Research Article

The phenotypic and genotypic characteristics of causal agents of potato bacterial soft rot in Ardabil province of Iran

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Abstract: Bacterial soft rot causes severe damage to potatoes and is responsible for considerable economic losses in potato crop during both growing season and storage period. The most common causal agent of potato soft rot worldwide is Pectobacterium carotovorum (Pc); however in Iran, two other species: P. atrosepticum and P. wasabiae have been reported. Identification and assessment of genetic variation in pathogen populations are very important to understanding taxonomy, epidemiology, and management of a pathogen. In this study, potato stems and tubers showing soft rot symptoms along with their surrounding soil were collected from fields and seed storages of Ardabil province. A total of 33 pectolytic bacterial strains were isolated on nutrient agar and eosin methylene blue culture media. The isolated strains and five standard strains belonging to P. atrosepticum, P. carotovorum and Dickeya dianthicola were studied. The strains were identified as P. carotovorum subsp. carotovorum on the basis of phenotypic characteristics including: gram-negative, facultative anaerobic, soft rot production on potato slices, growth at 37 °C, and inability to produce acid from α-methyl-D-glucoside and also molecular identification using species-specific primers. Based on Y1/Y2 and ExpccF/ExpccR primers, the expected amplicons (434 and 550 bp fragments, respectively) were obtained for all strains and the standard strains belonging to P. carotovorum. According to rep-PCR and cluster analysis using UPGMA and NTSYS 2.1 software, the selected strains were categorized into two main groups and four subgroups. Rep-PCR indicated different levels of genetic heterogeneity among Pcc strains, however, no clear correlation was found between clustering and the geographical origin of the strains.

Keyword: Pectobacterium carotovorum, rep-PCR, species-specific primers

Introduction

Iran produces 5.4 million tons of potatoes annually, and stands as the 12th largest potato producer in the world as well as the fourth largest in Asia, after China, India and Bangladesh (FAO, 2013). Potatoes are cultivated mostly under irrigation in nearly all provinces of Iran. However, three major potato growing regions are the southern shores of the Caspian Sea (the Elburz Mountains), the Zagros Mountains, and the southern lowlands (CIP, 2012). One of the production centers in the Elburz is Ardabil province located in northwest of the country and it ranks second in potato production across Iran.

Bacterial soft rots have significantly limited
potato production in the world. The main characteristic of this group of bacteria is the production of large amounts of pectolytic enzymes that induce rotting of plant tissues (Barras et al., 1994).

Soft rot coliforms belong to Enterobacteriaceae, Pectobacterium carotovorum subsp. carotovorum (Pcc), Pectobacterium atrosepticum (Pba) (Gardan et al., 2003), Pectobacterium carotovorum subsp. brasiliensis (Pcb) (Duarte et al., 2004), Pectobacterium wusabiae (Pwa) (Baghaee-Ravari et al., 2011, Pitman et al., 2008), P. carotovorum subsp. odoriferum (Pco) (Waleron et al., 2014) and P. betavasculorum (Pbt) (Nabhan et al., 2012) along with several Dickeya spp. including D. dianthicola, D. zeae, D. solani (Toth et al., 2011; van der Wolf et al., 2013). These bacteria can cause soft rots in potatoes and consequently heavy economic losses in many commercial crops both in the field and during storage (Czajkowski et al., 2015). Among them, Pcc is the most geographically diverse and has the widest host range (Charkowski, 2006; Toth et al., 2003). Different methods are employed to detect, identify and differentiate between pectinolytic Pectobacterium and Dickeya species using selective growth agar media, biochemical and physiological tests (Schaad et al., 2001), whole-cell fatty acid analysis (Dawnydt et al., 2006), serological (Gorris et al., 1994) and molecular techniques. Traditional methods are time-consuming and swayed by low sensitivity and specificity. Therefore PCR-based detection methods are used routinely to identify Pectobacterium spp. given their high specificity and rapid detection. The most common PCR-based detection methods include 16S rDNA sequence analysis (Kwon et al., 1997), ITS-PCR (Pitman et al., 2008; Toth et al., 2001), repetitive sequence-based PCR (rep-PCR) (Baghaee Ravari et al., 2013; Rezaei and Taghavi, 2010; Versalovic et al., 1991), restriction fragment length polymorphism (RFLP) (Darrasse et al., 1994; Gardan et al., 2003; Rahamanfar et al., 2012), amplified fragment length polymorphism (AFLP) (Avrova et al., 2002), multi locus sequence tagging (MLST) (Nabhan et al., 2012) and random amplification of polymorphic DNA (RAPD) (Mäki-Valkama and Karjalainen, 1994). In this study, phenotypic methods and PCR-based detection in combination with repetitive sequence-based PCR, the BOX-AIR primer (Treangen et al., 2009) were employed for identification and phylogenetic analysis of Pectobacterium spp. obtained from infected potato plants in Ardabil province. The species-specific primers used included Eca1f/Eca2r, specific for Pba (De Boer and Ward, 1995), EXPCCF/EXPCCR, specific for Pcc and Pwa strains (Kang et al., 2003), and Y1/Y2, specific for Pectobacterium spp. except for P. betavasculorum and Dickeya (Darrasse et al., 1994).

Materials and Methods

Sampling and strain collection

Suspected strains of Pectobacterium spp. were selected from the margin of infected potato stem and tuber samples which were collected from different fields and seed storages in Ardabil Province. Bacterial cultures were performed on nutrient agar (NA) and eosin methylene blue agar (EMB-agar) media. After incubation at 24 °C for 48-72 h, single colonies with a milky white and green metallic color were subcultured on NA medium. All strains were stored in the nutrient broth medium containing 25% (v/v) glycerol at -20 °C for further studies (Schaad et al., 2001). Reference strains used in this study were Pectobacterium atrosepticum SCR1043 provided by Dr. Minna Pirhonen (Helsinki University, Finland) and Dickeya dianthicola 2114, P. atrosepticum 1007, P. carotovorum 1955 and 1949 provided by Patricia van der Zouwen (Wageningen Plant Research Institute, Netherlands).

Biochemical and physiological tests

Biochemical tests conducted in the present study were gram reaction, potato soft rot, oxidation/fermentation of glucose, oxidase and catalase reactions, fluorescent pigment on King’s B medium, production of reducing substances from sucrose, phosphatase activity, sensitivity to erythromycin with 15µg per disk, indole production, starch, gelatin and esculin hydrolysis, lecithinase (Fahy and Hayward,
1983), H₂S production from cystein, arginine dihydrodase, nitrate reduction, utilization of citrate, D-tartrate and malonate, acid production from α-methyl-D-glucoside, trehalose, sorbitol, arabinot, arabinose, inulin, melibiase, raffinose, mannitrol and lactose (Schaad et al., 2001).

**Preparation of Bacterial DNA**

DNA extraction was performed using the whole cell alkaline lysis method (Rademaker and de Bruijn, 1997). Extracted DNA was analyzed by electrophoresis on a 1% agarose gel and stored at 4 °C or -20 °C until used.

**Molecular detection by specific primers**

Polymerase chain reaction (PCR) was performed using primer pair Y1 (5’-TTACCGGACGCC GAGCTGTGGCGT-3’) and Y2 (5’-CAG GAAG ATGTGGATTACGCCGAGT-3’) (Yahiaoui et al., 2003) in 25 μL of a reaction mixture containing 2.5 μL of PCR buffer 10X, 0.75 μL MgCl₂ (25 mM), 0.3 μL dNTPs (10 mM), 0.5 μL of each primer (10 pmol/μL), 0.3 μL of Taq DNA polymerase (5 u/μL) and 2 μL of the DNA extract.

PCR amplification was performed using a Thermocycler (Bio-rad, MJ Mini, USA) using the following program: initial denaturation for 5 min at 94 °C, 34 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 45 s, followed by a final extension at 72 °C for 7 min.

Also, PCR was carried out using ExpceF (5’-GAACCTTGCAACGCCGACCTTCTA-3’) and ExpceR (5’-GCGCATTAGCGCTGCTT AAG-3’) primers under the following conditions: initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturing at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min (Kang et al., 2003). The primers Eca1F (5’-CAGCATCGAAAAACACG-3’) and Eca2R (5’-GACACTCTCATCCAGCGA-3’) (De Boer and Ward, 1995) were used under the following conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 62 °C for 45 s and extension at 72 °C for 45 s, and a final extension at 72 °C for 8 min. Amplification products were analyzed by electrophoresis on 1% agarose gels.

**Repetitive Sequence-based PCR**

BOX-PCR was executed using the BOX A1R primer (5’-CTACgcaAgCgAcCTgACg-3’) (Versalovic et al., 1991) under the following conditions: each reaction (25 μL) containing 2.5 μL of PCR buffer 10X, 1.5 μL MgCl₂ (50 mM), 0.5 μL DMSO 100%, 16 μL ddH₂O, 1.25 μL mix of dNTPs (10 μM), 0.6 μL primer (50 pmol BOX-AIR), 0.4 μL of Taq DNA polymerase (5 μM). PCR consisted of an initial denaturation at 95 °C for 7 min, followed by 30-35 cycles of denaturing at 94 °C for 1 min, annealing at 53 °C for 1 min, extension at 65 °C for 6 min, and a final extension at 65 °C for 16 min. Cluster analysis was conducted by UPGMA method (unweighted pair-group method, Jaccard coefficient) using NTSSYSpc 2.1 software.

**Results**

**Biochemical and physiological tests**

Out of 60 strains isolated, biochemical and physiological tests were performed on 33pectolytic bacterial strains and five reference strains (Table 1). All the isolated strains were identified as *P. carotovorum*. Strains were gram-negative, able to produce soft rot on potato slices, aerobic/anaerobic growth, oxidase negative, catalase positive, non-fluorescent on King’s B (KB) medium. Starch hydrolysis and acid production from arabinose, inulin and sorbitol were negative, whereas gelatine and esculin hydrolysis were positive. Other characteristics are listed in Table 1. The strains exhibited similarity in physiological and biochemical tests. However, some had variations in a few characteristics such as lecithinase, phosphatase activity and utilization of a few carbon sources. All strains grew at 37 °C and were resistant to erythromycin. Most of the strains did not produce acid from alpha-methyl-D-glucoside. These findings support the identification of isolated strains as *P. carotovorum* rather than *P. atrosepticum*, *P. wasabiae* and *Dickeya* spp.
Pectolytic enterobacteria causing potato soft rot disease  

Table 1 Phenotypic characteristics for identification of causal agents of potato soft rot isolated in this study (obtained from Ardabil province, 2013) compared to reference strains.

<table>
<thead>
<tr>
<th>Tests</th>
<th>Pba</th>
<th>Pc</th>
<th>Dd</th>
<th>Positive strains (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation/fermentation of glucose</td>
<td>F/±</td>
<td>F/+</td>
<td>F/+</td>
<td>100</td>
</tr>
<tr>
<td>Potato soft rot</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>100</td>
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<tr>
<td>Blue pigment on PDA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
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<tr>
<td>Growth in 5% NaCl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>100</td>
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<tr>
<td>Growth at 37°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>100</td>
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<tr>
<td>Production of reducing substances from sucrose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
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<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>100</td>
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<tr>
<td>H₂S production from cysteine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>100</td>
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<tr>
<td>Lecithinase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>66.6</td>
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<td>Indole production</td>
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<td>+</td>
<td>+</td>
<td>0</td>
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<tr>
<td>Arginine dihydrolase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
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<tr>
<td>Sensitivity to erythromycin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
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<td>Phosphatase activity</td>
<td>+</td>
<td>+</td>
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<td>Acid production from</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Alpha-methyl-D-glucoside</td>
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<td>+</td>
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<td>100</td>
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<td>Trehalose</td>
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<td>+</td>
<td>+</td>
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<td>Arabinose</td>
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<td>+</td>
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<td>85.0</td>
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<tr>
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<td>-</td>
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<tr>
<td>Citrate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>100</td>
</tr>
</tbody>
</table>

+: positive strains; -: negative strains; Pba: Pectobacterium atrosepticum; Pc: Pectobacterium carotovorum; Dd: Dickeya dianthicola.

Cluster analysis based on the results of phenotypic features and strain grouping were performed using UPGMA method (Fig. 1). The strains were divided into four distinct groups. The similarity coefficients among strains ranged from 0.75 to 1.

Molecular detection by specific primers
PCR using species-specific primers (Y1/Y2, Eca1F/Eca2R and ExpccF/R) was used to determine whether Pectobacterium spp. or Dickeya spp. were present in individual plant samples. The expected amplicons totaling about 434 and 550 bp were obtained using Y1/Y2 and ExpccF/R primers for all isolated and standard strains belonging to P. carotovorum. The results obtained from some strains are shown in Figures 2 and 3. Also, a 690 bp fragment was only amplified using Eca1F/Eca2R primers for P. atrosepticum strains 1007 and SCRI1043.
**Figure 1** Cluster analysis of isolated strains based on the results of phenotypic data (+ and -) by NTSYS 2.1 software using UPGMA method (unweighted pair-group method, using simple matching coefficient). 2114: *D. dianthicola*; 1007: *P. atrosepticum*; SCRI1043: *P. atrosepticum*; 1955: *P. carotovorum*; 1949: *P. carotovorum*

**Figure 2** PCR products resulting from the amplification of 434 bp fragment using Y1 / Y2 primers on 1.2% agarose gel. L: 100bp DNA Ladder plus Bioron; 1: distilled water; 2, 3, 4, 5, 6 and 7: selected strains; 8: *D. dianthicola* 2114; 9: *D. dianthicola* 2114; 10: *P. carotovorum* 1955.
Figure 3 PCR products resulting from the amplification of 550 bp fragment using ExpccF/R primers on 1.2% agarose gel. L: 100bp DNA ladder plus Bioron; 1: distilled water; 2, 3, 4 and 5: selected strains; 6: *P. carotovorum* 1955; 7: *D. dianthicola* 2114.

Figure 4 Cluster analysis of selected strains of *P. carotovorum* based on the results of rep-PCR (using BOX-AIR primer by NTSYS 2.1 software and UPGMA method (unweighted pair-group method, using Jaccard’s coefficient).
Rep-PCR genomic fingerprinting
To study genetic diversity, 13 representative strains of Pcc were selected, and interspersed repetitive DNA sequences in their genomes were investigated by rep-PCR, using BOX-AIR primer (Treangen et al., 2009). The number and size of DNA fragments obtained in genomic fingerprinting were 11-13 bands, ranging from 150 to 3000 bp. Building on UPGMA method, the results revealed two main groups of strains with similarity value of approximately 72%. The first group was divided into three subgroups at 92% similarity where subgroup III was related to Pc 1955 standard strain. The second group showed one genotype (Fig. 4).

Discussion
In this research, we identified the pectolytic bacterial strains isolated from potato plants in Ardabil province using a combination of biochemical and molecular tests in order to accurately identify the relevant causal agent. The genetic diversity of the strains was determined by the rep-PCR technique. Based on biochemical tests, previous studies suggested that P. atrosepticum and P. wasabiae do not grow at 37°C and that P. atrosepticum and P. betavasculatorum are positive in the α-methyl glucoside test (Hauben et al., 1998; Gardan et al., 2003). In the present study, resistance of the strains to erythromycin, growth of all strains at 37 °C, and negative reaction of most of the strains (88%) in the α-methyl glucoside test support the identification of isolated strains as P. carotovorum rather than the closely related pectolytic P. atrosepticum, P. wasabiae and Dickeya spp.

Phenotypic discrimination using the traditional bacteriological methods is challenged due to various phenotypic characteristics among the strains of a species. Therefore, accurate identification solely based on biochemical tests has become more difficult. Studies have also shown that physiological and biochemical methods cannot clearly distinguish between related members of the Pectobacterium spp. (Fitman et al., 2008).

Molecular diagnostic techniques such as species-specific primers, ITS-PCR and PCR-RFLP have provided easy and rapid identification of bacterial strains (Toth et al., 2001). In this project, PCR was conducted using specific primers to the pectate lyase (pel) gene (Y1/Y2), which are able to produce a 434 bp fragment in all Pectobacterium spp. except for Pbt and Dickeya (Yahiaoui et al., 2003). Moreover, ExpccF/R primers were used to amplify a 550 bp fragment for Pcc and Pwa strains. According to previous studies, 434 and 550 bp DNA fragments were produced by all strains except for Dickeya dianthicola and Pba standard strains (Baghaee-Ravari et al., 2011; Kang et al., 2003; Yahiaoui et al., 2003). Isolated strains were screened using Eca1F/Eca2R primers (De Boer and Ward, 1995), and unlike some reports from Iran, they failed to amplify with Pba specific primers (Baghaee Ravari et al., 2011; Tavasoli et al., 2011).

Therefore, biochemical characterizations and species-specific primers differentiated the studied strains from different Pectobacterium and Dickeya species and identified them as Pcc. These subspecies were found to be the most important causal agent of soft rot disease of potatoes in Ardabil province. Some studies show that Pcc is the most prevalent soft rot bacteria of potatoes in Iran and worldwide (Amdan et al., 2015; Baghaee Ravari et al., 2013; Czajkowski et al., 2015; De Boer et al., 2012; Firouz, et al., 2006; Rahmani et al., 2012; Rezaei and Teghavi, 2010; Serfontein et al., 1991; Yahiaoui-Zaidi et al., 2003).

Repetitive extragenic palindromic elements (REP), enterobacterial repetitive intergenic consensus (ERIC) and BOX elements have been developed to target the repetitive sequences present in bacterial genomes and are commonly known as repetitive sequence-based PCR (REP-PCR) (Versalovic et al., 1991). These three fingerprinting techniques provide the banding profiles from the bacterial genome that can be used in clustering the pathogen isolates from genus down to strain level (Czajkowski et al., 2015). In the present study,
rep-PCR using BOX primer was conducted to study genetic heterogeneity within Pcc strains. BOX elements are widespread in the genomes of different bacterial groups and contribute to structural dynamics of the bacterial genome (Treangen et al., 2009; Versalovic et al., 1991). Results showed that Pcc strains and Pc1955 were grouped into four clusters at 92% similarity coefficient. Amplification of the sequences between each of these repetitive elements indicated that the identified Pcc strains were genetically variable. Therefore, the Pcc strains are phenotypically and genetically heterogeneous (Avrova et al., 2002; Baghaee-Ravari et al., 2013; Rezaei and Taghavi, 2010). Given that the strains from different locations, e.g., Arzan, Piraghom, Samian, Topthagho, Khalil Abad and Seid Abad of Ardebil were grouped into one cluster, there was no relationship between clustering based on the rep-PCR and geographical origin of the strains. Wide host range and geographical distribution may have caused genetic diversity in this species (Avrova et al., 2002). The present findings also confirmed that the rep-PCR technique is of reliable discriminatory power in evaluating the diversity of Pcc strains.

References


خصوصیات فنوتیپی و زنوتیپی پکتوباکتری های عامل بیماری پوسیدگی نرم سیب زمینی در استان اردبیل

چکیده: پوسیدگی نرم یکی از بیماری های مهم باکتریایی سایه زمینی در سیب از نظر اقتصادی و اجتماعی بسیار مهم می باشد. باکتری Pectobacterium carotovorum (Pc) مهم ترین عامل پوسیدگی نرم سیب زمینی است. اما در ایران P. atrosepticum و P. wasabiae (Pwa) نیز به عنوان عامل پوسیدگی نرم باکتریایی گزارش شده است. شناسایی و بررسی تنوع ژنتیکی در جمعیت عامل بیماری و اهمیت زیادی در تاکسونومی، اپیدمیولوژی و مدیریت بیماری است. در این مطالعه از آزمایشات گونه P. atrosepticum، P. carotovorum و Dickeya جدایه پکتوباکتری روند محیطی کشت نوترین ت آگار و ائوزین متیلن آگار جزئیاتی بهره برده شد. سپس با پرایمرهای Y1/Y2 و ExpccF/ExpccR به ترتیب قطعات مورد اندازه‌گیری، 434 و 550 جنت برای هر گروه انتخاب شدند. با پرایمرهای rep-PCR به ترتیب فلز سنگین rep-PCR مورد اندازه‌گیری، 434 و 550 جنت برای هر گروه انتخاب شدند. با پرایمرهای rep-PCR و Y1/Y2، expccF/expccR و Eca1F/Eca2R به ترتیب فلز سنگین rep-PCR مورد اندازه‌گیری، 434 و 550 جنت برای هر گروه انتخاب شدند.

واژگان کلیدی: آغازگرهای اتصالی گونه، Pectobacterium carotovorum، rep-PCR