

REVIEW ARTICLE

Detection, identification and differentiation of *Pectobacterium* and *Dickeya* species causing potato blackleg and tuber soft rot: a review

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Abstract

The soft rot *Enterobacteriaceae* (SRE) *Pectobacterium* and *Dickeya* species (formerly classified as pectinolytic *Erwinia* spp.) cause important diseases on potato and other arable and horticultural crops. They may affect the growing potato plant causing blackleg and are responsible for tuber soft rot in storage thereby reducing yield and quality. Efficient and cost-effective detection and identification methods are essential to investigate the ecology and pathogenesis of the SRE as well as in seed certification programmes. The aim of this review was to collect all existing information on methods available for SRE detection. The review reports on the sampling and preparation of plant material for testing and on over thirty methods to detect, identify and differentiate the soft rot and blackleg causing bacteria to species and subspecies level. These include methods based on biochemical characters, serology, molecular techniques which rely on DNA sequence amplification as well as several less-investigated ones.

Introduction

Soft rot *Enterobacteriaceae* (SRE) *Pectobacterium atrosepticum* (Gardan *et al.*, 2003) (formerly *Erwinia carotovora* subsp. *atroseptica* (Van Hall) Dye) (Pba), *Pectobacterium carotovorum* subsp. *carotovorum* (Gardan *et al.*, 2003) [*Erwinia carotovora* subsp. *carotovora* (Jones) Bergey *et al.*] (Pcc), *Pectobacterium carotovorum* subsp. *brasiliense* (*Erwinia carotovora* subsp. *brasiliense*) (Pcb) (Duarte *et al.*, 2004), *Pectobacterium wasabiae* (*Erwinia carotovora* subsp. *wasabiae*) (Pwa) (Pitman *et al.*, 2008) and several *Dickeya* spp. (*Erwinia chrysanthemi*), including *D. dianthicola* (*Erwinia chrysanthemi* pv. *dianthicola*), *Dickeya dadantii*, *Dickeya zae* (*Erwinia chrysanthemi* pv. *zae*) and the new species *Dickeya solani* (Toth *et al.*, 2011; van der Wolf *et al.*, 2013) are responsible for causing potato blackleg in the field and tuber soft rots in storage and in transit as well as in the field worldwide (Fig. 1). Additionally, some reports suggest that *P. carotovorum* subsp. *odoriferum* (Pco) (Waleron *et al.*, 2014) and *P. betavascularum* (Pbt) (Nabhan *et al.*, 2012) can also cause soft rot disease in potato. SRE

are recognised among the top 10 most important bacterial pathogens in agriculture limiting crop yield and quality (Mansfield *et al.*, 2012).

Of all *Pectobacterium* spp. infecting potato, Pcc has the widest host range worldwide, whereas Pba is found mainly on potato grown in temperate regions (Pérombelon, 2002). In 2004, Pcb, a highly aggressive bacterium, was shown to cause severe infection of potato crops in tropical and subtropical regions (viz. Brazil and South Africa) (Duarte *et al.*, 2004; van der Merwe *et al.*, 2010). Pwa which was first associated with horse radish in Japan (Pitman *et al.*, 2008) was later found also on potato in New Zealand, South Africa, Canada and various European countries, where it is responsible for severe economic losses (Nykyri *et al.*, 2012; Waleron *et al.*, 2013).

In 2005, *P. chrysanthemi* species was elevated to the genus level and renamed *Dickeya* which was divided into six genomospecies (*D. dianthicola*, *D. dadantii*, *D. zae*, *D. chrysanthemi*, *D. dieffenbachia* and *D. paradisiaca*) and nine biovars which largely resembled the initial

Soft rot *Erwinia* spp. (Burkholder et al. 1957) Soft rot *Pectobacterium* spp. (Gardan et al. 2003)

- *E. carotovora* subsp. *carotovora* → • *P. carotovorum* subsp. *carotovorum* (Pcc)
- *E. carotovora* subsp. *atroseptica* → • *P. atrosepticum* (Pba)
- *E. carotovora* subsp. *wasabiae* → • *P. wasabiae* (Pwa)
- *P. carotovorum* subsp. *brasiliense* (Pcb) (Duarte et al. 2004)

Soft rot *Dickeya* spp. (Samson et al. 2005)

- *E. chrysanthemi* → • *P. chrysanthemi* → • *Dickeya* species
 - *E. chrysanthemi* pv. *dianthicola* • *P. chrysanthemi* pv. *dianthicola* • *D. dianthicola*
 - *E. chrysanthemi* pv. *dadantii* • *P. chrysanthemi* pv. *dadantii* • *D. dadantii*
 - *E. chrysanthemi* pv. *zeae* • *P. chrysanthemi* pv. *zeae* • *D. zeae*
 - *E. chrysanthemi* pv. *chrysanthemi* • *P. chrysanthemi* pv. *chrysanthemi* • *D. chrysanthemi*
 - *E. chrysanthemi* pv. *dieffenbachiae* • *P. chrysanthemi* pv. *dieffenbachiae* • *D. dieffenbachiae*
 - *E. chrysanthemi* pv. *paradisiaca* • *P. chrysanthemi* pv. *paradisiaca* • *D. paradisiaca*
 - *D. solani* (van der Wolf et al. 2013)

Figure 1 Taxonomic classification of *Pectobacterium* spp. and *Dickeya* spp. associated with tuber soft rot and blackleg disease of potato (*Solanum tuberosum* L.).

Erwinia chrysanthemi biovar division (Samson et al., 2005). Recently, *D. dieffenbachiae* was reclassified as a subspecies of *D. dadantii* (Brady et al., 2012).

Dickeya spp. can affect a number of host species in different temperature zones (Toth et al., 2011). Over the last decade, most *Dickeya* spp. strains in Europe belong to biovars 1 and 7, and were classified as *D. dianthicola* (Janse & Ruissen, 1988). Subsequently, isolates belonging to the new genetic clade of biovar 3 have been found on potato in Europe (Sławiak et al., 2009). These strains constitute possibly a new *Dickeya* sp. named *D. solani*, which was found to be the prevalent *Dickeya* spp. on potato in Europe (Toth et al., 2011; van der Wolf et al., 2013).

During the last 50 years different aspects of *Pectobacterium* and *Dickeya* species causing blackleg and soft rot diseases have been extensively reviewed. However, procedures used to detect and identify the SRE have not been reviewed as much (Barras et al., 1994; De Boer, 2003; Charkowski, 2012; Czajkowski et al., 2012; Hauben et al., 1998). The purpose of this review is to present a comprehensive examination of the available methods. We present methods based on selective growth agar media, biochemical and physiological assays, serological methods and more recently developed nucleic acid sequence-based amplification methods. In addition, methods for species/strain differentiation and identification are examined including biochemical

profiling, fingerprinting (REP-PCR), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), multi locus sequence tagging (MLST), random amplification of polymorphic DNA (RAPD), fatty acid methyl ester (FAME) analyses and full genome sequencing in order to find either major or subtle differences in nucleotide genome sequences. The review also presents less established methods used that could be potentially useful in some situations, namely volatile metabolic profiling, conductometry, flow cytometry, microsphere immunoassays and phage typing.

Finally, using the present literature data on the topic and research experience we would like to propose and recommend set of methods and procedures that in our view would prove useful in the isolation, detection, identification and differentiation of SRE from environmental samples with special emphasis on potato.

Seed potato certification system and visual field inspections

Classification systems were introduced in virtually every country producing seed potato tubers to ensure high quality of the propagation material. These systems and schemes set the levels of tolerance to soft rot and blackleg diseases based on the visual inspections of growing crops as well as on inspections of the harvested tubers after

grading and dressing and before sale (Shepard & Claflin, 1975). Usually, inspectors visit potato fields at least two times during the season, looking for the presence of symptomatic plants. Records of the blackleg diseased plants provide some indications on the disease incidence and possible infection potential of the progeny tubers (seed) in the next generation but ignore latent progeny tuber contamination which tends to be more closely related to future disease development (Pérombelon & Hyman, 1995; Toth *et al.*, 2011).

It is important to point out that the tolerance for blackleg in the European seed potato tubers differs from country to country as there is no uniform policy in the European Union and the rest of the world (Toth *et al.*, 2011).

Sampling potato tubers for the presence of soft rot *Enterobacteriaceae*

A good sampling strategy is crucial for the fool-proof detection and identification of the bacteria present in environmental samples (Pickup, 1991). It is worth mentioning that in Europe there is no compulsory testing for *Pectobacterium* and *Dickeya* species in potato. In the case of potato seed stocks, we suggest to use a sampling scheme based on the one used to detect quarantine bacteria *Ralstonia solanacearum* and/or *Clavibacter michiganensis* subsp. *sepedonicus* in which routinely 200 tubers are tested per 25 tonne lots in four pooled samples of 50 tubers (alternatively 8 composite samples of 25 tubers each). Although this sampling scheme is not officially accepted, according to the data presented by the Euphresco Phytosanitary ERA-NET 'Dickeya species in potato and management strategies' (<http://www.euphresco.org/>), it allows estimation of infection level in (seed) tubers. This is based on the statistical assumption that there is a 95% probability of detecting at least one infected tuber in a seed lot if 1.5% tubers are infected, according to the Poisson distribution (EPPO, <http://www.eppo.int/>). This strategy is more suited to high than lower seed grades which can be extensively latently contaminated (Pérombelon, 2002).

Isolation of *Pectobacterium* and *Dickeya* species

Most methods used for identification and differentiation of soft rot and blackleg causing bacteria require isolation of viable cells from samples, and growth and purification of the bacteria prior to analyses.

Isolation from plant material

From symptomatic tissues, where bacterial densities are often greater than 10^6 cells g^{-1} , it is best to sample from the advancing front of the rot or from newly

diseased tissue to avoid interference and growth suppression by contaminating saprophytes. The sample is usually suspended and diluted in sterile water or buffer and a loopful streaked on a growth medium selective for SRE. To protect bacterial cells from oxidative stress due to the release of the plant compounds during tissue preparation or enrichment, an antioxidant 0.05% DIECA (diethyldithiocarbamic acid) is commonly used (Toth *et al.*, 2011).

Plates are incubated at different temperatures as the pathogens have different optimal growth temperatures (Pérombelon & van der Wolf, 2002). Depending on the medium, bacterial colonies appear after 24–48 h at 21–37°C.

In latent infection, the bacteria may be found in all tissues, stems, roots, leaves and (progeny) tubers; however, their density is usually low, rarely exceeding 10^3 cells g^{-1} plant tissue. In stems, the bacteria are more frequently found in the first 15–20 cm above ground level (Hélias *et al.*, 2000), whereas in tubers they are more commonly present in the stolon end (Czajkowski *et al.*, 2009), but are also frequently found in lenticels and suberized wounds (Pérombelon, 2002). As the diseases are seed-borne, (seed) tubers are tested in seed certification programmes for the bacteria either qualitatively or quantitatively. Extraction of the bacteria from tubers varies and is either from ground peel strips encompassing the stolon end or the stolon end tuber section of individual or replicated bulked tuber lots (Pérombelon & van der Wolf, 2002). However, what has not yet been agreed on is whether it is more useful: (a) to determine the presence or absence of contaminated tubers in a seed lot, (b) the average tuber contamination level of a seed lot or (c) the distribution frequency of individual tuber with different contamination levels, as disease development is related to the latter (Tsrör *et al.*, 2012). It can be speculated that the number of highly contaminated tubers is more important than the average level of lot contamination, as even relatively small population of heavily infected tubers may already result in diseased plants.

Isolation from soil, rain and irrigation water

Soft rot and blackleg bacteria survive poorly in the environment (Pérombelon & Kelman, 1980), and their survival depends mostly on temperature, humidity level and pH. Survival can be longer in potato debris postharvest and in the rhizosphere of certain plants and weeds, but even under favourable conditions, survival is restricted (Pérombelon & Hyman, 1989). The detection of low numbers of the bacteria in soil is further hampered by the presence of other (antagonistic) microorganisms that can overgrow the target bacteria during isolation.

In general, soil to be tested for the presence of SRE can be processed by suspending in sterile water or buffer and shaking for a couple of hours, or better still, first enriched by incubating in a selective broth (pectate enrichment broth – PEB; see below) (Pérombelon & van der Wolf, 2002) before dilution and plating onto agar selective media (see below).

Soft rot and blackleg bacteria can survive for more than 200 days in sterile water (Cothier & Gilbert, 1990), and have been detected in surface water (Quinn *et al.*, 1980). The use of contaminated surface water during irrigation can result in infection of potato crops (Cappaert *et al.*, 1988). Because of the low numbers present, often $<10^1$ cells mL⁻¹, test samples have to be enriched by incubation in a PEB or the bacteria need to be concentrated by centrifugation prior to plating (Pérombelon & van der Wolf, 2002).

Detection, identification and differentiation

For many years, detection and identification of pectinolytic *Pectobacterium* and *Dickeya* species bacteria have depended solely on the isolation of viable bacterial cells on (semi-selective) culture agar media followed by serological and biochemical analyses, bioassays and microscopic observations including Gram staining. Later, molecular techniques based on detection of nucleic acids have been introduced, which avoid the need for live cells and now are used routinely due to their high specificity and reproducibility. What is more, they are much faster than traditional methods and allow qualitative and (semi) quantitative detection of bacteria in complex environments.

It is often difficult to discriminate among the methods used exclusively for detection and identification of SRE from those used to differentiate the isolates of *Pectobacterium* and *Dickeya* into species and subspecies, as some may be used for both objectives simultaneously. Therefore, we decided, for the purpose of this review, to divide the methods according to their (technical) background; (a) morphological and biological methods, (b) serological (immunological) methods, (c) molecular detection methods and (d) other methods. They are critically assessed in terms of their practical applications in detection systems.

Morphological and biological methods

Immunomagnetic separation of soft rot *Enterobacteriaceae* before growth on selective media

Overcrowding of the target bacteria by saprophytes can be a real drawback with semi-selective media but can be avoided by introducing an immunomagnetic separation (IMS) step prior to plating (Van Der Wolf *et al.*, 1996a).

In IMS, complexes are formed consisting of the target bacterial cells bound to the target bacteria-specific surface-directed antibodies which then bind to protein A coupled with paramagnetic particles. These complexes can be recovered when subjected to a strong magnetic force, thereby allowing rapid and efficient recovery and concentration of the target bacterial cells, which can then be dilution-plated on selective media such as crystal violet pectate (CVP; see below). The recovery is affected by several factors including the antibodies, nature and type of the magnetic particles, the IMS system and the number of wash cycles applied to remove non-target components. Van der Wolf *et al.* (1996a,b) reported a recovery sensitivity of $<10^2$ Pba cells mL⁻¹ tuber peel extract with little interference from saprophytic bacteria, which is a significant improvement on direct plating on CVP.

Artificial media used for isolation and identification of *Pectobacterium* and *Dickeya* species

Different agar media have been developed for the isolation of SRE from plant tissue, soil and water; however, till present only one medium –CVP– has been used extensively worldwide (Cuppels & Kelman, 1974). Detection of SRE on CVP depends on the formation of characteristic deep cavities by the bacterial colonies. The selectivity of the CVP medium is based on the presence of crystal violet which inhibits growth of most Gram-positive bacterial species, and the use of polypectate as the sole carbon source. At present, only one source appears to be available commercially (Hélias *et al.*, 2011) that can be used also in single and double layer forms (Pérombelon & Burnett, 1991). Crystal violet pectate remains the preferred diagnostic selective medium for the isolation of SRE from plants and the environment (Laurila *et al.*, 2008; Tsror *et al.*, 2009).

The selectivity of the medium is sufficient for the isolation of the pathogen from symptomatic stems, but may be insufficient for an efficient isolation from tuber extracts with a high microbial background. Occasionally some *Pectobacterium* and *Dickeya* species isolates may not grow on the CVP medium or do not produce cavities; this phenomenon is not yet entirely understood (R. Czajkowski, unpublished observations).

Several other selective media for the isolation of SRE have been developed, but are not widely used. The most promising is that of Pierce (1992) who modified Miller-Schroth's medium. In this medium, agar was replaced with sodium polypectate and NaOH and MOPS (3-morpholinopropane-1-sulfonic acid) were used to buffer the pH. Pectinolytic *Pectobacterium* and *Dickeya* species colonies growing on this medium form pink-red-orange cavities, whereas growth of other

saprophytic bacteria is either suppressed or failed to produce cavities. Surprisingly, despite the better recovery rate for SRE than CVP (Pierce, 1992), it has not become popular, possibly due to the customary practice of the researchers.

Artificial media used for enrichment

When the pathogen populations are low, they need to be enriched above detection level (Pérombelon, 2002). The test material is incubated under anaerobic conditions in a liquid enrichment medium, PEB, containing sodium polypectate as the sole carbon source (Pérombelon & van der Wolf, 2002). The basic principle here relies on creating conditions that stimulate natural selection for SRE at the expense of other (saprophytic) bacteria. These procedures allow multiplication of the pathogens which facilitates isolation of the bacteria as well as the bacterial genomic DNA.

Biochemical characterisation of *Pectobacterium* and *Dickeya* species

In the past, biochemical tests were commonly used to differentiate *Pectobacterium* and *Dickeya* species from other bacteria. As the procedures are cumbersome and time consuming, they tended to be replaced by more rapid serological and molecular methods described below. Biochemical methods often are based on a restricted number of characters and, by ignoring the natural variability within taxa, run the risk of closely related species or subspecies being missed. Nowadays, the biochemical approach is mainly used to confirm results obtained with other less cumbersome methods. The tests used are usually those previously described (Hyman *et al.*, 1998). The most commonly applied biochemical and physiological tests are summarised in Table 1. These tests, however, can give rise to ambiguous results, which can be avoided by using freshly grown, pure cultures of the test bacteria. It is worth mentioning that at the moment there are no reliable biochemical tests available for distinguishing Pcb from Pcc and Pwa due to high strain variation (van der Merwe *et al.*, 2010).

As a shortcut, it is common to apply only a restricted number of key tests when the presumed identity of the target isolates is known: catalase and oxidase activity and oxidation/fermentation tests (oxidative/fermentative metabolism of carbohydrates; Pérombelon & van der Wolf, 2002). *Pectobacterium* and *Dickeya* species possess catalase but not oxidase activity and are able to utilise carbohydrates both via fermentation and oxidation. In addition, it is advisable to determine the ability to macerate potato tuber tissue in potato slice or tuber assays as well as to perform the Gram staining test.

Table 1 Selected physiological and biochemical tests used to discriminate the most common soft rot *Enterobacteriaceae* (Pba, Pcc, Pwa, Pcb and *Dickeya* spp.) (after Pérombelon & van der Wolf, 2002; Baghaee-Ravari *et al.*, 2011)

Test	Pba	Pcc	Pwa	Pcb	<i>Dickeya</i> spp.
Cavity formation on CVP (24 h, at 28°C)	+	+	+	+	+
Growth in nutrient agar at 37°C	–	+	–/+	+	+
Growth in 5% NaCl	+	+	–/+	+	–
Sensitivity to erythromycin	–	–	–	–	+
Production of reducing substances from sucrose	+	–	–	–	–
Production of indole	–	–	–	–	+
Production of phosphatase	–	–	–	–	+
Acid production from lactose	+	+	–	+	+
Acid production from maltose	+	–	–	+	–
Acid production from α -methyl glucoside	+	–	–	+	–
Acid production from trehalose	+	+	+	+	–
Acid production from sorbitol	–	–	–	–	–
Utilisation of malonate	–	–	–	–	+

'+', indicates positive reaction; '–', indicates negative reaction; '–/+', indicates ambiguous reaction.

Determination of *Dickeya* spp. biovars with biochemical methods

Former classification of *Dickeya* spp. was based on physiological/biochemical tests which allowed dividing the previous *Erwinia chrysanthemi* (*Dickeya* spp.) species into seven biovars (biochemically distinct groups). Later, two additional biovars (biovars 8 and 9) of *Dickeya* spp. were described (Samson & Nassan-Agha, 1978). Palacio-Bielsa *et al.* (2006) introduced a modified *Dickeya* sp. biovar determination system and later two further biochemical tests were added (Palacio-Bielsa *et al.*, 2006; Sławiak *et al.*, 2009). The results of the tests, however, can sometimes be ambiguous.

Although identification of biovars may still play an important role in the differentiation of the bacterial isolates in some cases, the biochemical tests' data cannot be relied on their own and other complementary tests are desirable, preferably based on molecular methods (Boccaro *et al.*, 1991). Nowadays, the biovar classification system tends to receive less attention due to the development of much more precise molecular detection and differentiation techniques (Pritchard *et al.*, 2012).

NGM medium for differentiation of *Dickeya* spp. from *Pectobacterium* spp.

In 1966, it was first reported that *Dickeya* spp. produce a blue pigment called indigoidine, which gives the

bacteria protection from reactive oxygen species during plant infection (Starr *et al.*, 1966). This observation led Lee & Yu (2005) to develop the NGM medium, containing glycerol and manganese chlorite, on which *Dickeya* spp. form blue colonies. Other SRE do not produce the blue pigment (Lee & Yu, 2005). The medium was used successfully to differentiate *D. solani* and *D. dianthicola* from *Pectobacterium* spp. isolated from symptomatic plants (Van Vaerenbergh *et al.*, 2012). However, not all strains of *Dickeya* spp. produce the blue pigment when grown on this medium (R. Czajkowski, unpublished observations), therefore its usefulness may be questionable and results must be confirmed by another method [e.g. species-specific (real-time) PCRs].

Fatty acid methyl ester analysis

Pectobacterium spp. was reported to produce at least 10 different fatty acids, with chain lengths between 12 and 18 carbon atoms (Dawyndt *et al.*, 2006), which have been used to develop an identification system (FAME). This allows differentiation of the various *Pectobacterium* spp. and identification of unknown pectinolytic bacterial strains based on the ratios between different fatty acids (de Boer & Sasser, 1986). Cother *et al.* (1992) used FAME to identify *Dickeya* spp. isolates in alpine water: all isolates contained *cis*-9-hexadecanoic, hexadecanoic, tetradecanoic acids and most strains contained also dodecanoic acid (Cother *et al.*, 1992). Although FAME analyses allow distinction of *Dickeya* spp. from *Pectobacterium* spp., the assay is not suitable to differentiate *Dickeya* to species and subspecies (van Der Wolf *et al.*, 2013)

Volatiles profiling

Volatile metabolites have been used for the detection of *Pectobacterium* and *Dickeya* species (Varns & Glynn, 1979). Several volatiles were reported to be produced by SRE, for example acetaldehyde, ethanol, 1-propanol, acetone 3-hydroxy-2-butanone, 2-butanone and ethanol (Lui *et al.*, 2005). However, not all can be attributed to *Pectobacterium* and *Dickeya* species as some were also detected in uninfected plant tissue. Only few could be related solely to the presence of the *Pectobacterium* spp. bacteria. Liu *et al.* (2005) reported that acetic acid ethenyl ester (vinyl acetate) was uniquely associated with Pba and cyclohexene, diazene and methoxy-(1,1-dimethyl-2-hydroxyethyl)-amine were exclusively associated with the Pcc presence on potato. Although several other specific volatile metabolites have been described, it is difficult to assess the practical impact of this discovery. The first electronic sensor system (so-called 'electronic nose') was developed in

2000 and contains three independent sensors to detect compounds produced by *Pectobacterium* spp. during potato tuber infections in storage. The sensor system was reported to detect even latent infection in potato tubers artificially contaminated with *Pectobacterium* spp. at 85% relative humidity and at 4°C (de Lacy Costello *et al.*, 2000). If this is confirmed, the method could be useful in commercial bulk-stored potatoes where massive tuber soft rot within the stock is a constant threat.

Serological (immunological) methods

In general, immunological methods for detection of *Pectobacterium* and *Dickeya* species are based on a comprehensive knowledge of the existing serogroups. For serological grouping of *Pectobacterium* and *Dickeya* species, LPS (lipopolysaccharides) O antigens have been considered as the most useful. For Pba, eight different serogroups have been described with the prevalence of the serogroup I strains in Scotland and the Netherlands (de Boer & McNaughton, 1987). For *Dickeya* spp., nine serogroups have been described although only serogroup O₁ *Dickeya* spp. strains were isolated from potato in Europe (Samson & Nassan-Agha, 1978). In the case of Pcc, the situation is more complex due to occurrence of a large number of different O serogroups present in strains infecting potato in Europe. So far, this heterogeneity of O serogroups has prevented the development of good Pcc detection methods based on antibodies and antigens interactions (de Boer *et al.*, 1979). The major problems related to the use of polyclonal and monoclonal antibodies are listed below.

For polyclonal antibodies, the major drawback is that they show low specificity as they can cross-react with some non-target pathogens due to the presence of shared somatic and flagella antigens as well as other cellular antigens/antibodies. Furthermore, *Pectobacterium* and *Dickeya* species are serologically heterogeneous and in consequence not all isolates would react specifically with polyclonal antibodies. In contrast to the polyclonal, monoclonal antibodies exhibit unique, overall high, specificity to a single epitope. This may, however, involve an increasing risk of false-negative reactions, in case of serological variation of the pathogen. The relatively high production costs of monoclonal antibodies are considered as another important obstacle for its use (de Boer *et al.*, 1987).

Monoclonal antibodies have been produced, evaluated and used in several studies relating to detection and identification of *Pectobacterium* spp. in general but specifically of Pba (Gorris *et al.*, 1994). Polyclonal and monoclonal antibodies specific to extracellular pectate lyases (PL) from Pcc have been produced, and tested

for their potential in the detection and identification of soft rot and blackleg causing Pcc, Pba and *Dickeya* spp. (Klopmeier & Kelman, 1988).

The polyclonal antibodies raised against PL from Pba not only reacted readily with the enzyme from *Pectobacterium* spp. and *Dickeya* spp. but also cross-reacted with PL from pectinolytic *Pseudomonas* spp. and *Xanthomonas* spp. However, the PL monoclonal antibodies showed high specificity to SRE but not with other PLs, neither from different genera, nor with commercially available enzyme preparations (Klopmeier & Kelman, 1988). Detection is possible only when PL has been produced, hence latent infection would be missed.

In general, the immunological assays do not require expensive laboratory equipment, therefore they can be used everywhere, where simple, cheap and relatively sensitive and specific detection on a large scale is desirable, especially under field conditions. Different antisera are available from several commercial sources.

Early use of antibodies: double immunodiffusion

Polyclonal antibodies have long been used for the detection and differentiation of soft rot and blackleg causing bacteria in agglutination and precipitation tests (Graham, 1964). One of the initially popular methods is double immunodiffusion (Crowle, 1961). Double immunodiffusion assays have been used to characterise SRE with pure cultures and in symptomatic tissues (De Boer, 1983). The technique is simple and may still be of value in field studies if specialised laboratories and procedures cannot be used. However, with the improvement in immunological methods which followed, these tests are rarely if ever used at present.

Immunofluorescence staining and immunofluorescence colony staining

Immunofluorescence staining (IF) or fluorescent antibody staining (FAS) method is based on the application of the antibodies conjugated to a fluorophore (e.g. fluorescein and its derivatives – isothiocyanate FITC; rhodamin) which bind to bacterial cell walls (e.g. proteins, exopolymeric substances, lipopolysaccharides) hence facilitating recognition.

Immunofluorescence staining was used in soft rot plant pathogenic bacteria, such as Pba and Pcc. Its sensitivity is poor, *ca.* 10^6 cells mL⁻¹, but it allows detection of SRE in mixed populations, 10^4 cells mL⁻¹ of Pcc and 4×10^3 cells cm⁻² of Pba on potato plant leaf surface (Allan & Kelman, 1977, Phillips & Kelman, 1982). In addition, immunofluorescence stained Pcc cells were detected in spiked soil samples after 1 day of incubation and in

artificially infected insects or rotting potato tubers (Allan & Kelman, 1977). Immunofluorescence staining was also used to study the role of insects in the dissemination of Pcc in potato fields (Phillips & Kelman, 1982).

Immunofluorescence colony staining (IFC) is a more sensitive variant of the immunofluorescence staining in which bacterial colonies, not individual bacterial cells, are stained with antibodies conjugated with fluorescent dyes. In addition, it allows isolation of viable bacteria if required while IF does not. Immunofluorescence colony staining was used to detect Pba in infested potato peel extracts with a detection limit of *c.* 10 – 50 cells mL⁻¹ and to detect *Dickeya* spp. in cattle slurry with sensitivity of 10^2 cells mL⁻¹ (Jones *et al.*, 1994).

The major problem of applying IF and IFC methods for diagnostic purposes is that antibodies used for detection of *Pectobacterium* and *Dickeya* species may not be sufficiently specific, thereby they allow the detection of non-target (saprophytic) bacteria present in the same environment or fail to detect serological variants of the bacterial species. Therefore, although the risks of cross-reactions between anti-*Dickeya* and anti-*Pectobacterium* spp. antibodies and antigens of saprophytic bacteria are low, it is advisable to confirm the results using non serological tests (van der Wolf *et al.*, 1996b).

Enzyme-linked immunosorbent assay and enrichment enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) has been mainly used for detection of SRE in a double antibody sandwich format. More complex ELISA systems (e.g. with IF) have been developed but they all suffer from low sensitivity, with a detection lower limit of 10^5 – 10^6 cells g⁻¹ of plant material (Pérombelon & Hyman, 1995). In addition, ELISA assays fail to distinguish between viable and nonviable *Pectobacterium* and *Dickeya* species cells and, as in case of IF and IFC, they are also prone to cross-reactions with non-target bacteria (van der Wolf & Gussenhoven, 1992).

The sensitivity could be increased 1000-fold by incubating the test sample in an enrichment medium selective for *Pectobacterium* and *Dickeya* species (usually PEB) prior to testing (Lopez *et al.*, 1997). The enrichment-ELISA can be made quantifiable by a most probable number approach in which replicates of serially diluted test samples are verified. Up till now, ELISA and enrichment ELISA are used routinely in plant protection laboratories worldwide to detect bacteria in agriculture.

Nucleic acid-based molecular detection methods

Molecular detection methods based on the analysis of bacterial genomic DNA have become most frequently used

to detect and differentiate tuber soft rot and blackleg pathogens in environmental samples. They consist of amplification of target-specific sequences by PCR assays of different formats including LAMP (loop-mediated isothermal amplification) (see below). The PCR-based assays enable faster and cheaper detection of the pathogen than the standard plating methods, but they require specific and often expensive equipment. In comparison with serological assays they are more specific as PCR primers can be designed with specificity for the target genus, or (sub) species level. There are, however, well-reported limitations. Firstly, the procedures are based on the detection of nucleic acids that can be often relatively more stable in the environment than viable bacterial cells (Willerslev & Cooper, 2005). Consequently, viable cells cannot be reliably distinguished from non-viable cells (Keer & Birch, 2003). This limitation has been partially overcome by the use of compounds that can specifically bind DNA released from dead cells such as propidium monoazide (Gorshkov *et al.*, 2009). Secondly, there is still the possibility of false-positive reactions with non-targets with similar sequences, yet undetermined or not published in international databases. Furthermore, variation in the bacterial population genome may result in false-negative results.

As from the 1990s, several methods based on DNA amplification for detection of *Pectobacterium* and *Dickeya* species have been developed as well as new PCR variations, including multiplex PCR, real-time PCR, padlock probes and LAMP. DNA suitable for specific amplification of SRE sequences can be isolated from plant extracts by several standard DNA extraction procedures as well as by protocols using commercial kits, among which some are suitable for robotized DNA extraction able to process 96 samples simultaneously (Smit *et al.*, 2001). Pure bacterial cell lysates obtained by boiling in water or 5 mM NaOH may also be used as templates for PCR reactions which would allow omitting the DNA isolation step. The detection threshold of procedures based on direct isolation of total bacterial genomic DNA from suspected plants or tubers is relatively high (Smit *et al.*, 2001). Detection limits vary from 1 to 15 cells mL⁻¹ bacterial suspensions in water (Pritchard *et al.*, 2012) to 10⁵ cells mL⁻¹ from some potato cultivars possibly due to the presence of PCR inhibiting factors (Diallo *et al.*, 2009). For increased sensitivity, bacteria may be enriched prior to DNA isolation as mentioned above (Pérombelon & van der Wolf, 2002).

Despite these drawbacks, DNA-based amplification methods are now the most widely used in modern laboratories for detection and differentiation of *Pectobacterium* and *Dickeya* species. New assays for specific detection of important species and subspecies of plant pathogenic SRE are continuously being developed. The most commonly

used primer sets developed for detection of *Pectobacterium* and *Dickeya* species are listed in Tables 2 and 3.

PCR-based detection with species-specific primers

Selection of appropriate target genes was performed with different approaches and in the past the most frequently selected genes were those associated with virulence including: *pelADE* (Nassar *et al.*, 1996), *pel* genes (Darrasse *et al.*, 1994), *pelY* (Fréchon *et al.*, 1998), *pell* (Diallo *et al.*, 2009) and *cfa6* (Li *et al.*, 2011), or the house-keeping genes, for example *rhsA* taking part in cell wall biogenesis (Park *et al.*, 2006). Others were determined as a result of an extensive phylogenetic study based on 16S rDNA and 16S-23S intergenic transcribed region (Duarte *et al.*, 2004). Some primers were developed not from a defined gene of interest but rather on selected piece of bacterial genomic DNA of unknown function. For example, the *Eca1f* and *Eca2r* primers, specific for *Pba* (de Boer & Ward, 1995), were selected on the basis of the sequence of the DNA hybridization probes for detection of *Pectobacterium* spp. described earlier (Darrasse *et al.*, 1994). Primers EXPCCF and EXPCCR, specific for *Pcc* and *Pwa* strains, were selected based on the URP-PCR fingerprinting result in which a common single band produced by all *Pcc* and *Pwa* strains was found (Kang *et al.*, 2003). Recently, Pritchard *et al.* (2012) presented a bio-informatic tool allowing easy prediction of primer sets for specific detection of species and subspecies of bacteria based on the raw genome sequence information. In this approach, the algorithm is used to predict regions in the genome which are useful to design the specific primers (Pritchard *et al.*, 2012). It seems that this approach may be advantageous over the classic one in which firstly the gene of interest needs to be found and then the primers developed on the basis of its nucleotide sequence. Notwithstanding the above, several PCRs developed in the early 1990s still remain widely used and recognised as the 'gold standard' in molecular detection of *Pectobacterium* and *Dickeya* species bacteria.

Multiplex PCR

There are a few multiplex PCR assays available for the detection of soft rot and blackleg pathogens (Peters *et al.*, 2007; Diallo *et al.*, 2009). The majority of the assays include simultaneous detection of several *Dickeya* spp. and *Pba*, which for a long time were considered as the foremost pathogens in both hot and temperate climatic regions, respectively. Now the situation is blurred due to worldwide potato seed trade and introduction of SRE from other hosts (Peters *et al.*, 2007; Diallo *et al.*, 2009). Recently, a more comprehensive multiplex PCR assay

Table 2 Methods used for the isolation, detection and differentiation of soft rot *Pectobacterium* and *Dickeya* spp. in practical applications and research

Method	Application	Sensitivity, Detection Level	Remarks	Reference
CVP plating	Isolation of pure bacterial colonies and viable bacterial cells	Depending on the sample source and presence of other microorganisms, recovery of bacteria on the medium up to 100%	Very rarely <i>Pectobacterium</i> and <i>Dickeya</i> spp. isolates may not grow on the medium or do not produce cavities; incubation up to 5 days at optimal temperature is required to assess the cavity formation; pectin source can affect the quality of the medium	Cuppels & Kelman (1974) and Hellas <i>et al.</i> (2011)
Enrichment in PEB medium	Enrichment of low bacterial populations of <i>Pectobacterium</i> spp. and <i>Dickeya</i> spp., useful for isolation of bacteria from environmental samples	Frequently even 1–10 bacterial cell are enriched to the detectable densities: usually 10 ² –10 ³ cells mL ⁻¹ bacteria should be present in starting material	Despite medium selectivity, other bacterial species antagonistic to <i>Pectobacterium</i> and/or <i>Dickeya</i> spp. maybe also enriched, hence false-negative results	Pérombelon & van der Wolf (2002)
PCR with species-specific primers	Differentiation of <i>Pectobacterium</i> and <i>Dickeya</i> spp. from other bacteria, differentiation of <i>Pectobacterium</i> from <i>Dickeya</i> spp. Can be applied in a singleplex and multiplex setting for detection of several pathogens	From 1 cfu mL ⁻¹ to 10 ⁵ cells mL ⁻¹ depending on assay, primer sets, conditions; an average detection limit is 10 ⁴ cells mL ⁻¹ in plant extracts	In general PCR works better on pure bacterial colonies or purified genomic DNA, false-positive reactions possible, the results should be confirmed with other methods, the assays detect also nonviable bacterial cells and DNA	Please see the Table 3 for reference
Real-time PCR (TaqMan™, SYBR green)	Quantitative and qualitative detection of bacteria, for TaqMan™, additionally assays with higher specificity due to the presence of probe complementary to the target DNA. TaqMan assays be applied in a singleplex and multiplex setting for detection of several pathogens	Pure genomic DNA of good quality is prerequisite	Relatively expensive for routine use, samples need to be investigated in duplicates or triplicates together with positive and negative controls	Please see the Table 3 for reference
Repetitive Sequence-Based PCR (REP-PCR)	Differentiation of <i>Pectobacterium</i> and <i>Dickeya</i> spp. isolates	High concentration of pure genomic DNA of good quality is prerequisite, viable bacterial cells used for DNA isolation are necessary	High resolution, even closely related species can be differentiated from each other, results on two different gels cannot be directly compared, the assay should include control designated strains	Versalovic <i>et al.</i> (1994)
Pulsed field gel electrophoresis (PFGE)	Differentiation of <i>Pectobacterium</i> and <i>Dickeya</i> spp. isolates	High concentration of pure genomic DNA of good quality is prerequisite,	High resolution, even strains can be differentiated from each other, results on two different gels cannot be directly compared, the assay should include control designated strains	Lee <i>et al.</i> (2006) and Kim <i>et al.</i> (2009)
Restriction Fragment Length Polymorphism-PCR (PCR-RFLP)	Differentiation of <i>Pectobacterium</i> and <i>Dickeya</i> spp. isolates	High concentration of pure genomic DNA of good quality is prerequisite, viable bacterial cells used for DNA isolation are necessary	Results on two different gels cannot be directly compared, the assay should include control designated strains, relatively good resolution	Boccaro <i>et al.</i> (1991) and Waleron <i>et al.</i> (2002)
Multi Locus Sequence Tagging (MLST)	Differentiation of <i>Pectobacterium</i> and <i>Dickeya</i> spp. isolates	Qualitative detection	Selection of good target genes may be difficult	Pitman <i>et al.</i> (2010), De Boer <i>et al.</i> (2012) and Waleron <i>et al.</i> (2013)

Table 3 PCR primer sets used to detect *Pectobacterium* spp. and *Dickeya* spp. bacteria with conventional, multiplex and real-time assays

Species/Subspecies	Assay Type	Primers (5'→3') (name, sequence)	PCR Product Size (bp)	References
<i>Dickeya</i> spp. and <i>Pectobacterium</i> spp.	Conventional	SR3F GGTGCAAGCGTTAATCGGAATG SR1cR AGACTCTAGCCTGTCAGTTTT	119	Toth et al. (1999b)
	Conventional RFLP	G1 GAAGTCGTAACAAGG L1 CAAGGCATCCACCGT		Toth et al. (2001)
<i>Dickeya</i> spp.	Conventional RFLP	ADE1 GATCAGAAAGCCCGCAGCCAGAT ADE2 CTGTGGCCGATCAGGATGGTTTTGTCGTGC	420	Nassar et al. (1996)
	Conventional RFLP	pelZ-1-F ATGAAACATACCCTTCTGTTTGC pelZ-1-R TTATTCCAGATCTTTGGCCAT	1263	Lee et al. (2006)
	Conventional	5A GCGGTTGTTACCAGGTGTTTT 5B ATGCACGCTACCTGGAAAGTAT	nd.	Chao et al. (2006)
	Real-time PCR	Df AGAGTCAAAAGCGTCTTG Dr TTTCACCCACCGTCAGTC	133	Laurila et al. (2010)
	Real-time PCR (TaqMan)	ECHf GAGTCAAAAGCGTCTTGCGAA ECHr CCCTGTTACCGCCGTGAA Probe ECH CTGACAAGTGATGTCCCTTCGTCTAGAGG	nd.	Pritchard et al. (2012)
	Real-time PCR (TaqMan)	DIA-Af GGCCGCTGAATACTACATT DIA-Ar TGGTATCTCTACGCCATCA Probe ATTAACGGCGTCAACCCGGC DIA-Cf CCAACGATTAGTCGGATCT DIA-Cr TAGTTGGTGCCAGGTTGGTA Probe DIA-C TCGACGTATGGGACGGTCGC	nd. nd.	Pritchard et al. (2012)
<i>Dickeya solani</i>	Real-time PCR (TaqMan)	SOL-Cf GCCTACACCATCAGGGCTAT SOL-Cr ACATACAGCGCGCATAAAC Probe Sol-C CCAGGCCGTGCTCGAAATCC SOL-Df GCCTACACCATCAGGGCTAT SOL-Dr CACTACAGCGCGCATAACT Probe SOL-D CCAGGCCGTGCTCGAAATCC	nd. nd.	Pritchard et al. (2012)
<i>Dickeya solani</i>	Real-time PCR (TaqMan)	dsf GCGAACTTCAACGGTAAA dsr CAGAGCTACCAACAGAGA Probe CTCTGCTGGACGGTTC	112	Van Vaerenbergh et al. (2012)
<i>Pectobacterium</i> spp.	Conventional	Y1 TTACCGGACGCCGAGCTGTGGCGT Y2 CAGGAAGATGTCGTTATCGCGAGT	434	Darrasse et al. (1994b)
Pba	Conventional	Y₄₅ TCACCGGACGCCGAACTGTGGCGT Y₄₆ TCGCCAACGTTTCAGCAGAAACAAGT Eca1 CGGCATCATAAAAACACG	439	Fréchon et al. (1998)
		Eca2 GCACACTTCATCCAGCGA PEAF CCGGTAATTCAAGCTAATCC PEAR CCTTACCTATCGTTCTCCT	690	de Boer & Ward (1995)
		904	Park et al. (2006)	
	LAMP	BIP (B1 TCCCACAGTGTACCAAGTTG + T linker + B2 TCGGCAGCCTATTCCTCTG) FIP (F1c GTCGCAGCTCCGTTGAAGA + T linker + F2c AGCAAGCCTACCATCCCA) B3 GCATTCTGTAGGCGAAGCG F3 TGCTGTTGATAGCGGCAAT LoopF1 GTTACGCCGTTACTCAAAGA LoopB1 GCGTCTCTTGGCTTAAATA	Variable	Li et al. (2011)
	Conventional	BR1f GCGTGCCGGGTTTATGACCT L1r CA(A/G)GGCATCCACCGT	690	Duarte et al. (2004)
	<i>Pectobacterium carotovorum</i> subsp. <i>brasiliensis</i>	Conventional		
Pcc and Pwa	Conventional, nested	ExpccF GAACTTCGCACCCGCCGACCTTCTA ExpccR GCCGTAATTGCCTACCTGCTTAAG INPCCF GGCCAAGCAGTGCCTGTATATCC INPCCR TTCGATCACGCAACCTGCATTACT	550 400	Kang et al. (2003)

Table 3 Continued

Species/Subspecies	Assay Type	Primers (5' → 3') (name, sequence)	PCR Product Size (bp)	References
Pwa	Conventional	Contig 1F CCTGCTGGCGTGGGGTATCG Contig 1R TTGCGGAAGATGTCGTGAGTGCG Contig 3F GCATTGACCAGTTTCGCCAGTTAC Contig 3R CTTTTGAGCAGCGCGGGTTGTG	258 132	de Haan <i>et al.</i> (2008)
	Conventional and real-time PCR	PW7011F CTATGACGCTCGCGGGTTGCTGTT PW7011R CGGCGGCGTCTAGTGAAAGTC	140	Kim <i>et al.</i> (2011)
Pba, <i>Dickeya</i> spp.	Conventional, multiplex	ERWFOR ACGCATGAAATCGGCCATGC ATROREV ATCGATAATTTGATTGTCCT CHRREV AGTGCTGCCGTACAGCACGT	389	Smid <i>et al.</i> (1995)
	Multiplex	Y₄₅ TCACCGGACGCCAACTGTGGCGT Y₄₆ TCGCCAACGTTTCAGCAGAACAAGT Ech1 TGGCGCGTCAGGAAGTTTAT Ech1' TCACCGGTCAGGGTGAAGTT	420 600	Diallo <i>et al.</i> (2009)
	Multiplex	Pca for GATCGGCATCATAAAAACACG Pca rev CGCACACTTCATCCAGCGAG Dcd Forw GAAAGCCCGCAGCCAGATC Dcd Rev TCAGGATGGTTTTGTCATGC	690 420	Peters <i>et al.</i> (2007)

nd., not determined (the information is absent in the publication).

has been developed which allows simultaneous detection of the four major blackleg and tuber soft rot pathogens of potato but not all to subspecies level, namely *Dickeya* spp., Pba, Pcc and Pwa (M. Potrykus, unpublished information).

The detection limits of the multiplex PCRs are usually comparable with the conventional single pathogen detection assays. In the presence of other bacteria (e.g. saprophytes), however, the limits may sometimes decrease ten-fold compared to simplex reactions (Smid *et al.*, 1995).

Real-time PCR

Real-time PCR procedures for detection of SRE were developed to enhance specificity, reliability and for quantification of the target pathogen. At present, there are only two real-time PCR assays using SYBR green developed for detection of SRE, that is *Dickeya* spp. (Laurila *et al.*, 2010) and Pwa (Kim *et al.*, 2011) (Table 3).

Real-time PCR assay has a great potential as it combines identification and quantification of pectinolytic *Pectobacterium* and *Dickeya* species simultaneously and directly from the plant material (Table 3). In contrast to conventional end-point PCR, the real-time assay allows quantification of the template at each cycle. The new primer sets for the real-time assays developed by Pritchard *et al.* (2012) on the basis of the raw genome sequence from various *Dickeya* spp. strains is able to differentiate *D. dianthicola* from *D. solani*.

The attempts to design primers for specific detection of Pcc and Pba in the real-time PCR assays have been undertaken (Takle *et al.*, 2007) and would be useful for

the routine detection of the bacteria in environmental samples. Meanwhile, several TaqMan[®] (Roche Molecular Systems, Pleasanton, California, USA)-based assays have also been developed and used for generic detection of SRE and for the specific detection of Pba, but have not been published in peer reviewed journals (J.G. Elphinstone, FERA, York, UK, unpublished results, J. M. van der Wolf, PRI, Wageningen, the Netherlands, personal communication). Thus, in 2009, NAK (the Netherlands General Inspection Service for Agricultural Seeds and Seed Potatoes) introduced a multiplex TaqMan-based assay (BioPlex Real-Time PCR) for the simultaneous detection of *Pectobacterium* and *Dickeya* species (de Haan & van den Bovenkamp, 2009). The assay was designed in fourplex, meaning that it can detect four groups of bacteria: SRE (*Pectobacterium* and *Dickeya* species but without differentiation of the genus), Pba, *Dickeya* spp. and Pwa using four specific TaqMan probes. Unfortunately, no information was provided about the probes and target genes used. The assay is used routinely by the NAK to check high grade potato seed in the Netherlands for contamination with *Pectobacterium* and *Dickeya* species. It is claimed that it is possible to detect 10⁴ cells mL⁻¹ plant extracts and 10² cells mL⁻¹ following enrichment in PEB medium prior to analysis (de Haan & van den Bovenkamp, 2009).

Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) assay was developed for the specific detection and identification of Pba (Li *et al.*, 2011) and Pcc (Kim *et al.*, 2011) but has not been used widely elsewhere. Li *et al.* (2011)

developed the LAMP assay using a gene cluster coding for a pathogenicity-related phytotoxin similar to coronafacic acid of *Pseudomonas syringae*. The assay allowed differentiation of Pba from other *Pectobacterium* and *Dickeya* species and could detect down to 10^2 cells mL⁻¹ bacteria.

Padlock probes

So far, there is only one publication describing the use of padlock probes (PLP) for the detection of SRE: Pba, Pwa and *Dickeya* spp. (Sławiak *et al.*, 2013). Sławiak *et al.* (2013) designed PLP assays on the basis of *recA* and 16S rDNA gene sequences, which resulted in a very sensitive (detection limit 5 pg of bacterial genomic DNA and/or 500 bacterial cells per reaction) PLP assay. The PLP were also used to discriminate between all target bacteria simultaneously. The method seems to have many advantages over other molecular detection methods; however, it is too early to say whether the PLP assays would be used routinely to detect SRE bacteria in the future.

Molecular fingerprinting methods

Bacterial differentiation methods based on the comparison of the number and size (pattern) of the DNA fragments obtained from the bacterial genome following separation in and visualisation on agarose gels, polyacrylamide gels or nitrocellulose sheets are commonly known as DNA fingerprinting.

For each of the techniques, it is crucial to use a set of reference strains as controls, which should at least include the type strains of the (sub)species being studied. The use of a globally accepted set of reference strains is advocated.

Repetitive sequence-based PCR

Repetitive sequence-based PCR (REP-PCR) has been developed to target the repetitive sequences present in bacterial genomes, namely Repetitive Extragenic Palindromic elements (REP), Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) and BOX elements (BOX-PCR). Versalovic *et al.* (1994) developed primers specific to the various repetitive sequences present in bacterial genomes. The relative resolution of the generated patterns is high and allows phylogenetic classification of SRE from genus down to strain level. REP-PCR-based techniques have been used extensively for the classification and characterisation of *Pectobacterium* and *Dickeya* species strains. The REP-PCR analyses were used for example in studies on the classification of a new clade of *Dickeya* spp. biovar 3, *D. solani* (Degefu *et al.*, 2013) and to characterise different *Pectobacterium* and *Dickeya* species in potato in South Africa and Zimbabwe (Ngadze *et al.*,

2012). The technique is easy to perform and its resolution is high.

Pulsed field gel electrophoresis

The application of pulsed field gel electrophoresis (PFGE) allowed identification of specific patterns common for all *Dickeya* spp. isolates from *Zantedeschia aethiopica* in Taiwan (Lee *et al.*, 2006). Pulsed field gel electrophoresis was also able to discriminate *Pectobacterium* spp. clades in diseased stems and tubers of potato plants originating from different fields in Korea (Kim *et al.*, 2009). Pulsed field gel electrophoresis was also successfully applied to confirm the identity and homogeneity of *D. solani* strains isolated in Finland and Israel (Degefu *et al.*, 2013; Tsror *et al.*, 2013).

Restriction fragment length polymorphism of amplified PCR product

Restriction fragment length polymorphism of amplified PCR product (PCR-RFLP) may be applied specifically for the identification and differentiation of closely related isolates within species and subspecies. To differentiate between *Pectobacterium* and *Dickeya* species, two groups of genes were mainly used; those coding for virulence factors (e.g. *pel* genes, *hrp* genes) and the housekeeping genes (e.g. 16S rDNA, 23S rDNA, *recA*, *gyrA*, *gyrB*, *rpoS*, and *dnaX*). Boccara *et al.* (1991) were the first to use PCR-RFLP based on pectinase coding genes to differentiate *Dickeya* spp. isolates. In comparison with biochemical assays used to classify the SRE, the PCR-RFLP was more discriminative. Darrasse *et al.* (1994) applied *pel* gene-based PCR-RFLP to classify *Pectobacterium* spp. isolates. The *pel* PCR product was digested with several restriction endonucleases in independent reactions and the products were compared on agarose gel. It was shown that with the PCR-RFLP method, previous results were confirmed that Pba isolates form a homogenous group while isolates of *P. carotovorum* are more genetically diverse suggesting a polyphyletic origin. To differentiate *Dickeya* spp., a *pelADE* gene cluster was used on 78 *Dickeya* spp. strains which could be separated into 16 RFLP patterns grouped in six clusters almost exclusively correlated with the biovar and pathovar grouping, as well as with the geographical distribution and relation to the plant host (Nassar *et al.*, 1996).

Waleron *et al.* (2002) applied a PCR-RFLP assay to differentiate pectinolytic *Pectobacterium* and *Dickeya* species on the basis of the housekeeping genes *recA* and *rpoS*. Restriction analyses of *recA* gene with four restriction endonucleases revealed the presence of 57 restriction groups (combined RFLP patterns). Additionally, application of the PCR-RFLP allowed the observation of two distinct RFLP profiles for Pba, 16 profiles for Pcc, one RFLP

profile characteristic for *P. betavasculorum*, Pco and Pwa (Waleron *et al.*, 2002). In the case of *Dickeya* spp., 14 separate *recA* PCR-RFLP restriction groups were identified. The high degree of differentiation within the Pcc and *Dickeya* spp. was later confirmed by other researchers and resulted in establishment of separate genera (Gardan *et al.*, 2003; Samson *et al.*, 2005).

Amplified fragment length polymorphism

Specific amplified fragment length polymorphism (AFLP) patterns were obtained for the majority of *Pectobacterium* and *Dickeya* species and subspecies, namely for Pcc, Pba, Pwa, *P. betavasculorum*, *P. carotovorum* subsp. *odoriferum* and *Dickeya* spp. (Avrova *et al.*, 2002). Yishay *et al.* (2008) used the AFLP method to characterise Pcc isolates from monocot and dicot plants from diverse geographical locations, Nabhan *et al.* (2012) to differentiate strains belonging to *Pectobacterium* spp. isolated in Germany and in South Africa, and Ngadze *et al.* (2012) to identify *Pectobacterium* spp. isolates from Zimbabwe, such as Pcb, Pcc, Pba and *D. dadantii*. What is more, AFLP analysis allowed clear division of Pcc from Pcb and grouping the Pcb into 12 clusters, reflecting their geographical origins (Yishay *et al.*, 2008; Nabhan *et al.*, 2012; Ngadze *et al.*, 2012).

In 2010 Pitman *et al.* introduced a modified AFLP method using fluorescent dye-labelled primers (fluorescent AFLP – fAFLP) for the differentiation of *Pectobacterium* spp. in New Zealand. Combining the detection of the fAFLP products with automatic DNA sequencing allowed mechanisation of the procedure without losing the resolution of the standard AFLP method (Pitman *et al.*, 2010).

Random amplified polymorphic DNA

Random amplified polymorphic DNA (RAPD) technique was used to differentiate isolates of Pba and *P. carotovorum* (Mäki-Valkama & Karjalainen, 1994) and Parent *et al.* (1996) applied it to differentiate the Pcc and Pba isolates from the pectinolytic fluorescent *Pseudomonas* spp. strains that can potentially inhabit the same environments.

Phylogenetic analysis based on rDNA gene sequences

Kwon *et al.* (1997) used the 16S rDNA to assess the phylogenetic relationships between *Erwinia amylovora*, *P. betavasculorum*, *P. carotovorum*, Pwa, *Dickeya* spp., *Escherichia coli*, *Pantoea agglomerans* and *Proteus vulgaris*. Soft rot *Enterobacteriaceae* (SRE) bacteria cluster together whereas human and animal pathogenic *Enterobacteriaceae* show higher phylogenetic heterogeneity (Kwon *et al.*, 1997). Fessehaie *et al.* (2002) used the

16S–23S intergenic spacer region to build *Pectobacterium* and *Dickeya* species phylogenetic trees and to compare this method with the use of 16S rDNA. The resolution of 16S–23S-based phylogeny was shown to be more efficient in separating *Pectobacterium* and *Dickeya* species subspecies than when 16S rDNA analysis was applied (Fessehaie *et al.*, 2002).

Ribotyping

A specific PCR-RFLP analysis of the amplified 16S and 23S rDNA sequences encoding the ribosome subunits called ribotyping was also used to differentiate soft rot and blackleg causing bacteria. Nassar *et al.* used ribotyping to successfully classify *Dickeya* spp. isolates into biovars and by the host (monocots and dicots) (Nassar *et al.*, 1994).

Multi locus sequence tagging

Multi locus sequence tagging has been applied to differentiate isolates of *Pectobacterium* and *Dickeya* species and it is recognised as a useful technique for this purpose (Pitman *et al.*, 2010; de Boer *et al.*, 2012; Waleron *et al.*, 2013). The genes that are more commonly utilised in this assay are the housekeeping genes: for example *acnA*, *gapA*, *mdh*, *pgi*, *milD*, *proA*, *rpoS*, *recA* and *dnaX* (Zeigler, 2003).

Young & Park (2007) investigated the phylogenetic relationships of plant pathogenic *Enterobacteriaceae*, namely *Brenneria* spp., *Dickeya* spp., *Enterobacter* spp., *Erwinia* spp., *Pectobacterium* spp. and *Samsonia* spp. using MLST analysis of nucleotide and peptide sequences of *atpD*, *carA* and *recA* genes. Nabhan *et al.* (2012) used the MLST in combination with other methods to distinguish *P. carotovorum* isolates originated from various locations and to prove that some of them need to be reclassified as Pwa. Koh *et al.* (2012) classified atypical *P. carotovorum* isolates to new species *P. carotovorum* subsp. *actinidiae* subsp. nov. on the basis of the MLST analysis on *atpD*, *carA* and *recA* nucleotide sequences.

Whole-genome sequencing, comparative genome analysis and its application for SRE detection

Development of the whole-genome sequencing and (automatic) annotation services resulted in rapid rise of genomic data readily available for SRE detection and identification purposes (Toth *et al.*, 2003; Pritchard *et al.*, 2012). Until now (July 2014), there are 24 *Dickeya* spp. and 22 *Pectobacterium* spp. genomes published at GOLD Genomes Online Database (<http://www.genomesonline.org/index>) and National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>). Some of these are complete annotated

data, but many others are just raw (draft) sequences (e.g. set of contigs) which often need some additional analysis. The average size of the genomes of *Pectobacterium* and *Dickeya* species is estimated to be *c.* 5 Mb which is within the range of most bacterial genomes sequenced so far. The genomic data have already proven to be useful in characterisation of (new) virulence factors (Bell *et al.*, 2002, 2004) and development of new primer pairs for detection of *D. dianthicola* and *D. solani* (Pritchard *et al.*, 2012). Genomics has also been successfully applied to properly identify strain Ecc SCC3193, which for years was a model strain to study molecular biology of Pcc (former *Erwinia carotovora* subsp. *carotovora* Pirhonen & Palva, 1988; Andersson *et al.*, 2000; Koskinen *et al.*, 2012) but recently was reclassified as Pwa (Nykyri *et al.*, 2012). In line, fifteen *Pectobacterium* and *Dickeya* species genomes have been fully sequenced, annotated and used for the comparative analyses (Nykyri *et al.*, 2012).

It is important to point out here, that the availability of complete and annotated genome sequences is expected to facilitate the future development of new whole-genome based detection and differentiation tools for *Pectobacterium* and *Dickeya* species (Pritchard *et al.*, 2012).

Other less established methods used for detection and differentiation of *Pectobacterium* and *Dickeya* species

In this section we briefly summarise the methods that were either developed for detection and differentiation of pectinolytic *Pectobacterium* and *Dickeya* species in the past and have not been widely used or those that, although developed recently, have not become as yet popular. Nevertheless, in our view they are of interest and should be mentioned here.

Flow cytometry

To date, only a couple of publications describe the use of flow cytometry to detect *Pectobacterium* and *Dickeya* species. Golan *et al.* (2010) used the method to count the Pcc cells tagged with the green fluorescent protein (GFP) in artificially inoculated *Ornithogalum dubium* (sun star) plants. The combined use of flow cytometry and GFP-tagging facilitated screening plantlets of a large number of varieties of sun star for resistance to Pcc (Golan *et al.*, 2010). Fröhling *et al.* (2012) used flow cytometry to measure the viability of Pcc after heat treatment to demonstrate that cells which cannot be resuscitated on agar media still remain viable and infectious.

Microsphere immunoassay (Luminex system)

The detection of *Pectobacterium* and *Dickeya* species via microsphere immunoassay (MIA) has been described in

only one publication (Peters *et al.*, 2007) and the method has not been widely used. Peters *et al.* (2007) utilised this system to compare the procedure with ELISA for the detection of *Pectobacterium* and *Dickeya* species bacteria. The microsphere immunoassay was superior to ELISA, being more sensitive with a detection limit of 10^2 cfu mL⁻¹ instead of 10^5 cfu mL⁻¹ and could be performed in 1 h instead of 6 h while detecting both pathogens simultaneously.

Conductometry-based detection of *Pectobacterium* and *Dickeya* species

In 1996, Fraaije *et al.* developed an automatic assay designed to detect viable *Pectobacterium* and *Dickeya* species cells in potato tuber peel extracts in the presence of other bacterial species including *Pseudomonas*, *Bacillus*, *Enterobacter* and *Flavobacterium*. The detection limits were 10^2 – 10^3 cells mL⁻¹ and 10^3 – 10^5 cells mL⁻¹ peel extract for Pba and *Dickeya* spp. respectively (Fraaije *et al.*, 1996). Although the potential success of this approach is linked to its simplicity, low running cost and robustness, it failed to be adopted in *Pectobacterium* spp. and *Dickeya* spp. research (Charkowski, 2006). It appears that it has also been ignored by the vegetable and ornamental industry and hence it is doubtful if the method had ever been validated under commercial production conditions.

Bacteriophage (phage) typing

The first bacteriophage typing system of *Pectobacterium* spp. and *Dickeya* spp. was described late in 1990s (Gross *et al.*, 1991). In that instance, 13 different phages displaying distinct host range specificity against Pcc and Pba isolates were isolated from soil and rhizosphere of potato plants and screened against a collection of 389 *Pectobacterium* spp. strains of known serogroups. It was clear that the phage typing would be of limited use to differentiate the pectinolytic *Pectobacterium* spp. due to the high genetic diversity of the bacterial isolates and hence the need to utilise a large enough number of phages. Thus, Gross *et al.* (1991) concluded that *Pectobacterium* spp. differentiation by the phage typing is less efficient than by serological methods. There is also the risk of development of resistance to a phage which would require finding replacement discriminating phages. In contrast, Toth *et al.* (1999), when comparing RFLP, ERIC, RAPD and phage typing methods to differentiate the more genetically homogeneous Pba isolates, found that the resolution of phage typing methods was the highest. Despite difficulties in isolating suitable range of phages, the method was believed to have a potential to be the method of choice to study Pba ecology (Toth *et al.*, 1999). To our knowledge, no phage typing system has been developed

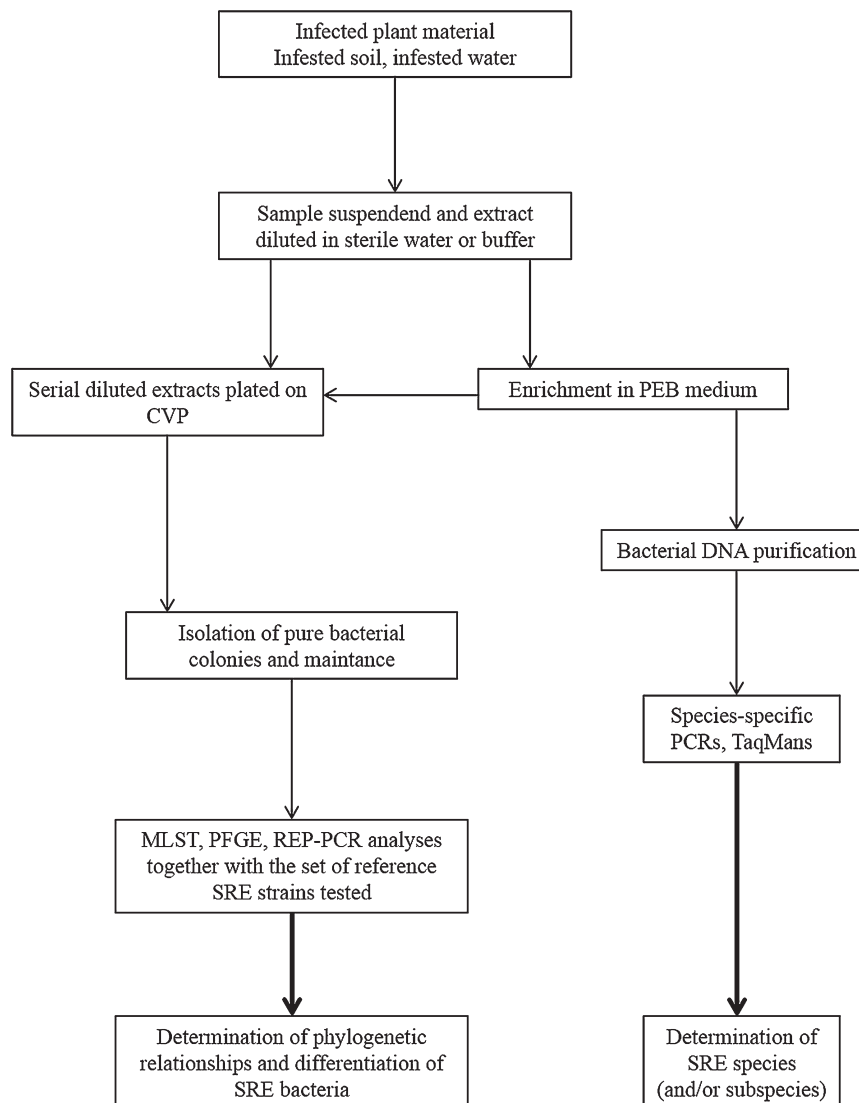


Figure 2 Protocol proposed for the detection and characterisation of SRE bacteria. Description of the methods is given in the text.

for the differentiation of *Dickeya* spp., and phage typing of *Pectobacterium* spp. has not been applied in ecological research.

A proposed set of procedures and methods for the isolation and characterisation of SRE

The protocol described below could be used as a blueprint for the reliable detection and characterisation of the SRE isolates. Briefly, once the test material has been selected, we advise to simultaneously isolate the target bacterial colonies on CVP and to enrich the possible low SRE numbers in latent infections in a selective PEB medium prior to application of PCRs followed by CVP plating or DNA extraction to identify the present *Pectobacterium* and

Dickeya species and subspecies. This should be followed by the utilisation of the molecular techniques: MLST, PFGE and/or REP-PCR to determine the phylogenetic relationships of the isolates (Fig. 2).

These methods are commonly used by researchers worldwide and have been published earlier. We hope that they would be adopted in future research concerning the isolation and characterisation of SRE.

Conclusions and perspectives

In the last 30 years there has been a migration from microbiological, serological and biochemical methods to molecular detection and differentiation techniques and in our view this trend will continue. Regardless of the approach,

the overall aim has remained primarily to detect and characterise *Pectobacterium* spp. and *Dickeya* spp. populations under different conditions accurately and reliably. However, this goal has been achieved only partially, as most of the methods, especially the immunological and molecular ones, usually rely on the use of probes based on a narrow range of features, such as epitopes or DNA sequences. Since most bacterial species exhibit a range of variability, probe specificity is inevitably limited.

Despite those limitations, a wide range of methods and procedures described in this review is now available to tackle the still outstanding problems in the ecology, epidemiology and taxonomy of SRE associated with potatoes and the diseases they cause. Research on such topics as distribution, survival, relative virulence of the different taxa as well as the source and spread of crop contamination over several generations can now be undertaken with greater confidence. The difficulty is mainly in the choice of the methods that would be more appropriate in each instance. Therefore it is vital to choose them depending on the objectives of the study, the expertise and facilities as well as on the financial resources available.

When the objective is a disease control through monitoring of the seed tuber contamination in certification programmes, a general consensus has not yet been reached, largely because of the cost involved. The time honoured method of verifying seed health by field inspection and rogueing of blackleg-affected plants, which is still widely practiced, can at best provide a rough notion of the possible infection. As disease development is largely weather-dependent, the absence of disease symptoms in a crop does not necessarily signify that the crop would be pathogen-free (Pérombelon, 2002). A more meaningful approach would be to determine seed tuber contamination, preferably their incidence too, by different pathogens. At present this approach has been adopted in only a few national certification programmes and then usually is restricted to the higher grade seed. Although suitable methods are now available, the limiting factor remains the cost involved when applied on a large scale. Of the methods which could be deployed commercially for rapid and specific detection and characterisation of the key pathogens, multiplex real-time PCR-based methods appear to fit the bill best by allowing the simultaneous detection of several pathogens. Since direct detection of target DNA in plant material with PCR-based methods often lack sensitivity, multiplication of the target cells in or on growth media prior to DNA detection is desirable. Until the molecular assays based on complete genome sequence of individual taxon become more readily available, identification and differentiation of the soft rot bacteria will

continue to rely on the simultaneous use of several complementary probes/methods whenever possible.

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