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Genotypic and phenotypic variability of *Pectobacterium* strains causing blackleg and soft rot on potato in Turkey

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Abstract Pectinolytic bacteria were isolated from 48 potato plants showing the symptoms of blackleg and collected in different fields of commercial potato production areas at Samsun, Amasya, Corum and Yozgat provinces in Turkey in 2015. The survey resulted in the isolation of 26 pectinolytic strains that belonged to *P. atrosepticum, P. carotovorum* subsp. *brasiliense, P. carotovorum* subsp. *carotovorum* and *P. parmentieri* species based on molecular identification with species-specific PCR and phenotypic characterization. The identified strains indicated typical biochemical characteristics of the assigned species. For 16 representative *Pectobacterium* isolates 10 unique rep-PCR band patterns were obtained. The *16S rRNA* and *recA* and *gapA* gene fragment sequencing confirmed the species identity of the isolates. The phenotypic characterization of the strains revealed that for all assays but one (cellulase, protease activity, swimming but not swarming), the tested *Pectobacterium* species were significantly different from each other proving the diversity of the strains belonging to these genera. Recent outbreaks of blackleg and/or soft rot in potato production areas in Turkey may pose a threat on other crops, as tomato, pepper, cucumber, onion, cabbage, broccoli and sugar beet are cultivated in the same provinces.

Keywords Pectinolytic *Erwinia* · *Pectobacterium atrosepticum* · *Pectobacterium carotovorum* subsp. *brasiliense* · *Pectobacterium carotovorum* subsp. *carotovorum* · *Pectobacterium parmentieri* · rep-PCR · *recA*

Introduction

Potato (*Solanum tuberosum* L.) is one of the most important plants cultivated all over the world with the production rate of 381 million tons, which was grown on about 19 million hectares of land (FAO 2014). Potato is affected by a number of pathogenic organisms such as e. g. pectinolytic bacteria from the genus *Dickeya* and *Pectobacterium* that were listed among the top 10 most important plant pathogens (Mansfield et al. 2012). Pectinolytic bacteria can cause wilting of the plant, rotting on daughter tubers, brown discoloration of vascular tissues and blackleg on potato stem base (Pérombelon and Kelman 1980). Seed potato production is adversely affected by pectinolytic bacteria due to downgrading or rejection of seed potatoes during certification process. Moreover, *Dickeya* spp. and *Pectobacterium* spp. can spread through latently infected seed tubers, which may facilitate their introduction to

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other countries (Perombelon 2002; Toth et al. 2011; Czajkowski et al. 2015). *Pectobacterium* and *Dickeya* species secrete a wide range of cell wall-degrading enzymes (PCWDE) such as pectinases, cellulases and proteases that are considered to be important virulence determinants responsible for disease symptoms development (Hugouvieux-Cotte-Pattat et al. 2014).

The pectinolytic bacteria used to be classified into *Enterobacteriaceae* family (Lelliot and Dickey 1984). As a result of recent reclassifications, Adeolu et al. (2016) introduced a new family Pectobacteriaceae, to which the *Dickeya* and *Pectobacterium* genus were transferred. Currently, the *Pectobacterium* genus comprises of 10 species, and most of them were reported to cause blackleg and/or soft rot on potato, namely *P. atrosepticum* (Gardan et al. 2003), *P. parmentieri* (Khayi et al. 2016), *P. carotovorum* subsp. *brasiliense* (Duarte et al. 2004), *P. carotovorum* subsp. *carotovorum* (Hauben et al. 1998) as well as newly established *P. polaris* (Dees et al. 2017a, b) and *P. peruviense* (Waleron et al. 2017). The genus *Dickeya* consists of 8 species but only strains of *D. dianthicola* and *D. solani* cause blackleg and soft rot on potato in temperate climate regions (Toth et al. 2011; Potrykus et al. 2016).

Bacteria from *P. atrosepticum* genus are found exclusively on potato plants. They seem to be still of major concern in countries such as Scotland, where the climate conditions are cooler and wetter (G. Saddler, personal information). Additionally, in Poland and Norway, the number of potato plants infected with *P. atrosepticum* is substantial reaching 40% of all the samples tested in different years (Sledz et al. 2000; Waleron et al. 2002; Dees et al. 2017a, b).

Recently, the importance of *P. parmentieri* strains for potato blackleg and soft rot symptoms occurrence in field conditions has been investigated in more details. Both *P. parmentieri* and *P. carotovorum* subsp. *brasiliense*, happened to remain unnoticed in Europe and temperate climate countries through years due to the unavailability of the effective identification methods and finally misclassification to *P. c.* subsp. *carotovorum* (Nabhan et al. 2012; Nykyri et al. 2012; Waleron et al. 2013, 2015). *P. carotovorum* subsp. *brasiliense* strains have been shown to be highly virulent and to cause severe blackleg incidences on potato in Brazil and South Africa (Duarte et al. 2004; Van der Merwe et al. 2010). During the last ten years aggressive strains of *P. c.* subsp. *brasiliense* have been reported on potato and they have caused increasing losses in European countries and other parts of the world (Ma et al. 2007; Panda et al. 2012; Ngadze et al. 2012; Nabhan et al. 2012; De Boer et al. 2012; Leite et al. 2014; Lee et al. 2014; Onkendi and Moleleki 2014; Waleron et al. 2015; van der Wolf et al. 2017). Nevertheless, about 30% of the isolates from potato plants showing symptoms of blackleg and/or soft rot are identified as *P. c.* subsp. *carotovorum*, which is a very heterologous group (Sledz et al. 2000; Dees et al. 2017a, b).

Soft rot bacteria were reported to cause disease symptoms in a number of plants in Turkey. In the previous investigations concerning potato plantations both *P. c.* subsp. *carotovorum* (*Erwinia carotovora* subsp. *carotovora)* and *P. atrosepticum* (*Erwinia carotovora* subsp. *atroseptica*) were causal agents of blackleg in field and soft rot in storage in Central Anatolian region of Turkey (Benlioglu 1991; Benlioglu et al. 1991). Moreover, *P. c.* subsp. *carotovorum* (*Erwinia carotovora* subsp. *carotovora)* and *Dickeya* spp. (*Erwinia chrysanthemi*) were reported on tomato plants (Aysan et al. 2005) and *Dieffenbachiae amonea* (Cetinkaya-Yildiz et al. 2004), while *P. carotovorum* strains were reported to cause disease on tulips (Boyraz et al. 2006) and several ornamental hosts: *Cactus* spp., *Dieffenbachia* spp., *Drecena massangena*, *Drecena marginata*, *Primula* sp., *Schefflera actinophylla*, *Senecio cruentus*, *Syngonium podphyllum* and *Yucca aloifolia* (Aysan et al. 2009). Additionally, bacteria from *P. atrosepticum* were reported to cause stalk and head rot on sunflower in Turkey (Bastas et al. 2009).

The current status of the causal agents of serious losses in Turkish potato production remains unknown since the last information about pectinolytic bacteria in potato production was published 25 years ago (Benlioglu 1991; Benlioglu et al. 1991). To our knowledge, this is the first up-to-date report detailing the occurrence and characterization of the pectinolytic bacteria isolated from potato in Turkey.

Materials and methods

Bacterial strains, isolation and media conditions

Twenty six pectinolytic strains were isolated from diseased plants with blackleg and/or soft rot symptoms in commercial potato production. Pieces of infected tissue were homogenized in sterile water and plated on Nutrient Agar (Himedia, India). Uniform bacterial colonies were

plated on Luria Agar (Himedia, India) or Crystal Violet Pectate (CVP) medium (Hélias et al. 2012). Bacteria were kept for long-term storage, in 40% glycerol at −80 °C. Prior to genomic DNA isolation and performance of the phenotypic and pathogenicity tests bacteria were cultured in Lysogeny Broth (Btl, Poland) with shaking (200 rpm) or LA at 28 °C for 24–48 h. Sixteen representative strains isolated in Turkey and reference strains used in this study are listed in Table 1.

Identification of pectinolytic strains with species-specific PCR

Identification of *P. atrosepticum, P. carotovorum/P. parmentieri* and *Dickeya* spp. was performed with multiplex PCR (Potrykus et al. 2014). To discriminate the strains of *P. parmentieri* and *P. c.* subsp. *brasiliense* species, species-specific PCR with PhF/R primers (De Boer et al. 2012) and Br1/L1r primers (Duarte et al. 2004) were performed, respectively.

Genotypic profiling of *Pectobacterium* strains using rep-PCR

Bacterial genomic DNA was isolated from bacterial suspensions using a Genomic Mini AX Bacteria Kit (A&A Biotechnology, Gdynia, Poland). The strains were analyzed using the primers corresponding to the conserved regions of the REP, ERIC and BOX repetitive sequences while the amplified products were separated with electrophoresis, visualized and compared according to Degefu et al. (2013).

16S rRNA, *rec*A and *gapA* genes analysis

To confirm the identification on species level, selected representative strains were subjected for *16S rRNA* gene fragment and/or *rec*A gene sequencing (Weisburg et al. 1991; Waleron et al. 2002). For one strain, namely *P. c.* subsp. *brasiliense* A4G1, we obtained a *gapA* gene fragment sequence (Cigna et al. 2017), as we were unable to amplify *recA* gene fragment for it. Amplicons were sequenced with forward and reverse primers by Genomed (Warsaw, Poland) or Medsantek (Turkey). The obtained *16S rRNA* sequences of five strains: *P. c.* subsp. *brasiliense* A4G1 (KX548227), *P. parmentieri* VK5G3 (KX557265), *P. atrosepticum* CA2G8 (KX557266), *P. c.* subsp. *carotovorum* A10G7 (KY071198) and A11G4 (KY071199) and *recA* sequences of 11 strains: *P. parmentieri* YS18Y5 (KX548226), VK7G11(KY067396), VK5G3 (KY067397), VK10Y13 (KY067399), CA3G6 (KY077482), CA1G7 (KY077483); *P. c.* subsp. *carovotorum* A16G3 (KY067398), A11G4 (KY067400), A8G4 (KY067401), VK10G14 (KY067403); *P. atrosepticum* CA9G1 (KY067402) and a *gap A* sequence of one strain: *P. c.* subsp. *brasiliense* A4G1 (MF539826) were deposited in GenBank and compared with available sequences using BLASTN search (http://www.ncbi.nlm.nih.gov/). Phylogenetic analysis was performed with *rec*A gene sequences. Maximum likelihood phylogenetic trees were generated with default parameters at www.phylogeny.fr (Dereeper et al. 2008, 2010).

Pathogenicity tests: disease symptoms development on potato plants and potato tubers

Potato plants (cv. Marabel) were grown in 20 cm diameter pots for 4 weeks with 16 h light at 26 °C and 8 h dark at 16 °C with 65% humidity during daytime. Two stems per potato plant, 5 cm above the stem base, were pierced by a sterile pipette tip and 20 μl of bacterial suspension ($OD_{600} = 0.1$) were inoculated into the stems and covered with a parafilm strip to avoid desiccation. The development of disease symptoms was observed daily for 4 days. The experiment was performed twice with three replicates for each of 13 strains used. Control plants were inoculated with sterile water.

Maceration ability of the eight strains was determined on the whole tubers (cv. Denar) as described previously (Potrykus et al. 2014). After 48 h, soft tissue was weighed to evaluate the amount of macerated tissue. The experiment was performed twice with six replicates. For negative control, tubers were inoculated with sterile water.

Phenotypic characterization with plate assays

Sixteen representative strains were subjected to biochemical characterization according to Schaad et al. (2001). The evaluation of the cell wall degrading enzymes (cellulase, protease) production was performed as described previously (Potrykus et al. 2014). Briefly, 2 μl of bacterial suspension (0.5 McFarland) were spotted onto a specific plate and were incubated for 48 h at 28 °C. LA media were used to assess the motility of the strains at different agar concentrations, 0.3% for

Table 1 Pectinolytic strains used in this study

No	Species	Strain	Other collections[a]	Potato cultivar and Plant part	Country, year of isolation	References
Strains isolated in Turkey						
1	*P. atrosepticum*	CA2G8		Potato stem, cv. van Gogh	2015, Turkey, Çorum	This work
2		CA9G1		Potato stem, cv. Melody	2015, Turkey, Çorum	This work
3		C10G2		Potato stem, cv. Melody	2015, Turkey, Çorum	This work
4		YS4G4		Potato stem, cv. Agria	2015, Turkey, Yozgat	This work
5	*P. c.* subsp. *brasiliense*	A4G1		Potato stem, cv. Melody	2015, Turkey, Amasya	Ozturk and Aksoy 2016
6	*P.c.*subsp. *carotovorum*	VK10G14		Potato stem, cv. Melody	2015, Turkey, Samsun	This work
7		A8G4		Potato stem, cv. Melody	2015, Turkey, Amasya	This work
8		A11G4		Potato stem, cv. Krone	2015, Turkey, Amasya	This work
9		A10G7		Potato stem, cv. Melody	2015, Turkey, Amasya	This work
10		A16G3		Potato stem, cv. Lady Rosseta	2015, Turkey, Amasya	This work
11	*P. parmentieri*	CA3G6		Potato stem, cv. Jelly	2015, Turkey, Çorum	This work
12		CA1G7		Potato stem, cv. Agria	2015, Turkey, Çorum	This work
13		YS18Y5		Potato tuber, cv. Jelly	2015, Turkey, Yozgat	Ozturk et al. 2016
14		VK10Y13		Potato tuber, cv. Melody	2015, Turkey, Samsun	This work
15		VK5G3		Potato stem, cv. Melody	2015, Turkey, Samsun	This work
16		VK7G11		Potato stem, cv. Musica	2015, Turkey, Samsun	This work
Reference strains						
1	*D. dianthicola*	IFB0103	NCPPB453^T, CFBP1200^T, IPO2114, SCRI4073	*D. caryophyllus*	1956, UK	Samson et al. 2005
2		IFB0485		Potato stem, cv. Igor	2013, Poland	Potrykus et al. 2016
3	*D. solani*	IFB0099	IPO2276, LMG28824	Potato stem, cv. Santa	2005, Poland	Slawiak et al. 2009
4	*P. atrosepticum*	IFB5444		Potato stem, cv. Irys	2013, Poland	Dees et al. 2017a, b
5	*P. c.* subsp. *brasiliense*	IFB5369	110A/6/37	Potato stem, cv. Lady Claire	2011, Poland	Waleron et al. 2015
6		IFB5390	LMG21371, Ecbr 2012	Potato	2002, Brasil	Duarte et al. 2004
7		IFB5506		Potato cv. Tajfun	2013, Poland	Dees et al. 2017a, b
8	*P. c.* subsp. *carotovorum*	IFB5128	SCRI162, UGC22	Potato stem	1970, Tasmania	Slawiak et al. 2013
9	*P. parmentieri*	IFB5400		Potato tuber, cv. Harpun	2013, Poland	Zoledowska et al. 2017
10		IFB5407		Potato stem, cv. Lord	2013, Poland	Zoledowska et al. 2017

[a] IFB Intercollegiate Faculty of Biotechnology, Gdansk, Poland; CFBP Collection Française des Bactéries Phytopathogénes, Angers, France; Invergowie, Dundee, Scotland; SCRI Scottish Crop Research Institute, Invergowie, Dundee, Scotland; LMG/BCCM Laboratorium voor Microbiologie Universiteit Gent/the Belgian Coordinated Collections of Microorganisms, IPO Plant Research International, Wageningen, The Netherlands

swimming and 0.6% for swarming (Harshey 2003). The diameters of the colonies were measured after 24 h at 28 °C. The experiments were conducted with eight replicates and performed twice.

Quantitative evaluation of the total pectate lyase activity

Pectate lyase activity was examined by monitoring spectrophotometrically the formation of unsaturated products from polygalacturonate (Tardy et al. 1997). One unit of pectate lyase activity was defined as the amount of the enzyme required to produce 1 μmol of unsaturated product per minute. Total pectate lyases activity was expressed as μmoles of unsaturated products (UP) liberated per 1 min per mg of bacterial dry weight. The measurements were performed using spectrophotometer (Beckman DU-640, USA) at 230 nm, 37 °C for 5 min. The experiment was performed twice with two replicates.

Statistical analysis

We compared the phenotypic features of the strains isolated in Turkey and the reference strains used in the study with ANOVA followed by the Tuckey post-hoc test that was carried out using Statistica 12.5 StatSoft Polska. Error bars representing standard error are shown in charts, the data represented are the mean values. The significance level used was $P < 0.05$.

Results

Identification of pectinolytic bacteria isolated from potato plants and tubers

During 2015 season, potato plants with typical symptoms of blackleg and soft rot were collected on the territory of Turkish Samsun, Amasya, Çorum and Yozgat provinces. Surveyed regions are under temperate climate where potato is planted February–May and harvested in July–October. Samsun region (Havza and Vezirköprü towns), is nearly under the same climate as Amasya province with a transition climate between Middle Anatolian and Black Sea region. The other two provinces, Yozgat and Çorum, are in general cooler and they receive less precipitation than Amasya and Samsun. Approximately 8–10 fields from each province were visually inspected and 48 symptomatic plants were collected, from which 26 pectinolytic bacterial isolates were obtained. Additionally, 10 symptomless plant samples were collected, but no pectinolytic isolates were obtained. Disease symptoms were mostly observed on one or rarely on two stems per infected plant.

Twenty six bacterial isolates were Gram-negative rods able to form cavities on CVP, oxidase, arginine and indole negative, positive for catalase, unable to show phosphatase activity, facultative anaerobes with an ability to macerate potato slices and to grow at 27 °C. On the basis of phenotypic characteristics, 20 of the isolates were assigned as *Pectobacterium carotovorum* and 6 isolates were assigned as *P. atrosepticum* species (Supplementary Table 1).

From 26 isolates, 16 were chosen for further, thorough characterization (Table 1). These isolates were tested with Multiplex PCR and with species-specific PCR. Out of 16 strains, four were identified as *P. atrosepticum*, one as *P. c.* subsp. *brasiliense*, five strains as *P. c.* subsp. *carotovorum* and six as *P. parmentieri* (Table 1).

rep-PCR genomic fingerprinting

To know more about the diversity of the strains belonging to the same species, rep-PCR was performed (Fig. 1, Table 2). In general, 16 different profiles were generated with REP primers. Six *P. parmentieri* strains produced the same profile with BOX and ERIC primers (data not shown) while they indicated three different REP profiles (Fig. 1, Table 2). *P. parmentieri* CA1G7 represented profile 1, the same as *P. parmentieri* IFB5400 isolated in Poland. Four other *P. parmentieri* isolates (YS18Y5, VK10Y13, VK5G3, VK7G11) had identical profiles, and represented profile 2, the same as *P. parmentieri* IFB5407 strain also isolated in Poland. One strain, *P. parmentieri* CA3G6 represented a distinct profile from all other *P. parmentieri* strains, however it was more similar to profile 1 than to profile 2. Three out of 4 *P. atrosepticum* isolates exhibited the same REP profile 3 together with *P. atrosepticum* IFB5444 isolated in Poland, while one *P. atrosepticum* strain (YS4G4) had a distinct profile. On the contrary, high variability in the profiles of the *P. c.* subsp. *carotovorum* strains was observed. Out of 6 *P. c.* subsp. *carotovorum* strains used in this study, only A10G7 and A16G3 isolated in Turkey represented the same profile 7. Other *P. c.* subsp. *carotovorum* strains (VK10G14,

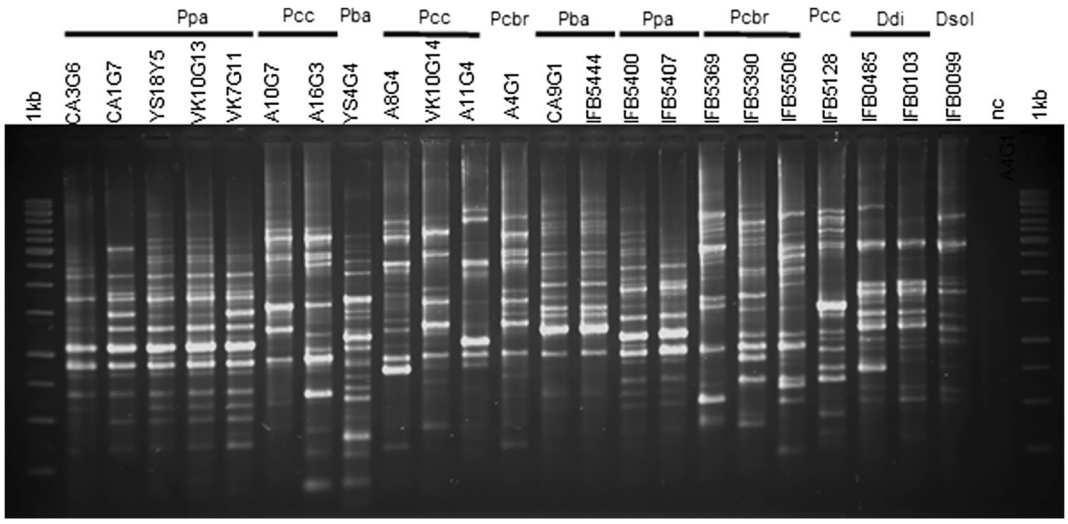

Fig. 1 Profiling of *Pectobacterium* sp. isolated in Turkey with regard to the reference strains with rep-PCR using REP 1 R-I and REP 2-I primers. Rep-PCR profiles were resolved and visualised with agarose gel electrophoresis. Pba – *P. atrosepticum*, Pcbr – *P. c.* subsp. *brasiliense*, Pcc – *P. c.* subsp. *carotovorum*, Ppa – *P. parmentieri*, Ddi – *D. dianthicola*, Dsol – *D. solani*, nc – negative control, 1 kb - 1 kb Gene Ruler Fermentas

A8G4, A11G4) exhibited diverse REP profiles (profile 4, 5, 6) and they were different from the profile obtained for *P. c.* subsp. *carotovorum* strain IFB5128 isolated in Tasmania (profile 10). The only *P. c.* subsp. *brasiliense* A4G1 strain represented different pattern than the reference *P. c.* subsp. *brasiliense* strains isolated in Poland and Brazil (IFB5369, IFB5390 and IFB5506) (Table 2).

*rec*A, *16S rRNA* and *gapA* based phylogenetic analysis

The *16S rRNA* sequences obtained for *P. atrosepticum* CA2G8, *P. c.* subsp. *carotovorum* A11G4 and A10G7, *P. parmentieri* VK5G3 and *P. c.* subsp. *brasiliense* A4G1 were identical to the sequences obtained for the strains from the corresponding species, which confirmed correct identification of the isolates with Multiplex PCR and species-specific PCR. Moreover, we have obtained a *gapA* gene fragment sequence for *P. c.* subsp. *brasiliense* A4G1, which was highly similar to *gapA* sequences found in the GenBank sequenced for two *P. c.* subsp. *brasiliense* SX309 and BC1 strains (99% sequence coverage, 97% sequence identity).

Pectobacterium strains representing different REP profiles and additionally all 6 *P. parmentieri* isolates were chosen for *recA* sequencing (Fig. 2). The phylogenetic tree based on *recA* sequences formed four clades. All identified Turkish strains clustered with appropriate species, which again confirmed earlier identification. Interestingly, when *recA* sequences of six *P. parmentieri* strains were compared to each other, T/A transition in the 470 position of the *recA* alignment, could be observed. For CA3G6 and CA1G7 strains, adenine was found in the place, while for the latter four *P. parmentieri* strains (YS18Y5, VK10Y13, VK5G3, VK7G11) thymine. Moreover, *recA* sequences of *P. carotovorum* strains formed one large clade, which was divided into four subclades. One subclade consisted of only *P. c.* subsp. *carotovorum* strains (A16G3 and VK10G14 and IFB5128). The other subclade was further divided into two groups, one consisting of only *P. c.* subsp. *brasiliense* strains, while the other of *P. c.* subsp. *carotovorum* ATCC15713 type strain and two more strains isolated in Turkey (A11G4 and A8G4).

Pathogenicity on potato plants and ability to macerate potato tuber tissue

Six *P. parmentieri* strains (VK5G3, VK7G11, VK10Y13, CA1G7, CA3G6, IFB5400), 5 *P. c.* subsp. *carotovorum* strains (VK10G14, A8G4. A10G7, A11G4, A16G3), three *P. atrosepticum* strains (CA9G1, C10G2, IFB5444) and two *P. c.* subsp. *brasiliense* strain (A4G1, IFB5506) were used for inoculation of potato plants. Typical blackleg or rotting on the stem appeared 2–3 days post inoculation (data not shown). For all *P. atrosepticum* and *P. parmentieri* strains, severe symptoms of the disease developed as soon as 2 days post infection. Four

Table 2 Phenotypic characterization of strains isolated in Turkey and reference strains used in the study

No	Species	Strain	Rep-PCR profile[a]	Cellulase activity (cm)	Protease activity (cm)	Swimming 0.3% agar (cm)	Swarming 0.6% agar (cm)
Strains isolated in Turkey							
1.	*P. atrosepticum*	CA2G8	3	0.76	1.63	0.87	0.42
2.		CA9G1	3	0.61	1.69	0.52	0.48
3.		C10G2	3	0.56	1.45	0.52	0.86
4.		YS4G4	15	0.53	1.46	0.44	0.46
5.	*P. c.* subsp. *brasiliense*	A4G1	8	1.38	1.94	2.00	0.44
6.	*P. c.* subsp. *carotovorum*	VK10G14	4	1.16	1.80	1.55	1.26
7.		A8G4	5	1.33	2.00	1.65	0.53
8.		A11G4	6	1.42	2.14	2.10	0.41
9.		A10G7	7	1.39	1.94	1.96	0.43
10.		A16G3	7	1.53	1.51	1.71	0.56
11.	*P. parmentieri*	CA1G7	1	1.12	1.69	0.68	0.49
12.		YS18Y5	2	1.03	1.14	1.53	0.36
13.		VK10Y13	2	0.95	1.21	1.45	0.66
14.		VK5G3	2	0.92	1.54	0.93	1.41
15.		VK7G11	2	0.88	1.47	0.90	0.50
16.		CA3G6	16	0.73	1.34	1.04	0.69
Reference strains							
1.	*D. dianthicola*	IFB0103	9	0.67	0.71	0.71	0.40
2.		IFB0485	9	1.25	1.24	0.81	0.47
3.	*D. solani*	IFB0099	14	1.93	1.83	1.58	1.03
4.	*P. atrosepticum*	IFB5444	3	0.58	1.70	0.39	0.46
5.	*P. c.* subsp. *brasiliense*	IFB5369	12	1.19	2.08	2.19	0.60
6.		IFB5390	13	0.82	1.62	2.54	0.79
7.		IFB5506	11	0.79	1.89	1.51	0.54
8.	*P. c.* subsp. *carotovorum*	IFB5128	10	0.94	1.41	1.11	0.50
9.	*P. parmentieri*	IFB5400	1	0.96	1.18	1.38	0.37
10.		IFB5407	2	0.81	0.83	1.17	0.49

[a] The rep-PCR profiles obtained for each tested strain were numbered

days after inoculation plants inoculated with all tested strains collapsed completely which led to the death of the plants. The control plants treated with sterile water did not develop disease symptoms.

The ability to macerate potato tubers was tested for eight strains isolated in Turkey which were chosen on the basis of rep-PCR analysis, and which represent eight different profiles (Table 2, Fig. 3a). The *P. c.* subsp. *carotovorum* strains isolated in Turkey (A16G3, A8G4, VK10G14, A11G4) exhibited 10 times greater ability to macerate potato tubers than *P. c.* subsp. *carotovorum* IFB5128 (Fig. 3a). On the contrary, the *P. c.* subsp. *brasiliense* A4G1 was less virulent than the reference strains (2.6 fold). For the *P. parmentieri* and *P. atrosepticum* strains, their maceration ability was comparable to that of their reference strains.

Phenotypic characterization

First, the biochemical characterization of the strains was performed. Four *P. atrosepticum* strains (CA2G8, CA9G1, C10G2, YS4G4) were exclusively able to utilize maltose, could reduce sucrose and produced acid from α-methyl glucoside (Supplementary Table 1). Five isolated

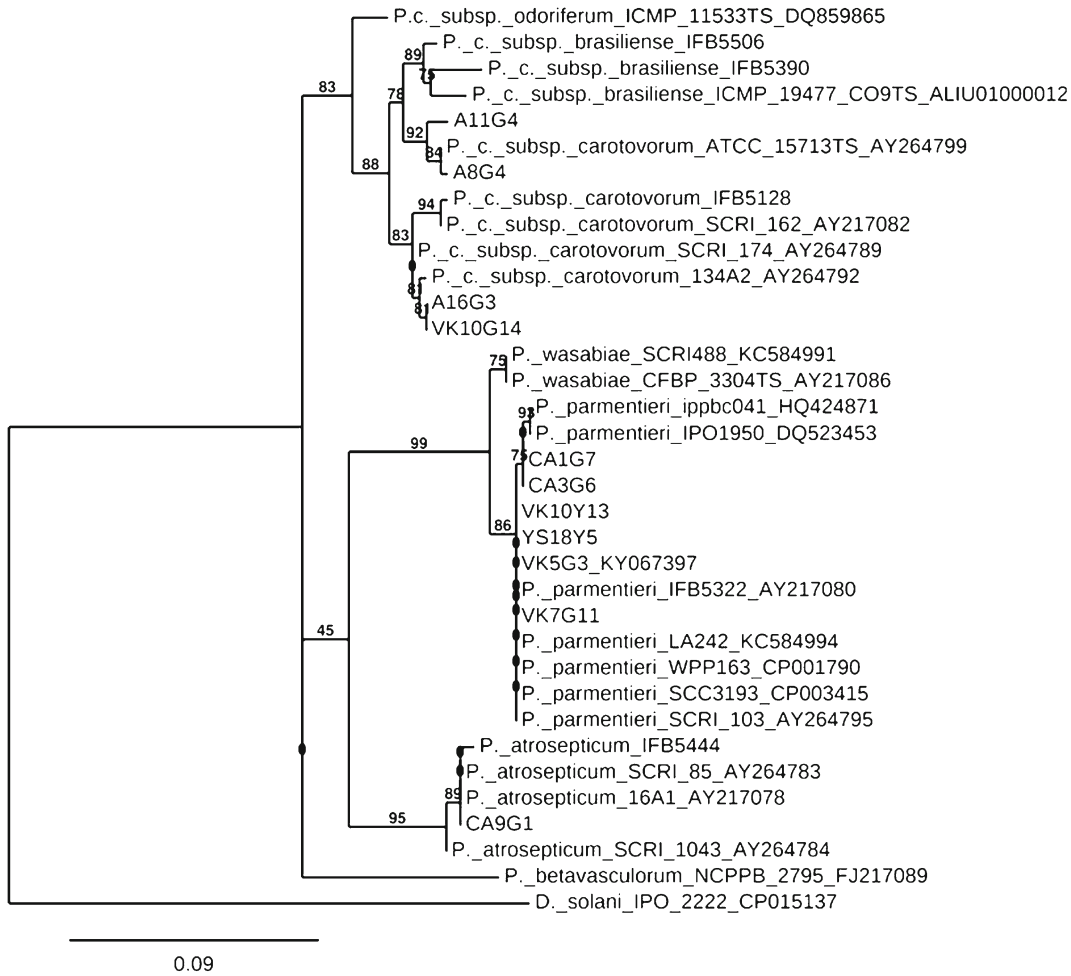

Fig. 2 Maximum Likelihood phylogeny showing evolutionary relationships of *Pectobacterium* sp. strains isolated in Turkey, based on *rec*A gene seqeunces. Bootstrap values are indicated at branch points. Accession numbers of reference strains in Genbank are at the end of sequence. *Dickeya solani* IPO2222T was included as an outgroup. The tree was generated using www.phylogeny.fr

P. c. subsp. *carotovorum* strains (VK10G14, A8G4, A11G4, A10G7, A16G3) exclusively utilized D + arabinose as a single carbon source, while three *P. c.* subsp. *carotovorum* strains (VK10G14, A8G4 and A16G3) could utilise also sorbitol (Supplementary Table 1). Moreover, all tested isolates could utilize L-rhamnose, D-mannitol, raffinose, D + cellobiose, D + xylose, D + galactose, D + trehalose, D-mannose, L + arabinose (data not shown). Ten isolates, namely six *P. parmentieri* (VK5G3, VK7G11, VK10Y13, YS18Y5, CA1G7 and CA3G6) and four *P. atrosepticum* (CA2G8, CA9G1, C10G2, YS4G4) were not able to grow at 37 °C, while all tested *P. c.* subsp. *carotovorum* strains could. The same six *P. parmentieri* strains were not able to grow in the presence of 5% NaCl (Supplementary Table 1). None of the tested *Pectobacterium* spp. isolates grew at 39 °C.

Sixteen *Pectobacterium* spp. isolates and 10 reference strains belonging to *Dickeya* or *Pectobacterium* genera were screened for their characteristics regarding pectate lyase, cellulase and protease activity, as well as swimming and swarming ability (Table 2, Fig. 3, Supplementary Fig. 1).

The pectate lyase production was verified with quantitative spectrophotometric method for eight *Pectobacterium* spp. strains representing different profiles in REP-PCR analysis and six reference strains. The lowest pectate lyase activity in the presence of polygalacturonic acid was exhibited by *P. c.* subsp. *carotovorum* IFB5128 reference strain, which was almost 3 times smaller than that observed for *P. c.* subsp. *carotovorum* strains isolated in Turkey (Fig. 3b). The largest pectate lyase activity was

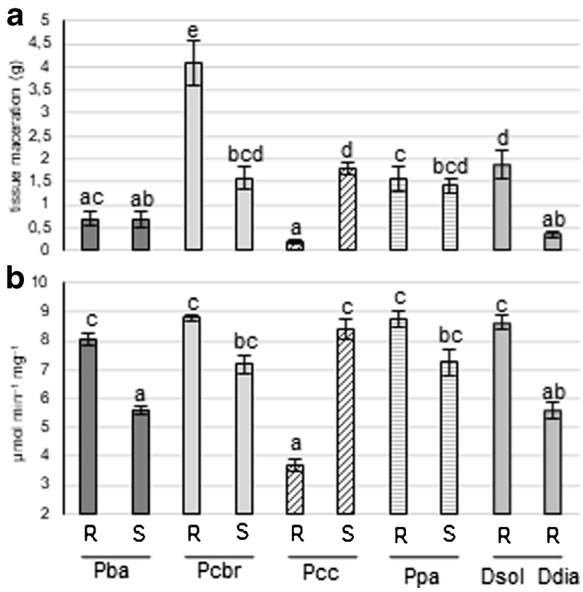

Fig. 3 Comparison of the tuber maceration ability and total pectinolytic activity of the selected *Pectobacterium* spp. strains. **a**. Maceration ability on potato tubers (cv. Denar), measured by fresh mass of macerated area (g) ($n = 6$); **b**. Total pectinolytic activity measured for bacteria grown in the presence of polygalacturonic acid (µmol min^{-1} mg^{-1}) ($n = 2$). The experiments were performed twice. Within each figure, columns respesent the mean value and error bars represent the standard error, columns with different letters are significantly different based on ANOVA followed by Tuckey post hoc test at $P < 0.05$. Pba – *P. atrosepticum*, Pcbr – *P. c.* subsp. *brasiliense*, Pcc – *P. c.* subsp. *carotovorum*, Ppa – *P. parmentieri*, Dsol – *D. solani*, Ddia – *D. dianthicola*, R – the mean value obtained for reference strain of the species, S – the mean value obtained for the strains isolated in Turkey (two *P. atrosepticum,* two *P. c.* subsp. *brasiliense,* four *P. c.* subsp. *carotovorum*, three *P. parmentieri* strains)

exhibited by *P. c.* subsp. *brasiliense* reference strain IFB5369, but it was not significantly different from the one observed for the *P. c.* subsp. *brasiliense* A4G1 (Fig. 3b). On the contrary, the *P. atrosepticum* isolates produced significantly lower amounts of pectate lyases than the *P. atrosepticum* reference strain IFB5444 (Fig. 3b).

The lowest cellulase activity was observed for *P. atrosepticum* strains, while for *D. solani* IFB0099 it was 3 times larger (Table 2, Supplementary Fig. 1). The *P. parmentieri* strains expressed intermediate cellulase activity, similar to *D. dianthicola*. *P. parmentieri* CA3G6 exhibited about 30% smaller cellulase activity than the rest of *P. parmentieri* strains tested. The reference strains and strains isolated in Turkey that belong to *P. atrosepticum* and *P. parmentieri* exhibited a similar level of cellulase activity, contrary to strains from *P. c.* subsp. *brasiliense* and *P. c.* subsp. *carotovorum* isolated in Turkey which exhibited a significantly higher cellulase activity than the reference strains used (Table 2, Supplementary Fig. 1A). The protease activity observed for the tested strains is variable. The lowest production among strains isolated in Turkey was represented by *P. parmentieri* YS18Y5, while the highest two *P. c.* subsp. *carotovorum* strains A11G4 and A8G4 (Table 2). In general, *P. c.* subsp. *brasiliense* and *P. c.* subsp. *carotovorum* and *D. solani* exhibited the largest protease actitivy, while *P. parmentieri* and *D. dianthicola* reference strains produced a significantly lower amount of proteases among the strains tested (Supplementary Fig. 1B).

The lowest swimming motility was presented by the *P. atrosepticum* YS4G4 and IFB5444, while the highest by *P. c.* subsp. *brasiliense* A4G1, IFB5369 and IFB5390; and one *P. c.* subsp. *carotovorum* A11G4. Generally, the *P. atrosepticum* strains were the least motile in LA medium containing 0.3% agar, while the strains belonging to *P. c.* subsp. *brasiliense* were the most motile (Table 2, Supplementary Fig. 1C). Regarding swarming ability, no difference between reference strains of *Pectobacterium* and the strains isolated in Turkey was visible (Supplementary Fig. 1D). However, variation between *P. c.* subsp. *carotovorum* strains was observed. *P. c.* subsp. *carotovorum* VK10G14 was able to swarm even 30% more rapidly than *D. solani* IFB0099, while *P. c.* subsp. *carotovorum* A8G4, A11G4 and A10G7 showed much lower swarming motility. They were approximately 2 times less motile than *D. solani* (Table 2). Among *P. parmentieri* strains, the highest swarming ability was presented by *P. parmentieri* VK5G3.

Discussion

Soft rot *Pectobacteriaceae* are reported as very important plant pathogens in a wide range of hosts (Ma et al. 2007). Turkey has large areas suitable for seed and ware potato production in different regions of the country (Aslanoglu et al. 2011). In 2015, the potato production in Turkey reached 4.7 million tons and the plants were cultivated on 1.5 million hectares of land. Growing potato production is also facilitated thanks to the possibility to store tubers in natural caves that serve as cheap storage facilities (Caliskan et al. 2010). The aim of this research was to detect, isolate, identify and characterize

the pectinolytic bacteria associated with recent outbreaks of blackleg and soft rot on potato in Turkey. With the presented study, it was verified that, indeed, the globally reported main potato pectinolytic pathogens *P. atrosepticum*, *P. c.* subsp. *brasiliense, P. c.* subsp. *carotovorum* and *P. parmentieri* are present in Turkey. In the current study, no bacteria from *Dickeya* genus were isolated, however, during the survey undertaken in 2016, bacteria from this genus were also detected on potato plants grown in Turkey (Ozturk and Aksoy 2017).

Rep-PCR fingerprinting method proved that for *Pectobacterium* spp. the REP primers were the most suitable, which is in agreement with the findings of Zoledowska et al. (2017). The analysis witth REP primers enabled us to distinguish three profiles among *P. parmentieri* strains, while at the same time with ERIC and BOX primers we obtained only one profile for the same set of *P. parmentieri* strains (data not shown). Low variability between *P. atrosepticum* strains was observed (two distinct profiles), contrary to high variability observed for *P. c.* subsp. *carotovorum* strains (five distinct profiles). Similarly, Faquihi et al. (2015) observed high variability of *P. c.* subsp. *carotovorum* strains and grouped 30 strains isolated in Morocco in nine different clades. On the other hand, *P. parmentieri* strains isolated in Turkey exhibited two rep-PCR profiles (1 and 2) that correspond to profile I and II described by Zoledowska et al. (2017). Profile I is also characteristic of the reference strain *P. parmentieri* SCC3193. The uniform REP profiles shown for Finish, Polish and Turkish strains indicated that they might have a common origin. Interesingly, in the *recA* sequence of six *P. parmentieri* strains isolated in Turkey one SNP position could be assigned, which was also described as T-A transition at 540 bp by Zoledowska et al. (2017). Here, two *P. parmentieri* strains with A in this position fell into profile 1 and 16 of rep-PCR analysis, while the latter four *P. parmentieri* strains were classified into profile 2.

When testing virulence of the strains on potato stems, slimy extended lesions mostly led to a faster collapse of the stem if plants were infected with *P. atrosepticum* and *P. parmentieri* strains than if they were inoculated with *P. c.* subsp. *brasiliense* and *P. c.* subsp. *carotovorum*. Similarly, de Boer et al. (2012) observed that strains of *P. c.* subsp. *carotovorum* and *P. c.* subsp. *brasiliense* were less virulent for stem inoculation than *P. atrosepticum* and *P. parmentieri*. Interestingly, *P. c.* subsp. *brasiliense* strains isolated in Brazil, South Africa and Poland show high tuber maceration. In our test, the *P. atrosepticum* isolates caused the lowest maceration, however the temperature of incubation was not optimal for the strains of this species (28 °C).

The phenotypic characterization of the tested strains revealed that for most of the assays (cellulase, protease production, swimming but not swarming) strains from different *Pectobacterium* species were significantly different from each other proving a large diversity in this genus. For comparison purpose also species from genus *Dickeya* were added. Apart from swimming motility, the reference strain *D. solani* IFB0099 showed higher cellulase, protease activity and swarming mobility than the reference and isolated *Pectobacterium* strains. The most motile in swimming were *P. c.* subsp. *brasiliense* strains, while the least motile were *P. atrosepticum* strains, which is in agreement with low motility phenotype observed for 23 *P. atrosepticum* strains isolated in Poland (Sledz, personal communication). The *P. parmentieri* strains showed the largest pectinase activity among *Pectobacterium* spp., while the activities of other plant cell wall degrading enzymes in the strains of this species were low or intermediate. Among the strains isolated in Turkey, the highest protease and cellulase activity as well as swimming ability were scored for *P. c.* subsp. *brasiliense* and *P. c.* subsp. *carotovorum* strains, while the lowest for *P. atrosepticum*, strains. This may lead to the conclusion, that *P. c.* subsp. *brasiliense* and *P. c.* subsp. *carotovorum* strains isolated in Turkey are better suited for spreading the infection among different plants than *P. atrosepticum* strains.

The regions of Samsun, Amasya, Corum and Yozgat, where the sampling of symptomatic potato plants was performed, can be assumed as areas of higher risk of potato blackleg and/or soft rot outbreaks as the number of fields devoted to potato production increases there steadily. Moreover, *Pectobacterium* spp. can be transmitted by latently infected seed potatoes and strains of this taxa are multi host pathogens, thus the presence of pectinolytic bacteria in potato fields may pose other crops, such as tomato, pepper, cucumber, onion, cabbage, broccoli and sugar beet that are cultivated in the same provinces, under threat. The presented study is the first step for the

determination and characterization of the population of *Pectobacterium* and *Dickeya* spp. on the territory of Turkey to assess the risk of pectinolytic bacteria transition between the same and different hosts.

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Compliance with ethical standards

Conflict of interest No conflict of interest.

Human and/or animals rights Not applied.

Informed consent Not applied.

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