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Diversity among Bacterial Isolates of *Pectobacterium* and *Dickeya* Causing Soft Rot of Ornamental Plants in Western Mazandaran, Iran

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Abstract Bacteria cause soft rot on a wide range of crops. The isolates involved in soft rot of many ornamental plants have been researched extensively due to their economic importance. Based on the phenotypic features and results of the molecular methods, it was evident that *Pectobacterium* spp and *Dickeya* spp are the main bacterial soft rot causal agent associated with ornamental plants in Western Mazandaran, Iran. In the present study 26 isolates were investigated for detection and determination of diversity among isolates of *Pectobacterium* and *Dickeya*. The variation within the isolates was studied in terms of physiological characteristics, pathogenicity and molecular methods in this paper *P. betavascularum* is reported on Aglaonema for the first time.

Keywords Detection, Identification, Pectobacterium, Dickeya, PCR

1. Introduction

Most *Pectobacterium* which cause soft rot, lack specificity of attacking the hosts, while certain *Pectobacterium* species has a narrow range of crops. The five soft rot pathogens of *Pectobacterium*: *P. atrosepticum*, *P. betavascularum*, *P. wasabiae*, *P. carotovorum*, and *P. odoriferum* have been thoroughly examined taxonomically[25,13,12]. *Pectobacterium* species are distinguished by their ability to produce large quantities of pectic enzymes (mainly pecticlyases) that enable them to cause soft rot in macerated parenchymatous tissue of a wide range of plant species. One of the famous bacterial diseases in potato is basal stem rot, also known as blackleg, which is associated with decay of the parent seed tuber. In many other crops, wilting and yellow leaves in early stages of attack may take place. In advanced stages chlorotic, necrotic and water soaked areas will occur. However, the symptoms of soft rot caused by *Pectobacterium* spp. in susceptible hosts under field conditions are indistinguishable. *Pectobacterium* produce extracellular enzymes such as proteases, polygalacturanase, pectatelyase which degrade the plant cell wall and membrane compounds lead to plant death[3,27]. Some chemicals have been proved for soft rot disease control but these have limitation in soft rot control[20,18]. Agricultural practices such as rotation, sanitation, removal of plant debris, appropriate plant nutrition, insect control, warehouse temperature control, use of clean irrigation water and planting of resistant cultivars and production of planting material free from pathogen by tissue culture technique, soft chemicals, natural chemicals, disinfectants, calcium application, growth regulators, chemical elicitors to induce natural host defenses, biological control, integrated control, hypobaric pressure, physical means such as ultraviolet illumination, radiation, hot water or heat shock treatments, modified atmosphere storage and packaging genetic modification of plants[4] are effective means for *Pectobacterium* soft rot control. To-date several methods had been developed for detection of soft rot *Pectobacterium*. Selective growth media[26,24], immunological[30] and PCR based techniques[32,8] are more reliable methods used in many laboratories for *Pectobacterium* characterization. However, there are some specific probes[6] for *Pectobacterium* characterization, but in routine tests they are limited by low signals when labeled with non-radioactive probes. A set of primer designed to amplification of 434-bp fragment of a pectatelyase encoding gene (*pel* gene) in order to detect and identify all *Pectobacterium* species (*atrosepticum*, *carotovorum*, *odoriferum*, and *wasabiae*), except *betavasculorum*[6]. Another pair of primers (EXPCCR and EXPCCF) was also designed to amplify a 550bp fragment in *P. carotovorum* and *P. wasabiae* strains. Digestion of obtained PCR product with *Rsa*I restriction

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enzyme represented 5 different RFLP patterns in agarose gel[15]. a banding pattern of 620-bp was produced from pure cultures of *P. atrosepticum* using Eca1f and Eca2r primers[7]. Powerful analytical tools (e.g. specific genes and DNA sequencing, DNA-DNA hybridization, isozyme analysis, randomly amplified polymorphic DNA, restriction fragment length polymorphism (RFLP) and rep-PCR are used for identification and differentiation of isolates even at strain level, which are important for ecological and epidemiological surveillance purposes. These procedures require only a small proportion of samples for examination. The discriminatory power of these markers is very high and closely related strains can be differentiated. The occurrence of soft rot bacterium of ornamental plants have been previously reported in northern Iran[16,2]. The aims of this study were detection and determination of genetic diversity of some soft rot isolates collected from ornamental plants using biochemical and PCR-based techniques.

2. Materials and Methods

Leaves and stems of ornamental plants (table 1)infected by soft rot bacterium with water soaked lesions, wilting, and soft rot symptoms were collected randomly from 50 commercial ornamental greenhouses in northern Iran during 2009-2010. In total, 60 samples were collected and selected for characterization assays. Infected tissues were surface sterilized using 70% ethyl alcohol. Small pieces of infected tissues were soaked in saline solution (0.85% NaCl) for 20 min to disperse the bacterial cells into the solution. Standard protocol using eosin methylene blue (EMB), nutrient agar (NA) and King's B (KB) media were used for isolation of bacteria from the plant samples[30,7]. Twenty six pure cultures were collected and stored at -80°C. The authentic cultures for *Pectobacterium atrosepticum* 1043 SCRI, *Dickeya dadanticola* 3937 SCRI (SCRI Scottish Crop Research), *Dickeya dianthicola* IPO 980, *Dickeya* sp. IPO 2222, *Pectobacterium carotovorum* subsp. *carotovorum* IPO 1949, *Dickeya chrysanthemi* DSM 4610 (German Resource Center for Biological Material) were used as standards. Isolates which were positive to pectolytic activity selected for the conventional biochemical and physiological tests including facultative anaerobic activity, gram reaction, erythromycin sensitivity, iodole production, oxidase, phosphatase, malonate utilization, reducing substance from sucrose, methyl red, nitrate reduction, growth on 5% sodium chloride (NaCl), lecithinase, urease and arginine dihydrolayse, casein, starch, Tween hydrolyses, H_2S production and assimilation of carbon sources using basal medium of Ayers[30]. Plants inoculation was done by injection of 10^8 CFU/ml bacterial suspensions from 24h slant culture into stem of young ornamental plants and incubated at 28°C with 85-90% relative humidity[10,1]. Controls were treated only with sterile water. Four primer pairs ADE1/ ADE2, Y1/Y2, EXPCCR/EXPCCF and ECA1f/ECA2r were used to detect *Dickeya* and *Pectobacterium* isolates in PCR assay[6,8,22,15]. PCR was performed in 25µl of reaction mixture. Amplified DNA fragments were run on 1.5% agarose gel at 90V for 1h and visualized under UV-Light following ethidium bromide staining. In rep-PCR analysis total bacterial genomic DNAs were extracted using Alkalin Lysis Method[28]. Colonies on agar plates were suspended in 10 µl of lysis buffer (100 µl of 0.05 M NaOH was added to 10 µl of cell suspension and incubated at 95°C for 15 min). The bacterial suspensions were then centrifuged for 2 min at 12,000 rpm. Genomic DNA (50ng/µl) was used as the template for all rep-PCR reactions[14]. Two primers of BOX and ERIC were used as described by Rademaker *et al.*[28]. The amplification cycles were consisted of 35 cycles for denaturation (94°C/1min), annealing (48°C/1min), extension (72°C/2min) and final extension (72°C/10min). Amplified DNAs were analyzed by electrophoresis in a 1.5% agarose gel in TBE 0.5X buffer. A 3kbp ladder was used as a size marker. Banding patterns were stained with ethidium bromide and visualized under UV light. To determine the relationships among strains, cluster analysis was made by UPGMA algorithm with the use of NTSYS software pc2.0[5]. Banding patterns were recorded using UviDoc® (version 99.02) software and **bands** were scored as '**1**' for presentand '**0**' for **absence**. The dendrograms were constructed from a similarity matrix using Dice's coefficient [29].

Table 1. Isolate code, Bacterial Genuses and host tissues

Isolates code	Bacteria	Host	Plant part
1	P. *carotovorum*	Chinese evergreen	Leaves
2	*Pectobacterium* spp.	Begonia	Leaves
3	P. *carotovorum*	Tvy-arum	Stem
4	*Pectobacterium* spp	Painted nettle	Stem
5	P. *carotovorum*	Dragon Tree	Leaves
6	*Pectobacterium* spp	Tvy-arum	plants
7	P. *carotovorum*	Goose foot plant	plants
8	P. *carotovorum*	Tvy-arum	Leave
9	P. *carotovorum*	Tuft root	plants
11	D. *chrysanthemi*	Chinese evergreen	Leave
12	*Pectobacterium* spp	Painted nettle	Leave
13	P. *betavascularum*	Begonia	Leave
14	*Pectobacterium* spp	Sago palm	Leave
15	P. *betavascularum*	Chinese evergreen	Leave
17	*Pectobacterium* spp	Persian parrotia	plants
18	*Pectobacterium* spp	Painted nettle	Stem
19	P. *atrosepticum*	Goose foot plant	Leave
21	*Dickeya* spp.	Tuft root	Stem
23	*Dickeya* spp.	Chinese evergreen	Stem
24	*Dickeya* spp.	Chinese evergreen	Stem
25	*Pectobacterium* spp	Painted nettle	Leave
26	D. *chrysanthemi*	Calla lily	plants
28	D. *chrysanthemi*	Ribbon plant	plants
34	*Dickeya* spp.	Begonia	Leave
35	*Pectobacterium* spp	Goose foot plant	Stem
36	P. *carotovorum*	Calla lily	plants

3. Results and Discussion

In total, twenty six isolates with cream-white colonies were purified from soft rotted plant tissues. Based on certain characteristics such as positive for indole production and phosphatase activity, reducing substances from sucrose, sensitive to erythromycin, positive to produce soft rot on potato and hyper sensitivity reaction on tobacco, able to growth in 5% NaCl, reduction of catalase and nitrate and production of H_2S from cystein, the isolates were identified as either *Pectobacterium* spp. or *Dickeya* spp. The *Pectobacterium* strains were further divided into three groups based on bacteriological characteristics. Seven strains belonging to group I, was negative for casein, VP, and indole production, as well as phosphatase activity, citrate utilization and lecitinase test. This group was clustered in single group of *Pectobacterium carotovorum*. One strain belonging to group II (*Pectobacterium atrosepticum*) was negative for casein, and VP production and growth at 36°C and positive to α-methyl glocoside, citrate and MR tests. The group III with single strain of *Pectobacterium betavascularum* gave negative response to casein, citrate and MR tests and was positive for VP. Three strains (11, 26, and 28) also were identified as *Dickeya chrysanthemi* and placed in a separate group. The Microlog system was also employed to identify two isolates (13, 15) as *Pectobacterium betavascularum* and isolate 11 as *Dickeya chrysanthemi* with similarity index of 67%, 49% and 63%, respectively. Inoculation of representative bacterial suspension into ornamental plants parts produced typical bacterial soft rot symptoms. The rotted tissues with mushy appearance were observed 2-5 days after inoculation. No symptoms were developed on control plants. A 434bp PCR-amplified fragments corresponding to pectatelyase-encoding gene was obtained in ten strains of *Pectobacterium* spp. by using Y1/Y2 primers. After PCR amplification with ECA1f/ECA2r primers, one isolates (19) produced a 690bp banding pattern designated to be *P. atrosepticum*. Likewise, the primer set (EXPCCF/ EXPCCR) amplified a single fragment of the expected size (550bp) for seven isolates of *Pectobacterium carotovorum* (36, 9, 8, 7, 5, 3, 1) an expected band of 420bp was also obtained from three *Dickeya* strains using ADE1/ADE2 primers. ERIC-PCR and BOX-PCR analysis were produced different fingerprinting profiles based on their origin and the primers used. The use of the PCR based methods for characterization of P. *carotovorum* subsp. *carotovorum* was strongly depicted with primers analogous to ubiquitous repetitive DNA sequences[9] to generate specific DNA fingerprints of *Pectobacterium* spp. ERIC-PCR and BOX-PCR analysis, showed that, bacteria isolated from different hosts gave different fingerprinting profiles, based on the primer used, the patterns of the reference isolates were different from those of isolates obtained from different plants, but identical in both methods. An UPGMA dendrogram constructed using the patterns generated with the two primers and the Dice index values confirmed that the isolates from ornamental plants were identical. The DNA fragment sizes varied between about 190 to 3800 bp with the use of the BOX primer. Both methods, showed observable diversity among isolates from every species of ornamental plants. Multiple and different DNA fragments yielded by amplification of chromosomal DNA with BOX and ERIC primers, revealed that there was divergence among isolates. Generally, the groups generated by ERIC-PCR and BOX-PCR are related to the plant species and geographical origins. Because of this, most of our isolates from specific areas were placed in the main groups. The influence of geographical origins on bacterial genetics was reviewed before[31,19]. Undoubtedly, rep-PCR has high reliability in epidemiological studies of bacterial diseases and the information derived from it, is applicable for detection and genetically characterization of bacterial pathogens. Based on BOX-PCR results, two main clusters were formed at 65% similarity. The first cluster including *Pectobacterium* spp. sub-divided into three subgroups with 68% similarity. The second cluster identified as *Dickeya* spp. divided into two subgroups with 71% similarity (figure 1). In ERIC-PCR analysis the similarity values between two main clusters was 52%, between subgroups of *Pectobacterium* spp. 59% and among *Dickeya* spp. subgroups was 70% (figure 2). From twenty six strains that were presumptively identified as *Pectobacterium* spp., ten were confirmed as *Pectobacterium* species and three *Dickeya chrysanthemi* using biolog and PCR methods. BiologMicrolog® system also identified isolates 13 and 15 as *Pectobacterium betavascularum* and 11 as *Dickeya chrysanthemi*. Molecular fingerprints based on BOX-PCR and ERIC-PCR, showed large genetic diversity within strains of *Pectobacterium* spp. The reason for this variability is unknown to many authors but might be related to some environmental and host conditions such as different levels of **relative humidity** and temperature and nutrition resources in greenhouses[4]. We here applied PCR-based technique with specific primers with this aim to increase detection sensitivity, simplicity and rapidity compared with phenotypic and biochemical assay. This led to accurate identification of *Pectobacterium carotovorum*, *Pectobacterium atrosepticum* and *Dickeya chrysanthemi*. However, thirteen strains with identical characteristics of the genus *Pectobacterium* failed to be amplified with specific bands. In this study, close correlations between biochemical characteristic and BOX-PCR based genetic finger prints among some of the strains were observed. The strains of similar genetic groups were exhibited similar phenotypic features and thus were placed in the same genetic/phenotypic group. Some similarities and differences were obtained from BOX-PCR and ERIC-PCR analyses. BOX primer grouped the strains more properly than ERIC primer and clustered *P. carotovorum* strains into 2 groups. Based our knowledge this is first report of bacterial soft rot on *Aglaonema* sp. (Chinese evergreen) caused by *Pectobacterium betavascularum* in Iran.

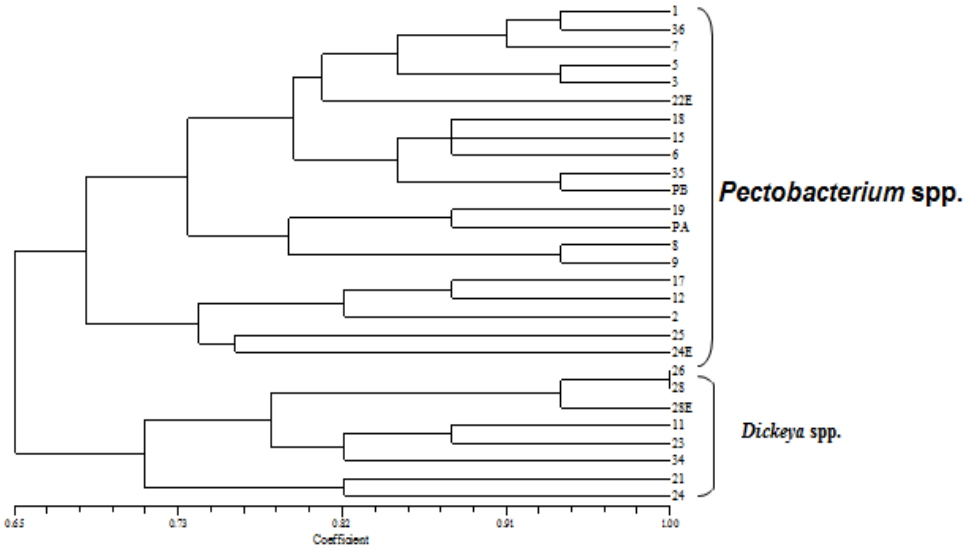

Figure 1. Dendrogram based on BOX-PCR of isolates from ornamental plants

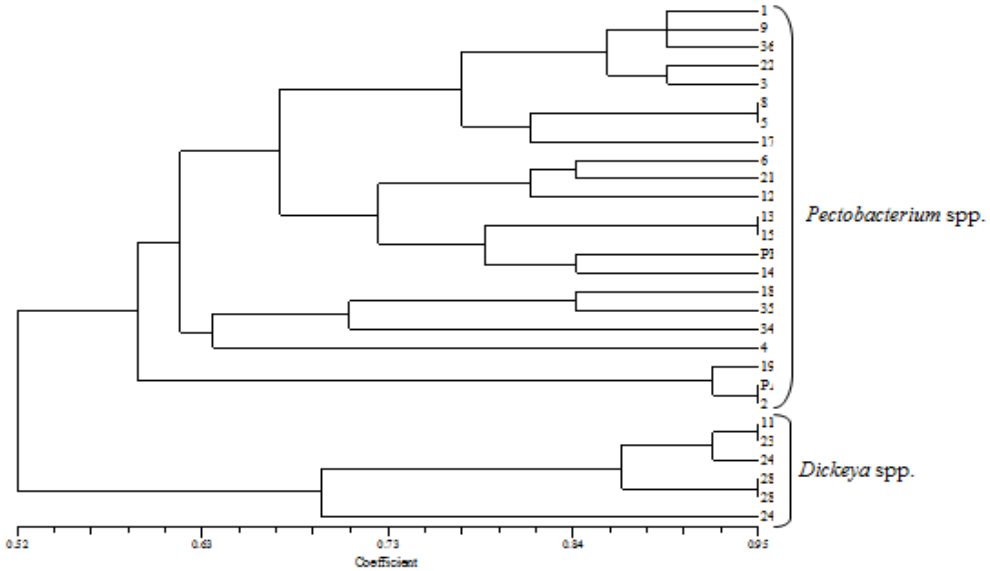

Figure 2. Dendrogrambased on ERIC-PCR fingerprint profiles

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