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ISOLATION, IDENTIFICATION AND PRESERVATION OF PECTINOLYTIC BACTERIA PATHOGENIC TO POTATO

ABSTRACT

Blackleg of potato plants and soft rot of tubers are caused by several species of pectinolytic bacteria from genera *Pectobacterium* and *Dickeya*. The text describes simple methods of isolating bacteria from symptomatic and symptomless organs of potato plants, their identification using Polymerase Chain Reaction (PCR) and preservation.

Keywords: Blackleg, *Dickeya*, *Pectobacterium*, Polymerase Chain Reaction, selective medium, soft rot

INTRODUCTION

There are several species of soft rot bacteria that are pathogenic to many cultivated plants. Blackleg of potato plants and soft rot of potato tubers are two diseases caused by different species of bacteria (Table 1) within to two genera, *Pectobacterium* and *Dickeya* – formerly called "soft rot erwinia". Bacterial species differ in range of host plants, from narrow *e.g. P. atrosepticum* (Pba), to broad-host-range pathogens, *e.g. P. carotovorum* subsp. *carotovorum* (Pcc). The significance of species in Europe changes in space and time. For a long time *P. atrosepticum* was the main causal agent of blackleg in potato crop (still is in Scotland). *Dickeya* spp. (including *D. solani* (Dsol)) was responsible for 50-100% of blackleg cases in France and The Netherlands in 2005 and 70% of blackleg plants were infected with Dsol in 2008 in Finland (Degefu *et al.,* 2013; van der Wolf *et al.,* 2007). *P. carotovorum* subsp. *brasiliense* (Pcb), which has been an emerging species since 2012 and it is already a major species of potato in some countries (Toth *et al.*, 2011; van der

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Wolf *et al.*, 2016). Diseases caused by pectinolytic bacteria cause severe losses in potato production worldwide. In countries with cold climates such losses are high mostly due to the downgrading of seed potato plantations, and the decline in potato yield, which in countries with warmer climates is even more severe (Sławiak *et al.*, 2009; Tsror *et al.*, 2009, 2012).

There are three phases of soft rot disease in the potato tubers: infection, after infection asymptomatic phase, called latent infection, and symptomatic one, when bacteria produce enzymes which degrade the cell wall in tubers and plants. The most important factors related to severity of the disease are the aggressiveness of the bacteria, susceptibility of plants, temperature and humidity. The symptoms of the disease caused by different bacterial species such as wilting of potato plants, stunted, yellowish foliage, dark brown or black lesions in the stem base of plants are similar and not distinguishable (De Boer *et al.*, 2012). Soft rot symptoms of potato tubers depend on the manner of infection. Rotting of the tuber tissue starts from the stolon end in tubers infected by the stolon, and at any place of the tuber when infected by lenticels or/and wounds.

Table 1

Pectinolytic bacteria pathogenic to the potato in Europe

Genus	Species	Reference
Pectobacterium	*P. atrosepticum*	Gardan *et al.,* 2003
	P. carotovorum subsp. *carotovorum*	Gardan *et al*., 2003
	P. c. subsp. *brasiliense*	Duarte *et al.,* 2004
	P. wasabiae (at present *P.parmentieri*)	Gardan *et al*., 2003; Khay *et al*., 2016
Dickeya	*D. solani*	Sławiak *et al*., 2009
	D. dianthicola	Samson *et al*., 2005

Control of the potato crop against these bacteria is difficult because of the lack of effective chemical products and the sources of high resistance to be used in breeding programs. Potato tubers infected with bacteria are the main source of infection. Therefore the determination of health status of potato seeds has a significant importance to make a decision about control measure to be used.

There are several methods used for identification of pectinolytic bacteria including determination of the type of growth on selective media, phenotypic properties, and DNA-based analysis. Using of serological tests is also recommended. The most common medium for isolation of bacteria is the crystal violet pectate medium (CVP), on which bacteria cause characteristic cavities as a result of pectin degradation. This medium is useful for growth of all species of pectinolytic bacteria originating from diseased plants, soil or water. It contains crystal violet, which inhibits the growth of Gram-positive bacteria (Helias *et al.*, 2012).

Based on the results of studies on genetic characterisation of pectinolytic bacteria several various PCR (Polymerase Chain Reaction) protocols have been developed. Multiplex PCR described by Potrykus *et al.* (2014) enables distinguishing three groups of pathogens: (1) *Pectobacterium atrosepticum* (Pba), (2) *Dickeya solani* (Dsol) and *D. dianthicola* (Ddth), (3) *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) and *P. wasabiae* (Pw) (at present:

P.parmentieri). Single species can be distinguished using other protocols (Figure 1). Species-specific real time PCR primers for Dsol and Ddth described by Pritchard *et al.*, (2012) were used in conventional PCR (Lebecka, unpublished). Multiplex PCR is a useful tool to identify a mixed infection from bacteria isolated directly from potato samples. For identification of the species of isolated bacteria from potato in pure culture the following methods can be applied: multiplex PCR in combination with specific PCR, or specific PCR alone (Fig.1).

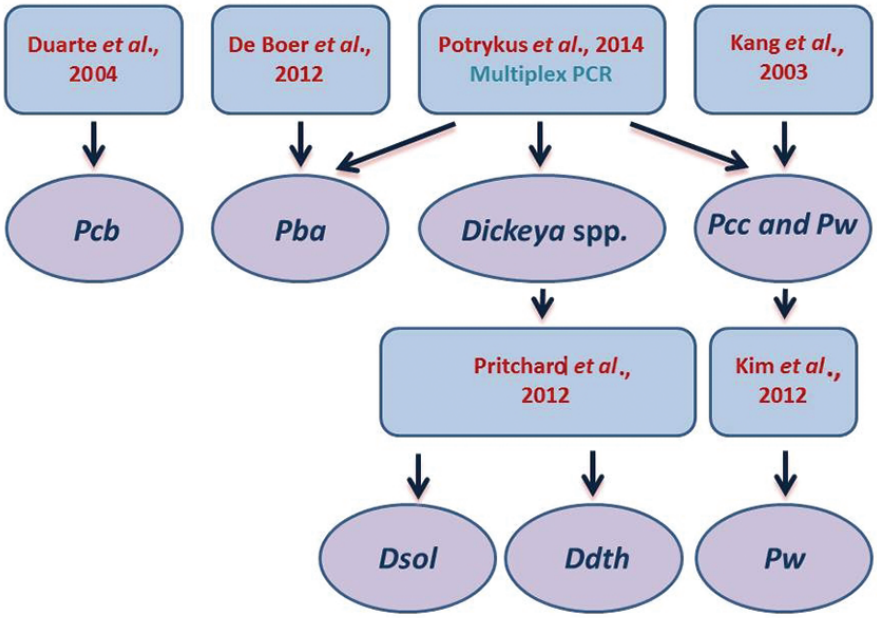

Fig. 1. The methods of detection pectinolytic bacteria pathogenic to the potato by conventional PCR
Pba - *P. atrosepticum*; Pcc - *P. carotovorum* subsp. *carotovorum*; Pcb - *P. c.* subsp. *brasiliense*; Pw - *P. wasabiae*; Dsol - *D. solani*; Ddth - *D. dianthicola*

MATERIALS AND REAGENTS

1) Agar (Sigma Aldrich, cat. No A1296)
2) Agarose, Prona Agarose, Basica LE (ABO, cat. No BGQT500)
3) Beaker 1000 ml (Danlab, cat. No 1101.01.900)
4) Boric Acid (Genoplast Biochemicals, cat. No BMGPBO0125-2)
5) Calcium chloride dihydrate (Avantor, cat. No ACRS42352)
6) Camping gas propan/butan C206 super
7) Cell spreader (Bionovo, cat. No B-4046)
8) Chelex® 100 sodium form (Sigma Aldrich, cat. C-7901)
9) Crystal Violet (Sigma Aldrich, cat. No C0775)
10) dNTP Mix, 10mM (Thermofisher Scientific, ABO, cat. No R0192)

11) DreamTaqT Green Buffer, 10X (Thermofisher Scientific, ABO, cat. No B714X)
12) DreamTaq™ Green DNA Polymerase (Thermofisher Scientific, ABO,cat. No EP0712)
13) EDTA (Genoplast Biochemicals, cat. No BMGPB0025-2)
14) Erlenmeyer flask (Bionovo, cat. No S-1137)
15) Eppendorf Semi-Micro Vis Cuvette (Eppendorf, cat. No 0030079353)
16) Eppendorf tubes 2 ml (Medlab Products, cat. No 25-2000-1)
17) Eppendorf PCR tubes (Meranco, cat. No 0030125215)
18) Ethanol 96 % (ALCHEM, cat. No 363-113964200)
19) Ethidium bromide (ABO, cat. No 0219020201)
20) Extractions bags 120 x 140 mm (Bioreba, Hornik, cat. No 9057290)
21) Fine permanent marker (Staedtler permanent, Lumacolor)
22) Glass bottle 100 ml (Bionovo, cat. No S-2071)
23) Glass bottle 1000 ml (Bionovo, cat. No S-2074)
24) Glass bottle 250 ml (Bionovo, cat. No S-2072)
25) Gloves, nitrile
26) Glycerol anhydrous pure (Avantor cat. No, 443320113)
27) Inoculation loop and handle (Bionovo, cat. No 1-2128 and 1-2120)
28) Isopropyl alcohol (ALCHEM, cat. No 363-327515001)
29) Liquid nitrogen
30) Lysogeny Broth (LB, Luria Bertani) (Sigma Aldrich, cat. No L3152)
31) MassRuler™ DNA Ladder (Thermofisher Scientific, ABO, cat. No SM0383)
32) Parafilm (Linegal, cat. No H666.1-R)
33) Pectin Dipecta (Agdia Biofords, cat. No AG366)
34) Petri dishes, diameter 9cm (Medlab, cat. No 51-0091-0SR)
35) Scalpel (Swann-Morton, Carbon Steel Surgical blades)
36) Sodium hydroxide (Sigma Aldrich, cat. No 71687)
37) Sodium hypochlorite (CAS: 7681-52-09, “Chemia” Sp. z o.o. Warszawa)
38) Sodium nitrate (Avantor, cat. No 792660111)
39) Spray bottle Turn'n'Spray 500 ml (Linegal, cat. No PX91.1)
40) Tips 0.5-20 µl (Eppendorf, Meranco, cat. No 0030.000.854)
41) Tips 2-200 µl (Eppendorf, Meranco, cat. cat. No No 0030 000.889)
42) Tips 50-1000 µl (Eppendorf, Meranco, cat. No 0030 000.927)
43) TRIS (Genoplast Biochemicals, cat. No BMGPB0026-2)
44) Tri-sodium citrate dehydrate (Avantor, cat. No 795780112)
45) Tryptone (Sigma Aldrich, cat. No T7293)
46) Viabank (BioMaxima S.A. Centrum Mikrobiologii)

EQUIPMENT

1) Autoclave (Prestige Medical, model Extended Plus 2100)
2) Automatic ice machine (Scotsman, model: AF 10 ASB 0600)
3) Centrifuge 5424R (Eppendorf)
4) Dewar with liquid nitrogen

5) Electronic scales (RADWAG, model: PS 210/c/2)
6) Electrophoresis set (Thermofisher Scientific, model: Easy cast B1, B2)
7) Freezer -80°C (Frigor)
8) Gas burner (WLD-TEC GmbH, model: gasprofi 2 scs)
9) Hand model homogeniser (Bioreba AG, Hornik, cat. No 9057392)
10) Incubator Shaker (Biosan, model: ES-20, cat. No BS-010111-AAA)
11) Laminar flow cabinet, Biohazard class cat. No A (ESCO, model: AC3-3E1)
12) Liquid nitrogen flask (Bionovo, catalog number: B-4110)
13) Microwave
14) pH meter
15) Pipette Reference 100-1000 µl (Eppendorf, cat. No 4920.000.083)
16) Pipette Reference 20-200 µl (Eppendorf, cat. No 4920.000.067)
17) Pipette Reference 2-20 µl (Eppendorf, cat. No 4920.000.032)
18) Shaker Lab Dancer (Bionovo, cat. No K-1670)
19) Thermomixer 5436 (Eppendorf)
20) Transilluminator (Vilber Lourmat, model: ECX-F26.MX)
21) Varioklav steam sterilizer (HP Medizintechnik)
22) Water purification system (PURELAB, Elga)

PROCEDURE

A. Isolation of bacteria

1) From symptomless tubers

Wash tuber in tap water, disinfect by immersing for 1min in 3% sodium hypochlorite, air dry. Cut out the stolon parts of the tubers with a scalpel and collect in a completely filled with the Lysogeny Broth medium (Oksińska *et al.*, 2016), to provide low oxygen conditions. Incubate at 28°C for 48 h.

2) From tubers with symptoms of rotting

Cut out a core of healthy looking tissue directly adjacent to symptomatic tissue, soak in sterile distilled water for 30 min to allow bacteria to leak out from the sample.

3) From symptomless plants or plants with symptoms

Cut out a piece of the stem starting from the ground up to 20 cm of a symptomless plant, or a piece of the stem from the healthy looking tissue directly adjacent to symptomatic tissue of a plant with symptoms. Disinfect by immersing in 0.3% sodium hypochlorite for 3 min (or in 70% ethanol for 1 min), wash in sterilised water. A piece of the stem from a symptomless plant can be treated in the same way as a tuber described in point 1), but pieces from symptomatic plant stem are ground with 1 ml of sterilised water in an extraction bag with the use of a hand model homogeniser.

4) Incubation of plant samples in liquid medium or water on selective medium

Dilute the bacteria obtained as described above, as a suspension or pellet, 10-fold and plate on Crystal Violet Pectate (SL-CVP_{AG366}) medium (Helias *et al.,* 2012). Before the use dry out the medium on the Petri dishes for 30 min. Spread

10 µl of the water suspension of the tested plant samples 10-fold diluted, distribute on each plate with a glass rod until dryness. Seal the plates with a parafilm. Incubate the colonies at 28ºC for 48 h. Grown colonies of pectinolytic bacteria form characteristic pits. Re-isolate the colony, repeat dilution in sterile water and streak on the LB agar medium.

B. Detection of pectinolytic bacteria by conventional PCR (Polymerase Chain Reaction)

1) Extraction of DNA with chelex (Pritchard *et al.*, 2012):
   a) centrifuge the suspension of bacteria in water at 9,000 × g for 5 min, discard the supernatant,
   b) resuspend the pellet in 300 µl 6% chelex 100,
   c) heat at 56°C for 20 min, then at 100°C for 8 min (or 95°C for 10 min),
   d) chill on ice,
   e) centrifuge at 20,000 × g for 5 min,
   f) transfer the purified DNA in aqueous supernatant to a new tube,
   g) measure the DNA content in a spectrophotometer at 260 nm wavelength (A_{260} = 0.2 = 100 µg × ml^{-1}).
2) Procedure of PCR performance

Table 2

The primers and conditions of the PCR used for detection of pectinolytic bacteria pathogenic to the potato

Species	Primer name	PCR product	Primer sequence	Reference
P. atrosepticum	ECA1f	690 bp	CGGCATCATAAAAACACG	de Boer *et al*., 2012
	ECA1r		GCACACTTCATCCAGCGA	
	95ºC / 5 min, 40x (95ºC / 30s, 62ºC / 30 s, 72ºC / 30 s) and 72ºC / 4 min			
P. carotovorum subsp. *carotovorum*/ *P. wasabiae*	EXPCCF	550 bp	GAACTTCGCACCGCCGACCTTCTA	Kang *et al.,* 2003
	EXPCCR		GCCGTAATTGCCTACCTGCTTAAG	
	94ºC / 4 min, 30x (94ºC/ 1 min, 60ºC / 1 min, 72ºC / 2 min) and 72ºC / 7 min			
P. wasabiae	PW7011F	140 bp	CTATGACGCTCGCGGGTTGCTGTT	Kim *et al*., 2012
	PW7011R		CGGCGGCGTCGTAGTGGAAAGTC	
	94ºC / 5 min, 35x (94ºC / 1 min, 67ºC / 30 s, 72ºC / 1 min) and 72ºC / 10 min			
P. c. subsp. *brasiliense*	BR1f	322 bp	GCG TGC CGG GTT TAT GCA CT	Duarte *et al*., 2004
	L1r		CAA GGC ATC CAC CGT	
	94ºC / 10 min, 25x (94ºC / 1 min, 67ºC / 1 min, 72ºC / 30 s) and 72ºC / 10 min			
D. solani	SOL-C	110 bp	GCCTACACCATCAGGGCTAT	Pritchard *et al*., 2012
			ACACTACAGCGCGCATAAAC	
	48ºC /30s, 95ºC / 10 min, 36x (95ºC / 15 s, 55ºC / 1 min)			
D. dianthicola	DIA-A	110 bp	GGCCGCCTGAATACTACATT	Pritchard *et al*., 2012
			TGGTATCTCTACGCCCATCA	
	48ºC /30s, 95ºC / 10 min, 36x (95ºC / 15 s, 55ºC / 1 min)			

Table 2

Continued

Species	Primer name	PCR product	Primer sequence	Reference
Dickeya spp	Df	130 bp	AGAGTCAAAAGCGTCTTG	Potrykus *et al*., 2014
	Dr		TTTCACCCACCGTCAGTC	
P. atrosepticum	Y45	420 bp	TCACCGGACGCCGAACTGTGGCGT	
	Y46		TCGCCAACGTTCAGCAGAACAAGT	
P. carotovorum subsp. *carotovorum*/ *P. wasabiae*	EXPCCF	550 bp	GAACTTCGCACCGCCGACCTTCTA	
	EXPCCR		GCCGTAATTGCCTACCTGCTTAAG	
95°C / 4 min, 30 × (94°C / 45 s, 62°C / 90 s, 72°C / 90 s) and 72°C / 3 min				

For one PCR reaction use the following mix: 13.3 µl of water, 10x concentrated buffer 2 µl, nucleotides (dNTPs 1.25 µM) 1.6 µl, primers (5µM) 1 µl of each, polymerase 0.1 µl. To 19 µl of the PCR mix add 1 µl of the extracted DNA (use 5 to 12.5 ng of DNA as a template for the PCR reaction).

3) Gel electrophoresis
   a) Gel Agarose (15 or 20 g × l^{-1} – for fragments of DNA of the size of 100 bp), dissolve in a microwave mixing a few times in the erlenmeyer flask (50 ml – 110 ml for 3 min)
   b) Add ethidium bromide (5 µg x l^{-1})
   c) Pour the gel solution into the levelled casting tray with a comb, allow the gel to solidify (15 - 30 min), remove the well comb, pour the buffer TBE 10 × (25 ml + 225 ml), load the ladder (100 bp) and the samples, set the running voltage – 5 V × cm^{-1}. When the bands are in ¾ of the gel stop the electrophoresis and visualize the gel in UV light (Fig. 2.).

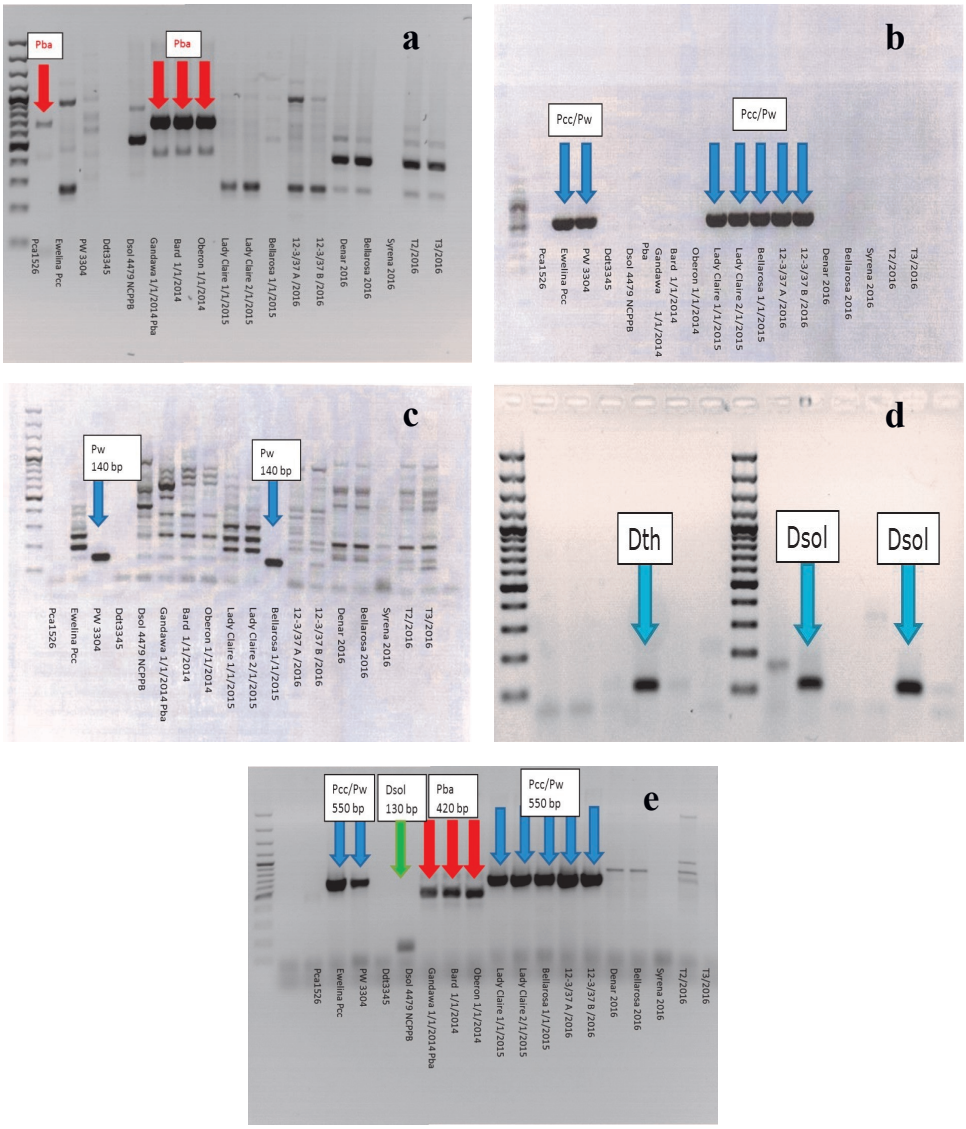

Fig. 2. Electrophorograms of :
a) *Pectobacterium atrosepticum,* 690 bp, De Boer *et al.*, 2012; b) *Pectobacterium carotovorum* subsp. *carotovorum/ P. wasabiae*, 561 bp, Kang *et al.*, 2003, c) *P. wasabiae*, Kim *et al.*, 2012; d) *Dickeya dianthicola* and *D. solani*, 110 bp, Pritchard *et al.*, 2012, e) *P. atrosepticum, P. carotovorum* subsp. *carotovorum/ P. wasabiae*, *Dickeya* spp. (in this figure *D. solani*) Potrykus *et al.*, 2014

C. Preservation

Bacteria of a fresh culture from a single colony are transferred either to eppendorf tubes with 15% of glycerol liquid LB broth medium or to Viabank

tubes (Biomaxima S. A. Copyright, 2013). The bacteria are frozen in liquid nitrogen and then kept in a freezer (for longer preservation it is better to keep the bacteria at -70 or -80°C).

RECIPES

1) Lysogeny Broth medium:
   a) Lysogeny Broth 20.0 g
   b) Agar 16.0 g
2) Crystal Violet Pectate medium:

Prepare two mixes. Dissolve the ingredients in 500 ml of distilled water in the following order and add each after the previous one has dissolved:

a) Calcium chloride dihydrate 1.02 g
b) Tryptone 1.0 g
c) Tri-sodium citrate dihydrate 5.0 g
d) Sodium nitrate 5.0 g
e) Crystal Violet (0.075%) 2.0 ml
f) Agar 4.0 g

Dissolve in 500 ml of distilled water and then heat (80-100^{o}C) to allow the pectin to dissolve without lumps.:

a) Sodium hydroxide (5M) 2.8 ml
b) Pectin Dipecta 18.0 g

Sterilise both mixes at 120°C for 15 min (longer sterilisation can destroy the pectins), pour the 1st mix slowly into the 2nd one while hot and stir gently. Check the pH (6.8-7.4), distribute in Petri dishes in a laminar flow cabinet.

3) TBE buffer 10 ×
   a) Boric Acid 55.0 g
   b) Tris 108.0 g
   c) EDTA 7.44 g

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