conventional PCR cycling conditions

Target organism	Step 1	Step 2	Step 3
Pectobacterium and *Dickeya* spp.	94° for 5 min	40 cycles: 94 °C for 30 s, 68 °C for 45 s, 72 °C for 45 s	72 °C for 7 min
Pectobacterium atrosepticum	94° for 5 min	36 cycles: 94 °C for 30 s, 62 °C for 45 s, 72 °C for 45 s	72 °C for 7 min
P. carotovorum subsp. *carotovorum*	94° for 5 min	30 cycles: 94 °C for 60 s, 60 °C for 1 min, 72 °C for 2 min	72 °C for 7 min
P. wasabiae	94° for 5 min	35 cycles: 94 °C for 60 s, 67 °C for 30 s, 72 °C for 60 s	72 °C for 7 min
"*P. carotovorum* subsp. *brasiliensis*"	94° for 5 min	25 cycles: 94 °C for 45 s, 62 °C for 45 s, 72 °C for 90 s	72 °C for 7 min
Dickeya spp.	94° for 5 min	25 cycles: 94 °C for 60 s, 72 °C for 2 min	72 °C for 7 min

6. Load 12 μL of the reaction if the PCR buffer has a loading dye added or 10 μL of the reaction mixed with 2 μL of loading dye if it doesn't.
7. Include a 100 bp or 1 kb DNA ladder.
8. The amplicon of interest can be visualized under UV light using a gel doc system, and the amplicon size can be established by comparing to a DNA ladder.

3.3 Quantitative Detection and Differentiation Using Real-Time PCR

Real-time PCR analysis allows high-throughput detection, quantification, and identification of *Pectobacterium* and *Dickeya* populations. The following section describes the detection of *P. atrosepticum* and *Dickeya* spp. using real-time PCR with TaqMan® probes. Currently there are no validated TaqMan® real-time protocols for *P. wasabiae* or *P. carotovorum*. However, there is a SYBR Green qPCR assay for the detection of *P. wasabiae* [30]. TaqMan® chemistry uses a customized fluorogenic probe complementary to the target DNA sequence to enable the detection of a specific PCR product as it accumulates during PCR.

The following section describes (a) nucleic acid extraction from homogenized samples, (b) primers and probes for detection of *P. atrosepticum* and *Dickeya* spp., (c) preparation of a standard curve for quantification and (d) real-time PCR analysis.

3.4 Nucleic Acid Extraction

1. Pipette off the extract from the homogenized sample (*see* Subheading 3.1.3) into a 15 mL centrifuge tube, and centrifuge at 4 °C for 10 min at 90×*g* to remove any remaining particulate matter.

2. Dispense two 5 mL aliquots of supernatant into separate 15 mL tubes and centrifuge at 4 °C and 2,236 × *g* for 15 min to pellet the bacteria.

One of the bacterial pellets can be stored at –20 °C (as a backup stock), and nucleic acid can be extracted from the other bacterial pellet using Promega Wizard Magnetic DNA purification for Food in combination with a Kingfisher 96 magnetic particle separator (Thermo Labsystems) following the manufacturers' instructions. Alternatively, nucleic acid can be extracted using the protocol below.

1. Resuspend the pellet in 1 mL of 0.25 strength Ringer's buffer.
2. To each tube, add 250 µL buffer B (Promega Cat No Z3201) and 750 µL precipitation solution (Promega Cat No Z3191).
3. Vortex tubes and incubate at room temperature for 5 min.
4. Centrifuge tubes at 2,236 × *g* for 15 min at room temperature.
5. Remove 750 µL of supernatant from the sample tubes while avoiding the pellet and add to an equal volume of ice cold isopropanol.
6. Add 75 µL sodium acetate (3 M) and gently invert the tubes to mix.
7. Incubate at room temperature for at least 1 h.
8. Centrifuge tubes at 11,688 × *g* for 4 min, after which pipette off supernatant while avoiding disturbing the pellet.
9. Wash the pellet with 150 µL of 70 % ethanol (aq., v/v) and vortex tube.
10. Centrifuge tubes at 11,688 × *g* for 2 min and pipette off the ethanol.
11. Allow the pellet to air-dry for 10 min.
12. Resuspend the pellet in 100 µL TE buffer. The neat NA can be stored at –20 °C until required.
13. The NA should be diluted 1:2 with molecular grade water before being used as a template for real-time PCR.

3.5 Preparation of a Standard Curve

1. A known reference culture of *P. atrosepticum* and *Dickeya* spp. should be grown in LB broth at 27 °C or 37 °C with shaking for 16 h to give a bacterial density of ~10^9 CFU/mL.
2. Prepare a tenfold dilution series of the bacterial suspension from 10^9 to 10^0 CFU/mL.
3. Determine total cell counts in each dilution by plating on CVP medium. All dilutions should be performed in triplicate and the average cell count of the three replicates determined.
4. Carry out nucleic acid extraction using 5 mL of the 10^9 CFU/mL bacterial suspension following the nucleic acid extraction method.

5. Determine the concentration of the extracted DNA using a NanoDrop or spectrophotometer.
6. Prepare a tenfold serial dilution of the extracted DNA.

Use the DNA dilution series as standards in the real-time PCR assays so that a standard curve can be produced and the amount of DNA in the unknown extracts determined. *P. atrosepticum* or *Dickeya* spp. infection can be expressed as log pg DNA.g^{-1} fresh wet weight of leaf, stem, or tuber. The approximate CFU/g of fresh wet weight of leaf, stem, or tuber can also be calculated using the cell counts from the corresponding tenfold dilution series of the bacterial suspensions.

3.6 Real-Time PCR (TaqMan®) for the Detection and Quantification of Pectobacterium atrosepticum and Dickeya solani

Selected primers and probes for the detection and quantification of *Pectobacterium atrosepticum* (Table 4: Eca), total pectolytic bacteria (Table 4: PEC), *Dickeya* spp. (Table 4: Ech), *Dickeya solani* (Table 4: SolC or *fusA*) and the potato cytochrome oxidase (Table 4: COX) gene are listed below. The COX primer probe combination is an internal positive control that can be used to amplify a fragment of the potato cytochrome oxidase gene to determine reliable and uniform yields of pure NA from all extracts.

Real-time PCR reactions should be set up in a contamination-free environment using TaqMan® Universal PCR MasterMix. All TaqMan® reagents should be protected from light until ready for use as excessive exposure to light may affect the fluorescent probes. Real-time PCR should be performed in 25 μL reactions using 96 well optical plates and optical PCR seals or caps.

1. Prepare enough master mix on ice for the number of samples to be tested following the recipe in Table 5 (*see* **Notes 10** and **11**). All samples should be tested in duplicate.
2. Aliquot 23 μL of master mix into the wells of an optical plate.
3. For each reaction, add 2 μL NA which has been diluted 1:2 with molecular grade water.
4. A range of standards containing known amounts of DNA (*see* Subheading 3.5) should also be included in the real-time PCR along with no template controls (NTCs) for each assay on the plate.
5. Cover the plate using optical PCR seals or caps and centrifuge briefly.
6. Run the real-time PCR amplification using a Real-Time PCR Detection System and reaction conditions of an initial 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60s.
7. After the run is completed, examine the amplification plots of the standard, tested samples and controls using the real-time machine software. Record concentrations of standards and corresponding Ct values and check that averaged Ct values for tested samples are within the range of the standard.

Table 4
Primers and probes for real-time PCR (*see* Note 11)

Target organism	Primer name	Primer and probe sequence (5′-3′)	Reference
Pectobacterium and *Dickeya* spp.	PEC-1F PEC-1R PEC-P	GTG CAA GCG TTA ATC GGA ATG CTC TAC AAG ACT CTA GCC TGT CAG TTT T CTG GGC GTA AAG CGC ACG CA	[29]
Pectobacterium atrosepticum	ECA-CSL-1F ECA-CSL-89R ECA-CSL-36T-P	CGGCATCATAAAAACACGCC CCTGTGTAATATCCGAAAGGTGG ACATTCAGGCTGATATTCCCCCTGCC	[29]
Dickeya spp.	ECH-1F ECH-1R ECH-P	GAG TCA AAA GCG TCT TGC GAA CCC TGT TAC CGC CGT GAA CTG ACA AGT GAT GTC CCC TTC GTC TAG AGG]	[29]
Dickeya dianthicola	DIA-A F DIA-A R DIA-A P	GGCCGCCTGAATACTACATT TGGTATCTCTACGCCCATCA ATTAACGGCGTCAACCCGGC	[31]
Dickeya solani	SOLC-F SOLC-R SOLC-P	GCCTACACCATCAGGGCTAT ACACTACAGCGCGCATAAAC CCAGGCCGTGCTCGAAATCC	[31]
Dickeya solani	*fusA*-F *fusA*-R *fusA*-P	GGTGTCGTTGACCTGGTGAAA ATAGGTGAAGGTCACACCCTCATC TGAAAGCCATCAACTGGAATGATTC	[37]
Potato (cytochrome oxidase gene)	COX-F COX-R COX-P	CGT CGC ATT CCA GAT TAT CCA CAA CTA CGG ATA TAT AAG AGC CAA AAC TG TGC TTA CGC TGG ATG GAA TGC CCT	[38]

Table 5
Master mix reagents for real-time PCR

Reagent	Working stock concentration	Volume per singleplex reaction (μL)	Volume per multiplex reaction (μL)	Final concentration
Molecular grade water	–	7	3.5	–
TaqMan® Universal PCR Master Mix	2×	12.5	12.5	1×
Forward Primer	5 μM	1.5	1.5 of each	0.3 μM
Reverse Primer	5 μM	1.5	1.5 of each	0.3 μM
TaqMan probe	5 μM	0.5	0.5 of each	0.1 μM
Template (1:2 dilution)	–	2.0	2.0	–