OCCURRENCE AND IDENTIFICATION OF *PECTOBACTERIUM CAROTOVORUM* SUBSP. *BRASILIENSIS* AND *DICKEYA DIANTHICOLA* CAUSING BLACKLEG IN SOME POTATO FIELDS IN SERBIA

Sanja Marković[1], Slaviša Stanković[2], Aleksandra Jelušić[1], Renata Iličić[3], Andrea Kosovac[4], Dobrivoj Poštić[4], Tatjana Popović[4]*

[1]*University of Belgrade, Institute for Multidisciplinary Research, Belgrade, Serbia*

[2]*University of Belgrade, Faculty of Biology, Belgrade, Serbia*

[3]*University of Novi Sad, Faculty of Agriculture, Novi Sad, Serbia*

[4]*Institute for Plant Protection and Environment, Belgrade, Serbia*

Corresponding author:

Dr Tatjana Popović

Phone: 00381112660049

Fax: 00381112669860

E-mail: tanjaizbis@gmail.com

ABSTRACT

Blackleg outbreaks were noticed on three fields (total c. 100 ha) during two consecutive years (2018, 2019) in one of the main potato growing areas in Serbia (Bačka region, Vojvodina). The percentage of infected plants reached 40-70% with 10.5% to 44.7% yield reductions. From the three fields out of 90 samples *Pectobacterium carotovorum* subsp. *brasiliensis* was most frequently identified and diagnosed as causal agent of potato blackleg in Serbia for the first time (29 isolates). *Dickeya dianthicola* was a less frequently causative bacterium, which was also noticed for the first time (nine isolates). A total of 38 isolates were characterized based on their phenotypic and genetic features, including a pathogenicity test on potato. The repetitive element Polymerase Chain Reaction (rep-PCR) using BOX, REP and ERIC primer pairs differentiated five genetic profiles among 38 tested isolates. Multilocus sequence analysis (MLSA) of four housekeeping genes, *acnA*, *gapA*, *icdA* and *mdh*, revealed the presence of three so far unknown *P. c.* subsp. *brasiliensis* multilocus genotypes and confirmed clustering into two main genetic clades as determined in other studies. MLSA also revealed the presence of a new genotype of *D. dianthicola* in Serbia.

Keywords: potato, *Pectobacterium*, *Dickeya*, characterization, MLSA

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the major cultivated world crops with the production rate of 368 million tons per year (FAO 2018). Deviation in potato yield significantly depends on the presence of potato pests and diseases, climate and agricultural standards (Oerke 2006). One of the most important and widely distributed diseases is potato blackleg, caused by plant pathogenic pectolytic bacteria, belonging to the genera *Pectobacterium* and *Dickeya* (formerly *Erwinia*) (Czajkowski et al. 2015; Charkowski et al. 2020). *Dickeya* spp. cause losses of up to 25% (Tsror et al. 2009). In temperate zones, *Pectobacterium atrosepticum* is the primary causative agent of blackleg, while *Pectobacterium carotovorum* subsp. *carotovorum* is less significant and only virulent strains of this bacterium can cause true blackleg symptoms under favorable conditions (De Haan et al. 2008). *Pectobacterium carotovorum* subsp. *brasiliensis*, as well as *Dickeya* spp., occur mainly in regions with warm climate or in warm growing seasons in temperate climates (Duarte et al. 2004; Van der Merwe et al. 2010; Toth et al. 2011; Oulghazi et al. 2017; Van der Wolf et al. 2017; Wright at al. 2018; Nasaruddin et al. 2019).

Potato blackleg caused by the *P. c.* subsp. *brasiliensis* is so far reported worldwide (Duarte et al. 2004; Ma et al. 2007; Van der Merwe et al. 2010; Ma et al. 2018). *Dickeya* sp. (formerly *Erwinia chrysanthemi*) causes diseases in a wide range of economically important crops, including potato (Toth et al. 2011; Degefu et al. 2013). *Pectobacterium* and *Dickeya* species are regulated non-quarantine pests (RNQP) for the potato seed and consumption of potato in the EU (Commission Implementing Regulation (EU) 2019/2072).

The development of potato blackleg disease depends on inoculum concentration in seed tubers and volunteers, susceptibility of the potato cultivar, environmental conditions and soil moisture

content (Pérombelon 2002). The main symptom in above ground plant parts is black discoloration at the base of potato stems, in the beginning often appearing only on one stem per plant (Van der Merwe et al. 2010; Ma et al. 2018). The pathogen starts to develop symptoms in infected mother tubers at various times during the vegetative season and spreads through the vascular system into the stem followed by stem tissue maceration and pith necrosis. Wilting first appears on top of leaves which roll and become necrotic on the margins, then the wilting progresses to lower leaves. Infected stems are soft and slimy in wet conditions, while in drought lesions are dry and brittle. Under favorable environmental conditions, the disease leads to plant decay (Pérombelon 2002; Van der Merwe et al. 2010; De Boer et al. 2012). One of the major sources of inoculum for blackleg infection are (latently) infected potato seed tubers (Czajkowski et al. 2009; Van der Merwe et al. 2010; Toth et al. 2011).

In Serbia, *P. atrosepticum* and *P. c.* subsp. *carotovorum* were reported as potato blackleg pathogens during the 1990s (Arsenijević et al. 1994; Obradović 1996) and since that time the disease was not known to have occurred. Recently, potato blackleg outbreaks (cultivar Lady Claire) were observed in two consecutive years (2018 and 2019) in three potato fields in the Bačka region, one of the major potato production areas in Serbia. The field area covered by the disease was more than 100 ha. Therefore, the main aim of the present study was to determine and characterize the causal agents of the recent potato blackleg outbreak in Serbia using conventional bacteriological methods and molecular characterization tools as an aid to efficiently prevent and control the disease in the future.

MATERIALS AND METHODS

Potato field monitoring

In 2018 an outbreak of potato blackleg was recorded in one field coded as T-N1/2, with 58 ha of plot size (GPS 45°21'05.0" N, 19°22'47.8" E), located in Obrovac. In 2019 the disease appeared in two fields coded as T-25, with 30 ha of plot size (GPS 45°37'11.7" N, 19°52'06.3" E) and T-28, 14 ha of plot size (GPS 45°38'85.6" N, 19°52'65.5" E), located in Maglić and Kulpin, respectively (Table 1). All fields were in the Bačka region (Vojvodina), the main potato growing area in Serbia. In all monitored fields, the potato cultivar was Lady Claire. The previous crops were seed corn in T-N1/2, corn in T-25 and wheat in T-28. The type of soil in this region is chernozem and a sprinkler move linear system was used for irrigation. Potato was planted in the middle of April in 2018 and beginning of May in 2019. Monitoring, visual inspection and disease development were performed bi-weekly during the potato vegetation period from the beginning of June onwards. The percentages of infection in the fields were determined three times during vegetative seasons by counting diseased plants, viz. 100 plants from 10 diagonally selected points. From each point (3 m length in size) 10 plants were rated. Two diagonals were chosen, from bottom left to upper right corner, and vice versa, considering that the fields were rectangular. The distance between points was calculated and measured in the field with a measuring rope. These points were marked by reflective field flags, and used when the counting was repeated and for sampling. Weather conditions (temperature and rainfall) were obtained from the nearest meteorological station (http://www.hidmet.gov.rs/podaci/agro/godina.pdf) for the locality of Novi Sad. Rainfall was measured also in the fields under observation by a rain gauge. For both years, irrigation was reduced (for 3-4 applications with approx. 70 mm water less) compared to healthy potato crops. Commonly used pesticide treatments were performed in

116 the observed fields. Potato was harvested using potato harvester in early September under dry
117 and warm weather conditions.
118
119 **Assessment of yield loss**
120
121 To determine the impact of the disease on yield, potato mass was weighed and yield was
122 expressed as a ton per hectare for each year and field. The yields obtained from the diseased
123 crops in 2018 and 2019 were compared separately with the average yield obtained from potato
124 crops (cv. Lady Claire) in a seven-year period (2011-2017) in the Bačka region (Ž. Bijelić,
125 *personal communication*). Yield losses of the diseased crops were calculated according to the
126 following formula:

$$\text{Yield loss (\%)} = \frac{(Ya - Yd)}{Ya} \times 100$$

127 where, Ya - the average yield achieved in a seven-year period (Bačka)
128 Yd - the yield determined in the diseased field.
129
130 **Sample collection and bacterial isolation**
131
132 In June, the potato plants with characteristic blackleg symptoms, stem necrotic lesions, blackness
133 at the stem basis, wilting and rotting of tubers, were collected from 10 different points in each of
134 the three observed fields, with a total of 90 plants. The samples were first washed in tap water
135 and dried on filter paper at room temperature. Isolation was performed with plant segments,
136 taken from the margins of healthy and diseased tissue, kept in a sterile phosphate buffer (PBS)
137 for 30-60 min and then plated onto the Crystal Violet Pectate (CVP) media (Hélias et al. 2011).

Thereafter, Petri plates were incubated at 26 °C for 48 h. The bacterial colonies which formed characteristic cavities on CVP were transferred onto Nutrient Agar (NA) and purified. A total of 38 isolates were selected for further study (Table 1). Pure cultures were stored in Luria Bertani (LB) broth (Difco, USA) supplemented with 20% (v/v) of sterile glycerol (Centrohem, Serbia) at -20 °C for short term storage.

The bacterial isolates were checked for their pectolytic activity on surface-sterilized healthy potato tuber slices cv. Lady Claire (Schaad et al. 2001). Holes were bored in the center of slices and inoculated with a loopful of fresh bacterial culture (24 h). The tuber slices were incubated in Petri dishes at room temperature under high humidity and checked for the presence of macerated tissue over the next 48 h.

Preliminary identification

Polymerase chain reaction (PCR) was used for rapid identification of all isolates by using the following specific primer sets for *Pectobacterium* spp. viz., for *P. c.* subsp. *carotovorum* primer set F0145/E2477 (Kettani-Halabi et al. 2013), for *P. c.* subsp. *brasiliensis* BR1f/L1r (Duarte et al. 2004), *P. atrosepticum* ECA1f/ECA2r (De Boer and Ward 1995) and for *Dickeya* spp. primer set ADE1/ADE2 (Nassar et al. 1996) (Table 2).

As control strains, *P. c.* subsp. *carotovorum* strain Pcc10 (Institute for Plant Protection and Environment, Serbia) and *Dickeya solani* strain MK10 (SASA, Scotland; Toth et al. 2014) were used for all comparisons in the study.

DNA extraction was performed from a full loop (HIMEDIA, Nichrome Loop-D-2 Diameter: 2 mm, double wound, calibrated to 0.005 mL) of bacterial isolates grown for 24 h on NA,

161 suspended in test tubes containing 500 µL of sterile distilled water (SDW). Suspensions were
162 heated for 10 min at 95 °C in a water bath, cooled on ice, and centrifuged for 5 min at 7,600 *g*.
163 Supernatants were used for PCR amplification.
164 A total of 25 µL PCR mix contained 2.5 µL of 10 × KAPA Taq Buffer with 1.5 mM $MgCl_2$, 0.5
165 µL of dNTPs (10 mM), 1 µL of each of the primers (10µM), 0.25 µL 5U/µl Taq DNA
166 polymerase (KAPA Biosystems, USA), 18.75 µL of ultrapure DNase/RNase-free water (Gibco,
167 UK), and 1 µL DNA. PCR was carried out using the amplification programs given in Table 2.
168 All amplified PCR products were electrophoresed in a 1% agarose gel stained with ethidium
169 bromide and checked for the presence of respective specific bands (666 bp for F0145/E2477, 322
170 bp for BR1f/L1r, 690 bp for ECA1f/ECA2r, and 420 bp for ADE1/ADE2 primer sets) under UV
171 light.
172
173 **Phenotypic features**
174
175 Thirty eight isolates were characterized using the following biochemical tests: Gram reaction in
176 3% KOH, oxidative-fermentative metabolism of glucose, indole production, nitrate reduction,
177 hydrogen sulphide (H_2S) production from peptone, the presence of arginine dihydrolase, gelatine
178 liquefaction, aesculin, starch, and casein hydrolysis, tyrosinase activity, utilization of (D+)
179 tartrate, lactic, tartaric, aspartic acids, and L-leucine utilization, growth at 37 °C and 4 °C, salt
180 tolerance (NaCl, 5%), acid production from D-glucose, D-mannitol, inositol, D-sorbitol, L-
181 rhamnose, D-sucrose, D-melibiose, amygdalin and L-arabinose (Schaad et al. 2001).
182 Pathogenicity of thirty eight isolates was checked on young potato plants (cv. Lady Claire) using
183 three plants per isolate. Potato tubers were planted in the pots with sterile substrate (Klasmann-

Deilmann GmbH) and placed in a greenhouse at temperature 22-25 °C under natural light, with regular watering. After five weeks of growing, the plants were in the phase of the 3rd and the 4th true leaf stage on the main stem defined by Biologische Bundesanstalt, Bundessortenamt and CHemical industry (BBCH 103-104). Isolates were grown on NA for 48 h and suspended in SDW. The pathogenicity test was performed by: (i) injection of bacterial suspension (adjusted to 10^7-10^8 CFU mL^{-1}) with a sterile hypodermic syringe and needle of 23G × 1 into the stem at the third node from the stem base, and (ii) soil inoculation with 20 mL of bacterial suspension given once (adjusted to 10^9 CFU mL^{-1}) to each pot in four holes (Tsror et al. 2009). Experiments were performed in three replicates. As a positive control treatment, *P. c.* subsp. *carotovorum* strain Pcc10 and *D. solani* strains MK10 were used. SDW served as a negative control treatment. Inoculated plants were kept in plastic boxes under controlled conditions at temperature 25 °C under high humidity (70-80%) and 16-/8-h (day/night) photoperiod. Symptom development was observed visually on a daily basis.

Re-isolations were performed on CVP and re-isolates were purified on NA. The identification of re-isolates, to be the same as the original ones, was performed using PCR with primer pairs BR1f/L1r and ADE1/ADE2.

Genotypic features

Total DNA extraction

Total DNA from thirtyeight isolates was extracted using a modified CTAB method given by Ausubel et al. (2003). Pure bacterial colonies (grown on NA for 24 h at 26 °C) of the isolates were suspended in 500 µL (approx. 10^6 CFU mL^{-1}) of SDW and centrifuged at 10,000 *g* for 10

min. The obtained pellet was re-suspended in TE buffer (50 mM TRIS, pH 8.0, 1 mM EDTA) (567 µL), 10% (w/v) of sodium dodecyl sulphate (SDS) (30 µL) with 20 mg mL^{-1} of proteinase K (3 µL). The mix was incubated for 30 min at 37 ºC where after 100 µL of 5 M NaCl was added. The next step consisted of addition of 300 µL of 3% hexadecyltrimethylammonium bromide (CTAB, pH 8.0) and incubation for 20 min at 65 ºC. For additional purification of DNA 750 µL of chloroform was added and centrifuged at 10,000 *g* for 10 min. The upper phase was collected, transferred to new tubes, mixed with ice-cold isopropanol (750 µL) and centrifuged at 10,000 *g* for 15 min. The pellet was washed with 1 mL of 96% ice-cold ethanol, centrifuged at 10,000 *g* for 10 min and dried at room temperature for 30 min. The obtained DNA was dissolved in 50 µL of TE buffer and stored at - 20 ºC.

DNA fingerprinting

The rep-PCR fingerprinting was performed with 38 isolates using three oligonucleotide primers: BOX (BOXA1R), ERIC (ERIC1R/ERIC2) and REP (REP1R-I/REP2-I) (Versalovic et al. 1994). The concentrations of DNA and their purity were measured using a Qubit Fluorometric Quantitation (Qubit 4 Fluorometer, Invitrogen™, USA) and equalized. PCR reactions were performed by Versalovic et al. (1994) (Table 2). A total volume of 25 µL of mix contained the following: 2.5 µL of 10 × KAPA Taq Buffer B; 0.5 µL of dNTP mixture (10 mM) (KAPA Biosystems, USA), 2 µL (10 µM) of each forward and reverse primer, 0.2 µL of (5U μL^{-1}) KAPA Taq polymerase (KAPA Biosystems, USA), 16.8 µL of ultrapure DNase/RNase-free water (Gibco, UK), and 1 µL of total sample DNA. After PCR amplification, DNA products were electrophoresed in a 1.5% agarose gel stained with ethidium bromide (0.5 µg mL^{-1}). To calculate the differences in band positions and the level of genetic similarity between the

obtained profiles, an unweight pair group method with arithmetic mean (UPGMA) trees were constructed using PyElph 1.4 software (Pavel and Vasile 2012).

Multilocus sequence analysis (MLSA)

Based on the results of rep-PCR performed with 38 isolates, a total of 25 isolates (five from each representative pattern group) were chosen for MLSA with four housekeeping genes: *acnA* (aconitate hydratase 1), *gapA* (glyceraldehyde-3-phosphate dehydrogenase A), *icdA* (isocitrate dehydrogenase, specific for NADP+) and *mdh* (malate dehydrogenase) (Ma et al. 2007; Moleleki et al. 2013) (Table 2). The PCR amplifications were performed with 12.5 µL of DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), 1 µL of each of the used primers (10 µM), 9.5 µL of ultrapure DNase/RNase-free water (Gibco, UK), 1 µL of sample total DNA, and to obtain the final reaction volume of 25 µL. QIAquick/250 Gel Extraction and Purification Kits (QIAGEN GmbH, Hilden, Germany) were used for purification of the obtained PCR products. After the purification, PCR products were sequenced in the Macrogen sequencing service (Amsterdam, the Netherlands). The obtained nucleotide sequences were checked for their quality in FinchTV v.1.4.0 (Geospiza, http://www.geospiza.com/finchtv) and aligned by the ClustalW program implemented in BioEdit (ver. 7.0.5). The sequences were trimmed to the following sizes [252 nt (*acnA*), 382 nt (*gapA*), 451 nt (*icdA*), 283 nt (*mdh*)] for phylogenetic analysis. They were simultaneously compared with the available *Pectobacterium* spp and *Dickeya* spp. sequences retrieved from the NCBI (National Center for Biotechnology Information) database (Table 3), using nucleotide BLAST*n* search tool. Representative isolates of all the detected haplotypes were deposited into the NCBI GenBank (Table 1).

The evaluation of the phylogenetic relations and genetic divergence among the bacterial isolates were assessed treating analyzed strains as single multilocus genotypes. The concatenated sequences used in further MLSA analysis were comprised of four housekeeping genes (*acnA, gapA, icdA* and *mdh*) and were 1368 bp long (Table 3). The best evolutionary model of nucleotide substitution was determined in jModelTest v.2.0.2 (Posada 2008) following the Bayesian Information Criterion (BIC). The most appropriate substitution model suggested by the jModelTest was further selected in Maximum likelihood phylogeny reconstructed in MEGA 6 applying 1,000 bootstrap replications (Tamura et al. 2013). The sequences of strain *Yersinia pestis* Yp91001 were used for tree rooting in all phylogenetic analyses based on Ma et al. (2007). In order to perceive evolutionary relatedness and genealogy of *P. c.* subsp. *brasiliensis* and *D. dianthicola*, two separate median-joining networks were calculated using software Network v. 5.0.1.1 (www.fluxus-engineering.com) (Bandelt et al. 1999), keeping parameter ε at its 0 value and applying maximum parsimony (MP) post-processing in order to obtain a network containing all the shortest trees. Per one representative isolate of each of the detected genotypes of *P. c.* subsp. *brasiliensis* and *D. dianthicola* in Serbia, as well as the type strains from the other species of the genera *Pectobacterium* and *Dickeya*, were employed in the analysis, all selected by the availability of the appropriate genes deposited in the NCBI database. The same *P. c.* subsp. *brasiliensis* and *D. dianthicola* strains were used in phylogenetic and network analysis in order to compare obtained results. Average genetic distances based on pairwise analysis (uncorrected p-distances) among *Pectobacterium* spp. and *Dickeya* spp. multilocus strains were estimated in MEGA 6 software (Tamura et al. 2013).

RESULTS

275

276 **Potato field monitoring and assessment of yield loss**

277

278 Blackleg symptoms occurring in potato crops in 2018 and 2019 (Figure 1) had the same
279 dynamics of disease progress. The first symptom appeared in the form of sporadic wilting of the
280 youngest stem segment (top) observed in the middle of May 2018 or two weeks later in 2019. At
281 the beginning of June, potato crops were in the phase of intensive growth (BBCH 209-301) and
282 disease incidence was 5-10%, uniformly distributed in the fields. In the phase of tuber formation
283 (BBCH 407-408), wilting was more widespread and recorded on 1-2 stems among the total of an
284 average of 6 stems per plant. The youngest leaves on plants rolled upwards, wilted, and necrosis
285 of leaves started to progress from the top part and leaf edges. At the same time, a small number
286 of plants developed typical symptoms of blackleg on stems, viz. a light to dark brown
287 discoloration of tissue at the stem base. In some cases, necrotic lesions were formed externally
288 along the whole stem (2-3 cm in size) and internally necrosis of the vascular system was visible.
289 By removing the diseased plants from the soil, rotting of mother tubers was observed. At the end
290 of June, wilting of lower leaves was noticed, followed by an expanded infection to previously
291 healthy stems on the same plants. Stems with typical blackleg symptoms decayed completely.
292 Foci of diseased plants were noticed in the fields and disease incidence in all three fields was
293 around 30%. In July (from the middle onwards) when the potato crops entered the end of the
294 vegetative period (BBCH 905-907), the number and diameter of foci with completely dried and
295 decayed plants increased. The disease incidence reached 45% in 2018; 40% and 70% for T-25
296 and T-28, respectively in 2019. After the potato harvest, rotten progeny tubers were found.

297 The achieved yield in the observed field was 29 ton ha^{-1} in 2018; in 2019 were 34 ton ha^{-1} and 21
298 ton ha^{-1} for T-25 and T-28, respectively. In the Bačka region, the average of seven-year yield
299 data of cultivar Lady Claire was 38 ton ha^{-1}. Yield was reduced by 23.7% in 2018 and in 2019 by
300 10.5% and 44.7% in the T-25 and T-28 fields, respectively.
301 During the vegetative period of potato in 2018 and 2019, rain occurred in short intervals and
302 abundantly during the whole growing season (in 2018, locality Obrovac: 132 mm in May, 163
303 mm in June and 71 mm in July; in 2019 for localities Kulpin and Maglić: 85 mm in May, 200
304 mm in June and 77 mm in July). According to the data from Republic Hydrometeorological
305 Service of Serbia, the average daily temperature was 2.6 °C higher compared to the annual
306 average, with 10% higher precipitation, while climate conditions in 2019 were within the annual
307 average.
308
309 **Bacterial isolation and potato rot test**
310
311 After the isolation of bacteria from the diseased potato stems and tubers, most colonies formed
312 characteristic cavities on CVP due to pectin degradation. Upon purification, two types of
313 colonies were formed on NA after 48 h incubation period: 1) round, small colonies, 1-2 mm in
314 diameter, smooth, creamy in color, obtained in both years and 2) irregular colonies, 2-3 mm in
315 diameter, slimy and with creamy white coloration, obtained only in 2018 (Supplementary Figure
316 1a).
317 Tissue maceration of inoculated potato slices appeared 24 h after the inoculation. Two types of
318 tissue decomposition were noticed: 1) one group of isolates (9 isolates coded with Dd prefix)
319 produced cream-colored rotting tissue with dark brown margins in the zone between the healthy

and decomposed tissue and 2) the second group (29 isolates coded with Pcb prefix) devastated the tissue causing cream-colored rotting but with no visible margins (Supplementary Figure 1b).

Preliminary identification

Eight isolates from 2018 (coded as Pcb33, Pcb34, Pcb38, Pcb39, Pcb61, Pcb62, Pcb64, Pcb67) and 21 isolates from 2019 (coded as Pcb2531, Pcb2538, Pcb2544, Pcb2549, Pcb2562, Pcb2563, Pcb2568, Pcb2811, Pcb2812, Pcb2813, Pcb2815, Pcb2817, Pcb2819, Pcb2833, Pcb2838, Pcb2839, Pcb2841, Pcb2842, Pcb2844, Pcb2847, Pcb2861) amplified the products at 322 bp using BR1f/L1r primer pair specific for *P. c.* subsp. *brasiliensis*. Nine isolates obtained in 2018 (Dd31, Dd32, Dd35, Dd37, Dd41, Dd42, Dd44, Dd46, Dd47) and control strain MK10 produced an amplicons at 420 bp using primer pair ADE1/ADE2 specific for *Dickeya* spp (Supplementary Figure 1c). Control strain Pcc10 amplified the product of 666 bp using F0145/E2477 primer pair.

Phenotypic features

All 38 isolates were Gram-negative, facultative anaerobic (O+/F+ test), positive for indole and hydrogen sulphide production, nitrate reduction, gelatine liquefaction, aesculin hydrolysis and utilization of aspartic acid. They were also tolerant to 5% of NaCl and able to grow at 37 ºC. All tested isolates showed negative reactions to starch hydrolysis, tyrosinase activity and L-leucine utilization. Additionally, all isolates produced acid from D-glucose, D-mannitol, L-rhamnose, D-sucrose, amygdalin and L-arabinose, but not from D-sorbitol. The differences among the tested isolates were obtained in the tests for arginine dihydrolase, utilization of tartrate, lactic and

343 tartaric acid, where nine isolates coded with Dd prefix were positive, while 29 isolates coded
344 with Pcb prefix were negative. Casein hydrolysis, acid production from inositol and D-melibiose
345 and growth at 4 ºC were positive for Pcb prefix isolates and negative for Dd prefix isolates. The
346 results of biochemical tests indicated the presence of the genus *Pectobacterium* in 29 isolates
347 with Pcb prefix and *Dickeya* in 9 isolates with Dd prefix.
348 Pathogenicity was confirmed on young potato plants cv. Lady Claire. When the injection method
349 was used, the initial symptoms on stems appeared two days after inoculation the (DAI) in the
350 form of dark brown lesions at the sites of inoculation. Wilting symptoms occurred on the third
351 DAI, while the lesions on the stems increased externally and were followed by internal necrosis
352 of vascular tissue. Five DAI, necrosis extended causing whole plant decay. When the soil
353 inoculation method was used, the first symptoms were noticed 10 DAI in the form of wilting of
354 the leaves and necrotic blackening of the stem bases. Later, 15 DAI, necrosis spread from the
355 lower plants parts leading to drying of the whole plant. Similar symptoms were observed for all
356 38 tested isolates as well as for the control strains Pcc10 and MK10. The negative control plants
357 were symptomless.
358 Re-isolations from symptomatic plants for all 38 isolates were successful on CVP media. The
359 reisolated bacteria caused pitting on CVP and exhibited the same morphology as the original
360 isolates on NA, and were confirmed to be the same as the original using PCR with specific
361 primer sets BR1f/L1r and ADE1/ADE2. Thus, Koch's postulates were fulfilled.
362
363 **Genotypic features**
364

The rep-PCR results using BOX, ERIC and REP primer sets showed five different patterns among 38 tested isolates. The obtained UPGMA phylogenetic trees also distinguished five groups of isolates, placing them in the five separated clusters (Figure 2).

The comparison of the isolates analyzed in this study with NCBI database strains using BLAST*n* revealed the presence of four multilocus haplotypes of *P. c.* subsp. *brasiliensis* among 20 analyzed isolates and a single haplotype of *D. dianthicola* present in all 5 genotyped isolates from Serbia. Isolates Pcb2833, Pcb2838, Pcb2842, Pcb2844 and Pcb2861 were attributed to the haplotype PCB-1 and showed 100% homology with *P. c.* subsp. *brasiliensis* strain kbs-1 (potato, Japan) for all four genes used in multilocus genotyping. Additionally, three new multilocus haplotypes of *P. c.* subsp. *brasiliensis* were detected: PCB-2 (Pcb33, Pcb34, Pcb62, Pcb64 and Pcb67), PCB-3 (Pcb2811, Pcb2812, Pcb2813, Pcb21815 and Pcb2817) and PCB-4 (Pcb2544, Pcb2549, Pcb2562, Pcb2563 and Pcb2568). Haplotype PCB-2 showed 98.80-99.82% per locus identity with the *P. c.* subsp. *brasiliensis* strains: SX309 (cucumber, China) (99.42% *acnA*), BZA12 (cucumber, China) (99.79% *gapA* and 99.82% *icdA*), BC1 (Chinese cabbage, China) (99.00% *mdh*). Isolates belonging to haplotype PCB-3 showed per locus homology of 99.48-100% with *P. c.* subsp. *brasiliensis* strains: SX309 (cucumber, China) (100% *acnA*), BZA12 (cucumber, China) (99.48% *gapA*), A45 (potato, Syria), (99.78% *icdA*) and kbs-1 (potato, Japan) (100% *mdh*). The fourth isolate group PCB-4 has shown to be genetically closest to the *P. c.* subsp. *brasiliensis* strains JKP4.3.22 (potato, Germany) (99.6% *acnA*), A45 (potato, Syria) (99.74% *gapA* and 99.78% *icdA*) and kbs-1 (potato, Japan) (100% *mdh*).

Potato isolates Dd31, Dd32, Dd42, Dd44 and Dd46 were identified as the same multilocus haplotype of *D. dianthicola*, coded as DD-1, that shares 96.67-100% per locus identity with the

D. dianthicola strains from NCBI: ME23 (potato, the USA, Maine) and RNS04.9 (potato, France) with 96.67% homology for *acnA* and 100% for *gapA*, *icdA* and *mdh*.

The estimation of the best substitution model in jModelTest for the *Pectobacterium* spp. phylogeny was done based on 15 *P. c.* subsp. *brasiliensis* ingroup sequences: 3 newly detected haplotypes (PCB-2, PCB-3 and PCB-4) and 13 *P. c.* subsp. *brasiliensis* NCBI strains (1692^{T}, kbs1=PCB-1, BZA12, SX309, HG1501090302, A45, JKP4.3.22, BC1, 88/157-2, 1033, C18, 1073 and 213; Table 3). The proposed best model according to the Bayesian criterion was K80 (Kimura 2 parameter) model with invariable sites (Kimura 1980) and it was further used for the Maximum likelihood phylogenetic analysis. The reconstruction of *Pectobacterium* spp. phylogeny via Maximum likelihood analysis and genealogical relations of *P. c.* subsp. *brasiliensis* isolates in Median joining network have shown congruent results (Figure 3A; 3B). Two major *P. c.* subsp. *brasiliensis* genetic clusters are revealed in both, the obtained phylogenetic tree and network, and are marked following the previous notation by Nabhan et al. (2012a) (Figure 3A; 3B). Clade I of the obtained network, genetically distant from the rest of the analyzed haplotypes, includes three South American *P. c.* subsp. *brasiliensis* strains: the type strain 1692^{T} from Brazil, 213 also from Brazil and strain 1073 from Peru (Figure 3B). The second haplogroup is separated by 20 nucleotide differences and assembled of significantly more isolates (kbs1=PCB-1, PCB-2, PCB-3, PCB-4, SX309, HG1501090302, A45, JKP4.3.22, BZA12, BC1, 88/157-2, C18 and 1033). This genetic cluster corresponds to *P. c.* subsp. *brasiliensis* Clade II according to the affiliation of strains 1033 and C18 described by Nabhan et al. (2012a). Closely positioned to these two strains (1033 and C18) are isolates BC1, 88/157-2 and JKP4.3.22. Two haplotypes detected in Serbia, PCB-1=kbs-1 and PCB-2, are centrally positioned in the Clade II of the network and, jointly with the strain BZA12, apparently

interconnect previous five isolates (1033, C18, BC1, 88/157-2 and JKP4.3.22) with another haplogroup. This haplogroup is comprised of new haplotypes PCB-3 and PCB-4 from Serbia, along with strains SX309, HG1501090302 and A45 (Figure 3B). Topology of the Maximum likelihood tree shows highly supported (99) basal positioning of the *P. c.* subsp. *brasiliensis* Clade I strains, from which diverse Cluster II may have evolved (Figure 3A). Further branching of the Clade II mainly does not have good bootstrap supports, but still reflects *P. c.* subsp. *brasiliensis* isolate clustering revealed in the Median joining network. All three *P. c.* subsp. *brasiliensis* haplotypes from Serbia are clustered into a subclade with strains kbs-1 (potato, Japan), BZA12, SX309 and HG1501090302 (cucumber, China), A45 (potato, Syria), and JKP4.3.22 (potato, Germany). All *P. c.* subsp. *brasiliensis* isolates from Serbia and strains from the NCBI have formed a well-supported (100) genetic group in the phylogenetic tree, showing inner genetic divergence between Clade I and Clade II (Figure 3A). Strains of the *P. c.* subsp. *carotovorum* (ATCC 15713^{T} and Pcc10) and *P. c.* subsp. *odoriferum* (CFBP 1878^{T}) are shown as closely related and along with *P. actinidiae* (KKH3^{T}) and all *P. c.* subsp. *brasiliensis* strains are segregated as a monophyletic lineage with a high bootstrap support (97). *P. atrosepticum* (CFBP 1526^{T}) and *P. betavasculorum* (CFBP 2122^{T}) are grouped in a separate genetic lineage, similarly to the secluded *P. wasabiae* (CFBP 3304^{T}) and *P. parmentieri* (SCC 3193^{T}) as a more distant genetic branch. The determination of pairwise genetic distances showed a divergence between *P. c.* subsp. *brasiliensis* Clade I and Clade II of 2.3%, while their genetic distance in relation to the *P. c.* subsp. *carotovorum* is 3.9% and 3.4%, respectively (Table 4). Genetic distance between *P. c.* subsp. *brasiliensis* and other *Pectobacterium* species varies from 4.4% (*P. actinidiae*) to 7 % (*P. parmentieri*).

In case of the *Dickeya* spp. phylogenetic analysis, a suggested best fit model was HKY (Hasegawa-Kishino-Yano) (Hasegawa et al. 1985) proposed for the 3 *D. dianthicola* in-group genotypes: DD-1 genotype detected in potato in Serbia, strain GBBC2039 from Belgium, and the type strain of *D. dianthicola* NCPPB 453^{T} from the UK that is genetically identical as the strains M23 (USA), RNS04.9 (France) and IPO980 (Netherlands). *D. dianthicola* multilocus genotype DD-1 from Serbia showed the same genetic divergence from the NCPPB 453^{T}, RNS04.9 IPO980 and M23 strains and strain GBBC2039 of 0.4% (Table 4). This equal distance and central position of the haplotype DD-1 (potato, Serbia) between the other two *D. dianthicola* genotypes as an interconnecting link is shown in the Median joining network (Figure 4B). The maximum likelihood phylogenetic tree has shown highly supported (100) joint clustering of the *D. dianthicola* strains with well supported inner divergence between the two branches: one consisting of Serbian DD-1 isolate and Belgium strain GBBC2039, and another branch with the type strain NCPPB 453^{T} and another three identical strains: M23, RNS04.9 and IPO980 (Figure 4A). Further genetic relations of other *Dickeya* species are unambiguous. Three species have shown genetic similarity to *D. dianthicola*: *D. fangzhongdai* (DSM 101947 ^{T}), *D. solani* (IPO 2222 ^{T} and MK10) and *D. dadantii* (DSM 18020 ^{T}) are being jointly highly supported (100) as a secluded genetic group with the *D. dianthicola* isolates. Strains of the *D. chrysanthemi* (NCPPB 516 ^{T}) and *D. zeae* (Ech586) are basally positioned (Figure 4A). The obtained p-distances between *D. dianthicola* isolates and *D. fangzhongdai*, *D. solani* and *D. dadantii* vary from 5% to 5.8%, whilst *D. chrysanthemi* and *D. zeae* expresses twice as big divergence from *D. dianthicola* varying from 10.3% to 11.5% (Table 4).

DISCUSSION

455

456 Blackleg disease in potato crops in Serbia, recorded for the first time in the 1990s, was at that
457 time found to be caused by two bacterial species, *P. atrosepticum* and *P. c.* subsp. *carotovorum*
458 (Arsenijević et al. 1994; Obradović 1996). Today, more than twenty years later, our results
459 indicate *P. c.* subsp. *brasiliensis* as the causative agent of blackleg in Serbia, which appeared in
460 two consecutive years (2018-2019). In 2018 this pathogen was found to a low extent in
461 combined infection with *D. dianthicola.* To our knowledge, this is the first report on the presence
462 of both pathogenic bacteria on potato in Serbia, and it indicates that the causal pathogen
463 population has changed over the years. According to Van der Wolf (2018) the population
464 structure of blackleg causing organisms can change rapidly. Recently, a shift in pathogen
465 population structure has been noticed in the Netherlands, where *P. c.* subsp. *brasiliensis* has
466 recently replaced *Dickeya* sp. as the main causal agent of blackleg (Van der Wolf et al. 2007;
467 Van der Wolf et al. 2017; Van der Wolf 2018). Since the first isolation of *P. c.* subsp.
468 *brasiliensis* from potato in Brazil (Duarte et al. 2004), an increasing number of outbreaks has
469 been noticed worldwide in countries such as Peru, USA, Canada, South Africa, Germany, Japan,
470 Israel, Syria (Duarte et al. 2004; Ma et al. 2007; Nabhan et al. 2012a, b) and New Zealand
471 (Panda et al. 2012). Portier et al. (2019) has proposed that *P. c.* subsp. *brasiliense* should be
472 renamed or elevated to species level to *Pectobacterium brasiliense*. *D. dianthicola*, originally
473 described as a pathogen of *Dianthus,* was later also found to cause blackleg in potato and has
474 been reported for this crop by European countries as well as worldwide, e.g. the USA, Australia,
475 Morocco and Pakistan (Tsror et al. 2009; Oulghazi et al. 2017; Ma et al. 2018; Sarfraz et al.
476 2018; Wright at al. 2018; Nasaruddin et al. 2019). This bacterium is still not found on *Dianthus*
477 in the EU.

Latently infected potato seed imported from different parts of the world could explain the outbreaks caused by both pathogens in many countries (Tsror et al. 2009; Czajkowski et al. 2011). During both years of our potato monitoring, climatic conditions were favorable for blackleg development. In 2018 daily temperatures were higher as compared to 2019, with abundant rainfalls, providing more favorable environment for *D. dianthicola* development, as it was also observed during warm summers in North Finland (Degefu et al. 2013).

Blackleg disease in the three infested fields reached a high percentage of disease incidences (40-70%). The symptoms in the observed potato fields, in a single or combined infection, were identical and corresponded to those described previously (Pérombelon 2002; De Haan et al. 2008; Van der Merwe et al. 2010). Yield loss of 23.7% was established in case of the combined infection (*P. c.* subsp. *brasiliensis* and *D. dianthicola*) in 2018, while in 2019 losses were 10.5% and 44.7% respectively, when *P. c.* subsp. *brasiliensis* was present as the only one causal agent. No observed correlation was found between yield and detected bacteria in the field. According to Tsror et al. (2009) *Dickeya* spp. can cause, under favorable climatic conditions in Israel, a potato yield decrease from 20-25%. However, it is not possible to differentiate losses caused by *Pectobacterium* and *Dickeya* (Toth et al. 2011).

The results of conventional bacteriological tests of Serbian potato isolates mainly matched the characteristics of *Pectobacterium* sp. and *Dickeya* sp. as described previously (Duarte et al. 2004; Czajkowski et al. 2009; Tsror et al. 2009; Van der Merwe et al. 2010; Baghaee-Ravari et al. 2011; Nabhan et al. 2012a). Deviations were obtained for *D. dianthicola* isolates in tests such as negative reaction in casein hydrolysis and acid production from D-meliobiose, and positive reactions for arginine dihydrolase and utilization of tartrate (Czajkowski et al. 2009; Tsror et al. 2009; Baghaee-Ravari et al. 2011). Pathogenicity, confirmed on young potato plants, manifested

identical symptoms of wilting, blackleg and decaying of plants, as was observed in fields and cited by other authors (Tsror et al. 2009; Van der Merwe et al. 2010).

In preliminary identification of Serbian potato isolates, we obtained positive results with species-specific primers ADE1/ADE2 (Nassar et al. 1996) and Br1f/L1r (Duarte et al. 2004). In work with these primer sets Czajkowski et al. (2015) recommended the use of pure bacterial colonies or purified genomic DNA considering that sometimes false-positive reactions could be yielded. Anyhow, the authors suggested that 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.

DNA fingerprinting using rep-PCR provided five different groups among 38 potato isolates proving to be a useful tool for discrimination of different blackleg causing bacteria. The MLSA approach used for the identification and characterization of Serbian *P. c.* subsp. *brasiliensis* and *D. dianthicola* isolates enabled insight into the genetic relatedness of the detected isolates and previously described strains belonging to the same and other related taxa in phylogenetic analyses. Four housekeeping genes (*acnA, gapA, icdA* and *mdh*) used in this study have shown to be equally informative for revealing the phylogenetic and genealogical relations of *P. c.* subsp. *brasiliensis* and *D. dianthicola*, by confirming overall genetic clustering as previously done by employing as many as 7-8 housekeeping genes (Ma et al. 2007; Nabhan et al. 2012a, 2012b). Almeida et al. (2010) stated that MLST consists of sequencing of multiple loci, typically 4 to 8 housekeeping genes, and usually allows strains to be distinguished below the species level. Phylogenetic analysis based on *gapA,* and *mdh* housekeeping genes is an accurate method to characterize and differentiate *Pectobacterium* isolates (Baghaee-Ravari et al. 2011) or to distinguish *Dickeya* species (Palacio-Bielsa et al. 2010). Sławiak et al. (2009) developed a

method for rapid characterization of *Dickeya* species based on *dnaX* sequence which has proven to yield accurate clustering and identification. Ma et al. (2018) stated that the phylogenetic relationships reconstructed using *dnaX* data alone are congruent with the results using MLSA data with housekeeping genes when characterized North American blackleg-associated bacteria.

The analysis of the genetic structure of Serbian potato *P. c.* subsp. *brasiliensis* isolates and strains from NCBI based on *acnA, gapA, icdA* and *mdh* housekeeping genes, confirmed overall isolates clustering into two main genetic groups previously proposed by Nabhan et al. (2012a). Three new genotypes from Serbia (PCB-2, PCB-3 and PCB-4) showed their affiliation to the Clade II and suggest possible further sub-clustering in this haplogroup. Based on presented the MLSA results, any assumption on infection origin would be very speculative. Geography should not be taken as a decisive variable when interpreting phylogeny and network results due to the intensive global trade of the planting material, but still it should be taken into account as shown by the fact that North American *P. c.* subsp. *brasiliensis* strain 1033 was detached by a 19 nucleotide difference from the heterogeneous Clade II, as well as the other two strains from South America (1073 and 213) that form separate Clade I. In case of *D. dianthicola*, high bootstrap values support the phylogenetic relatedness of the *D. fangzhongdai*, *D. solani* and *D. dadantii* to the *D. dianthicola* forming a genetic clade, of which the in-group relations are presently insufficiently understood.

In conclusion, our study identified *P. c.* subsp. *brasiliensis* as the main causing pathogen of blackleg disease of potato in some fields in Northern Serbia, as well as *D. dianthicola,* found in the combined infection in 2018. Given results present the first detailed study about the genetic structure of the detected isolates of both bacteria. This new group of plant pathogens will be further studied for epidemiological features, including survival and dissemination.

ACKNOWLEDGMENT

This work was supported by the Ministry of Education, Science and Technological Development, Republic of Serbia. The authors are thankful to Živojin Bijelić for help in the current work with valuable data. We would like to thank both anonymous reviewers and the editor of this journal for their generous time in constructive comments, contribution with criticism, questions and valuable insightful on the paper, as led us to an improved manuscript.

REFERENCES

Almeida, N.F., Yan, S., Cai, R., Clarke, C. R., Morris, C. E., Schaad, N.W., Schuenzel, E.L., Lacy, G.H., Sun, X., Jones, J.B., Castillo, J.A., Bull, C.T., Leman, S., Guttman, D.S., Setubal, J.C., and Vinatzer, B.A. 2010. PAMDB, a multilocus sequence typing and analysis database and website for plant-associated microbes. Phytopathology 100:208-215.

Arsenijević, M., Đurišić, S., and Milošević, D. 1994. Karakteristike sojeva *Erwinia carotovora* subsp. *atroseptica* i *Erwinia carotovora* subsp. *carotovora* izolovanih iz obolelih stabljika i krtola krompira (in Serbo-Croatian) [Characteristics of *Erwinia carotovora* subsp. *atroseptica*, potato pathogen in our country]. Zast. Bilja 45:169-178.

568 Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl,
569 K. 2003. Current protocols in molecular biology (pp. 208-212). John Wiley & Sons. Inc.,
570 Hoboken. NJ.
571
572 Baghaee-Ravari, S., Rahimian, H., Shams-Bakhsh, M., Lopez-Solanilla, E., Antúnez-Lamas, M.,
573 and Rodríguez-Palenzuela, P. 2011. Characterization of *Pectobacterium* species from Iran using
574 biochemical and molecular methods. Eur. J. Plant Pathol. 129:413-425.
575
576 Bandelt, H.J., Forster, P., and Röhl, A. 1999. Median-joining networks for inferring intraspecific
577 phylogenies. Mol. Biol. Evol. 16:37-48.
578
579 Charkowski, A., Sharma, K., Parker, M.L., Secor, G.A., and Elphinstone, J. 2020. Bacterial
580 Diseases of Potato. In The Potato Crop (pp. 351-388). Springer, Cham. Switzerland.
581
582 Czajkowski, R., Grabe, G.J., and van der Wolf, J.M. 2009. Distribution of *Dickeya* spp. and
583 *Pectobacterium carotovorum* subsp. *carotovorum* in naturally infected seed potatoes. Eur. J.
584 Plant Pathol. 125:263-275.
585
586 Czajkowski, R., Pérombelon, M.C.M., van Veen, J.A., and van der Wolf, J.M. 2011. Control of
587 blackleg and tuber soft rot of potato caused by *Pectobacterium* and *Dickeya* species: a review.
588 Plant Pathol. 60:999-1013.
589

590 Czajkowski, R., Pérombelon, M.C.M., Jafra, S., Lojkowska, E., Potrykus, M., Van Der Wolf,
591 J.M. and Sledz, W. 2015. Detection, identification and differentiation of *Pectobacterium* and
592 *Dickeya* species causing potato blackleg and tuber soft rot: a review. Ann. Appl. Biol. 166:18-38.
593
594 De Boer, S.H., and Ward, L.J. 1995. PCR detection of *Erwinia carotovora* subsp. *atroseptica*
595 associated with potato tissue. Phytopathology 85:854-858.
596
597 De Boer, S.H., Li, X., and Ward, L.J. 2012. *Pectobacterium* spp. associated with bacterial stem
598 rot syndrome of potato in Canada. Phytopathology 102:937-947.
599
600 De Haan, E.G., Dekker-Nooren, T.C., van den Bovenkamp, G.W., Speksnijder, A.G., van der
601 Zouwen, P.S., and van der Wolf, J.M. 2008. *Pectobacterium carotovorum* subsp. *carotovorum*
602 can cause potato blackleg in temperate climates. Eur. J. Plant Pathol. 122:561-569.
603
604 Degefu, Y., Potrykus, M., Golanowska, M., Virtanen, E., and Lojkowska, E. 2013. A new clade
605 of *Dickeya* spp. plays a major role in potato blackleg outbreaks in North Finland. Ann. Appl.
606 Biol. 162:231-241.
607
608 Duarte, V., De Boer, S.H., Ward, L.J., and De Oliveira, A.M. 2004. Characterization of atypical
609 *Erwinia carotovora* strains causing blackleg of potato in Brazil. J. Appl. Microbiol. 96:535-545.
610
611 FAO (Food and Agriculture Organization) 2018. FAOSTAT.
612 http://www.fao.org/faostat/en/#data/QC.

Hasegawa, M., Kishino, H., and Yano, T.A. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22:160-174.

Hélias, V., Hamon, P., Huchet, E., Wolf, J.V.D., and Andrivon, D. 2011. Two new effective semi-selective crystal violet pectate (CVP) media for isolation of *Pectobacterium* and *Dickeya.* Plant Pathol. 61:339-345.

Kettani-Halabi, M., Terta, M., Amdan, M., Bouteau, F., and Ennaji, M.M. 2013. An easy, simple inexpensive test for the specific detection of *Pectobacterium carotovorum* subsp. *carotovorum* based on sequence analysis of the pmrA gene. BMC Microbiol. 13:176.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16:111-120.

Ma, B., Hibbing, M.E., Kim, H.S., Reedy, R.M., Yedidia, I., Breuer, J., Breuer, J., Glasner, J., Perna, N., Kelman, A., and Charkowski, A.O. 2007. Host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacterium* and *Dickeya*. Phytopathology 97:1150-1163.

Ma, X., Schloop, A., Swingle, B., and Perry, K.L. 2018. *Pectobacterium* and *Dickeya* responsible for potato blackleg disease in New York State in 2016. Plant Dis. 102:1834-1840.

635 Moleleki, L.N., Onkendi, E.M., Mongae, A., and Kubheka, G.C. 2013. Characterisation of
636 *Pectobacterium wasabiae* causing blackleg and soft rot diseases in South Africa. Eur. J. Plant
637 Pathol. 135:279-288.
638
639 Nabhan, S., De Boer, S.H., Maiss, E., and Wydra, K. 2012a. Taxonomic relatedness between
640 *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium carotovorum* subsp.
641 *odoriferum* and *Pectobacterium carotovorum* subsp. *brasiliense* subsp. nov. J. Appl. Microbiol.
642 113:904-913.
643
644 Nabhan, S., Wydra, K., Linde, M., and Debener, T. 2012b. The use of two complementary DNA
645 assays, AFLP and MLSA, for epidemic and phylogenetic studies of pectolytic enterobacterial
646 strains with focus on the heterogeneous species *Pectobacterium carotovorum*. Plant Pathol.
647 61:498-508.
648
649 Nassar, A., Darrasse, A., Lemattre, M., Kotoujansky, A., Dervin, C., Vedel, R., and Bertheau, Y.
650 1996. Characterization of *Erwinia chrysanthemi* by pectinolytic isozyme polymorphism and
651 restriction fragment length polymorphism analysis of PCR-amplified fragments of pel genes.
652 Appl. Environ. Microbiol. 62:2228-2235.
653
654 Nasaruddin, A.S., Charkowski, A.O., Babler, B.N., Perna, N.T., and Glasner, J.D. 2019. First
655 report of *Dickeya dianthicola* causing blackleg on potato in Texas. Plant Dis. 103:2121-2121.
656

Obradović, A. 1996. Patogene i biohemijsko-fiziološke karakteristike bakterija grupe "*Erwinia carotovora*" parazita krompira (in Serbian=Croatian) [Pathogenic, biochemical and physiological characteristics of the bacteria „*Erwinia carotovora*" group, the potato pathogens]. Zast. Bilja 215:57-70.

Oerke, E.C. 2006. Crop losses to pests. J. Agric. Sci. 144:31-43.

Oulghazi, S., Khayi, S., Lafkih, N., Massaoudi, Y., El Karkouri, A., El Hassouni, M., Denis, F., and Moumni, M. 2017. First Report of *Dickeya dianthicola* Causing Blackleg Disease on Potato in Morocco. Plant Dis. 101:1671-1672.

Palacio-Bielsa, A., Mosquera, M.E.R., Álvarez, M.A.C., Rodríguez, I.M.B., López-Solanilla, E., and Rodríguez-Palenzuela, P. 2010. Phenotypic diversity, host range and molecular phylogeny of Dickeya isolates from Spain. Eur. J. Plant Pathol. 127:311-324.

Panda, P., Fiers, M.A.W.J., Armstrong, K., and Pitman, A.R. 2012. First report of blackleg and soft rot of potato caused by *Pectobacterium carotovorum* subsp. *brasiliensis* in New Zealand. New Dis. Rep. 26:2044-0588.

Pavel, A.B., and Vasile, C.I. 2012. PyElph-a software tool for gel images analysis and phylogenetics. BMC Bioinform. 13:9-15.

679 Pérombelon, M.C. 2002. Potato diseases caused by soft rot erwinias: an overview of
680 pathogenesis. Plant Pathol. 51:1-12.
681
682 Portier, P., Pédron, J., Taghouti, G., Fischer-Le Saux, M., Caullireau, E., Bertrand, C., Laurent,
683 A., Chawki, K., Oulgazi, S., Moumni, M., and Andrivon, D. 2019. Elevation of *Pectobacterium*
684 *carotovorum* subsp. *odoriferum* to species level as *Pectobacterium odoriferum* sp. nov., proposal
685 of *Pectobacterium brasiliense* sp. nov. and *Pectobacterium actinidiae* sp. nov., emended
686 description of *Pectobacterium carotovorum* and description of *Pectobacterium versatile* sp. nov.,
687 isolated from streams and symptoms on diverse plants. Int. J. Syst. Evol. Microbiol.
688 69(10):3207-3216.
689
690 Posada, D. 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25:1253-1256.
691
692 Sarfraz, S., Riaz, K., Oulghazi, S., Cigna, J., Alam, M.W., Dessaux, Y., and Faure, D. 2018. First
693 report of *Dickeya dianthicola* Causing blackleg disease on potato plants in Pakistan. Plant Dis.
694 102:2027.
695
696 Schaad, N.W., Jones, J.B., and Chun, W. 2001. Laboratory guide for the identification of plant
697 pathogenic bacteria. No.Ed.3. The American Phytopathological Society, St. Paul, MN., USA.
698
699 Sławiak, M., van Beckhoven, J.R., Speksnijder, A.G., Czajkowski, R., Grabe, G. and van der
700 Wolf, J.M. 2009. Biochemical and genetical analysis reveal a new clade of biovar 3 *Dickeya* spp.
701 strains isolated from potato in Europe. Eur. J. Plant Pathol. 125:245-261.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30:2725-2729.

Toth, I.K., van der Wolf, J.M., Saddler, G., Lojkowska, E., Hélias, V., Pirhonen, M., Tsror, L., and Elphinstone, J.G. 2011. *Dickeya* species: an emerging problem for potato production in Europe. Plant Pathol. 60:385-399.

Toth, I., Saddler, G., and Elphinstone, J. 2014. Investigating the biology and appropriate control of *Dickeya* species affecting GB potatoes. Kenilworth: Potato Council, 87. https://potatoes.ahdb.org.uk/sites/default/files/publication_upload/R437%20Final%20Report%20April%202014.pdf

Tsror, L., Erlich, O., Lebiush, S., Hazanovsky, M., Zig, U., Slawiak, M., Grabe, G., van der Wolf, J.M., and Van de Haar, J.J. 2009. Assessment of recent outbreaks of *Dickeya* sp. (syn. *Erwinia chrysanthemi*) slow wilt in potato crops in Israel. Eur. J. Plant Pathol. 123:311-320.

Van der Merwe, J.J., Coutinho, T.A., Korsten, L., and van der Waals, J.E. 2010. *Pectobacterium carotovorum* subsp. *brasiliensis* causing blackleg on potatoes in South Africa. Eur. J. Plant Pathol. 126:175-185.

Van der Wolf, J.V., Speksnijder, A., Velvis, H., Haar, J.V., and Doorn, J.V. 2007. Why is *Erwinia chrysanthemi* (*Dickeya* sp.) taking over? The ecology of a blackleg pathogen. In New

725 and old pathogens of potato in changing climate: Proceedings of the EAPR Pathology Section
726 seminar, 2.-6th of July 2007, Hattula, Finland/Asko Hannukkala and Marjo Segerstedt (eds.).
727 MTT.
728
729 Van der Wolf, J.M., De Haan, E.G., Kastelein, P., Krijger, M., De Haas, B.H., Velvis, H.,
730 Mendes, O., Kooman-Gersmann. M., and Van Der Zouwen, P.S. 2017. Virulence of
731 *Pectobacterium carotovorum* subsp. *brasiliense* on potato compared with that of other
732 *Pectobacterium* and *Dickeya* species under climatic conditions prevailing in the Netherlands.
733 Plant Pathol. 66:571-583.
734
735 Van der Wolf, J.M. 2018. Shifting populations of blackleg causing organisms: Significance and
736 possible control strategies. Phytopathology 108:281-281.
737
738 Versalovic, J., Schneider, M., De Bruijn, F.J., and Lupski, J.R. 1994. Genomic fingerprinting of
739 bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol. Cell. Biol.
740 5:25-40.
741
742 Wright, D., Bwye, A., Banovic, M., Baulch, J., Wang, C., Hair, S., Hammond, N., Coutts, B.,
743 and Kehoe, M. 2018. First Report of *Dickeya dianthicola* in Potatoes in Australia. Plant Dis.
744 102:2029-2029.
745
746
747

748 **FIGURE CAPTIONS:**

749

750 **Figure 1.** Blackleg symptoms on potato cv. Lady Claire collected in the Bačka region, Serbia
751 (2018, 2019). (A) lesions formed on the below ground part of stem, (B) brown discoloration of
752 tissue at the stem base at soil/air level, (C) lesions on upper part of stems, (D) focus of decaying
753 plants in the field, (E) soft rot of progeny tuber.

754

755 **Figure 2.** Dendrogram generated using UPGMA clustering method based on (A) BOX-PCR, (B)
756 ERIC-PCR and (C) REP-PCR results for 38 *P. c.* subsp. *brasiliensis* and *D. dianthicola* isolates
757 from Serbia. Reference strains Pcc10 and MK10 are marked with a red dot and blue rectangle,
758 respectively. Genetic distances are presented with numbers placed on the branches.

759

760 **Figure 3.** Reconstruction of the phylogenetic relations evaluated among and between four gene-
761 multilocus genotypes (*acnA, gapA, icdA* and *mdh*) of 4 *P. c.* subsp. *brasiliensis* haplotypes
762 detected in Serbia and sequence data of a selection of 21 *Pectobacterium* spp. strains from the
763 NCBI database (12 from potato). Geographic origin of the haplotypes is color-marked as given
764 in the legend. (A) Maximum likelihood phylogenetic tree reconstructed using the K2P+I model
765 with bootstrap support values >50 given at the nodes; bar: the estimated nucleotide substitutions
766 per site are 0.02. (B) Median joining network obtained for the *P. c.* subsp. *brasiliensis*
767 haplotypes. Circle sizes are proportional to the number of strains belonging to a specific
768 haplotype. Each black dot on the lines connecting the haplotypes marks one mutation; more than
769 5 nucleotide differences are shown with the corresponding number and abbreviation “n.df.”
770 (nucleotide differences). Red interconnecting dots are median vectors that represent missing or

771 unsampled intermediate haplotypes. Matching of the genetic Clades I and II on the phylogenetic
772 tree and network is noted in-between.
773
774 **Figure 4.** Reconstruction of the phylogenetic relations based on four gene-multilocus genotypes
775 (*acnA, gapA, icdA* and *mdh*) evaluated among and between 6 *D. dianthicola* isolates (1 Serbian
776 haplotype and 5 *D. dianthicola* NCBI database strains) and 5 strains of other *Dickeya* spp.
777 selected from the NCBI database. Geographic origin of the isolates is marked in specific color as
778 given in the legend. (A) Maximum likelihood phylogenetic tree reconstructed using the HKY
779 model with bootstrap support values >50 given at the nodes; bar: the estimated nucleotide
780 substitutions per site are 0.02. (B) Median joining network obtained for the *D. dianthicola*
781 haplotypes. Circle sizes are proportional to the number of isolates belonging to a specific
782 haplotype. Each black dot on the lines connecting the haplotypes marks one mutation.

Table 1. Serbian potato *P. c.* subsp. *brasiliensis* and *D. dianthicola* isolates used in the present study, locality, year of isolation, organ, DNA fingerprinting group affiliation and GenBank accession numbers.

Isolate code	Year of isolation	Locality	Organ	DNA fingerprinting group			Accesion number			
				BOX	ERIC	REP	*acnA*	*gapA*	*icdA*	*mdh*
Dd31	2018	Obrovac	Stem	III	III	III	MK604559	MK604561	MK604563	MK604569
Dd32	2018	Obrovac	Stem	III	III	III	-	-	-	-
Dd35	2018	Obrovac	Stem	III	III	III	-	-	-	-
Dd37	2018	Obrovac	Stem	III	III	III	-	-	-	-
Dd41	2018	Obrovac	Stem	III	III	III	-	-	-	-
Dd42	2018	Obrovac	Stem	III	III	III	-	-	-	-
Dd44	2018	Obrovac	Stem	III	III	III	MK604560	MK604562	MK604564	MK604570
Dd46	2018	Obrovac	Stem	III	III	III	-	-	-	-
Dd47	2018	Obrovac	Stem	III	III	III	-	-	-	-
Pcb33	2018	Obrovac	Stem	II	II	II	MK604547	MK604549	MK604551	MK604557
Pcb34	2018	Obrovac	Stem	II	II	II	-	-	-	-
Pcb38	2018	Obrovac	Stem	II	II	II	-	-	-	-
Pcb39	2018	Obrovac	Stem	II	II	II	-	-	-	-
Pcb61	2018	Obrovac	Stem	II	II	II	-	-	-	-
Pcb62	2018	Obrovac	Stem	II	II	II	MK604548	MK604550	MK604552	MK604558
Pcb64	2018	Obrovac	Stem	II	II	II	-	-	-	-
Pcb67	2018	Obrovac	Stem	II	II	II	-	-	-	-
Pcb2531	2019	Maglić	Stem	I	I	I	-	-	-	-
Pcb2538	2019	Maglić	Stem	I	I	I	-	-	-	-
Pcb2544	2019	Maglić	Stem	I	I	I	MT134020	MT134026	MT134032	MT134038
Pcb2549	2019	Maglić	Stem	I	I	I	-	-	-	-
Pcb2562	2019	Maglić	Stem	I	I	I	MT134019	MT134025	MT134031	MT134037
Pcb2563	2019	Maglić	Stem	I	I	I	-	-	-	-
Pcb2568	2019	Maglić	Stem	I	I	I	-	-	-	-
Pcb2811	2019	Kulpin	Tuber	IV	IV	IV	MT134018	MT134024	MT134030	MT134036
Pcb2812	2019	Kulpin	Tuber	IV	IV	IV	-	-	-	-
Pcb2813	2019	Kulpin	Tuber	IV	IV	IV	-	-	-	-
Pcb2815	2019	Kulpin	Tuber	IV	IV	IV	-	-	-	-
Pcb2817	2019	Kulpin	Tuber	IV	IV	IV	MT134017	MT134023	MT134029	MT134035
Pcb2819	2019	Kulpin	Tuber	IV	IV	IV	-	-	-	-
Pcb2833	2019	Kulpin	Stem	V	V	V	-	-	-	-
Pcb2838	2019	Kulpin	Stem	V	V	V	-	-	-	-
Pcb2839	2019	Kulpin	Stem	V	V	V	-	-	-	-
Pcb2841	2019	Kulpin	Stem	V	V	V	-	-	-	-
Pcb2842	2019	Kulpin	Stem	V	V	V	MT134016	MT134022	MT134028	MT134034
Pcb2844	2019	Kulpin	Stem	V	V	V	-	-	-	-
Pcb2847	2019	Kulpin	Stem	V	V	V	-	-	-	-
Pcb2861	2019	Kulpin	Stem	V	V	V	MT134015	MT134021	MT134027	MT134033

Isolates marked in bold were used in MLSA. Isolates marked in grey field have been deposited in NCBI.

1 **Table 2.** Primers used in this study and their corresponding profiles

Primer name	Primer sequences	Fragment length (bp)	Annealing temperature (°C)	Reference
	A specific primer sets for detection of Pectobacterium spp. *and Dickeya* spp.			
ADE1 ADE2	5′-ATCAGAAAGCCCGCAGCCAGAT-3′ 5′-CTGTGGCCGATCAGGATGGTTTTGTCGTGC-3′	420	72	Nassar et al. (1996)
F0145 E2477	5′-TACCCTGCAGATGAAATTATTGATTGTTGAAGAC-3′ 5′-TACCAAGCTTTGGTTGTTCCCCTTTGGTCA-3′	666	55	Kettani-Halabi et al. (2013)
ECA1f ECA2r	5′-CGGCATCATAAAAACACG-3′ 5′-GCACACTTCATCCAGCGA-3′	690	62	De Boer and Ward (1995)
Br1f L1r	5′-GCGTGCCGGGTTTATGACCT-3′ 5′-CAAGGCATCCACCGT-3′	322	62	Duarte et al. (2004)
	The repetitive-PCR fingerprinting primer sets			
BOXA1R	5′-CTACGGCAAGGCGACGCTGACG-3′	-	52	Versalovic et al. (1994)
ERIC1R ERIC2	5′-ATGTAAGCTCCTGGGGATTCAC-3′ 5′-AAGTAAGTGACTGGGGTGAGCG-3′	-	52	
REP1R-I REP2-I	5′-IIIICGICGICATCIGGC-3′ 5′-ICGICTTATCIGGCCTAC-3′	-	40	
	Multilocus sequence analysis primer sets			
acnA3F acnA3R	5′-CMAGRGTRTTRATGCARGAYTTTAC-3′ 5′-GATCATGGTGGTRTGSGARTCVGT-3′	300	52	Ma et al. (2007)
icdA400F icdA977R	5′-GGTGGTATCCGTTCTCTGAACG-3′ 5′-TAGTCGCCGTTCAGGTTCATACA-3′	520	52	
gapA326F gapA845R	5′-ATCTTCCTGACCGACGAAACTGC-3′ 5′-ACGTCATCTTCGGTGTAACCCAG-3′	450	52	
mdh2 mdh4	5′-GCGCGTAAGCCGGGTATGGA-3′ 5′-CGCGGCAGCCTGGCCCATAG-3′	500	52	Moleleki et al. (2013)

2

Table 3. NCBI GenBank MLSA data of different *Pectobacterium* spp. and subspecies and *Dickeya* spp. used for comparison with our MLSA results for 25 Serbian potato blackleg isolates using the same set of genes (*acnA*, *gapA*, *icdA* and *mdh*).

Strain name	Host	Origin	GenBank Accession number			
			acnA	*gapA*	*icdA*	*mdh*
Pectobacterium carotovorum* subsp. *brasiliensis						
BZA12	Cucumber	China	CP024780	CP024780	CP024780	CP024780
SX309	Cucumber	China	CP020350	CP020350	CP020350	CP020350
BC1	Chinese cabbage	China	CP009769	CP009769	CP009769	CP009769
HG1501090302	Cucumber	China	KX010008	KX010017	KX010026	KX010035
kbs-1	Potato	Japan	LC145701	LC145702	LC145703	LC145704
A45	Potato	Syria	HM156766	HM156826	HM156887	HM156948
C18	Potato	Syria	HM156768	HM156828	HM156889	HM156950
JKP4.3.22	Potato	Germany	HM156792	HM156851	HM156913	HM156974
88/157-2	Potato	Switzerland	KP404135	KP404136	KP404137	KP404138
1033	Potato	Canada	JF926764	JF926774	JF926784	JF926794
1073	Potato	Peru	HM156787	HM156848	HM156910	HM156969
213	Potato	Brazil	JF926771	JF926781	JF926791	JF926801
1692^{T}	Potato	Brazil	NZ_CP047495	NZ_CP047495	NZ_CP047495	NZ_CP047495
Pectobacterium carotovorum* subsp. *carotovorum						
ATCC 15713^{T}	Potato	Denmark	FJ895848	FJ895849	FJ895850	FJ895851
Pcc10	Cabbage	Bosnia and Herzegovina	MT452473	MT188696	MT452474	MT188698
Pectobacterium atrosepticum						
CFBP 1526^{T}	Potato	UK	JN600333	JN600336	JN600339	JN600342
Pectobacterium wasabiae						
CFBP 3304^{T}	*Eutrema wasabi*	Japan	CP015750	CP015750	CP015750	CP015750
Pectobacterium parmentieri						
SCC 3193^{T}	Potato	Finland	NC_017845	NC_017845	NC_017845	NC_017845
Pectobacterium carotovorum* subsp. *odoriferum						
CFBP 1878^{T}	*Witloof chicory*	France	JF926763	JF926773	JF926783	JF926793
Pectobacterium betavasculorum						
CFBP 2122^{T}	*Beta vulgaris*	USA	JN600334	JN600337	JN600340	JN600343
Pectobacterium actinidiae						
KKH3^{T}	Kiwi	South Korea	JRMH01000001	JRMH01000001	JRMH01000001	JRMH01000001
Dickeya dianthicola						
RNS04.9	Potato	France	CP017638	CP017638	CP017638	CP017638
IPO980	Potato	Netherlands	NZCM002023	NZCM002023	NZCM002023	NZCM002023
GBBC2039	Potato	Belgium	NZCM001838	NZCM001838	NZCM001838	NZCM001838
ME23	Potato	USA Maine	CP031560	CP031560	CP031560	CP031560
NCPPB 453^{T}	Dianthus	UK	NZ_CM001841	NZ_CM001841	NZ_CM001841	NZ_CM001841
Dickeya dadantii						
DSM 18020^{T}	Geranium	Comoros	CP023467	CP023467	CP023467	CP023467

		Dickeya fangzhongdai				
DSM 101947^T	*Pyrus pyrifolia*	China	NZ_CP025003	NZ_CP025003	NZ_CP025003	NZ_CP025003
		Dickeya solani				
IPO 2222^T	Potato	Netherlands	NZ_CP015137	NZ_CP015137	NZ_CP015137	NZ_CP015137
MK10	Potato	Israel	NZCM001839	NZCM001839	NZCM001839	NZCM001839
		Dickeya zeae				
Ech586	Philodendron	USA Florida	CP001836	NC013592	NC013592	NC013592
		Dickeya chrysanthemi				
NCPPB 516^T	*Parthenium argentatum*	Denmark	NZ_CM001904	NZ_CM001904	NZ_CM001904	NZ_CM001904

4 Type strains are marked with sign ^{T}.

Table 4. Average genetic divergence according to pairwise analysis (p-distance method) based on concatenated partial sequences of genes *acnA, gapA, icdA* and *mdh* between *P. c.* subsp. *brasiliensis* / *D. dianthicola* and selected strains belonging to other subspecies/species. Standard errors are shown above the diagonal and were obtained by a bootstrap procedure (1.000 replicates).

Species / Subspecies	***P* / SE**									
	1	2	3	4	5	6	7	8	9	10
1. *P. c.* subsp. *brasiliensis* Clade II		0.003	0.004	0.004	0.005	0.007	0.006	0.007	0.007	0.010
2. *P. c.* subsp. *brasiliensis* Clade I	0.023		0.004	0.005	0.005	0.007	0.006	0.007	0.007	0.010
3. *P. c.* subsp. *carotovorum*	0.034	0.039		0.003	0.004	0.006	0.006	0.006	0.007	0.010
4. *P. c.* subsp. *odoriferum*	0.036	0.040	0.022		0.005	0.006	0.006	0.006	0.007	0.010
5. *P. actinidiae*	0.044	0.044	0.040	0.035		0.006	0.006	0.006	0.007	0.010
6. *P. atrosepticum*	0.060	0.065	0.058	0.057	0.058		0.006	0.007	0.007	0.010
7. *P. betavasculorum*	0.061	0.067	0.063	0.062	0.070	0.060		0.006	0.007	0.010
8. *P. wasabiae*	0.067	0.070	0.066	0.064	0.061	0.066	0.067		0.005	0.010
9. *P. parmentieri*	0.070	0.069	0.069	0.064	0.064	0.066	0.074	0.032		0.010
10. *Y. pestis*	0.179	0.180	0.177	0.178	0.176	0.179	0.177	0.176	0.179	

Species / Subspecies	***P* / SE**								
	1	2	3	4	5	6	7	8	9
1. *D. dianthicola* SRB		0.002	0.002	0.006	0.006	0.006	0.008	0.008	0.011
2. *D. dianthicola* Belgium	0.004		0.002	0.006	0.006	0.006	0.008	0.008	0.011
3. *D. dianthicola* USA-UK^{T}-France-Netherlands	0.004	0.007		0.006	0.006	0.006	0.008	0.008	0.011
4. *D. fangzhongdai*	0.050	0.051	0.050		0.005	0.005	0.008	0.008	0.011
5. *D. dadantii*	0.055	0.056	0.054	0.053		0.006	0.007	0.008	0.010
6. *D. solani*	0.056	0.058	0.054	0.045	0.056		0.008	0.008	0.010
7. *D. chrysanthemi*	0.104	0.105	0.103	0.104	0.105	0.104		0.008	0.010
8. *D. zeae*	0.114	0.115	0.113	0.110	0.110	0.107	0.098		0.010
9. *Y. pestis*	0.200	0.199	0.200	0.195	0.195	0.194	0.196	0.190	

P, p-distance over sequence pairs between groups; SE, standard error.

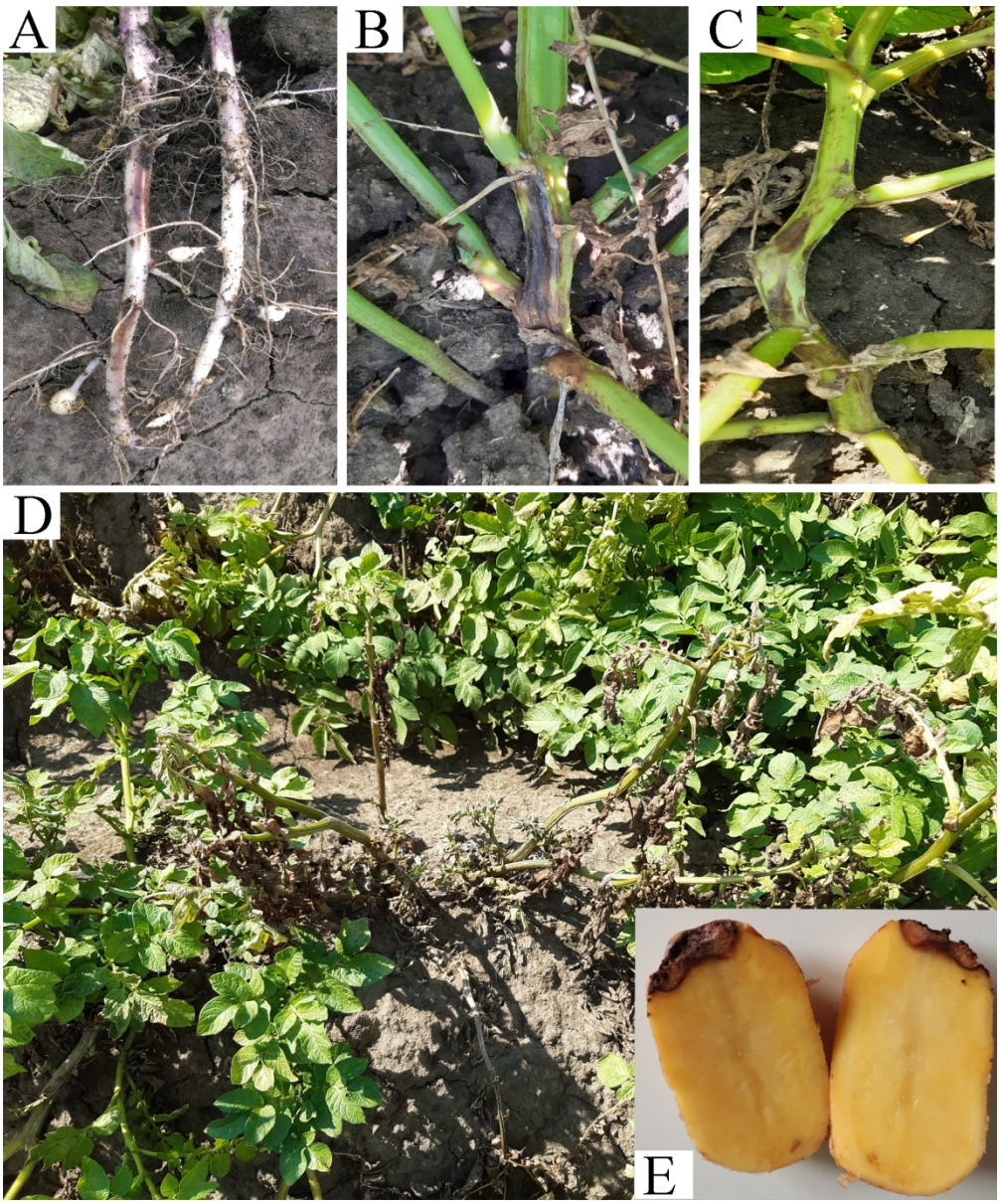

Figure 1. Blackleg symptoms on potato cv. Lady Claire collected in the Bačka region, Serbia (2018, 2019). (A) lesions formed on the below ground part of stem, (B) brown discoloration of tissue at the stem base at soil/air level, (C) lesions on upper part of stems, (D) focus of decaying plants in the field, (E) soft rot of progeny tuber.

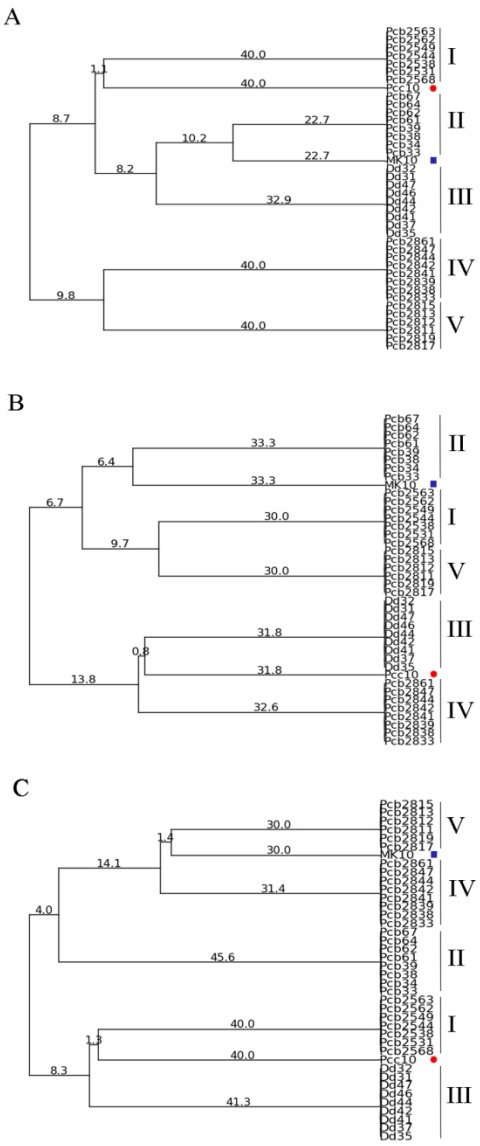

Figure 2. Dendrogram generated using UPGMA clustering method based on (A) BOX-PCR, (B) ERIC-PCR and (C) REP-PCR results for 38 P. c. subsp. brasiliensis and D. dianthicola isolates from Serbia. Reference strains Pcc10 and MK10 are marked with a red dot and blue rectangle, respectively. Genetic distances are presented with numbers placed on the branches.

99x199mm (300 x 300 DPI)

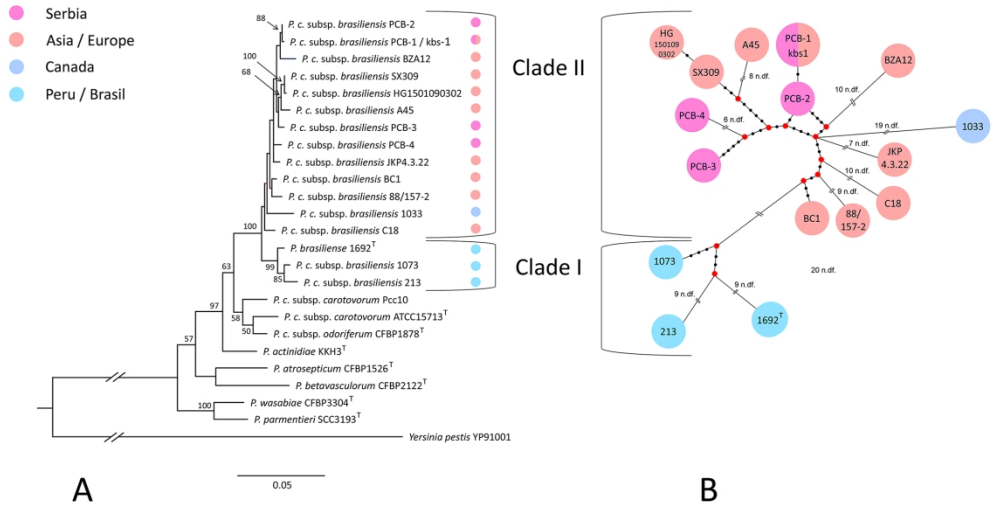

Figure 3. Reconstruction of the phylogenetic relations evaluated among and between four gene-multilocus genotypes (acnA, gapA, icdA and mdh) of 4 P. c. subsp. brasiliensis haplotypes detected in Serbia and sequence data of a selection of 21 Pectobacterium spp. strains from the NCBI database (12 from potato). Geographic origin of the haplotypes is color-marked as given in the legend. (A) Maximum likelihood phylogenetic tree reconstructed using the K2P+I model with bootstrap support values >50 given at the nodes; bar: the estimated nucleotide substitutions per site are 0.02. (B) Median joining network obtained for the P. c. subsp. brasiliensis haplotypes. Circle sizes are proportional to the number of strains belonging to a specific haplotype. Each black dot on the lines connecting the haplotypes marks one mutation; more than 5 nucleotide differences are shown with the corresponding number and abbreviation “n.df.” (nucleotide differences). Red interconnecting dots are median vectors that represent missing or unsampled intermediate haplotypes. Matching of the genetic Clades I and II on the phylogenetic tree and network is noted in-between.

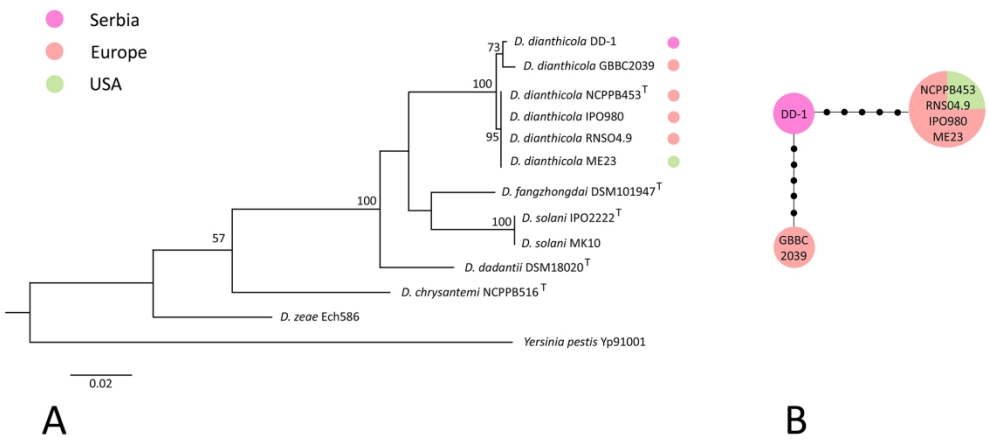

Figure 4. Reconstruction of the phylogenetic relations based on four gene-multilocus genotypes (acnA, gapA, icdA and mdh) evaluated among and between 6 D. dianthicola isolates (1 Serbian haplotype and 5 D. dianthicola NCBI database strains) and 5 strains of other Dickeya spp. selected from the NCBI database. Geographic origin of the isolates is marked in specific color as given in the legend. (A) Maximum likelihood phylogenetic tree reconstructed using the HKY model with bootstrap support values >50 given at the nodes; bar: the estimated nucleotide substitutions per site are 0.02. (B) Median joining network obtained for the D. dianthicola haplotypes. Circle sizes are proportional to the number of isolates belonging to a specific haplotype. Each black dot on the lines connecting the haplotypes marks one mutation.