DOI: 10.1111/epp.12935

EPPO STANDARD ON DIAGNOSTICS

PM 7/155 (1) *Pectobacterium* spp. and *Dickeya* spp.

Specific scope: This Standard describes a diagnostic protocol for *Pectobacterium* spp. and *Dickeya* spp.[1]
This Standard should be used in conjunction with PM 7/76 (5) *Use of EPPO diagnostic protocols*.
Specific approval and amendment: Approved in 2023–04. Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Pectobacterium and *Dickeya* are pectinolytic enterobacterial plant pests which were first characterized in the early 20th century (Jones, 1901). For decades they were classified as pectinolytic *Erwinia* species (Winslow et al., 1920). However, their taxonomical categorization has been reconsidered several times in recent years and this is still ongoing. Taxonomic information relevant for diagnosis is presented in Appendix 1.

Pectobacterium and *Dickeya* are broad host range pathogens on crops, vegetables and ornamentals and widespread in both temperate and tropical regions. Their global distribution is favoured by their latent presence in vegetative planting material such as tubers, bulbs, rhizomes and corms. Members of both genera may also be spread by water movement through the soil when infected tissues release the bacteria which are then transmitted to the roots of other plants which they can invade (Czajkowski et al., 2012; Kubheka et al., 2013).

For decades, *P. atrosepticum* has been the classic bacterial seed potato pest in cool temperate climates of Europe and Northern America, causing blackleg disease. The emergence of distinct new and more aggressive *Pectobacterium* and *Dickeya* species since 2010 (Czajkowski et al., 2015; Humphris et al., 2015; van der Wolf et al., 2017) forced seed potato growing countries (e.g. Belgium and the Netherlands) to reduce the tolerances set in the different classes of the certification schemes. Detection and identification are complicated by the similarity of disease symptoms and the fact that the primary bacterial species in seed potato cultivation changes over time and varies between different locations, largely determined by environmental conditions (Dees, Lebecka, et al., 2017; Dees, Lysøe, et al., 2017; Motyka-Pomagruk et al., 2021). The implementation of a certification scheme is the only effective measure to prevent the development of pests over a certain threshold. Although these pests are not recommended for regulation by EPPO as quarantine pests, *Pectobacterium* and *Dickeya* are designated as EU Regulated Non-Quarantine Pests with zero tolerance in potato microplants (for a definition of microplants, see EPPO, 2019a, 2019b) as determined by testing and tolerance levels are recommended in EPPO Standard PM 4/28 *Certification scheme for seed potatoes* (EPPO, 1999; under revision). Testing is also recommended in the framework of EPPO Standard PM 3/21 *Post-entry quarantine for potato* (EPPO, 2019a, 2019b) and in the framework of trade of potato plant material. A diagnostic protocol was considered useful for seed potatoes moving in trade and was developed to provide guidance for the diagnosis of *Pectobacterium* and *Dickeya* mainly on *Solanum tuberosum* (potato), but some information on other host plants and non-plant matrices is provided.

Pectobacterium and *Dickeya* can also be detected in open natural water systems used for irrigation, i.e. rivers and ponds (e.g. Ben Moussa et al., 2021; Hugouvieux-Cotte-Pattat et al., 2019; Laurila et al., 2008; Norman et al., 2003; Oulghazi, Pédron, et al., 2019; Parkinson et al., 2014; Pédron et al., 2019) and the use of contaminated water for irrigation may result in infection of potato crops (Cappaert et al., 1988). However, the potential for infecting potato and transmission through seed potatoes has not yet been determined for many *Pectobacterium* and *Dickeya* species isolated from water. Furthermore, several insects can carry the pests (Rossmann et al., 2018).

The flow diagram describing the diagnostic procedures is presented in Figure 1.

2 | IDENTITY

Preferred name: *Pectobacterium*
Taxonomic position: Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Pectobacteriaceae
EPPO Code: 1PECBG
Phytosanitary Categorization: RNQP (Annex IV)

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

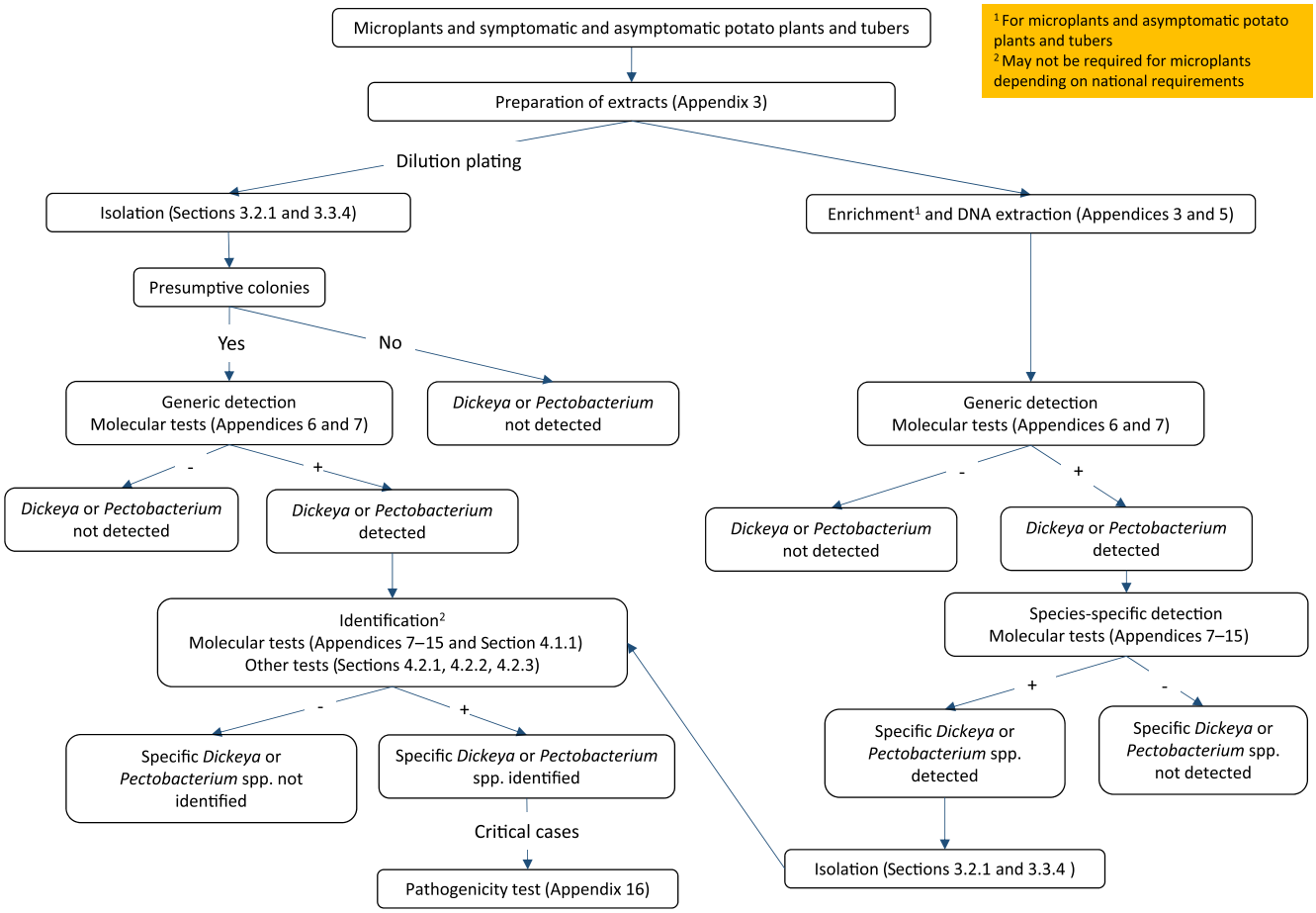

FIGURE 1 Flow diagram describing the diagnostic procedure for *Pectobacterium* and *Dickeya* spp on *Solanum tuberosum* (potato).

Preferred name: *Dickeya*
Taxonomic position: Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Pectobacteriaceae
EPPO Code: 1DICKG
Phytosanitary Categorization: RNQP (Annex IV)

More information on the taxonomy of *Pectobacterium* and *Dickeya* species is presented in Appendix 1.

3 | DETECTION

A large number of plant species across the world can be infected by *Pectobacterium* and *Dickeya* (Charkowski, 2018; Ma et al., 2007). The host range of *Pectobacterium* is considered to be the widest of all the necrotrophic bacteria although potato is probably the most important host (Pérombelon, 2002; Toth et al., 2003). *P. atrosepticum* appears to be quite potato specific and there are only a few strains isolated from other plant species, e.g. from *Apium graveolens* var. *dulce* (celery), *Solanum lycopersicum* (tomato) and *Helianthus annuus* (sunflower). Disease caused by *Dickeya* species has also been reported on a wide variety of dicotyledonous hosts, e.g. on potato, *Cichorium intybus* (chicory), *Daucus carota* subsp. *sativus* (carrot) and *Streptocarpus ionanthus* (African violet) and from monocotyledonous hosts, e.g. on *Oryza sativa* (rice), *Zea mays* (maize), *Ananas comosus* (pineapple) and *Musa* x *paradisiaca* (banana) (Ma et al., 2007; Samson et al., 2005). The most distinctive feature of *Pectobacterium* and *Dickeya* pathogenicity is the secretion of enzymes, such as pectinases and polygalacturonases, which degrade plant cell walls and cause characteristic necrosis and rotting symptoms (Charkowski, 2018).

3.1 | Symptoms

3.1.1 | Symptoms on *Solanum tuberosum* (potato)

The typical symptom of *Pectobacterium* and *Dickeya* on potato plants is a wet, blackish lesion on the stem with maceration of the pith, commonly named blackleg.

3.1.1.1 | *Symptoms on leaves*

An early symptom, due to colonization of vascular root tissues, is wilting of top leaves which develop a greasy dark green-grey appearance (Figure 2). This is commonly most visible at the warmest time of day. The

FIGURE 2 Early wilting of potato foliage diagnosed with *Dickeya dianthicola*. Courtesy of J. Van Vaerenbergh (ILVO, Belgium).

FIGURE 3 Permanent wilting and desiccation of potato foliage diagnosed with *Dickeya dianthicola*. Courtesy of J. Van Vaerenbergh (ILVO, Belgium).

wilting is reversible in the first stage of infection, but later plants fail to recover, and wilting is permanent followed by desiccation of foliage (Figure 3). Usually, it is not feasible to visually attribute blackleg symptoms to a specific *Pectobacterium* or *Dickeya* species because symptom expression is mostly linked to environmental conditions and potato cultivar. However, a typical symptom for *P. atrosepticum* is the yellowing of the foliage with necrotic patches in the leaves with small, stiff leaves at the top of affected stems and leaf margins curled inwards (Figure 4). When weather conditions are not favourable to development of the pests, appearance of typical symptoms in the foliage will be delayed and reduced and plants may not collapse but simply appear stunted and lacking vigour (Figure 5). Potato plants that show no obvious signs of infection may still produce infected tubers.

3.1.1.2 | *Symptoms on stems*

When the infection develops in the stem, oily lesions are visible at the base of the stem which blends into large dark patches as the infection progresses. This symptom is called 'blackleg'. The stem pith decays and darkens, and vascular tissue in and above the lesion may be discoloured. In wet weather, the decay tends to be slimy. In dry weather, stems may be hollow and desiccated, with maceration of the pith (Figures 6 and 7). Technically, the term 'blackleg' applies only when the symptom arises from infected seed potatoes. Otherwise, it is called 'aerial stem rot' and this can develop late in the growing season and under long lasting rainy conditions, when stems tend to lie on the ground. It starts from the top or the middle of the stem and progresses downwards (Figure 8). This aerial stem rot is usually caused by *Pectobacterium carotovorum* and can be confused with blackleg symptoms.

FIGURE 4 Stiff leaves with margins curled inwards and yellowing of potato foliage diagnosed with *Pectobacterium atrosepticum* Courtesy of J. Van Vaerenbergh (ILVO, Belgium).

FIGURE 5 Stunted growth of a potato plant diagnosed with *Dickeya dianthicola*. Courtesy of S. Bobev (University of Plovdiv, Bulgaria).

FIGURE 6 Blackleg lesion developing on the stem and degradation of pith tissue of a potato plant diagnosed with *Dickeya dianthicola*. Courtesy of J. Van Vaerenbergh (ILVO, Belgium).

3.1.1.3 | *Symptoms on tubers*

Symptoms on infected tubers may be visible from the outside of the tuber depending on the severity of infection and soil conditions. Cutting open the symptomatic

FIGURE 7 Vascular infection in potato stem diagnosed with *Pectobacterium atrosepticum* (left) and *Dickeya dianthicola* (right). Courtesy of J. Van Vaerenbergh (ILVO, Belgium).

tuber will reveal macerated tissue with a cheesy or jelly like consistency (Figure 9) which can show purple to black margins when exposed to air (Figure 10) and can develop an unpleasant odour. However, symptomatic tubers infected by *D. dianthicola* or *D. solani typically* produce little or no odour. Tuber soft rot can be initiated at lenticels (Figure 11), stolon end and/or in wounds under wet conditions and the rotting can spread to the whole tuber. When seed tubers start rotting in the field before emergence, blanking occurs in the field. In a wet potato season, progeny tubers can rot in the field (Figure 12).

FIGURE 8 Aerial stem rot in potato diagnosed with *Pectobacterium carotovorum*.

FIGURE 9 Liquefaction of a naturally infected mother tuber of potato diagnosed with *Dickeya solani* Courtesy of J. Van Vaerenbergh (ILVO, Belgium).

FIGURE 10 Rotting of the mother tuber of potato diagnosed with *Dickeya solani* with expression of indigoidine and tuber rot of potato diagnosed with *Pectobacterium atrosepticum* showing blackening at the margin of the affected tissue. Courtesy of J. Van Vaerenbergh (ILVO, Belgium).

3.1.2 | Symptoms on other plant species

Pectobacterium and *Dickeya* cause disease in plant species from 50% of angiosperm orders (Ma et al., 2007) and the known host ranges of these pests partially overlap. In many cases there is just one report of a single host plant species per family and order. There are some host range limitations associated with subgroups of both genera and *Pectobacterium* species, unlike *Dickeya* species, have not yet been reported to cause disease in cereals. *Pectobacterium carotovorum* soft rot of vegetables, e.g. *Daucus carota* subsp. *sativus* (carrot), *Brassica oleracea* (cabbage), *Lactuca sativa* (lettuce) and *Allium cepa* (onion), is common across climatic zones including those found in Europe (Cariddi & Sanzani, 2013; Gardan et al., 2003; Pérombelon, 2002; Waleron et al., 2014, 2015; Zaczek-Moczydłowska et al., 2019).

Both *Pectobacterium* and *Dickeya* may be found in flower bulb and rhizome cultivation, such as in production of *Hyacinthus* spp. (hyacinth), *Iris* spp. (iris), *Freesia* spp. (freesia), *Muscari* spp. (muscari), *Dahlia* spp. (dahlia) and *Zantedeschia* spp. (zantedeschia) (Van Doorn et al., 2011).

FIGURE 11 Lenticel rot on tubers of potato diagnosed with *Pectobacterium carotovorum*. Courtesy of V. Hélias and Y. Le Hingrat (INOV3PT, France) and J. Van Vaerenbergh (ILVO, Belgium).

FIGURE 12 Stem and tuber rot of potato diagnosed with *Pectobacterium parmentieri*. Courtesy of S. Bobev (University of Plovdiv, Bulgaria).

Symptoms on different plant species are shown in Figures 13–19. Generally, symptoms of bacterial soft rot begin as small, water-soaked, translucent lesions on the epidermis of the plant tissue. They grow in size over time and develop as soft and pulpy discoloured depressions, commonly seeping liquid containing bacteria. Entire fleshy fruits, roots, tubers, stems and rhizomes, bulbs, corms, buds, leafstalks, and leaves may rot and collapse, sometimes leaving only the epidermis. The decomposing tissues frequently give off a characteristically putrid odour.

A typical indication of decaying underground organs is the weak and chlorotic foliage with upward turned leaves and lesions on the stem (blight). Finally, the stem also rots and becomes soft and pulpy.

On occasion particular symptoms are seen, e.g. a dark brown decay of rice tillers caused by *Dickeya zeae*, bleeding canker of pear trees caused by *Dickeya fangzhongdai*, or core rot of the onion bulb caused by *Pectobacterium carotovorum*.

3.2 | Detection in symptomatic plant material

The test procedures described in this protocol have not been standardized nor validated through inter-laboratory comparisons.

3.2.1 | Isolation from symptomatic host plants

Generally, non-selective nutrient media such as Nutrient Agar (NA), Yeast Peptone Glucose Agar (YPGA),

FIGURE 13 Break down of stem and leaves on *Zea mays* diagnosed with *Dickeya zeae*. Courtesy of J. Van Vaerenbergh (ILVO, Belgium).

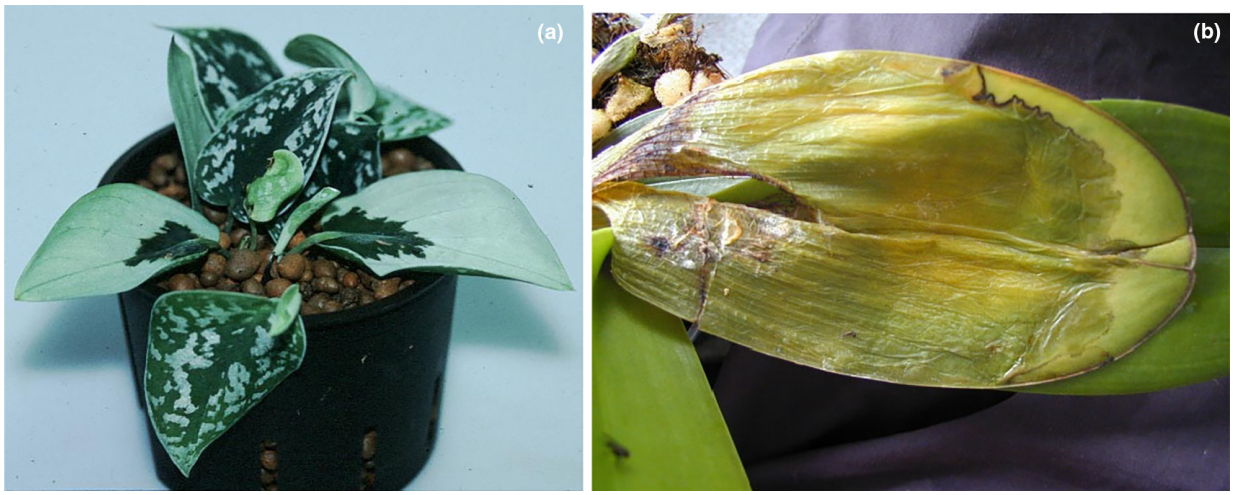

FIGURE 14 (a) Water-soaked leaf lesions on *Scindapsus pictus* diagnosed with *Dickeya dadantii*. (b) Breakdown of the leaf of *Phalaenopsis* diagnosed with *Dickeya dadantii*. Courtesy of J. Van Vaerenbergh (ILVO, Belgium).

FIGURE 15 Stem and leaf necrosis on *Chrysanthemum* cuttings diagnosed with *Dickeya chrysanthemi*. Courtesy of J. Van Vaerenbergh (ILVO, Belgium).

King's B medium, Nutrient Agar plus Glycerol (NGM), Liquid Enrichment (LEM366), Casamino and Peptone-Glucose (CPG) and Potato Dextrose Agar (PDA) (see Appendix 4) can be used for isolation from symptomatic plant material. However, since secondary infections and saprophytic bacteria are often present when rotting symptoms are more advanced, dilution plating is required. Dilution should be assessed based on the turbidity of the extract, however, given the numbers of bacterial cells in an extract from symptomatic material, dilutions such as 1/1000 and 1/10 000 are suitable for isolation using an appropriate plating technique. Separate plates can be prepared with diluted cell suspensions of reference strains as positive controls, as provided in Section 5. Semi-selective media such as Crystal Violet Pectate (CVP) are widely used for isolation of pectinolytic enterobacteria from plants, tubers

FIGURE 16 (a) Soft rot of cabbage leaves diagnosed with *Pectobacterium carotovorum*. (b) Soft rot of iceberg lettuce diagnosed with *Pectobacterium carotovorum*. (c) Soft rot of *Citrullus lanatus* (watermelon) diagnosed with *Pectobacterium carotovorum*. Courtesy of S. Bobev (University of Plovdiv, Bulgaria).

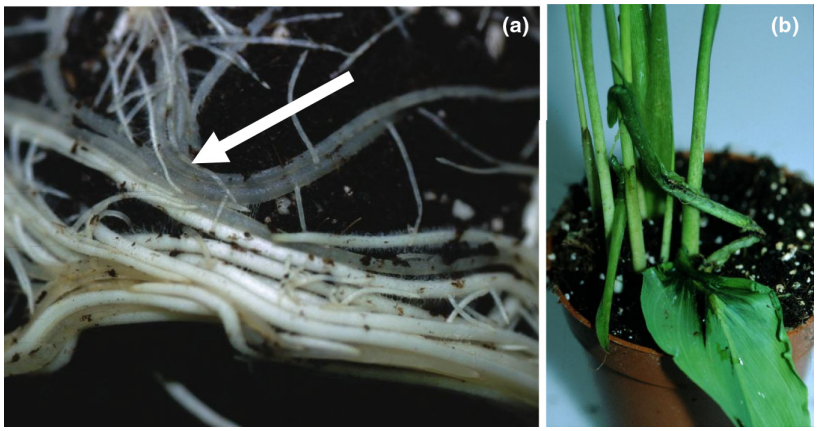

FIGURE 17 (a) Translucent roots on *Zantedeschia aethiopica* (calla lily) diagnosed with *Pectobacterium zantedeschiae*. (b) Tissue collapse on *Zantedeschia aethiopica* (calla lily) diagnosed with *Pectobacterium zantedeschiae*. Courtesy of J. Van Vaerenbergh (ILVO, Belgium).

FIGURE 18 Rotting of basal plate and bulb scales of hyacinth diagnosed with *Dickeya solani*. Courtesy of J. Van Vaerenbergh (ILVO, Belgium).

FIGURE 19 Rotting of *Alium cepa* bulb diagnosed with *Pectobacterium carotovorum*. Courtesy of S. Bobev (University of Plovdiv, Bulgaria).

and environmental samples (Cuppels & Kelman, 1974; Hélias et al., 2012; Hyman et al., 2001; Pérombelon & Burnett, 1991). Pectinolytic bacteria form characteristic cavities in the medium (Figure 20), due to their ability to enzymatically metabolize pectin. The plates can be incubated for two to six days at 20–22°C and 25–28°C to allow growth of different species. *P. atrosepticum* and *P. parmentieri* grow slowly at 28°C on CVP.

NGM, a non-selective medium (differential medium) containing glycerol and manganese chloride, and on which a bluish diffusible pigment indigoidine is produced (Lee & Yu, 2006) can be used for isolation of *Dickeya* from symptomatic potato plants and allow the differentiation

of *D. solani* and *D. dianthicola* from *Pectobacterium* spp. (Van Vaerenbergh et al., 2012). However, not all strains of *Dickeya* spp. produce the blue pigment on this medium (Charkowski, 2015). Some strains can also produce a brownish pigment (e.g. on King's B medium), or no pigment at all (Figures 21 and 22).

3.2.2 | Molecular tests

3.2.2.1 | *Generic detection*

Several molecular tests have been developed for the detection of *Pectobacterium* and *Dickeya* at genus level. Those routinely used in the EPPO region are described in the Appendices.

- Conventional PCR for the detection of *Pectobacterium* at genus level from Darrasse et al. (1994) described in Appendix 6

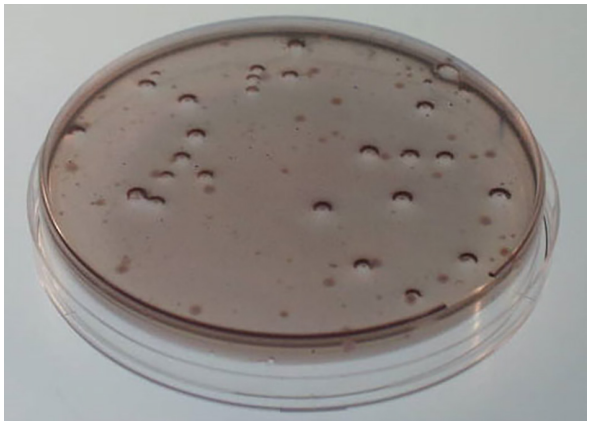

FIGURE 20 Pectolytic isolation on double layer crystal violet pectate (DLCVP) medium: *Pectobacterium* and *Dickeya* form characteristic cavities in the medium. Courtesy of V. Hélias (FN3PT, France).

- Conventional PCR for the detection of *Dickeya* at genus level from Nassar et al. (1996) described in Appendix 7

are recommended for testing microplants of *Solanum tuberosum* when identification of the bacterium at species level is not needed. However, it should be noted that presence of symptoms on microplants is highly unlikely.

Other molecular tests for the generic detection of *Dickeya* and *Pectobacterium* spp. are presented in Appendix 2.

3.2.2.2 | *Species-specific detection*

Several molecular tests have been developed for the species-specific detection of *Pectobacterium* and *Dickeya*. Those routinely used in the EPPO region are described in Appendices.

- Conventional PCR for *Dickeya* spp. from Nassar et al. (1996) described in Appendix 7
- Real-time PCR for *Pectobacterium brasiliense* from van der Wolf et al. (2017) described in Appendix 8
- Real-time PCR for *Pectobacterium brasiliense* from Muzhinji et al. (2020) described in Appendix 9
- Real-time PCR for *Pectobacterium parmentieri* from van der Wolf et al. (2017) described in Appendix 10
- Real-time PCR for *Pectobacterium atrosepticum* from Brierley et al. (2008); Humphris et al. (2015) described in Appendix 11
- Real-time PCR for *Dickeya solani* from Van Vaerenbergh et al. (2012) described in Appendix 12
- Real-time PCR for *Dickeya solani* from Pritchard et al. (2013) described in Appendix 13
- Real-time PCR for *Dickeya dianthicola* from Pritchard et al. (2013) described in Appendix 14
- Conventional PCR for *Pectobacterium brasiliense* from Duarte et al. (2004) described in Appendix 15

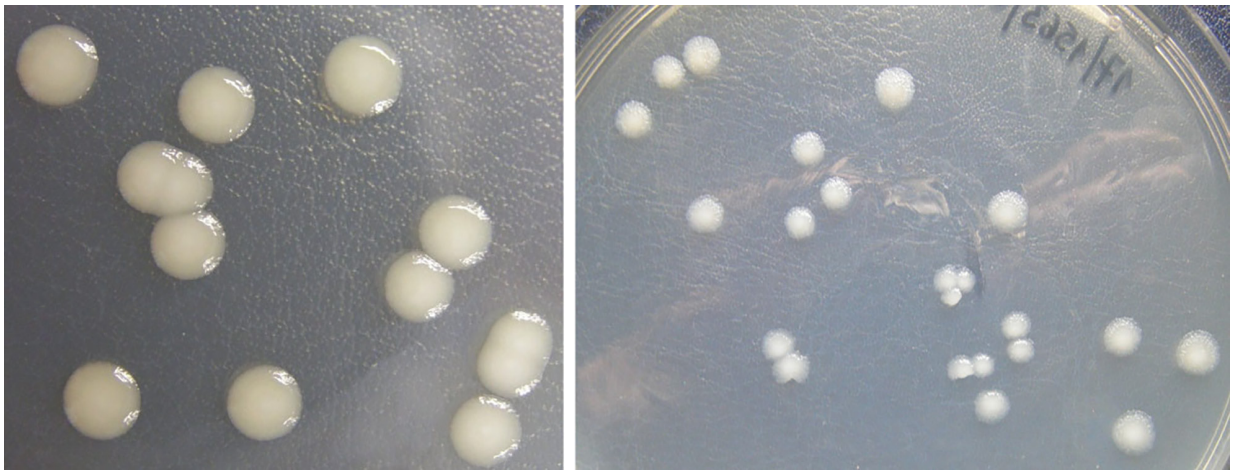

FIGURE 21 Colonies of *Pectobacterium brasiliense* (left) and *Pectobacterium parmentieri* (right) on King's B medium after incubation at 28°C for 72 h. Courtesy of J. Van Vaerenbergh (ILVO, Belgium).

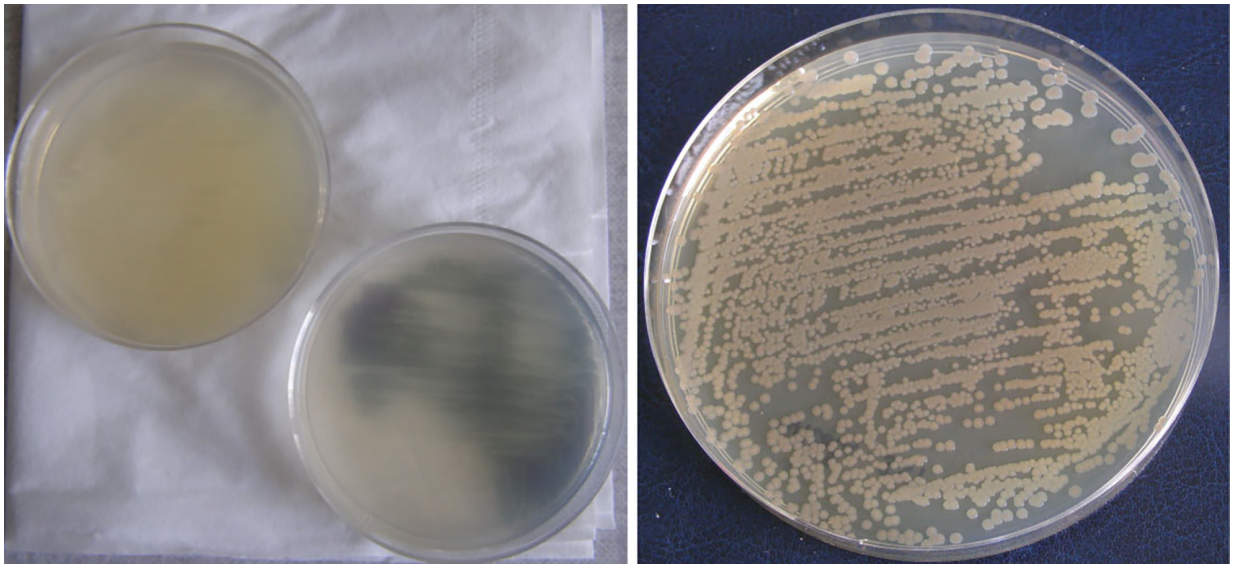

FIGURE 22 Dark blue diffusible pigment produced by *Dickeya dianthicola* on NGM medium (left) and brownish colonies of *Dickeya solani* on King's B medium (right). Courtesy of J. Van Vaerenbergh (ILVO, Belgium).

Other molecular tests for the species-specific detection of *Dickeya* and *Pectobacterium* are presented in Appendix 2.

3.3 | Detection in asymptomatic plant material

The tests described in this protocol have not been standardized nor validated through inter-laboratory comparisons. Detection in asymptomatic plant material relies essentially on molecular tests.

3.3.1 | Microplants sample preparation

Samples can be prepared following the procedures presented in Appendix 3.

3.3.2 | Seed potato tubers sample preparation

Samples can be prepared with an enrichment step, following different protocols.

Enrichment of the tuber extract is performed in pectin-based medium. Procedures are presented in Appendix 3.

3.3.3 | Sample preparation for other plants

Dickeya and *Pectobacterium* can be detected in other asymptomatic plants. For ornamental bulbs, vascular tissue from the basal plate is homogenized and is tested after enrichment. It is recommended to spike a duplicate homogenate with reference strains to test for inhibition of multiplication in the enrichment step by components in the homogenate.

3.3.4 | Isolation

Semi-selective media based on pectate, such as CVP are widely used to recover soft rot bacteria from various asymptomatic tissues. Their selectivity is based on the inclusion of crystal violet, which inhibits the growth of Gram-positive bacteria, and on the presence of polypectate as the single carbon source. The source of pectate is essential because pectinolytic enterobacteria are not able to degrade all of them (Cuppels & Kelman, 1974; Hélias et al., 2012; Hyman et al., 2001; Pérombelon & Burnett, 1991). The degradation of pectate is visualized by characteristic cavities in the medium. CVP can be used in single- and double-layer forms (Pérombelon & Burnett, 1991). Plates are incubated at 20–22°C and 25–28°C to allow growth of different species. *P. atrosepticum* and *P. parmentieri* grow slowly at 28°C on CVP.

Media are described in Appendix 4.

3.3.5 | Molecular tests

3.3.5.1 | *Generic detection*

Several molecular tests have been developed for the detection of *Pectobacterium* and *Dickeya* at genus level. Those routinely used in the EPPO region are described in Appendices.

- Conventional PCR for the detection of *Pectobacterium* at genus level from Darrasse et al. (1994) described in Appendix 6
- Conventional PCR for the detection of *Dickeya* at genus level from Nassar et al. (1996) described in Appendix 7

are recommended for testing microplants of *Solanum tuberosum* when identification of the pest at species level is not needed.

Other molecular tests for the generic detection of *Dickeya* and *Pectobacterium* are presented in Appendix 2.

3.3.5.2 | *Species-specific detection*

Several molecular tests have been developed for the species-specific detection of *Pectobacterium* and *Dickeya*. Those routinely used in the EPPO region are described in Appendices.

- Conventional PCR for *Dickeya* spp. from Nassar et al. (1996) described in Appendix 7
- Real-time PCR for *Pectobacterium brasiliense* from van der Wolf et al. (2017) described in Appendix 8
- Real-time PCR for *Pectobacterium brasiliense* from Muzhinji et al. (2020) described in Appendix 9
- Real-time PCR for *Pectobacterium parmentieri* from van der Wolf et al. (2017) described in Appendix 10
- Real-time PCR for *Pectobacterium atrosepticum* from Brierley et al. (2008); Humphris et al. (2015) described in Appendix 11
- Real-time PCR for *Dickeya solani* from Van Vaerenbergh et al. (2012) described in Appendix 12
- Real-time PCR for *Dickeya solani* from Pritchard et al. (2013) described in Appendix 13
- Real-time PCR for *Dickeya dianthicola* from Pritchard et al. (2013) described in Appendix 14
- Conventional PCR for *Pectobacterium brasiliense* from Duarte et al. (2004) described in Appendix 15

Other molecular tests for the species-specific detection of *Dickeya* and *Pectobacterium* are presented in Appendix 2.

3.4 | Detection in non-plant matrices

3.4.1 | Surface or recirculation water

Surface water should be ideally sampled when water temperatures are at or above 15°C. At selected sampling points, surface water can be collected by filling sterile tubes or bottles, ideally at a depth below 30 cm and in the vicinity of any known host plants. For industrial or sewage effluents, samples should be collected from the point of effluent discharge.

It is advisable to take duplicated samples of at least 50 mL, taken at least at 3 different moments in time per sampling point. Samples should be transported in cool (range of 10–15°C) and dark conditions and tested preferably within 24 h. To improve the likelihood of detecting *Pectobacterium* or *Dickeya* spp. in water, it is recommended to first concentrate bacterial populations using one of the following methods:

(i) centrifugation of 30–50 mL sub-samples at 10 000 *g* for 10 min (or 7000 *g* for 15 min) preferably at 4–10°C, discarding the supernatant and resuspending the pellet in 1 mL sterile water.
(ii) membrane filtration (1 L through a maximum pore size of 0.22 µm) followed by washing the filter in 5–10 mL 10 mM phosphate buffer (PB, pH 7.2) and retention of the washings. This method is suitable for larger volumes of water containing low numbers of saprophytes. If the samples are turbid, this may block the filtration process and saturate the filter. A 1/10 dilution of the sample in sterile water is then highly recommended.

Dilution plating of the concentrated samples can then be performed.

Concentration is usually not advisable for samples of sewage/industrial effluents since increased populations of competing saprophytic bacteria will inhibit detection of *Pectobacterium* and *Dickeya* spp.

Generic detection can be performed by dilution plating and conventional PCR tests from Darrasse et al. (1994) and Nassar et al. (1996). For plating, 50–100 µL of sample and each dilution are spread on CVP medium and incubated for 48–72 h at 20–22°C and 25–28°C to allow growth of different species. The plates should then be observed for the formation of cavities. The cavity producing colony can be cultured on a common nutrient medium.

For the generic detection using conventional PCR, the pellet or the filtration membrane is suspended in 10 mM PB, pH 7.2 and the sample is tested with or without prior enrichment in PolyGalacturonate Enrichment Medium (PGEM).

3.4.2 | Soil

Testing soil is not recommended as it is considered to give erratic results due to the highly dispersed and low population of bacteria. Analysis of known weed hosts (e.g. *Cyperus rotundus*) and volunteers growing in the soil is considered to be more reliable (Fikowicz-Krosko et al., 2017).

4 | IDENTIFICATION

4.1 | Molecular tests

Several molecular tests have been developed for the identification of *Pectobacterium* and *Dickeya* spp. Those routinely used in the EPPO region are described in Appendices. The list of molecular tests that can be used for the identification of *Pectobacterium* and *Dickeya* spp. is provided in Sections 3.2.2.2 and 3.3.5.2 *Species-specific detection*.

Identification is recommended for symptomatic and asymptomatic potato plants and tubers. Identification in microplants may not be required depending on national requirements.

4.1.1 | DNA barcoding

Sequenced PCR amplicons from selected gene loci allow accurate differentiation of *Pectobacterium* and *Dickeya* strains to species level. Multilocus sequence typing (MLST) has also been applied to differentiate isolates of *Pectobacterium* and *Dickeya* species and it is recognized as a useful technique for this purpose (De Boer, 2012; Pitman et al., 2010; Waleron et al., 2013). Soft rot Enterobacteriaceae can be classified with sequences of various housekeeping genes such as *dnaX* (Ma et al., 2018; Portier et al., 2019; Sławiak et al., 2009), *gapA* (Cigna et al., 2017; Moleleki et al., 2013), *gyr*B (Moretti et al., 2016; Ngadze et al., 2012); *leu*S and *rec*A (Portier et al., 2019) but also on genes involved in the pectin degradation process (an extensive database is maintained by ILVO, Belgium, on the *pel*Y gene for *Pectobacterium* spp. and on the *pel*I gene for *Dickeya* spp.). Multilocus sequence analysis (MLSA), based on partial sequences of *gyr*B, *rpo*B, *inf*B and *atp*D genes allows separation of close phylogenetic groups within *Brenneria*, *Pectobacterium*, *Dickeya*, *Erwinia*, *Pantoea* and *Samsonia* species (Brady et al., 2012), as confirmed by Adeolu et al. (2016) when revising taxonomy of the 'Enterobacteriales' order.

Guidance for sequence analysis is given in Appendices 7 and 8 of EPPO Standard PM 7/129 *DNA Barcoding as an identification tool for a number of regulated plant pests* (EPPO, 2021).

4.2 | Other tests

4.2.1 | Genomic fingerprinting tests

REP-PCR-based methods can be used for the classification and characterization of *Pectobacterium* and *Dickeya* strains (Tsror et al., 2009; EPPO, 2010; Ngadze et al., 2012; Degefu et al., 2013).

4.2.2 | Proteomic analysis based on Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS)

MALDI-TOF for proteomic analysis allows rapid, reliable and robust identification of *Pectobacterium* and *Dickeya* spp.

4.2.3 | Fatty acid analysis

Although fatty acid methyl ester (FAME) analyses allow distinction of *Dickeya* spp. from *Pectobacterium* spp., the test is not suitable to differentiate *Dickeya* to species and subspecies (van der Wolf et al., 2014).

4.3 | Pathogenicity test

A pathogenicity test is used for critical cases. The procedure for the pathogenicity test is described in Appendix 16.

5 | REFERENCE MATERIAL

The following collections can provide *Pectobacterium* and *Dickeya* reference strains:

1. NCPPB=National Collection of Plant Pathogenic Bacteria, Fera, Sand Hutton, York, YO411LZ, UK; https://www.fera.co.uk/ncppb
2. Centre International de Ressources Microbiennes-Collection Française de Bactéries associées aux Plantes, Angers, France (CIRM-CFBP); 42 Rue Georges Morel, CS60057, 49071 Beaucouzé Cedex, France; https://cirm-cfbp.fr/page/Home
3. LMG=Belgian Co-ordinated Collections of Micro-organisms (BCCM)/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, K.L. Ledeganckstraat 35, B-9 000 Gent, Belgium; https://bccm.belspo.be/about-us/bccm-lmg
4. DSMZ=Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7B, 38124 Braunschweig, Germany; https://www.dsmz.de/collection

An extensive list of type strains is provided in Table 1.

6 | REPORTING AND DOCUMENTATION

Guidance on reporting and documentation is given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7 | PERFORMANCE CHARACTERISTICS

When performance characteristics are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc).

8 | FURTHER INFORMATION

Further information on these organisms can be obtained from:

TABLE 1 List of type strains from *Dickeya* and *Pectobacterium* taxa. Proposed but not yet approved species and subspecies are shown in quotation marks.

Taxon	Type strain reference in different collections	Isolation year	Biological and geographical origin	References for species description
Dickeya aquatica	CFBP 8348, NCPPB 4580, LMG 27354	2008	River water in the United Kingdom	Parkinson et al. (2014)
Dickeya chrysanthemi pv. *chrysanthemi*	CFBP 2048, DSM 4610, NCPPB 402, LMG 2804	1956?	*Chrysanthemum morifolium* in the USA	Burkholder et al. (1953), Samson et al. (2005)
Dickeya chrysanthemi pv. *parthenii*	CFBP 1270, LMG 2486, NCPPB 516	1957	*Partenium argentatum* in Denmark	Starr (1947) comb. nov.
Dickeya dadantii subsp. *dadantii*	CFBP 1269, DSM 18020, LMG 25991, NCPPB 898	1960	*Pelargonium capitatum* on the Comoro Islands	Brady et al. (2012), Samson et al. (2005)
Dickeya dadantii subsp. *dieffenbachiae*	CFBP 2051, LMG 25992, NCPPB 2976	Unknown	*Dieffenbachia* spp. in the USA	Samson et al. (2005), Brady et al. (2012), comb. nov.
Dickeya dianthicola	CFBP 1200, LMG 2485, NCPPB 453	1956	*Dianthus caryophyllus* in the UK	Samson et al. (2005)
'Dickeya fangzhongdai'	CFBP 8607, DSM 101947	2009	*Pyrus pyrifolia* in the People's Republic of China	Tian et al. (2016)
Dickeya lacustris	CFBP 8647, LMG 30899	2017	Lake water in France	Hugouvieux-Cotte-Pattat et al. (2019)
Dickeya oryzae	ZYY5, JCM 33020, ACCC 61554	2019	Roots of rice in the People's Republic of China	Wang et al. (2020)
Dickeya poaceiphila	CFBP 8731, NCPPB 569	<1958	*Saccharum officinarum* in Australia	Hugouvieux-Cotte-Pattat et al. (2021)
Dickeya solani	CFBP 7345, CFBP 8199, LMG 25993, NCPPB 4479	2007	*Solanum tuberosum* in the Netherlands	van der Wolf et al. (2014), sp. nov
Dickeya undicola	CFBP 8650, LMG 30903	2014	Lake water in Malaysia	Oulghazi, Cigna, et al. (2019), Oulghazi, Pédron, et al. (2019)
Dickeya zeae	CFBP 2052, LMG 2505, NCPPB 2538	1960	*Zea mays* in the USA	Samson et al. (2005), sp. nov.
Dickeya parazeae	CFBP 8716T, LMG 32070T	2021	Water in France	Hugouvieux-Cotte-Pattat et al. (2021) sp. nov.
Pectobacterium aquaticum	CFBP 8637, NCPPB 4640	2016	River water in France	Pédron et al. (2019)
Pectobacterium actinidiae	LMG26003	2008	*Actinidia chinensis* cv. Hort16A in the Republic of Korea	Koh et al. (2012)
Pectobacterium aroidearum	CFBP 8168, LMG 2417, NCPPB 929	1959	*Zantedeschia aethiopica* in South Africa	Nabhan et al. (2013)
Pectobacterium atrosepticum	CFBP 1526, DSM 18077, LMG 2386, NCPPB 549	1957	*Solanum tuberosum* in the UK	Van Hall (1902), Gardan et al. (2003)
Pectobacterium betavasculorum	CFBP 1539, CFBP 2122, LMG 2466, NCPPB 2795	1972	*Beta vulgaris* in the USA	Gardan et al. (2003)
Pectobacterium brasiliense	CFBP 6617, LMG 21371, NCPPB 4609	1999	*Solanum tuberosum* in Brazil	Duarte et al. (2004), Portier et al. (2019)
Pectobacterium cacticida	CFBP 3628, DSM 21821, LMG 17936, NCPPB 3849	1944	*Carnegiea gigantean* in the USA	Alcorn et al. (1991), Hauben et al. (1998)
Pectobacterium carotovorum	CFBP 2046, DSM 30168, LMG 2404, NCPPB 312	1952	*Solanum tuberosum* in Denmark	Jones (1901), Portier et al. (2019)
Pectobacterium fontis	CFBP 8629, LMG 30744	2013	Waterfall in Malaysia	Oulghazi, Cigna, et al. (2019), Oulghazi, Pédron, et al. (2019)
Pectobacterium odoriferum	CFBP 1878, LMG 5863, NCPPB 3839	1978	*Cichorium intybus* in France	Gallois et al. (1992), Gardan et al. (2003), Portier et al. (2019)
Pectobacterium parmentieri	CFBP 8475, LMG 29774, NCPPB 4649	2008	*Solanum tuberosum* in France	Khayi et al. (2016)

(Continues)

TABLE 1 (Continued)

Taxon	Type strain reference in different collections	Isolation year	Biological and geographical origin	References for species description
Pectobacterium parvum	CFBP 8630, LMG 30828	2004	*Solanum tuberosum* in Finland	Pasanen et al. (2020)
'Pectobacterium peruviense'	CFBP 5834, LMG 30269	1977–1979	*Solanum tuberosum*	Waleron et al., 2018
Pectobacterium polaris	CFBP 8603, DSM 105255, NCPPB 4611	2010	*Solanum tuberosum*	Dees, Lebecka, et al. (2017), Dees, Lysøe, et al. (2017)
Pectobacterium polonicum	LMG31077	2016	Ground water from potato field	Waleron, Misztak, Waleron, Jonca, et al. (2019)
Pectobacterium punjabense	CFBP 8604	2017	*Solanum tuberosum*	Sarfraz et al. (2018)
Pectobacterium quasiaquaticum	CFBP 8805=LMG 32181	2021	From river water in France	Ben Moussa et al., 2021
Pectobacterium versatile	CFBP 6051, NCPPB 3387		*Solanum tuberosum*	Portier et al., 2019
Pectobacterium wasabiae	CFBP 3304, DSM 18074, LMG 8404, NCPPB 3701	1985	*Wasabia japonica*	Gardan et al., 2003
'Pectobacterium zantedeschiae'	DSM 105717	2005	*Zantedeschia aethiopica*	Waleron, Misztak, Waleron, Franczuk, et al. (2019)

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9 | FEEDBACK ON THIS DIAGNOSTIC PROTOCOL

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int

10 | PROTOCOL REVISION

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website. When *errata* and *corrigenda* are in press this will also be marked on the website.

ACKNOWLEDGEMENTS

This protocol was drafted by: J. Van Vaerenbergh, Flanders' Institute for Agricultural, Fisheries and Food Research (ILVO), Merelbeke, Belgium, A. Aspin, Fera Science Ltd, York, United Kingdom, G. Cahill, SASA, Edinburgh, United Kingdom. R. Gottsberger, Austrian Agency for Health and Food Safety GmbH (AGES), Vienna, Austria. V. Hélias and J. Cigna, French Federation of Seed Potato Growers (FN3PT), France, M. Roselló Pérez, Plant Pathology Diagnostic Laboratory (GVA), Valencia, Spain. E. Stefani, University of Modena and Reggio Emilia (UNIMORE), Reggio Emilia, Italy. R. Vreeburg, Dutch Food and Consumer Product Safety Authority (NVWA), the Netherlands.

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How to cite this article: EPPO (2023) PM 7/155 (1) *Pectobacterium* spp. and *Dickeya* spp. *EPPO Bulletin*, 53, 309–353. Available from: https://doi.org/10.1111/epp.12935

APPENDIX 1 - TAXONOMIC INFORMATION ON *DICKEYA* AND *PECTOBACTERIUM* SPP.

1. *Pectobacterium* spp.

In 1998, the genus *Erwinia* was divided into three phylogenetic groups (Hauben et al., 1998) and the pectinolytic *Erwinia* were moved out of the genus into a new genus *Pectobacterium* as proposed for the first time by Waldee in 1945. Four main species were delineated, i.e. *Pectobacterium carotovorum* including the subspecies *atrosepticum*, *betavasculorum*, *carotovorum*, *odoriferum* and *wasabiae*, *Pectobacterium chrysanthemi*, *Pectobacterium cacticida* and *Pectobacterium cypripedii*.

In the 2000's, Samson et al. (2005) transferred *Pectobacterium chrysanthemi* to a novel genus named *Dickeya* (see the Dickeya dedicated section below) while three subspecies of *P. carotovorum* were raised to species level, i.e. *P. atrosepticum*, *P. betavasculorum* and *P. wasabiae* (Gardan et al., 2003). The *P. wasabiae* strains isolated from potato were later transferred to the novel species *P. parmentieri* (Khayi et al., 2016). Additionally, two new subspecies were proposed in *P. carotovorum*, *P. carotovorum* subsp.

brasiliensis (Duarte et al., 2004) and *P. carotovorum* subsp. *actinidiae* with strains causing canker-like symptoms in yellow kiwi trees (*Actinidia chinensis*) (Koh et al., 2012). Availability of whole-genome sequences has confirmed the high genetic diversity within the genus *Pectobacterium* (Zhang et al., 2016) and has also contributed to the definition of novel *Pectobacterium* species in the past few years:

- *P. aroidearum*, strains mainly infecting monocotyledonous plants (Nabhan et al., 2013).
- *P. polaris*, isolated from soft rot of potato tubers in Norway (Dees, Lysøe, et al., 2017) and Poland (Waleron et al., 2018), the Netherlands (Zhang et al., 2016) and from *Brassica napus* (rape) (Zhang et al., 2016).
- *P. peruviense*, isolated from potato at high altitudes (Waleron et al., 2018).
- *P. punjabense*, first isolated from blackleg symptoms of potato plants in Pakistan (Sarfraz et al., 2018) and later also obtained from potato field in Europe (Cigna et al., 2021; Loc et al., 2021) and in China (Handique et al., 2022).
- *P. polonicum*, isolated from ground water on a vegetable field in Poland (Waleron, Misztak, Waleron, Jonca, et al., 2019).
- *P. zantedeschiae*, isolated from Calla lily bulbs cultivated in Poland and from leaves of Calla lily grown in Serbia (Waleron, Misztak, Waleron, Franczuk, et al., 2019).
- *P. fontis*, isolated from a waterfall source in Malaysia (Oulghazi, Pédron, et al., 2019).
- *P. aquaticum*, isolated from waterways in France (Pédron et al., 2019).
- *P. versatile* (Portier et al., 2019), initially proposed as genomospecies '*Candidatus* P. maceratum' isolated from macerated tissue of *Brassica oleracea* (cabbage) and potato tubers in Russia (Shirshikov et al., 2018). This species includes strains isolated from a wide range of plants and various waterways and has a worldwide distribution (Portier et al., 2019).
- *P. parvum* (Pasanen et al., 2020), strains closely related to *P. polaris* but with low maceration ability on potato and slowly growing on culture medium.
- *P. quasiaquaticum* (Ben Moussa et al., 2021), isolated from waterways and lake water in France.

The revision of the taxonomic status of the *P. carotovorum* subspecies resulted in elevating *P. carotovorum* subsp. *odoriferum* to species level and to propose *P. brasiliense* and *P. actinidiae* as new species (Portier et al., 2019). As of October 2019, the genus *Pectobacterium* is divided into 19 recognized or proposed species and subspecies. It is expected that in the next few years novel *Pectobacterium* species will be proposed/discovered resulting in re-arrangements in the genus classification.

Of all *Pectobacterium* species, *P. carotovorum* has the widest known host range, whereas *P. atrosepticum* is reported almost exclusively on potato and *P. betavasculorum* almost exclusively on *Beta vulgaris* (beet) (Pérombelon, 2002). Furthermore, pectinolytic enterobacteria principally present in tropical and subtropical regions have been diagnosed in Europe, e.g. *P. parmentieri* (as *P. wasabiae*) (Dees, Lebecka, et al., 2017; Nabhan et al., 2012a, 2012b; Waleron et al., 2013) and *P. brasiliense* (de Werra et al., 2015; Nunes Leite et al., 2014; Waleron et al., 2015).

P. wasabiae was originally identified as a pest of *Wasabia japonica* (Japanese horseradish, i.e. wasabi) (Goto & Matsumoto, 1987). However, it also received attention as a potato pest in several countries around the world and ultimately was recognized as a novel species, *P. parmentieri*. It is suspected that the species has long been present in potato crops (e.g. in Northern Europe) but only recently have sequence-based methods enabled its differentiation from *P. carotovorum*.

P. brasiliense was originally isolated from potato plants in Brazil (Duarte et al., 2004). It is now also known to cause soft rot in *Capsicum* spp., perennial plants, wild carrots and *Cucurbita pepo* (pumpkin) (Ma et al., 2007; Nabhan et al., 2012b; Gottsberger & Huss, 2016). It has subsequently been detected in potato cropping systems on all continents (De Boer, 2012; del Pilar Marquez-Villavicencio et al., 2011; Leite et al., 2014; Nabhan et al., 2012a; Ngadze et al., 2012; van der Merwe et al., 2010).

2. *Dickeya* spp.

Erwinia chrysanthemi was assigned to the genus *Erwinia* as a pest of *Chrysanthemum* × *morifolium* (florists' chrysanthemum) (Burkholder et al., 1953). However, as the wide host range of the pest became recognized, the need for subdivision became necessary. Six pathovars were designated based on host specificity, i.e. pvs. *chrysanthemi*, *dianthicola*, *dieffenbachiae*, *paradisiaca*, *parthenii* and *zeae* (Lelliott & Dickey, 1984). A biovar test format with stable physiological and biochemical features was developed for identification (Samson et al., 1987; Samson Ngwira & Rivera, 1990). After relocation of *Erwinia chrysanthemi* into the genus *Pectobacterium* (Hauben et al., 1998), further taxonomic analysis separated *P. chrysanthemi* from *Pectobacterium* and it was finally transferred to the new genus *Dickeya* (Samson et al., 2005). Initially six species were classified in *Dickeya*, corresponding more or less to both the pathovar and biovar classification, i.e. *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. dieffenbachiae*, *D. paradisiaca*, and *D. zeae* (Samson et al., 2005). Later on, *D. dieffenbachiae* was reclassified as *D. dadantii* subsp. *dieffenbachiae* (Brady et al., 2012). More recently, *Dickeya paradisiaca* was reclassified as *Musicola paradisiaca*, and

Dickeya zeae was split in two species: *Dickeya zeae* and *Dickeya parazeae*.

Originally, *Dickeya* strains were commonly found in tropical and subtropical climates affecting a wide diversity of plants, particularly potato and tomato, *Ipomoea batatas* (sweet potato), banana, corn, rice, pineapple and many ornamentals, in particular African violet and aroids, such as *Philodendron* and *Dieffenbachia* (Ma et al., 2007; Toth et al., 2011). For a long time, their presence in temperate climates was almost exclusively confined to greenhouse cultivation of ornamentals. However, in the past few decades, *D. dianthicola* has been occasionally isolated from symptomatic potato tubers and plants in Western Europe showing that it is well adapted to temperate conditions (Samson et al., 2005). Furthermore, in the early years of the 21st century, a new highly virulent *Dickeya* genomospecies was identified from potato in many European countries and described as *D. solani* (van der Wolf et al., 2014). The emergence of *D. solani* reported in various countries during the same period (Toth et al., 2011), indicated that the pest was spread through international trade. Initially also found in hyacinth (Chen et al., 2012), *D. solani* is thought to have been transferred from hyacinth to potato in the recent past, possibly via contaminated irrigation water (Czajkowski et al., 2012; Sławiak et al., 2009).

More recently, other *Dickeya* strains have been isolated and identified that are being assigned to novel species:

- *Dickeya aquatica*, isolated from freshwater rivers in the UK (Parkinson et al., 2014).
- *D. fangzhongdai*, originally isolated from bleeding canker on pear trees in China (Tian et al., 2016), but also diagnosed on *Phalaenopsis* orchids and isolated from river water (Alic et al., 2018).
- *D. lacustris*, isolated from water and associated plants from lakes in France (Hugouvieux-Cotte-Pattat et al., 2019).
- *D. undicola*, originally isolated from freshwater lake in Malaysia (Tan et al., 2015) and later obtained from freshwater samples collected in Asia and Europe (Oulghazi, Cigna, et al., 2019).
- *D. oryzae* has been recently described from roots of rice in China (Wang et al., 2020).

In addition, the genomospecies *D. poaceiphila* is suggested for a strain isolated from sugar cane in Australia (Duprey et al., 2019). Additionally, several sequenced *Dickeya* strains have also been reclassified but public databases may not have been updated yet following these changes.

APPENDIX 2 - LIST OF SPECIES AND TESTS USED FOR THEIR DETECTION AND IDENTIFICATION

	Detection and identification	Appendix
Dickeya spp. & *Pectobacterium* spp.	Conventional PCR (Toth et al., 1999)	–
	Real-time PCR (Pritchard et al., 2013)	–
	Conventional multiplex PCR (Diallo et al., 2009)[a]	–
Dickeya spp.	Conventional PCR and RFLP of PCR amplicon (Nassar et al., 1996)	Appendix 7
	Conventional PCR (Chao et al., 2006)	–
	Real-time PCR (Laurila et al., 2010)	–
	Real-time PCR (Humphris et al., 2015; Pritchard et al., 2013)	–
	Real-time PCR (Zijlstra et al., 2020)	–
	Real-time PCR (Dobhal et al., 2020)	–
	Multiplex real-time PCR (Dobhal et al., 2020)	–
	LAMP test (Yasuhara-Beel et al., 2017)	–
	Agdia AmplifyRP	–
Dickeya dianthicola	Real-time PCR (Pritchard et al., 2013)	Appendix 14
	Real-time PCR (van der Wolf et al., 2014)	–
	Real-time PCR (Karim et al., 2019)	–
	Real-time PCR (Dobhal et al., 2020)	–
	Multiplex real-time PCR (Dobhal et al., 2020)	–
	LAMP test (Ocenar et al., 2019)	–
Dickeya fangzhongdai	Real-time PCR (Tian et al., 2020)	–
Dickeya solani	Real-time PCR (Van Vaerenbergh et al., 2012)	Appendix 12
	Real-time PCR (Pritchard et al., 2013)	Appendix 13
	Real-time PCR (van der Wolf et al., 2014)	–
Pectobacterium spp.	Conventional PCR (Darrasse et al., 1994)	Appendix 6
	Multiplex real-time PCR (Arizala et al., 2022)	–
Pectobacterium atrosepticum	Conventional PCR (De Boer & Ward, 1995)	–
	Conventional PCR (Frechon et al., 1998)	–
	Real-time PCR (Brierley et al., 2008; Humphris et al., 2015)	Appendix 11
Pectobacterium brasiliense	Conventional PCR (Duarte et al., 2004)	Appendix 15
	Real-time PCR (van der Wolf et al., 2017)	Appendix 8
	Real-time PCR (Muzhinji et al., 2020)	Appendix 9
Pectobacterium carotovorum	Conventional PCR (Kang et al., 2003)	–
Pectobacterium parmentieri	Conventional PCR (Kim et al., 2011)	–
	Real-time PCR (De Boer, 2012)	–
	Real-time PCR (Kim et al., 2011)	–
	Real-time PCR (van der Wolf et al., 2017)	Appendix 10
	Multiplex real-time PCR (Arizala et al., 2022)	–
Pectobacterium punjabense	Real-time PCR (Cigna et al., 2021)	–
Pectobacterium wasabiae	Conventional PCR (Kim et al., 2011)	–
	Real-time PCR (Kim et al., 2011)	–

[a] *Dickeya* species pathogenic on potato and *Pectobacterium atrosepticum*.

APPENDIX 3 - SAMPLE PREPARATION FOR TESTING

Protocols for sample preparation have not been standardized nor validated through inter-laboratory comparisons, thus the procedures used in a number of laboratories in the EPPO region are provided in this Appendix.

1. Microplants

Up to 10 well developed (4–6 weeks) microplants can be pooled. Cuttings of stems, leaves and roots of the microplants are prepared and macerated as individual or composite samples for 2 h in 5 mL of 50 mM phosphate buffer (pH 7.0). 200 µL of the macerate is added

to 1.8 mL of LEM366 and incubated at 27°C for 48 h. Alternatively, the sample is placed in a maceration bag (e.g. Bioreba bags) and suspended in a small volume (2–3 mL) of 10 mM phosphate buffer and homogenized by hand with a rubber mallet or using a semi-automated homogenizer (Homex 6, Bioreba, or similar equipment). The enriched macerate is used for further testing.

2. Symptomatic potato samples and other symptomatic host plants

Discoloured vascular tissue is removed from the stem of the wilting plant or from the margin of rotting tissue (stem, leaves, tuber, parenchymatous tissue). The sample is placed in a maceration bag (e.g. Bioreba bags) and suspended in a small volume (2–3 mL) of 10 mM phosphate buffer and homogenized by hand with a rubber mallet or using a semi-automated homogenizer (Homex 6, Bioreba, or similar equipment). The macerate is soaked for 5–10 min at ambient temperature before performing the subsequent tests. The suspension is then diluted 1/10, 1/100, 1/1000 and 1/10 000.

3. Asymptomatic seed potato

Laboratory samples commonly contain 100–200 seed tubers that can be processed in bulk or processed in subsamples.

3.1. Sample processing at ILVO (BE)

Any excessive soil is first removed from the tubers. Then the peel is removed with a disinfected peeler knife from the heel (stolon) end of each tuber and a small core of the exposed vascular tissue is taken. 25 tissue cores are mechanically squashed in a maceration bag (such as Bioreba) using a rubber mallet after which 5 mL of 10 mM phosphate buffer is added. The macerate is soaked for 30 min at ambient temperature after which 200 µL of the macerate is added to 1.8 mL of PGEM (Polygalacturonate Enrichment Medium) in a 2 mL microvial, followed by 48 h incubation at 25°C under hypoxic conditions. The tubes are then centrifuged for 5 min at 10 000 rpm after which the liquid is poured out gently and 1 mL of sterile 10 mM phosphate buffer is added, followed by mild vortexing. The pellet suspensions are kept at −20°C for testing. Positive controls of a strain of the appropriate *Pectobacterium*/*Dickeya* species (see section of the main text) in PGEM are prepared to verify detection with a concentration step before enrichment of 10^2–10^3 cells/mL.

3.2. Sample processing at IVIA (ES)

Stolon ends and peels are used simultaneously. Up to 200 seed tubers are cleaned to remove any excessive soil. Stolon ends and peels are removed from each tuber with a disinfected scalpel.

For stolon ends, a small core of the exposed vascular tissue is taken. Tissue cores are mechanically squashed in a maceration bag (e.g. Bioreba) using a rubber mallet or using a semi-automated homogenizer (Homex 6, Bioreba, or similar equipment) with 5 mL of 50 mM phosphate buffer. Then, 35 mL of 50 mM phosphate buffer are added and the homogenate is macerated for 30 min at ambient temperature. Alternatively, the macerate bag is shaken in an orbital shaker at 100 rpm, at 4°C for 12–18 h. The macerate is centrifuged at 180 *g* for 10 min. The supernatant is recovered in a new centrifuge tube and centrifuged at 10 000 *g* for 10 min. Centrifugation steps are preferably performed at 4–10°C. The obtained pellet is resuspended in 1.5 mL of 10 mM phosphate buffer and plated, including at least two serial decimal dilutions of the resuspended pellet, on recommended media. An enrichment step can be also performed. 200 µL of the resuspended pellet is mixed with 1.8 mL PGEM in a 2 mL microtube and incubated at 28°C for 48–72 h. The tubes are then centrifuged for 10 min at 10 000 *g*, the supernatant is discarded and the pellet is resuspended in 1 mL of sterile 10 mM phosphate buffer and plated, including at least two serial decimal dilutions, on recommended media.

For the pieces of peels, 4 cm of peel with abundant lenticels are used, obtained from four sides of a tuber (1 cm each) and placed into a maceration bag with 4 mL of 50 mM phosphate buffer. Pieces of peels from up to 25 tubers can be pooled in a maceration bag with 40 mL of 50 mM phosphate buffer. The peel macerate is then processed as described for stolon end samples.

3.3. Sample processing at INOV3PT (FR)

200 seed tubers samples are washed under running tap water and air-dried at room temperature. A small core (including the peel) is taken from the heel end of each tuber and 4 composite subsamples corresponding to 50 tubers each are made. Each subsample is placed in a sterile vial containing 20 mL of maceration buffer. The tubes are incubated under constant gentle shaking at room temperature for 2 h or 4–6°C overnight. An aliquot of 200 µL of this suspension is poured into a 2 mL micro-centrifuge tube containing 1.8 mL of the enrichment medium LEMAG366 (Hélias et al., 2012), providing low oxygen conditions, and incubated at 27°C for 48 h.

3.4. Sample processing at SASA (GB)

Each sample (max. 200 tubers) is divided into 4 equal subsamples. After removing any excess soil, a strip of peel running from the stolon end to the rose end is removed using a disinfected potato peeler. From the stolon end of each tuber a small core of the exposed vascular

TABLE 2 Test conditions used in different laboratories.

	ILVO (BE)	IVIA (ES)	INOV3PT (FR)	SASA (GB)	Agroscope (CH)
nb tubers/sample	100	200	200	200	300
nb tubers/subsample	25	1–25	50	25	50
Tissue tested	Stolon end	Stolon end and peel	Heel end and peel	Stolon end and peel	Stolon end
Tissue processing	Homogenization	Homogenization	Shaking	Homogenization and shaking	Shaking
Enrichment medium	PGEM	PGEM	LEMAG366	PEM	LEMAG366
Enrichment temperature	25°C	28°C	27°C	25°C or 36°C	28°C
Enrichment time	48 h	48–72 h	48 h	48–72 h	48 h
Enrichment condition	Hypoxic	Hypoxic	Hypoxic	Aerobic and hypoxic	Hypoxic

tissue is taken. Stolon cores and peel strips are placed separately into either Bioreba bags or plastic 125 mL sample pots, depending on the processing method chosen below:

- Samples are homogenized in a Bioreba bag by hand using a rubber mallet, 40 mL of 50 mM phosphate buffer is added and the liquid left to macerate for 30–45 min.
- Stolon ends and peelings in sample pots are shaken in 40 mL 50 mM phosphate buffer in an orbital shaker at 100 rpm, 4°C for 12–18 h, as per EU Directive for Ring/Brown rot.

The liquid is transferred to a 50 mL centrifuge tube and centrifuged twice, firstly at a low speed to clarify the supernatant and secondly at a high speed to form a concentrated bacterial pellet. After centrifugation, the pellet is resuspended in 1.5 mL of Ringer's solution and serially diluted 1/10, 1/100, 1/1000. 100 µL of each dilution are spread onto duplicate CVP plates previously dried to remove excess surface moisture. CVP plates are incubated upside down: one set of plates at 27°C and one at 37°C for 48–72 h. Depending on bacterial species, characteristic cavities formed by *Pectobacterium* or *Dickeya* spp. appear after 24–48 h. Selected cavity forming colonies are purified on Nutrient Agar before being used for further molecular/phenotypic characterization.

If an enrichment step is necessary, equal amounts (v/v) of Pectate Enrichment Medium, PEM (Meneley & Stanghellini, 1976) and sample are mixed in a centrifuge tube followed by 48 h incubation at the desired temperature under hypoxic conditions. Dilution plating is performed from 1/10 to 1/1 000 000.

3.5 Sample processing at Agroscope (CH)

For each seed lot, a 300-tuber sample is divided into 6 composite subsamples of 50 tubers each. Seed tubers are washed under running tap water and air-dried at room temperature for two days. The peel at the stolon end is removed with a sterile scalpel before a 0.5 cm-deep tissue sample from the stolon end (including the vascular bundles) of each tuber is taken. 50 stolons are pooled and placed in a 50 mL tube containing 20 mL of maceration buffer. The tubes are incubated under constant gentle shaking at 6–8°C overnight. After this maceration step, tubes are left standing for 2 min to allow the tuber parts to settle. The supernatant is collected in a new clean 50 mL tube and centrifuged again at 4000 *g* for 10 min. The supernatant is discarded, and the pellet suspended in 200 µL of sterilized distilled water. An aliquot of 200 µL of this suspension is poured into a 2 mL Eppendorf tube containing 1.8 mL of the enrichment medium LEMAG366 (Hélias et al., 2012), under low oxygen conditions, and incubated at 28°C for 48 h (Table 2).

4. Asymptomatic onions and other Amaryllidaceae

No specific procedure is available for onions and other Amaryllidaceae.

APPENDIX 4 - BUFFERS AND MEDIA

All media are sterilized by autoclaving at 121°C for 15 min, except when stated otherwise. When expecting problems with fungal contamination it is recommended to add antifungal compounds (such as cycloheximide 100 mg/L).

1. Buffers

Phosphate buffer (50 mM PB, pH 7.0) for the extraction and dilution of bacteria from infected tissues

Na_2HPO_4	4.26 g
KH_2PO_4	2.72 g
Distilled water	1 L
Adjust pH to 7.2	

Phosphate buffer (10 mM PB, pH 7.2) for resuspension of pelleted extracts

$Na_2HPO_4 \cdot 12H_2O$	2.70 g
$NaH_2PO_4 \cdot 2H_2O$	0.40 g
Distilled water	1 L
Adjust pH to 7.2	

Phosphate buffer saline (10 mM PBS buffer, pH 7.2)

NaCl	8.0 g
KCl	0.2 g
$Na_2HPO_4 \cdot 12H_2O$	2.9 g
KH_2PO_4	0.2 g
Distilled water to	1 L
Adjust pH to 7.2	

Homogenization buffer for CTAB method adapted for onion and other Amaryllidaceae species

Tris–HCl (1 M autoclaved solution pH 8.0)	10 mL
NaCl 5 M (autoclaved solution)	10 mL
Ethylene diamine tetraacetic acid - EDTA (0.5 M) (autoclaved solution)	10 mL
2-mercaptoethanol	10 mM
Distilled water up to	100 mL

CTAB buffer (pH 8.0)[a] *for potato and other host plants, except onion and other Amaryllidaceae species*

Tris–HCl (1 M autoclaved solution pH 8.0)	20 mL
NaCl 5 M (autoclaved solution)	56 mL
Ethylene diamine tetraacetic acid – EDTA (0.5 M) (autoclaved solution)	8 mL
Cetyl-trimethyl-ammonium Bromide – CTAB	2 g
Poly vinyl pyrrolidone – PVP-40	1 g
Distilled water up to	100 mL

[a] Do not autoclave. It is recommended to keep the buffer for no longer than 1 week.

CTAB buffer (pH 8.0)[a] *for onion and other Amaryllidaceae species*

Tris–HCl (1 M autoclaved solution pH 8.0)	10 mL
NaCl 5 M (autoclaved solution)	29 mL
Ethylene diamine tetraacetic acid – EDTA (0.5 M) (autoclaved solution)	10 mL
Cetyl-trimethyl-ammonium Bromide – CTAB	2 g
Poly vinyl pyrrolidone – PVP-40	1 g
2-mercaptoethanol	0.2 mL
Distilled water up to	100 mL

[a] Do not autoclave. It is recommended to keep the buffer for no longer than 1 week.

¼ Strength Ringer's solution for the extraction and dilution of bacteria from infected tissues and resuspension of pelleted extracts

Dissolve 1 tablet (Oxoid) in 500 mL of distilled water.

2. Non-selective media

2.1. Nutrient Agar (NA) (Lelliott & Stead, 1987)

Nutrient agar	28.0 g
Distilled water	1.0 L

pH is adjusted to 7.2.

Alternative recipes can be used but validation by the laboratory is recommended.

2.2. Yeast peptone glucose agar (YPGA) (Lelliott & Stead, 1987)

Yeast extract	5.0 g
Oxoid proteose peptone	5.0 g
Glucose	10.0 g
Oxoid agar No. 3	12.0 g
Distilled water	1 L

pH is adjusted to 6.5–7.0.

2.3. King's B medium (Lelliott & Stead, 1987)

Proteose peptone No: 3	20.0 g
Glycerol	10.0 mL
K_2HPO_4	1.5 g
$MgSO_4 \cdot 7H_2O$	1.5 g
Agar (Oxoid Technical No. 2)	15.0 g
Distilled water	1 L

pH is adjusted to 7.2.

Alternative recipes can be used but validation by the laboratory is recommended.

2.4. NGM medium (Lee & Yu, 2006)

The medium consists of Nutrient Agar supplemented with 1% glycerol, that induces pigment production, and 2 mM $MnCl_2 \cdot 4\ H_2O$, that further enhances colour development.

Meat extract	1.0 g
Yeast extract	2.0 g
Bactopeptone	5.0 g
NaCl	5.0 g
$MnCl_2 \cdot 4\ H_2O$	0.4 g
Glycerol	10.0 mL
Microbiological grade agar	15.0 g
Distilled water	1 L

pH is adjusted to 7.4±0.2 at 25°C.

2.5. Liquid Enrichment Medium LEM366 (Hélias et al., 2012)

$MgSO_4 \cdot 7H_2O$	0.375 g
$(NH_4)_2SO_4$	0.1 g
K_2HPO_4	0.1 g
5 N NaOH	20 µL
Pectin (Dipecta AG366, Agdia)	0.17 g
Distilled water	100 mL

pH is adjusted to 6.8–7.4.

2.6. PolyGalacturonate Enrichment Medium (PGEM)

$MgSO_4 \cdot 7H_2O$	0.375 g
$(NH_4)_2SO_4$	0.1 g
K_2HPO_4	0.1 g
5 N NaOH	20 µL
Polygalacturonic acid (Sigma P3850)	0.19 g
Distilled water	100 mL

Dissolve at ~60°C. pH is adjusted to ~7.2. Dispense by filter sterilization in 1.8 mL volumes in sterile 2 mL reaction tubes.

2.7. Potato Dextrose Agar (PDA)

Manufacturer's instructions should be followed when preparing the medium.

Potato Dextrose Agar (PDA) Oxoid CMO139	39.0 g
Distilled water	1 L

2.8. Casamino acid Peptone-Glucose (CPG) (Cuppels & Kelman, 1974)

The medium is used to grow the bacterial inoculum.

Glucose	10.0 g
Bactopeptone	10.0 g
Bacto-Casaminoacids	1.0 g
Microbiological grade agar	15.0 g
Distilled water	1 L

pH is adjusted to 7.4±0.2 at 25°C.

2.9. Pectate Enrichment Medium, PEM (Meneley & Stanghellini, 1976)

Sodium polypectate	0.625 g
10% $(NH_4)_2SO_4$	2.5 mL
10% K_2HPO_4	2.5 mL
5% $MgSO_4 \cdot 7H_2O$	1.5 mL
Distilled water	225 mL

pH is adjusted to 7.4±0.2 at 25°C.

3. Semi-selective isolation media

3.1. Single Layer CVP medium: SL-CVPAG366 (Hélias et al., 2012)

The SL-CVP_{AG366} is a single layer polypectate-based medium. It is prepared from two mixes that are sterilized separately before being combined.

Crystal violet mix

In 500 mL distilled water:	
$CaCl_2 \cdot 2H_2O$	1.02 g
Tryptone	1.0 g
Tri-sodium citrate	5.0 g
Na NO_3	2.0 g
Crystal violet (0.075%)	2.0 mL
Agar	4.0 g

Pectin mix

In 500 mL distilled water:	
NaOH (5 M)	2.0 mL
Pectin Dipecta AG366 (Agdia)	18.0 g

Introduce ingredients of both mixes in the order of the component list.

Dissolve each ingredient of the crystal violet mix by stirring before adding the following one.

Stir the second mix and heat it up to 80–100°C to allow the pectin to be dissolved avoiding formation of lumps.

Sterilize both mixes at 120°C for 15 min.

After autoclaving, pour the crystal violet mix into the pectin mix while the media is still hot, by gently stirring the medium with a magnetic stirrer (to avoid bubble formation). Verify the pH is 6.8–7.4 (adjust the NAOH quantity if needed).

Distribute the medium immediately in Petri dishes in a laminar flow cabinet. When the surface is dry, store the SL-CVP plates at 4°C.

3.2. Double Layer CVP medium: DL- CVPAG366 (Hélias et al. 2012)

The DL-CVP_{AG366} is a double layer medium. The two corresponding mixes are prepared separately.

Basal layer medium

$CaCl_2 \cdot 2H_2O$	5.5 g
Tryptone	1 g
Crystal violet 0.1%	1.5 mL
$NaNO_3$	1.6 g
Agar	15 g
Distilled water	1 L

Introduce ingredients in the order of the recipe and dissolve each component by stirring the medium before adding the following one.

Sterilize the basal layer medium at 120°C for 15 min.

Pour 15 mL of the basal layer medium in Petri dishes and allow them to set in a laminar flow cabinet before pouring the overlayer medium.

Overlayer medium

EDTA 5.5% (pH 8.0)	2.0 mL
NaOH 5 M	4.8 mL
Distilled water	800 mL
Pectin Dipecta (Agdia)	20 g

Heat up to 80–100°C and stir the overlayer mix to allow the pectin to be dissolved.

Distribute the medium in 2 × 400 mL bottles before autoclaving (120°C for 15 min).

Verify the pH (6.8–7.4) before pouring.

Distribute 7 mL of the overlayer medium onto the set basal layer.

When the surface is dry, store the DL-CVP at 4°C until used, eliminate the condensation before use.

Note:

1. Plates should be free from surface condensation before use.
2. Avoid excess drying of plates.
3. Quality control can be performed after preparation of each new batch of medium by plating a suspension of a reference culture of a relevant *Pectobacterium*/*Dickeya* strain and observing formation of typical cavities after incubation at 28°C for two to six days.

Performance characteristics available from Hélias et al. (2012)

Matrices tested:
Recovery rates comparison were conducted using pure bacterial cultures.

Effectiveness of CVP media on *Pectobacterium* and/or *Dickeya* isolation from infected natural samples was evaluated from a range of host plants, including potato, carrots, tobacco, onions, radishes as well as ornamentals such as *Syngonium*, *Lilium*, *Aconitum*, *Hosta*, *Zantedeschia*, *Ornithogalum* and several flower bulbs. Both SL-CVPAG366 and DL-CVPAG366 formulations used in various laboratories (Hélias et al., 2012) have successfully allowed recovery of isolates of Soft Rot *Pectobacteriacae*.

Analytical sensitivity:
CVP media were evaluated for recovery rate on 4 *P. atrosepticum*, 3 *P. carotovorum* and 3 *Dickeya* strains. Bacterial suspensions calibrated to 10^3 and 10^2 cfu/mL) were plated on five replicate dishes of SL-CVP and of DL-CVP. NBA (0.3% beef extract, 0.5% bactopeptone, 15% agar) was used as a nonselective control medium. Colony counts were used to calculate recovery rates. The average recovery rates on the SL-CVP formulations varied from 55% for *P. carotovorum* strains to 98% for *Dickeya* strains. The lower recovery rate of *P. carotovorum* on SL-CVPAG366 was the result of the poor performance on this medium of one of the two strains tested (34% vs. 78.4%). The average recovery rates on the DL-CVP formulations varied from 84% for *P. atrosepticum* strains to 97% for *Dickeya* strains. Recovery rates did not differ significantly between single-layer and double-layer CVPAG366 formulations ($t = 1.234$, 8 d.f., $p > 0.05$).

Analytical specificity:
Inclusivity:
The ability of 30 *Pectobacterium* spp. and 9 *Dickeya* spp. reference strains to produce cavities on the SL-CVP medium was evaluated. Among these, 12 *Pectobacterium* spp. and 9 *Dickeya* spp. were also tested on DL-CVP formulation. 97.5% of the Soft Rot Pectobacteriacae were able to form deep and typical cavities on SL-CVP whereas 100% of the strains produced cavities on the double layer DL-CVP.

APPENDIX 5 - DNA EXTRACTION

1. Microplants

The macerate is centrifuged and the pellet resuspended in 1 mL of 10 mM phosphate buffer, pH 7.2, into a 1.5 mL microtube. The suspension is frozen at −20°C and heat-shocked at 100°C for 10 min.

2. Potato

2.1. DNA extraction from symptomatic potato tubers with AGOWA sbeadex mag Maxi plant DNA isolation kit

DNA extraction can be performed with the AGOWA sbeadex mag Maxi plant DNA isolation kit (LGC) in a KingFisher extraction robot (ThermoFisher Scientific) according to the manufacturer's instructions.

2.2. CTAB method adapted for symptomatic potato

2.2.1. Fresh or lyophilized samples

Small pieces of fresh tissue or lyophilized material are put into the extraction bags with the proportional amount of CTAB buffer: 1:5 (w:v) for fresh material and 0.25:5 (w:v) for lyophilized samples. The sample is homogenized using a homogenizer (e.g. manual rubber mallet, semi-automatized Homex 6, Bioreba, or similar devices). One mL of these extracts is transferred into a 1.5 mL microtube, heated at 65°C for 30 min, and then centrifuged at 16 000 *g* for 5 min. The supernatant is transferred into a new 2 mL microtube, 1 mL of chloroform:isoamyl alcohol (24:1) is added and shaked.

After centrifugation at 16 000 *g* for 10 min, 700 µL of the supernatant are transferred into a 1.5 mL microtube and 490 µL (approximately 0.7 volumes) of cold 2-propanol are added. After mixing by inverting twice, the microtube is incubated at −20°C for 20 min. The microtube is centrifuged at 16 000 *g* for 20 min and the supernatant discarded to recover the pellet. The pellet is washed with 1 mL ice-cold 70% ethanol and centrifuged at 16 000 *g* for 10 min. The washing with 1 mL ice-cold 70% ethanol and centrifugation at 16 000 *g* for 10 min is repeated. After gently discarding the ethanol, the pellet is air-dried (the microtube is put upside down overnight), then resuspended in 100 µL of TE buffer or RNase- and DNase-free water and stored at −20°C until use.

2.2.2. Extracts obtained after processing the samples (Appendix 3)

One mL of the extract is centrifuged at 16 000 *g* for 10 min in a 1.5 mL microtube and the supernatant is discarded. The pellet is reconstituted with 1 mL of CTAB buffer.

The 1.5 mL microtube is heated at 65°C for 30 min, and then centrifuged at 16 000 *g* for 5 min. One mL of the supernatant is transferred into a new 2 mL microtube, 1 mL of chloroform:isoamyl alcohol (24:1) is added and shaken to mix. After centrifugation at 16 000 *g* for 10 min, 700 µL of the supernatant are transferred into a 1.5 mL microtube and 490 µL (approximately 0.7 volumes) of cold 2-propanol are added. After mixing by inverting twice, the microtube is incubated at −20°C for 20 min. The microtube is centrifuged at 16 000 *g* for 20 min and the supernatant discarded to recover the pellet. The pellet is washed with 1 mL ice-cold 70% ethanol and centrifuged at 16 000 *g* for 10 min. The ethanol wash and centrifugation steps are repeated. After gently discarding ethanol, the pellet is air-dried (the microtube upside down overnight), then resuspended in 100 µL of TE buffer or RNase- and DNase-free water and is stored at −20°C until use.

2.3. DNA extraction from asymptomatic potato tubers

The protocols described below are used for composite samples of asymptomatic potato tubers, using stolon ends or tuber peel extracts.

2.3.1. CTAB protocol

1 mL of the extract (enriched or not) obtained after processing the samples from stolon ends, pieces of peel or enriched samples (see Appendix 3) is centrifuged at 16 000 *g* for 10 min in a 1.5 mL microtube. After discarding the supernatant, the pellet is resuspended in 1 mL CTAB buffer. The 1.5 mL microtube is incubated at 65°C for 30 min, and then centrifuged at 16 000 *g* for 5 min. 1 mL of the supernatant is transferred into a fresh 2 mL microtube, 1 mL of chloroform:isoamyl alcohol (24:1) is added and the sample is mixed by shaking. After centrifugation at 16 000 *g* for 10 min, 700 µL of the supernatant are transferred into a 1.5 mL microtube and 490 µL of cold 2-propanol are added. After mixing by inverting twice, the microtube is incubated at −20°C for 20 min. The microtube is centrifuged at 16 000 *g* for 20 min and the supernatant discarded. The pellet is washed with 1 mL ice-cold 70% ethanol and centrifuged at 16 000 *g* for 10 min. This washing step is repeated. After gently discarding the ethanol, the pellet is air-dried (with the microtube upside down) overnight, then resuspended in 100 µL of TE buffer or RNase- and DNase-free water and stored at −20°C until use.

2.3.2. DNA extraction based on columns, e.g. DNeasy Plant Mini Kit (Qiagen)

For the extracts described in Appendix 3, 1 mL of the extract (enriched or not) from stolon ends, pieces of peel or enriched samples is centrifuged at 16 000 *g* for 10 min, the supernatant is discarded, and the pellet is resuspended in the quantity of lysis buffer recommended by the manufacturer and, after following their instructions, the DNA extract is stored at −20°C until use.

2.3.3. DNA extraction based on magnetic beads, e.g. BioSprint 96 workstation and Biosprint 96 DNA Plant Kit (Qiagen)

For the extracts described in Appendix 3, 1 mL of the extract (enriched or not) is centrifuged at 16 000 *g* for 10 min, the supernatant is discarded, and the pellet is resuspended in the quantity of lysis buffer recommended by the manufacturer and, after following their instructions, the DNA extract is stored at −20°C until use.

3. Other host plants

3.1. CTAB method adapted for symptomatic onion and other Amaryllidaceae species

Onion and Amaryllidaceae plant tissues are very rich in mucilage. Mucilage may cause problems when using columns (e.g. DNeasy Plant MiniKit by Qiagen) to extract and purify DNA. Therefore, it is recommended to extract DNA using an adapted CTAB method.

500 mg of plant tissue are homogenized in 1 mL of extraction buffer. The homogenate (300 µL) is mixed with 80 µL of lysozyme (50 mg mL^{-1} in 10 mM Tris–HCl, pH 8.0) and incubated at 37°C for 30 min. After incubation, 500 µL of CTAB buffer is added to the homogenate and incubated at 65°C for 30 min. The sample is allowed to cool at room temperature for 3 min before the addition of 500 µL of ice-cold chloroform. Samples are mixed by vortexing and then centrifuged at 13 000 *g* for 10 min. The upper aqueous layer is transferred to a new microfuge tube, 0.6 volume of isopropanol is added, and the tube is placed on ice for 20 min to precipitate the DNA. DNA is recovered by centrifugation as described above. The pellet is washed with ice-cold 75% ethanol

and centrifuged at 13 000 *g* for 2 min. After removal of ethanol, the pellet is air-dried and resuspended in 100 µL of sterile water.

3.2. CTAB method adapted for other symptomatic host plants, except onion and Amaryllidaceae species

3.2.1. Fresh or lyophilized samples

Small pieces of fresh tissue or lyophilized material are put into the extraction bags with the proportional amount of CTAB buffer: 1:5 (w:v) for fresh material and 0.25:5 (w:v) for lyophilized samples. The sample is homogenized using a homogenizer (e.g. manual rubber mallet, semi-automatized Homex 6, Bioreba, or similar devices). One mL of these extracts is transferred into a 1.5 mL microtube, heated at 65°C for 30 min, and then centrifuged at 16 000 *g* for 5 min. The supernatant is transferred into a new 2 mL microtube, 1 mL of chloroform:isoamyl alcohol (24:1) is added and shaked. After centrifugation at 16 000 *g* for 10 min, 700 µL of the supernatant are transferred into a 1.5 mL microtube and 490 µL (approximately 0.7 volumes) of cold 2-propanol are added. After mixing by inverting twice, the microtube is incubated at −20°C for 20 min. The microtube is centrifuged at 16 000 *g* for 20 min and the supernatant discarded to recover the pellet. The pellet is washed with 1 mL ice-cold 70% ethanol and centrifuged at 16 000 *g* for 10 min. The washing with 1 mL ice-cold 70% ethanol and centrifugation at 16 000 *g* for 10 min is repeated. After gently discarding the ethanol, the pellet is air-dried (the microtube is put upside down overnight), then resuspended in 100 µL of TE buffer or RNase- and DNase-free water and stored at −20°C until use.

3.2.2. Extracts obtained after processing the samples (Appendix 3)

One mL of the extract is centrifuged at 16 000 *g* for 10 min in a 1.5 mL microtube and the supernatant is discarded. The pellet is reconstituted with 1 mL of CTAB buffer.

The 1.5 mL microtube is heated at 65°C for 30 min, and then centrifuged at 16 000 *g* for 5 min. One mL of the supernatant is transferred into a new 2 mL microtube, 1 mL of chloroform:isoamyl alcohol (24:1) is added and shaken to mix. After centrifugation at 16 000 *g* for 10 min, 700 µL of the supernatant are transferred into a 1.5 mL microtube and 490 µL (approximately 0.7 volumes) of cold 2-propanol are added. After mixing by inverting twice, the microtube is incubated at −20°C for 20 min. The microtube is centrifuged at 16 000 *g* for 20 min and the supernatant discarded to recover the pellet. The pellet is washed with 1 mL ice-cold 70% ethanol and centrifuged at 16 000 *g* for 10 min. The ethanol wash and centrifugation steps are repeated. After gently discarding ethanol, the pellet is air-dried (the microtube upside down overnight), then resuspended in 100 µL of TE buffer or RNase- and DNase-free water and is stored at −20°C until use.

3.3. DNA extraction based on columns, e.g. DNeasy Plant Mini Kit (Qiagen)

This DNA extraction procedure is recommended for symptomatic plant hosts other than onion and other Amaryllidaceae species.

100 mg of fresh tissue or 20 mg of dry tissue are mixed with lysis buffer (quantities can differ among several kit brands) in an extraction bag and homogenized. For extracts described in Appendix 3, 1 mL of the extract (enriched or not) is used and samples are centrifuged at 16 000 *g* for 10 min. The supernatant is discarded, and the pellet is resuspended in the quantity of lysis buffer recommended by the manufacturer and, after following their instructions, the DNA extract is stored at −20°C until use.

3.4. DNA extraction based on magnetic beads, e.g. BioSprint 96 workstation and Biosprint 96 DNA Plant Kit (Qiagen)

30–50 mg of fresh symptomatic plant material other than onion and the other Amaryllidaceae species, 24–40 mg of frozen plant material or up to 30 mg of lyophilized plant material are ground mechanically to get a fine powder. The powder is resuspended in 200 µL lysis buffer, carefully mixed, and then centrifuged at 6 000 *g* for 5 min. For extracts described in Appendix 3, 1 mL of the extract (enriched or not) is centrifuged at 16 000 *g* for 10 min, the supernatant is discarded, and the pellet is resuspended in the quantity of lysis buffer recommended by the manufacturer and, after following their instructions, the DNA extract is stored at −20°C until use.

4. DNA extraction from surface or recirculation water

50 mL of water sample are centrifuged at 16 000 *g* for 20 min and the pellet is resuspended in 2 mL of 10 mM phosphate buffer, pH 7.2. It is also possible to concentrate the sample by filtering 50 mL water sample using a 0.22 µm membrane. The membrane is then put in a 50 mL centrifuge tube and soaked for 30 min with 10 mM phosphate buffer, pH 7.2 (to completely cover the membrane). After a vigorous vortexing, the liquid phase is recovered and centrifuged at 16 000 *g* for 20 min. The supernatant is discarded and the pellet resuspended in 2 mL of 10 mM phosphate buffer, pH 7.2. 1 mL of the extract can be used for testing.

5. DNA extraction from isolates

5.1. Heat-shock DNA extraction

A colony is suspended in 1 mL of 10 mM phosphate buffer, pH 7.2, into a 1.5 mL microtube. The suspension is heat-shocked at 100°C for 10 min and frozen at −20°C until use.

5.2. Chelex extraction from pure culture (according to FERA Science Ltd.)

Bacteria are suspended in 1 mL sterile distilled water in a 1.5 mL microtube to obtain a suspension with a light turbidity. The suspension is centrifuged at 9500 *g* for 5 min. The supernatant is discarded and the pellet resuspended in 300 µL of 6% Chelex 100 suspension by vortexing. The Chelex 100 should remain in suspension when pipetting, this can be achieved by vortexing or placing it on a magnetic stirrer. The microtubes are placed in a pre-heated block set at 100°C for 8 min. Immediately after boiling, the microtubes are vortexed at high speed for 10 s before chilling them on ice or in a frozen tube rack. The chilled microtube are centrifuged at 17000 *g* for 5 min and 200 µL of the supernatant are transferred into a clean microtube and stored at −20°C until use.

APPENDIX 6 - CONVENTIONAL PCR (DARRASSE ET AL., 1994)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The test can be used for the generic detection of *Pectobacterium* spp. on various matrices and hosts
1.2. The test is adapted from Darrasse et al. (1994)
1.3. The target sequence is located on the *pel*Y gene coding for the Pectate lyase Y protein
1.4. Oligonucleotides:

Primer	Sequence	Amplicon size
Y_1	5′ TTA CCG GAC GCC GAG CTG TGG CGT 3′	435 bp
Y_2	5’ CAG GAA GAT GTC GTT ATC GCG AGT 3′	

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. Tissue source: potato microplants and leaves, stems, parenchymous tissues, pieces of peel and stolon end of potato plants
2.1.2. DNA extraction procedures are described in Appendix 5.
2.1.3. DNA should preferably be stored at approximately −20°C.
2.2. Conventional PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	13.8	N.A.
Buffer II (Applied Biosystems)	10×	2.5	1×
$MgCl_2$ (Perkin-Elmer)	25 mM	2.5	2.5 mM
dNTPs (Promega)	1.25 mM	2	0.1 mM
Forward primer Y1	25 µM	0.5	0.5 µM
Reverse primer Y2	25 µM	0.5	0.5 µM
AmpliTaq DNA Polymerase (Applied Biosystems)	5 U/µL	0.2	1 U
Subtotal		22	
Genomic DNA extract		3	
Total		25	

2.2.2. PCR conditions: 94°C for 5 min, then 35 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 45 s. Final extension of 10 min at 72°C.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole

genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification controls).

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: in order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- NIC and NAC should produce no amplicons
- PIC, PAC (and if relevant IC) a band of the expected size (435 bp) is visualized

When these conditions are met:

- A test will be considered positive if amplicons of 435 bp are produced
- A test will be considered negative, if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validation list).

Validation data from inov3PT (FR)
Analytical specificity data
Inclusivity: 93.6% tested on 47 *Pectobacterium* strains: *P. polaris* (9), *P. versatile* (8), *P. parmentieri* (8), *P. atrosepticum* (7), *P. brasiliense* (5), *P. odoriferum* (3), *P. wasabiae* (2), *P. parvum* (1), *P. carotovorum* (1), *P. betavasculorum* (2), *P. punjabense* (1). The 2 strains of *P. betavasculorum* and *P. punjabense* were not detected. The use of a degenerate reverse primer (5' CAG GAA GAT YTC GTT ATC GCG MGT 3′) can improve the analytical specificity of the test, as shown by in silico analysis (ILVO, unpublished).

Exclusivity: 100% tested on 26 *Dickeya* strains: *D. dianthicola* (7), *D. solani* (7), *D. dadantii* subsp. *dieffenbachiae* (2), *D. dadantii* subsp. *dadantii* (2), *D. paradisiaca* (2), *D. zeae* (2), *D. chrysanthemi* bv *chrysanthemi* (2), *D. chrysanthemi* bv *parthenii* (2).

APPENDIX 7 - CONVENTIONAL PCR (NASSAR ET AL., 1996)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The test can be used for the detection and identification of *Dickeya* spp. on several matrices such as potato (*Solanum tuberosum*), corn (*Zea mays*), onion (*Allium cepa*) and rice (*Oryza sativa*)
1.2. The test is adapted from Nassar et al. (1996)
1.3. The target sequence is located on the pectate lyase *pel*ADE gene cluster
1.4. Oligonucleotides:

Primer	Sequence	Amplicon size
Primer ADE1	5′-GAT CAG AAA GCC CGC AGC CAG AT-3′	420 bp
Primer ADE2	5′-CTG TGG CCG ATC AGG ATG GTT TTG TCG TGC-3′	

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. Matrices: potato microplants and matrices from potato (*Solanum tuberosum*), corn (*Zea mays*), onion (*Allium cepa*) and rice (*Oryza sativa*)
2.1.2. Nucleic acid extraction procedures are described in Appendix 5
2.1.3. DNA should preferably be stored at approximately −20°C
2.2. Conventional PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	15.925	N.A.
PCR buffer (BioTools)	10×	2.5	1×
$MgCl_2$ (BioTools)	50 mM	0.75	1.5 mM
dNTPs	20 mM	0.125	0.1 mM
Forward primer ADE1	10 µM	0.25	0.1 µM
Reverse primer ADE2	10 µM	0.25	0.1 µM
Polymerase (BioTools)	5 U/µL	0.2	1 U
Subtotal		20	
Genomic DNA extract		5	
Total		25	

2.2.2. PCR conditions: 94°C for 4 min, followed by 40 cycles of 94°C for 30 sec and 72°C for 1 min, and 1 cycle of 72°C for 10 min

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification controls).

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: in order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- NIC and NAC should produce no amplicons
- PIC, PAC (and if relevant IC) a band of the expected size (420 bp) is visualized

When these conditions are met:

- A test will be considered positive if a band of the expected size (420 bp) is visualized
- A test will be considered negative, if no band or a band of a different size than expected is visualized
- Tests should be repeated if any contradictory or unclear results are obtained

4. Performance characteristics available

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

Validation data from Nassar et al. (1996)
Analytical specificity data

Inclusivity: 100% tested on 78 strains of *Dickeya* spp.
Exclusivity: 100% tested on *Pectobacterium atrosepticum* (20 strains), *Pectobacterium. betavasculorum* (2 strains), *Pectobacterium. carotovorum* subsp. *carotovorum* (10 strains), *Pantoea agglomerans* (2 strains), and *Pectobacterium. rhapontici* (1 strain). Other pectinolytic bacteria that were associated (or not) with soft rot symptoms, such as *Bacillus subtilis* (one strain), *Pseudomonas marginalis* (three strains), *Pseudomonas viridiflava* (one strain), *Pseudomonas fluorescens* (one strain), and *Yersinia ruckeri* (one strain), also were included. Nonpectinolytic microorganisms, including *Ralstonia solanacearum* (three strains), *Xanthomonas campestris* (one strain), *Clavibacter* sp. (three strains), *Agrobacterium tumefaciens* (one strain), nitrogen-fixing bacteria (six strains), fungi and yeasts (five strains).

Pseudomonas sp. (two strains), *Comamonas* sp. (two strains), and *Enterobacter* sp. (one strain), were also tested.

APPENDIX 8 - REAL-TIME PCR (VAN DER WOLF ET AL., 2017)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The test can be used for the detection of *Pectobacterium brasiliense* on microplants, potato tubers and identification of bacterial colonies
1.2. The test is based on van der Wolf et al., 2017
1.3. The target sequence is located in the *araC* gene coding for the arabinose operon regulatory protein
1.4. Oligonucleotides:

Primer/probe	Sequence
PcbrFw	5′ TGC GGG TTC TGC GTT TC 3′
PcbrRv	5′ TGG CGC GTT CGC AAT AT 3′
PcbrP	5′ FAM – CAA GGC ACG ATA CG – MGB 3′

1.5. The real-time PCR test was validated using an ABI 7500 real-time PCR system (Applied biosystems, USA)

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. Matrices: microplants, potato tubers and bacterial colonies
2.1.2. DNA extraction procedures are described in Appendix 5.
2.1.3. DNA should preferably be stored at approximately −20°C.

2.2. Real-time Polymerase Chain Reaction – real-time PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	2.275	N.A.
Premix Ex Taq (Takara)	2×	5	1×
ROX dye II	50×	0.1	0.5×
Forward Primer PcbrFw	10 µM	0.125	0.125 µM
Reverse Primer PcbrRv	10 µM	0.3	0.3 µM
Probe PcbrP	5 µM	0.2	0.1 µM
Subtotal		8	
DNA solution		2	
Total		10	

2.2.2. PCR conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism,

total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of nucleic acid that has been spiked to the sample and no relation with the target nucleic acid (e.g. synthetic internal amplification controls).

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: in order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

Validation data from van der Wolf et al. (2017)

Analytical sensitivity data

A minimum of 100 fg of purified bacterial DNA in milli-Q water and in potato peel extracts, with Ct-values <35 in all test replicates.

Analytical specificity data

Inclusivity: The test for *Pectobacterium brasiliense* reacted with 16 out of 17 *Pectobacterium brasiliense* strains, including two strains isolated in South Africa, 13 strains isolated in The Netherlands and the type strain isolated in Brazil. A potato strain of *Pectobacterium brasiliense* isolated in Peru in 1979 did not react (IPO 590 = LMG 6670, is not *P. brasiliensis* according to genomic analysis – note from ILVO).

Exclusivity: None of the 85 non-target strains belonging to other sub-species of soft rot Enterobacteriaceae, including other *Pectobacterium* strains reacted.

APPENDIX 9 - REAL-TIME PCR (MUZHINJI ET AL., 2020)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The test can be used for the detection of infections of *Pectobacterium brasiliense* in microplants, potato plants and tubers and identification of bacterial colonies

1.2. The test is adapted from Muzhinji et al. (2020)

1.3. The target sequence is located in the ITS and the *tRNA-glu* gene

1.4. Oligonucleotides:

Primer/probe	Sequence
Forward primer Pb1F	5′ CCT TAC CAA GAA GAT GTG TGT TGC 3′
Reverse primer Pb2R	5′ CAT AAA CCC GGC ACG CT 3′
Probe PbPr	5′ FAM – CAA GCG CAC CTG TTG ATG TCA TGA GTG – BHQ1 3′

1.5. The real-time PCR test was validated using a BIORAD T100 thermocycler with CFX96 real-time module

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. Matrices: microplants, potato tubers and bacterial colonies

2.1.2. DNA extraction procedures are described in Appendix 5

2.1.3. DNA should preferably be stored at approximately −20°C

2.2. Real-time Polymerase Chain Reaction – real-time PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	6.4	N.A.
Go Taq Probe PCR Master mix (Promega)	2×	10.0	1×
Forward Primer Pb1F	10 µM	0.6	0.3 µM
Reverse Primer Pb2R	10 µM	0.6	0.3 µM
Probe PbPr	10 µM	0.4	0.2 µM
Subtotal		18	
DNA sample		2	
Total		20	

2.2.2. PCR conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of nucleic acid that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification controls).

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: in order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

(A) Validation data from Muzhinji et al. (2020)

4.1. Analytical sensitivity data

10 fg genomic DNA/µL of isolate JJ68 from South Africa with Ct < 40.

Cell concentration of 10^3 cfu/mL with mean Ct of 34.9 ± 0.9

4.2. Analytical specificity data

Inclusivity: 100%, tested on 11 target strains of *P. brasiliense* (8 isolated in the Netherland, two isolated in South Africa and the type strain isolated in Brazil).

Exclusivity: 100%, tested on 24 non-target strains of *P. carotovorum* subsp. *carotovorum* (11), *P. atrosepticum* (3), *P. parmentieri* (4) and *Dickeya* spp. (6).

(B) Validation data from NAK (NL)

Analytical specificity data obtained with Qiagen QuantiTect mix instead of the GO Taq Probe mix, with 0.2 µM for primers and probes.

Inclusivity: 99% tested on 76 target strains (Isolate LMG 6670, originating from Peru) did not react (see note in Appendix 8).

Exclusivity: 95% tested on 92 non-target strains (Taxonomic names as known at time of validation: *Dickeya chrysanthemi biovar chrysanthemi, Dickeya chrysanthemi bv. parthenii, Dickeya dadantii, Dickeya dadantii subsp. dadantii, Dickeya dadantii subsp. dieffenbachiae, Dickeya dianthicola, Dickeya paradisiaca, Dickeya solani, Dickeya* spp., *Dickeya zeae, Dickeya/Pectobacterium* spp., *Erwinia carotovora subsp. atroseptica, Erwinia chrysanthemi/Dickeya dianthicola, Pectobacterium* spp., *Pectobacterium aquaticum, Pectobacterium aroidarum, Pectobacterium atrosepticum, Pectobacterium betavasculorum, Pectobacterium cacticida, Pectobacterium carotovorum* subsp. *actinidiae, Pectobacterium carotovorum subsp. carotovorum, Pectobacterium carotovorum* subsp. *odoriferum, Pectobacterium fontis, Pectobacterium maceratum, Pectobacterium parmentieri, Pectobacterium peruviense, Pectobacterium polaris, Pectobacterium polaris* subsp *parvum, Pectobacterium polonicum, Pectobacterium versatile, Pectobacterium wasabiae / P. parmentieri, Pectobacterium zantedeschiae*). Cross-reaction was recorded with one unknown *Dickeya or Pectobacterium* spp. strain (NAK 477), one unknown strain of *Pectobacterium* sp. (NAK253), one strain of *P. aquaticum* (NAK 467), one strain of *P. cacticida* (CFBP 3628) and one strain of *P. polaris* (CFBP 8603).

APPENDIX 10 - REAL-TIME PCR (VAN DER WOLF ET AL., 2017)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. **General Information**

1.1. The test can be used for the detection of *Pectobacterium parmentieri* on microplants, potato tubers and identification of bacterial colonies

1.2. The test is based on van der Wolf et al. (2017)

1.3. The targeted sequence is located in the *mdh* gene coding for the malate dehydrogenase protein

1.4. Oligonucleotides:

Primer/probe	Sequence
PwF1	5′ TCT GTT CAA TGT CAA CGC AGG TA 3′
PwR1	5′ AGG TAA CCG CAA TTT GCT CAA 3′
PwP1	5′ FAM – TGT GCG CAA CCT G – MGB 3′

1.5. The test was developed and validated on ABI 7500 Real-Time System (Applied Biosystems)

2. **Methods**

2.1. Nucleic Acid Extraction and Purification

2.1.1. Matrices: microplants, potato tubers and bacterial colonies

2.1.2. DNA extraction procedures are described in Appendix 5

2.1.3. DNA should preferably be stored at approximately −20°C

2.2. Real-time Polymerase Chain Reaction – real-time PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	2.1	N.A.
Perfect real Time (Takara)	2×	5.0	1×
ROX dye II (Thermo Fisher Scientific)	50×	0.1	0.5×
Forward Primer PwF1	10 µM	0.3	0.3 µM
Reverse Primer PwR1	10 µM	0.3	0.3 µM
Probe PwP1	5 µM	0.2	0.1 µM
Subtotal		8	
DNA dilution		2	
Total		10	

2.2.2. PCR conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification controls).

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: in order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

Validation data from van der Wolf et al. (2017)

4.1. Analytical sensitivity data

100 fg of purified DNA in milli-Q water or in potato peel extract

4.2. Analytical specificity data

Inclusivity: The test reacted with 18 out of 18 potato isolates of *Pectobacterium parmentieri*, including 16 from the Netherlands, one from Switzerland and one from the United Kingdom and with the type strain of *Pectobacterium wasabiae* from *Eutrema wasabi*.

Exclusivity: The test did not react with 106 non-target strains belonging to other *Pectobacterium* or *Dickeya* species (94 strains) and with 12 other plant-associated bacterial species.

APPENDIX 11 - REAL-TIME PCR (BRIERLEY ET AL., 2008; HUMPHRIS ET AL., 2015)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The test can be used for the detection of *Pectobacterium atrosepticum* on microplants, leaves and stem of potato plants and peel and stolon end of tubers and identification of bacterial colonies. The test is optimized for the detection on asymptomatic plants, but it can also be used on symptomatic plants
1.2. The test is based on Brierley et al., 2008
1.3. The test targets the formate C-acetyletransferase (pyruvate formate lyase) gene
1.4. Oligonucleotides:

Primer/probe	Sequence
ECA-CSL-1F	5′ CGG CAT CAT AAA AAC ACG CC 3′
ECA-CSL-89R	5′ CCT GTG TAA TAT CCG AAA GGT GG 3′
ECA-CSL-36T-P	5′ FAM – ACA TTC AGG CTG ATA TTC CCC CTG CC – TAMRA 3′

1.5. Cycler or real-time PCR system: Applied Biosystem 7700 Sequence Detector

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. Matrices: microplants, peel and stolon end of tubers and bacterial colonies
2.1.2. DNA extraction procedures are described in Appendix 5
2.1.3. DNA should preferably be stored at approximately −20°C.
2.2. Real-time Polymerase Chain Reaction – real-time PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	7	N.A.
TaqMan® Universal PCR Master Mix (Applied Biosystem)	2×	12.5	1×
Forward Primer ECA-CSL-1F	5 µM	1.5	0.3 µM
Reverse Primer ECA-CSL-89R	5 µM	1.5	0.3 µM
Probe 1 ECA-CSL-36T-P	5 µM	0.5	0.1 µM
Subtotal		23	
DNA dilution		2	
Total		25	

2.2.2. PCR conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification controls).

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: in order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

4.1. Validation data from SASA (GB)

Analytical specificity data: The test was validated against a panel of 69 isolates of *Pectobacterium* spp. and *Dickeya* spp. and 37 isolates of other related Enterobacteriaceae. The test correctly identified all strains of *Pectobacterium atrosepticum*. No amplification was obtained from the other isolates of *Pectobacterium* spp. or *Dickeya* spp. or from any of the strains of related *Enterobacteriaceae*.

APPENDIX 12 - REAL-TIME PCR (VAN VAERENBERGH ET AL., 2012)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The test can be used for the detection of *Dickeya solani* on microplants, symptomatic seed potatoes and in latent infected potato tubers after enrichment in PGEM and identification of bacterial colonies

1.2. The test is based on van Vaerenbergh et al. (2012)

1.3. The target sequence is located on the *fliC* gene coding for flagellin

1.4. Oligonucleotides:

Primer/probe	Sequence
ds-F	5′ GCG AAC TTC AAC GGT AAA 3′
ds-R	5′ CAG AGC TAC CAA CAG AGA 3′
ds-P	5′ FAM – CTC TGC TGG ACG GTT C– MGB 3′

1.5. The test was developed and validated on ABI Prism 7900HT Sequence Detection System (Life Technologies, now ThermoFisher Scientific)

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. Matrices: microplants, (latently) infected potato tubers and bacterial colonies

2.1.2. DNA extraction procedures are described in Appendix 5

2.1.3. DNA should preferably be stored at approximately −20°C

2.2. Real-time Polymerase Chain Reaction – real-time PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	9.0	N.A.
Taqman Gene Expression master mix (Applied Biosystems)	2×	12.5	1×
Forward Primer ds-F	15 µM	0.5	0.3 µM
Reverse Primer ds-R	15 µM	0.5	0.3 µM
Probe 1 ds-P	10 µM	0.5	0.2 µM
Subtotal		23	
DNA dilution		2	
Total		25	

2.2.2. PCR conditions: 95°C for 10 min, then 40 cycles of 95°C for 15 s and 63°C for 1 min

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of nucleic acid that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification controls).

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: in order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

Validation data from ILVO (BE)

4.1. Analytical sensitivity data

Potato tuber macerates (as in Appendix 3) were spiked with diluted cell suspensions from 10 strains/isolates of *D. solani*, including the type strain PRI2222, and after 48 h incubation under hypoxic conditions at 28°C in PGEM, the test gave Ct-values <30 for all macerates with at least 10–20 cells/mL.

4.2. Analytical specificity data

Inclusivity was tested on strains/isolates identified as *D. solani* by the *fli*C DNA barcode. 124 out of 124 cultures were correctly identified (116 from potato, 8 from other plant species).

Exclusivity. No cross-reactions occurred with any of the 96 strains tested from other *Dickeya* taxa nor for the 48 *Pectobacterium* strains tested.

APPENDIX 13 - REAL-TIME PCR (PRITCHARD ET AL., 2013)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The test can be used for the detection of *Dickeya solani* on microplants, leaves and stem of potato plants, peel and stolon end of tubers and identification of bacterial colonies. The test is optimized for the detection on asymptomatic plants, but it can also be used on symptomatic plants
1.2. The test is adapted from Pritchard et al. (2013)
1.3. Oligonucleotides:

Primer/probe	Sequence
SOLC-F	5′ GCC TAC ACC ATC AGG GCT AT 3′
SOLC-R	5′ ACA CTA CAG CGC GCA TAA AC 3′
SOLC-P	5′ FAM – CCA GGC CGT GCT CGA AAT CC– TAMRA 3′

1.4. Cycler or real-time PCR system: ABI7500 real-time PCR system (Applied Biosystems)

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. Matrices: microplants, leaves and stem of potato plants, peel and stolon end of tubers and bacterial colonies
2.1.2. DNA extraction procedures are described in Appendix 5
2.1.3. DNA should preferably be stored at approximately −20°C.
2.2. Real-time Polymerase Chain Reaction – real-time PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	7	N.A.
TaqMan® Universal PCR Master Mix (Applied Biosystems)	2×	12.5	1×
Forward Primer SOLC-F	5 µM	1.5	0.3 µM
Reverse Primer SOLC-R	5 µM	1.5	0.3 µM
Probe 1 SOLC-P	5 µM	0.5	0.1 µM
Subtotal		23	
DNA dilution		2	
Total		25	

2.2.2. PCR conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 55°C for 1 min.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of nucleic acid that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification controls).

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: in order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

Validation Data from Pritchard et al. (2013)

4.1. Analytical specificity data

Inclusivity: 100%, tested on 16 strains of *Dickeya solani*

Exclusivity: 98.4%. The test gave negative results for 61/62 strains of *D. dianthicola*, *D. dadantii*, *D. dieffenbachiae*, *D. chrysanthemi* pv. *chrysanthemi*, Dickeya chrysanthemi pv. parthenii, *D. paradisiaca*; *D. zeae*, *P. atrosepticum*, *P. carotovorum*, *P. betavasculorum*, *P. carotovorum* subsp. *odoriferum*, *P. wasabiae*, *Pantoea agglomerans*, *Brenneria quercina*, *Erwinia amylovora* and other pathogenic bacteria. Cross-reaction was observed with a strain of *D. dadantii*.

APPENDIX 14 - REAL-TIME PCR (PRITCHARD ET AL., 2013)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The test can be used for the detection of *Dickeya dianthicola* on microplants, leaves and stem of potato plants, peel and stolon end of tubers and identification of bacterial colonies. The test is optimized for the detection on asymptomatic plants, but it can also be used on symptomatic plants

1.2. The test is adapted from Pritchard et al. (2013)

1.3. Whole genome sequencing was used to predict potential primer sets

1.4. Oligonucleotides:

Primer/probe	Sequence
DIA-A F	5′ GGC CGC CTG AAT ACT ACA TT 3′
DIA-A R	5′ TGG TAT CTC TAC GCC CAT CA 3′
DIA-A P	5′ FAM – ATT AAC GGC GTC AAC CCG GC– TAMRA 3′

1.5. Cycler or real-time PCR system: ABI7500 real-time PCR system (Applied Biosystems)

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. Matrices: microplants, leaves and stem of potato plants, peel and stolon end of tubers and bacterial colonies

2.1.2. DNA extraction procedures are described in Appendix 5

2.1.3. DNA should preferably be stored at approximately −20°C.

2.2. Real-time Polymerase Chain Reaction – real-time PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	7	N.A.
TaqMan® Universal PCR Master Mix (*Applied Biosystems*)	2×	12.5	1×
Forward Primer (DIA-A F)	5 µM	1.5	0.3 µM
Reverse Primer (DIA-A R)	5 µM	1.5	0.3 µM
Probe 1 (DIA-A P)	5 µM	0.5	0.1 µM
Subtotal		23	
DNA dilution		2	
Total		25	

2.2.2. PCR conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 55°C for 1 min.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification controls).

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: in order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

Validation data from Pritchard et al., 2013.

4.1. Analytical specificity data

Inclusivity: 100% tested on 7 strains of *Dickeya dianthicola*.

Exclusivity: 100% tested on 71 of *D. solani*, *D. dadantii*, *D. dieffenbachiae*, *D. chrysanthemi* pv. *chrysanthemi*, *Dickeya chrysanthemi* pv. *parthenii*, *D. paradisiaca*; *D. zeae*, *P. atrosepticum*, *P. carotovorum*, *P. betavasculorum*, *P. carotovorum* subsp. *odoriferum*, *P. wasabiae*, *Pantoea agglomerans*, *Brenneria quercina*, *Erwinia amylovora* and other pathogenic bacteria.

APPENDIX 15 - CONVENTIONAL PCR (DUARTE ET AL., 2004)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other

equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The test can be used for the identification of *Pectobacterium brasiliense* from microplants, potato tubers and identification of bacterial colonies
1.2. The test is based on Duarte et al. (2004)
1.3. The target sequence is the intergenic spacer region region of Brazilian potato blackleg-causing bacterium
1.4. Oligonucleotides:

Primers	Sequence	Amplicon size
BR1f	5′ GCG TGC CGG GTT TAT GAC CT 3′	322 bp
L1r	5′ CAR GGC ATC CAC CGT 3′	

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. Matrices: microplants, potato tubers (enrichment phase is necessary if taken from asymptomatic material) and bacterial colonies
2.1.2. DNA extraction procedures are described in Appendix 5
2.1.3. DNA should preferably be stored at approximately −20°C
2.2. Conventional PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	13.3	N.A.
PCR buffer (Promega)	5×	5	1×
$MgCl_2$ (Promega)	25 mM	3	3 mM
dNTPs (Promega)	10 mM	0.5	0.2 mM
Forward primer BR1f	50 µM	0.5	1 µM
Reverse primer L1r	50 µM	0.5	1 µM
GoTaq Polymerase (Promega)	5 U/µL	0.2	1 U
Subtotal		23	
Genomic DNA extract		2	
Total		25	

2.2.2. PCR conditions: 94°C for 5 min, then 30 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 2 min. Final extension of 5 min at 72°C.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of nucleic acid that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification controls).

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: in order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- NIC and NAC: no band is visualized
- PIC, PAC (and if relevant IC) a band of the expected size (322 bp) is visualized

When these conditions are met:

- A test will be considered positive if a band of the expected size (322 bp) bp is visualized
- A test will be considered negative, if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validation list).

(A) Validation data from Duarte et al. (2004).

4.1. Analytical specificity data

Inclusivity: amplification was observed on 16 strains of *Pectobacterium brasiliense* (8, 29, 54, 101, 106, 137, 138, 142, 153, 200, 201, 205, 212 (ATCC BAA-417), 213 (ATCC BAA-418), 219, 371 (ATCC BAA-419).

Exclusivity: no cross-reaction was observed with five strains of *P. carotovorum* or with *D. chrysanthemi*.

(B) Validation data from ILVO (BE)

4.2. Analytical specificity data

Inclusivity: specific amplicon obtained from 9/9 *P. brasiliense*.

Exclusivity: no amplicon obtained from 40 other *Pectobacterium* strains: *P. atrosepticum* (6), *P. betavasculorum* (2), *P. aroidearum* (3), *P. carotovorum* (20), *P. odoriferum* (3), *P. versatile* (4), *P. parmentieri* (1), *P. wasabiae* (1) and from 6 *Dickeya* strains (*D. dianthicola*, *D. solani*, *D. dadantii*, *D. chrysanthemi*, *D. zeae* and *D. fangzhongdai*).

APPENDIX 16 - PATHOGENICITY TESTS

1. Pathogenicity test on tomato, potato or Chinese cabbage

A range of host plants can be used for the pathogenicity test for *Pectobacterium* and *Dickeya* strains.

Solanum lycopersicum cv 'Moneymaker' (tomato), *Solanum tuberosum* (potato) and, *Brassica rapa* var. chinensis (Chinese cabbage) can be used for testing (Costa et al., 2006; Jaramillo et al., 2017). Bacterial cells grown for 24 h on Nutrient Agar (see Appendix 3) at 27°C are suspended in sterile distilled water (SDW) at a concentration of 10^8 cfu/mL and 100 µL are used to inoculate by injection the plant stem at the second and third axial leaf from the base. Inoculated plants are covered with plastic bags to maintain high humidity for 48 h in growth chambers at 22°C and 16 h photoperiod and evaluated for symptom development up to 21 days after inoculation (Figure 23).

Strains isolated from potato plants may display a higher virulent response on inoculated potato plants, although they also induce symptoms in other hosts, such as wilt, leaf chlorosis and stem soft rot. *Dickeya* strains display high virulence response on tomato and Chinese cabbage leading to plant death (Figure 24). The use of tomato is recommended for the species that require an acidic pH for an optimal growth, such as *Dickeya aquatica* (Duprey et al., 2019).

2. Pathogenicity test on potato slices (NVWA, NL)

The maceration ability of *Pectobacterium* and *Dickeya* strains at different inoculum densities (10^3, 10^4, 10^5, 10^6, 10^7, 10^8 cfu mL^{-1} in sterile water) can be compared

FIGURE 23 Pathogenicity test: symptoms caused by *Pectobacterium carotovorum* on tomato plants 21 days after inoculation. Courtesy of A.B. Costa (INIAV, PT).

FIGURE 24 Pathogenicity test: symptoms caused by *Dickeya chrysantemi* on chinese cabbage (*Brassica sinensis*) plants 21 days after inoculation. Courtesy of A.B. Costa (INIAV, PT).

FIGURE 25 Pathogenicity test: symptoms on detached chicory leaves infiltrated with a cell suspension of *Pectobacterium brasiliense* strains.

using a potato slice assay as described in Czajkowski et al. (2010).

3. Pathogenicity test in detached chicory leaves (ILVO, BE)

About 100 µL of a cell suspension at about 10^8 cells/mL is infiltrated in the midrib of a chicory leaf using a 1 mL syringe without needle. The midrib is pricked to allow infiltration. The inoculated leaves are arranged on moist laboratory wipes in a box and incubated with the lid closed for 24–72 h at 25°C (Figure 25).