

Chapter 2

Detection and Identification of *Phoma* Pathogens of Potato

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Abstract

Phoma foveata, *Phoma exigua* var. *exigua*, and *Phoma eupyrena* are fungal pathogens of potato, causing gangrene or pit rot symptoms in tubers, and they are responsible for significant crop losses. Various techniques are available to identify these pathogens in the laboratory. A multiplex Plexor® real-time PCR method which can detect and identify these pathogens in a single reaction will be presented.

Key words Multiplex, Fungal, *Phoma*, Gangrene, Potato, Bioassay, In vitro culture, Real-time PCR, Plexor®, PCR

1 Introduction

Gangrene is a storage dry rot of potato (*Solanum tuberosum*) caused predominantly by the soil-borne fungus *Phoma foveata* (Foister) [1], which infects largely through wounds arising at harvest or grading. The less aggressive pathogen, *Phoma exigua* var. *exigua* [1], is also associated with gangrene-type symptoms, although these do not progress so extensively. Gangrene symptoms start with small sunken thumbprint-like lesions at wounds, lenticel or tuber eyes, which then enlarge, becoming irregular in shape, often with a characteristic wavy edge externally (Fig. 1a–c). Internally, a dark rot develops with a well-defined edge between the diseased and healthy tissue, and large cavities can be present (Fig. 1b, c). The size of the external lesion is rarely indicative of the dimensions of the internal lesion (Fig. 1c). Primarily a seed-borne disease, gangrene is responsible for crop losses in most potato-producing regions of the world including Europe, Northern Africa, New Zealand, Australia [2], and China [3].

Phoma eupyrena is a less aggressive pathogen responsible for causing pit rot symptoms in potato which can look like immature gangrene lesions. Cases of gangrene and pit rot have been increasing during the last few years (SASA, unpublished data), possibly due to the loss of the fungicide 2-AB as a control measure and



Fig. 1 (a) External and (b) internal symptoms of gangrene (*Phoma exigua* var. *foveata*). (c) On the right of this tuber, a large external lesion can be seen, but the internal rot is not proportional in size as it has not progressed deeply into the tuber. (d) Characteristic wavy edge on the left hand side of this gangrene lesion. (e) Internal gangrene symptoms showing characteristic jet black discoloration, irregular shape, large cavities filled with white-gray fungal growth, and rusty reddish-brown discoloration. (f) Yellow colony of *Phoma foveata*. On close inspection, small specks of anthraquinone pigment production can be seen

sulfuric acid as a haulm desiccant, impacting on the spread and development of the pathogens responsible for these diseases.

Morphologically, *Phoma foveata* and *Phoma exigua* var. *exigua* are very similar and cannot be distinguished based on their pycnidia or conidia [1]. Pycnidia are globoid (90–200 μM) and dark brown to black in color, and their cylindrical conidia are 4–5 μM $\times$ 2–3 μM [4]. *Phoma eupyrena* is more easily identified from the other two *Phoma* species but all three have similar colony morphology, so laboratory expertise is required to differentiate them. *Phoma foveata* produces pigments known as anthraquinones (Fig. 1f) which exhibit as small yellow-brown flecks on the underside of the culture plate after approximately 7–10 days of growth. *Phoma exigua* var. *exigua* does not produce anthraquinone pigments, and this can be used to distinguish it from *Phoma foveata*. However, pigment production can be variable or lost completely, so colony morphology requires skilled interpretation, can be unreliable, and takes 7–14 days until colonies are mature. Therefore, it is necessary to be able to differentiate between these two species in a more reliable way. DNA-based techniques can assist with the rapid identification of these fungal species: No expertise is required in fungal morphology; these techniques are rapid to undertake and results are accurate in terms of identification.

However, at the molecular level there is considerable similarity between these two closely related *Phoma* varieties. MacDonald et al. [5] developed a RAPD-generated PCR-RFLP marker to distinguish between the two varieties, but the use of restriction enzymes makes this assay time-consuming and relatively expensive. Cullen et al. [6] developed conventional and quantitative PCR assays for the detection of *P. exigua* var. *foveata*, but the primers also detected the closely related *P. exigua* var. *exigua*. Aveskamp et al. [7] developed specific primers which can differentiate the two varieties with conventional PCR, but this is more time-consuming and less sensitive than real-time PCR. In order to facilitate the rapid identification of these three potato pathogens, for both diagnostic and research purposes, we describe below a multiplex assay, based on a real-time qPCR method known as Plexor® technology, which can distinguish between three *Phoma* potato pathogens in one reaction, thus saving on time, labor, and reagents.

2 Materials

2.1 Incubation Test

- Sterile masonry nail, approximately 4 mm diameter, or other blunt utensil to wound the tuber flesh.
- Sterile distilled water to create a humid environment.
- Paper toweling or capillary matting to wet with sterile water.
- Plastic container with lid to incubate the tubers in.
- Refrigerator at 5 °C.
- Methylated spirits and Bunsen burner to sterilize equipment.

2.2 Plating Media for Culturing *Phoma* sp.

2.2.1 Malt Extract Agar

Malt extract (Oxoid)	30.0 g/l
Mycological peptone	5.0 g/l
Agar	15.0 g/l
Adjust pH to 5.4	

Suspend 50 g in 1 l of distilled water and boil to dissolve. Sterilize by autoclaving at 115 °C for 10 min. Once cooled to approximately 55 °C, dispense into Petri dishes and allow to cool. Store at 4 °C until required.

2.2.2 Potato Dextrose Agar (PDA)

Potato extract (Oxoid)	4.0 g/l
Glucose	20.0 g/l
Agar	15.0 g/l
Adjust pH to 5.6	

Suspend 39 g in 1 l of water (purified as requested). Bring to the boil to dissolve completely. Sterilize by autoclaving at 121 °C for 15 min. Mix well before pouring. Once cooled to approximately 55 °C, dispense into Petri dishes and allow to cool. Store at 4 °C until required.

2.2.3 Aseptic Techniques

Methylated spirit, a Bunsen burner, and scalpels/knife are required in order to sterilize the equipment.

2.3 Real-Time PCR

2.3.1 Nucleic Acid Extraction Using Kit

- DNeasy plant mini kit (Qiagen).
- Kingfisher 96 magnetic particle separator (Thermo Labsystems).

2.3.2 Plexor® Detection and Differentiation

- MOPS/EDTA buffer (Promega).
- Plexor® primers (Table 2).
- Plexor® Master Mix (Promega): the reaction mix is supplied at 2× concentration and the reaction mix includes deoxynucleotides and iso-dGTP modified with the quencher dabcyI.
- Real-time PCR machine (such as Stratagene MX3005p).

3 Methods

3.1 Visual Inspection

If mature rots are present, a visual examination may be sufficient to identify gangrene symptoms and this provides the most rapid diagnosis. However, care has to be taken as the appearance of gangrene symptoms in tubers can be mistaken for other dry rot diseases, such as those caused by *Fusarium* species. Certain characteristic symptoms of gangrene infection can aid diagnosis:

1. Firstly, the presence of a wavy edge on the external lesion (Fig. 1a, d).
2. Secondly, when the affected tuber is cut in half through the center of the external lesion, if the size of the external lesion is not proportional to the size of the internal lesion, i.e., if the external lesion is large, but there is little internal rotting or vice versa, this is a good indication that the disease is gangrene (Fig. 1b, c, e).
3. Thirdly, if the internal symptoms are very irregular in shape, this can indicate gangrene. The internal lesion will be dry (*see* **Note 1**) and dark in color, with an extremely well-defined edge between the diseased and healthy tissue. Cavities will be present, often filled with fluffy fungal growth, which can vary in color; however, if there is black tissue present, this is likely to be due to pycnidia formation, which indicates gangrene infection (Fig. 1b, e). Likewise, if there is rusty reddish-brown tissue present, this is also indicative of gangrene.

The combined presence of these symptoms can be used to rule out *Fusarium* dry rot infections. Less well-developed infections would need to be subcultured in order to identify the fungal pathogen responsible.

3.2 Incubation Test

To detect for latent (no symptoms) contamination of tubers with *Phoma* species, a 100-tuber sample is tested

1. Utensils should be dipped in methylated spirit and flamed to sterilize before and between samples.
2. Wound tubers to a depth of 5 mm using a modified masonry nail (*see* **Note 2**). Tubers are wounded ten times, five wounds in an “X” formation on each side of the tuber.
3. Incubate tubers at 5 °C for a period of 4–6 weeks.
4. Once any rots develop, they will need to be subcultured to identify the causal organism, as described below.

3.3 Plating Medium

1. Using aseptic techniques, a knife should be used to cut into the lesion perpendicular to the center of the external lesion.
2. Once the full extent of the internal lesion is known, at the boundary between the diseased and healthy tissue, a sterile scalpel should be used to cut away a thin layer of tissue from the surface of the cut tissue, as this will remove contaminants that may have been dragged from the external skin of the tuber as the initial cut was made with the knife.
3. A final sterile scalpel is then used to scrape away a small amount of diseased tissue at the leading edge of infection (the boundary between diseased and healthy tissue) as this is where the fungus will be actively multiplying. Contaminants are more likely to be present near the initial starting point for the rot, particularly if there is an open wound, so this area should be avoided where possible.
4. Place the extracted tissue in the center of a Petri dish containing malt extract agar (Oxoid). On malt agar medium, *Phoma foveata* will display pigment production after approximately 7 days.
5. A second scraping of diseased tissue should be placed in the center of a potato dextrose agar (Oxoid) (PDA) plate. The use of PDA assists with the identification of other non-*Phoma* potato pathogenic fungi.
6. Incubate the plates for 7–14 days at ambient temperature.

3.4 Plexor® Assay

Based on real-time PCR technology, Plexor® (Promega) involves labeling one species-specific primer with a fluorescent dye and modified it with a methylisocytosine (iso-dC) residue at the 5'-end. The corresponding species-specific primer is not modified. Each set of species-specific primers is labeled with a different fluorescent

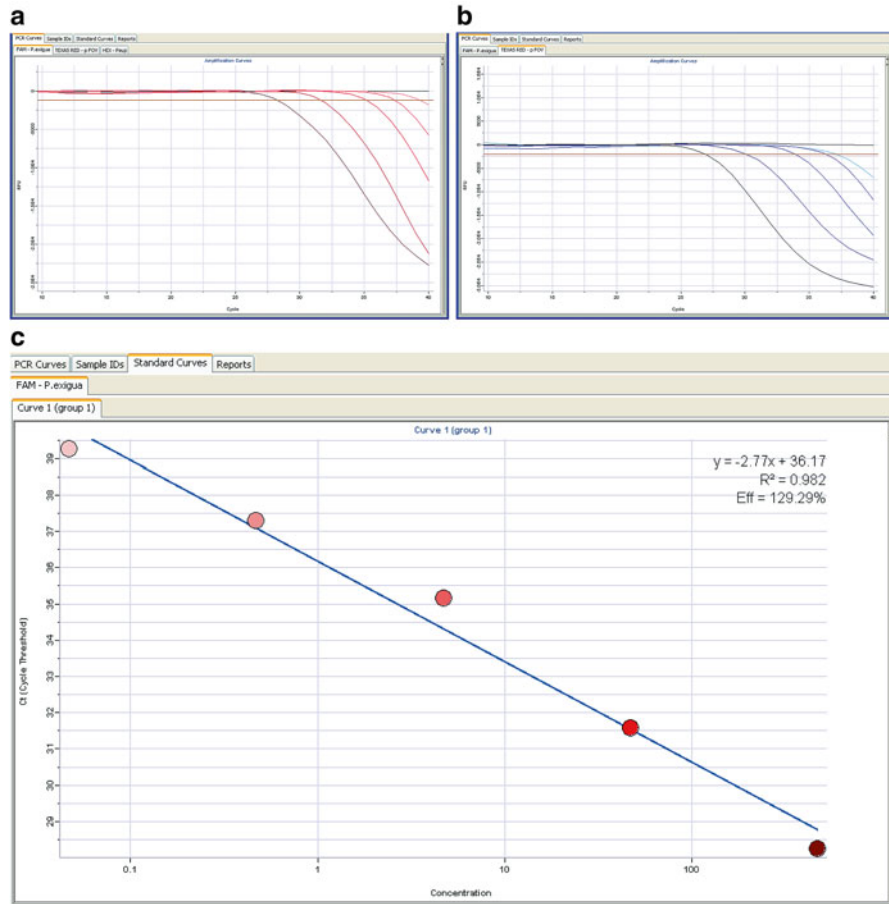


Fig. 2 (a) Plexor results showing a decrease in fluorescence as cycling continues. Higher concentrations of template DNA show a decrease in fluorescence earlier in the cycling process than lower template DNA concentrations. Negative control sample shows no decrease in fluorescence. These results are for *Phoma exigua* var. *exigua*, using a tenfold dilution series starting with 2 ng DNA (amplified with *Phoma* complex primers). (b) Amplification curves obtained, in multiplex reactions, for *Phoma foveata* on a tenfold dilution series of target DNA starting with 2 ng DNA. Higher concentrations of template DNA show a decrease in fluorescence earlier in the cycling process than lower template DNA concentrations. Negative control sample shows no decrease in fluorescence. (c) Standard curve obtained on a tenfold dilution series of DNA from *Phoma exigua* var. *exigua* in a multiplex reaction. Concentration of DNA shown is in picograms

label to create a multiplex reaction. The qPCR reaction buffer includes dabcyI-iso-dGTP (iso-dG); during thermocycling this becomes incorporated at the position complementary to the iso-dC label, effectively quenching the fluorescence over time [8], as shown in Fig. 2.

3.4.1 Primer Design

To design primers to detect and differentiate *Phoma foveata*, *Phoma exigua* var. *exigua*, and *Phoma eupyrena*, DNA sequences were generated from the respective fungal isolates held in SASA's culture collection by comparative sequence analyses using primers

Table 1**Primers used to amplify *Phoma* DNA for sequencing purposes**

Primer name	Specific target	Sequence 5' to 3'	Amplicon size (bp)
Phoma 2	RAPD-PCR primer	GGACCCCTGTACTGACGTC	474
Phoma 7	<i>Phoma</i> sp. marker	AGCGGCTAGGATAGACAGGCG	
EF1-1Fa	EF-1 α gene	GCTGGTATCTCCAAGGATG	~870
EF1-1Ra	EF-1 α gene	TCRGTGAARGCCTCAAC	

Table 2**Plexor® primers generated to detect *Phoma foveata*, *Phoma eupyrena*, and *Phoma* species**

Primer name	Target organism	Sequence 5' to 3'	Product size (bp)
PFoveataF	<i>P. foveata</i>	GGTGA ACTCTGTGCTCGATATGC ^a	80
PFoveataR		ATGACAGGAGTGAGACGATGATAGT	
PhomaF	<i>P. exigua</i>	GCCCGTTGGTCTCCACTTGTA ^b	96
PhomaR	var. <i>exigua</i> and <i>P. foveata</i>	AGAAAGCCCGAAATCTAGAGCAAC	
PeupyrenaF PeupyrenaR	<i>P. eupyrena</i>	CAAGTGCCACGAATGTACTGAG ^c TGATCTGACCTGTAAACAGCATCG	121

^aModified at the 5'-end with iso-dC and Texas Red^bModified at the 5'-end with iso-dC and FAM^cModified at the 5'-end with iso-dC and Hex

derived from RAPD-PCR fragments (Phoma 2 and Phoma 7) [5] and translation elongation factor 1 α (EF-1 α) gene-specific primers (Schoch, unpublished data) (Table 1). In addition to the sequences generated above, the *Phoma* sequences published on GenBank as accession numbers EU880838 (*P. exigua* var. *exigua*) and EU880839 (*P. exigua* var. *foveata*) [7] were also used during the design of diagnostic primers. The Plexor® primer design software (Promega) was used to select suitable primer pairs.

The first primer pair, PhomaF and PhomaR, detects *Phoma exigua* var. *exigua* and *Phoma foveata* DNA sequences which are highly homologous. The forward primer was modified at the 5'-end with a fluorescein phosphoramidite (FAM; peak emission at 516 nm and peak excitation at 492 nm) label and an iso-dC residue. The second primer pair (PfoveataF and PfoveataR) detects *Phoma foveata* DNA sequences, and the third primer pair detects *Phoma eupyrena* (PeupyrenaF and PeupyrenaR) DNA sequences (Table 2). These were labeled at the 5'-end of the forward primer with Texas Red (peak emission at 620 nm and peak excitation at

584 nm) and Hex (peak emission at 556 nm and peak excitation at 535 nm), respectively. Additionally, both forward primers were modified at the 5'-end with an iso-dC residue. Reverse primers PFOveataR and PeupyrenaR were not labeled or modified.

3.4.2 DNA Extraction

A portion of diseased tuber flesh is scraped and homogenized using a reusable pestle (*see Note 3*) in a 1.5 ml microfuge tube. 400 µl AP1 buffer (Qiagen) is then added and the DNA extraction is performed according to the DNeasy plant mini kit's user instructions (Qiagen).

3.4.3 Real-Time Plexor™ Assay

Quantitative PCR amplifications are performed in 25 µl reactions in a Stratagene MP3005P thermocycler (*see Note 4*) using a master mix recipe shown in Table 3, with all three primer sets at a final concentration of 200nM (*see Note 5*) and <100 ng purified template DNA. Reactions are carried out using 2× Plexor® qPCR system master mix (Promega). The following amplification protocol is used: initial denaturation of 2 min at 95 °C, followed by 40 cycles of 95 °C for 5 s and 60 °C for 35 s, then 1 cycle of 60 °C for 15 s and 95 °C for 5 s. Following amplification, results are analyzed using Plexor® Analysis Software (Promega) which is available from their website (www.promega.com).

Procedure:

1. Thaw Plexor® Master Mix and primers on ice.
2. Briefly vortex master mix and primers, and store on ice.
3. Prepare master mix as indicated below (Table 3).
4. Samples are tested in duplicate/triplicate.
5. Vortex reaction mixture briefly.
6. Add 20 µl of reaction mix to an optical-grade PCR plate for each sample.

Table 3
Plexor® reaction setup

	Volume per reaction (µl)
Plexor® Master Mix, 2×	12.5
5 µM primer pair <i>P. foveata</i> (<i>see Note 6</i>)	1.0
5 µM primer pair <i>Phoma</i> species	1.0
5 µM primer pair <i>P. europyrena</i>	1.0
MOPS/EDTA buffer	4.5
Template DNA	5.0
Total volume	25

7. Add 5 µl of template (for non-template control, add 5 µl MOPS/EDTA buffer).
8. Spin plate briefly and place in Stratagene MX3005p machine.
9. Set up program according to Promega technical manual.
10. Select “SYBR Green (with dissociation curve),” then select “OK.”
11. Select “plate set up.”
12. Highlight wells to be used.
13. Select “unknown” as the well type.
14. Select “none” for the reference dye.
15. Click dyes to be used (Hex, FAM, Texas Red).
16. Select “thermal profile setup.”
17. Change plateau in segment 1–95 °C for 2 min.
18. Change plateau 1 in segment 2–95 °C for 5 s.
19. Change plateau 2 in segment 2–60 °C for 35 s.
20. Delete 3rd plateau (highlight, then click “delete”).
21. Ensure cycle number for segment 2 is 40.
22. Save the file.
23. Start the run.
24. After the run is complete, extract the data and analyze on Plexor® software (according to the Promega user instructions).

3.4.4 Dilution of Standard Reference Template

Inclusion of a dilution series of a standard reference in the Plexor® assay will provide an indication on the assay's performance (Fig. 2a–c) and, when required, the limit of detection of the assay (Fig. 2b). This is provided when purchasing the 2× Plexor® Master Mix buffer.

1. Thaw and vortex a standard reference template.
2. Quantify DNA concentration.
3. Prepare serial dilutions of the standard reference template by adding 10 µl DNA to 90 µl MOPS/EDTA buffer. Change tips between dilutions.
4. Run Plexor® assay as above.
5. Extract data and analyze on Plexor® software.

3.4.5 Interpretation of Results

The assay is conclusive if all of the controls give the expected result: It is recommended to include a non-template control (no amplification expected; used to monitor potential contamination), a positive extraction control (amplification of each *Phoma* species target should occur in extraction control samples), and a positive amplification control (*Phoma* target DNA to monitor the performance of

Phoma real-time amplification). All tested samples are run in triplicate. The multiplex assay achieves the same level of sensitivity as the individual singleplex reactions. The multiplex detection limit is 80 fg for *P. foveata* (Fig. 2b), 160 fg for *Phoma* species, and 80 fg for *P. eupyrena*. The standard curves produced for each species show high correlation coefficient (R^2) values of 0.99, 0.98, and 0.98 for *P. foveata*, *Phoma* complex, and *P. eupyrena*, respectively, indicating linear responses in detection related to the increasing DNA concentration, which can be used to estimate DNA concentration of unknown samples. Figure 2c shows the standard curve obtained for *Phoma* complex amplification of *P. exigua* var. *exigua* DNA.

4 Notes

1. Gangrene symptoms produce a dry rot, but the presence of secondary bacterial activity may cause wet rots to develop.
2. A 4 mm-wide masonry nail with the sharp end removed provides an ideal tool with which to puncture the skin of tubers when assessing for latent infection. The nails are robust and can be flame sterilized easily.
3. A plastic pestle designed for use with a 1.5 ml microfuge tube is used to homogenize the tuber tissue prior to DNA extraction. The pestles are used once only for each sample and then are washed in soapy liquid, rinsed in tap water, and then left to soak in 0.2 M sodium hydroxide overnight. A thorough rinse in tap water and then autoclaving at 115 °C for 15 min is performed before they can be reused.
4. If using a Stratagene machine, ensure that the lamp is warmed up prior to loading the samples. Switching the lamp on prior to setting up the master mix solution is usually sufficient time.
5. Prepare a primer stock solution by diluting individual primers to 100 μ M and store in the dark (primers are light sensitive) at -20 °C. It is recommended to prepare a working dilution of primers from the 100 μ M stock solutions to minimize freeze-thawing cycles from stock solution. Prepare a working dilution of all three primer pairs together at 5 μ M. This is a 20-fold dilution, therefore 1 μ l each primer in 17 μ l of MOPS/EDTA buffer or 10 μ l each primer in 170 μ l MOPS/EDTA buffer. Diluted primers are stored in the dark at -20 °C.
6. Dilute primers and templates in MOPS/EDTA buffer (Promega), which is provided at pH 7.4. It is critical that this MOPS/EDTA buffer be used with the iso-dC-containing primers used in the Plexor® assay, as these primers are sensitive to pH below 7.0.

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Plant Pathology

Techniques and Protocols

Lacomme, C. (Ed.)

2015, XI, 352 p. 67 illus., 47 illus. in color., Hardcover

ISBN: 978-1-4939-2619-0

A product of Humana Press