Phenotypic and molecular characterization of white calla lily soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum*

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**ABSTRACT**

A destructive disease on white calla lily was observed in commercial ornamental plants greenhouses in Markazi province, Iran. Disease symptoms on leaves and stems were gray to pale brown discoloration and expanding water soaked lesions on stem. PCR amplification of pectatelyase encoding gene produced a 434bp banding pattern on 1% agarose gel. The causal agent identification was confirmed by sequencing of the *Pel* gene. The ITS-PCR products were digested with *Rsa*I restriction enzyme. For discrimination of the *P. carotovorum* from *Pectobacterium odoriferum*, all isolates were subjected to α-methyl glucoside test. All isolates were identified as *Pectobacterium carotovorum* subsp. *carotovorum*.

**Keywords**: Molecular detection, Identification, White calla lily, Soft rot.

**INTRODUCTION**

Calla lily (*Zantedeschia* spp.; family *Araceae*), is a valuable ornamental crop grown worldwide and it is used commonly as cut flowers. Several species of plant pathogenic bacteria are able to cause soft rot diseases in calla lily, mostly classified in the genus *Pectobacterium*, *Dickeya* and *Pseudomonas* (Wright and Burge, 2000). Among these, soft rot caused by *Pectobacterium carotovorum* is the most important worldwide (Snijder and van Tuyl, 2002; Cho et al., 2005) because of the lack of effective control methods and fast dissemination either in storage of tubers or growing time of the plants in the field (Vanneste, 1996; Wright et al., 2002). *P. carotovorum* was first described as *Erwinia carotovora* by Jones in 1901. The bacterium present in the intercellular spaces at the infection sites start multiplying and infections caused result in extensive paranchymatus maceration due to the production of extracellular enzymes, i.e. pectatelyase (PL), polygalacturonase, cellulase, and protease. Infection may happen with the convergence of cell deaths. Maceration depends on factors such as temperature and moisture (Perombelon, 1980). The symptoms of soft rot initiate with the loss of turgidity in the whole plant. The disease spread from the leaf petiole toward the base of flower peduncles, which result break down of the flower. In this situation, leaf blade turned yellow and extensive Water-soaked lesions on tubers lead plants to dead with the exudation of a foul-smelling slime (Cho et al., 2005). Disease severity increases when temperature ranges around 25°C (Pérombelon and Salmond, 1995). The relationship between virulence and genetic diversity in *P. carotovorum* subsp. *Carotovorum* was associated with the existence of a co-evolutionary specialization in the interaction between pathogen and the host. Phenotypic tests (Lelliot and Dickey, 1984) have been developed for the identification and characterization of *P. carotovorum* but it cannot discriminate between all...
**Pectobacterium** causing soft rot. A PCR test based on amplification of the pectatelyase-encoding gene (**pel** gene) followed by restriction fragment length polymorphism (RFLP) was developed for the detection and identification of all soft rot **Pectobacterium** except **P. carotovorum** subsp. **betavascularum** (Darrasse et al., 1994). Amplification of the 16S-23S intergenic transcribed spacer (ITS) which can separate the rRNA genes, showed a well definition of length variation especially when combined with restriction fragment length polymorphism (RFLP) and is suitable for the differentiation at the strain level (Toth et al., 2001). Bacterial soft rot of calla lily is difficult to control. Chemical bactericides are not effective on disease control. As environmental factors affect both host susceptibility and parasite virulence, optimizing environmental conditions by agronomical measures is recommended to reduce or eliminate the disease (Ni et al., 2010). Appropriate irrigation, soil drainage and mulching, can improve soft rot control (Wright and Burge, 2000), as well as suitable calcium or phosphorous fertilizations. Increased calcium uptake due to improved plant resistance, whereas excess phosphorous enhances the disease due to increased virulence of the pathogen. **P. carotovorum** causes economic losses in different districts of Iran, where calla lily has been cultivated for over 50 years. The aims of this study were detection and identification of pathogenic bacteria associated with soft rot of white calla lily using phenotypic and molecular methods.

**MATERIALS AND METHODS**

**Isolation of** **Pectobacterium carotovorum**

Twenty nine samples of steam and leaves with a light yellow color and exhibiting extensive water soaked lesions consistent with infection by **Pectobacterium** sp. were collected from a range of greenhouses and brought into the laboratory during 2013 for analysis. Diseased tissues used for the isolation were surface sterilized by 70% ethyl alcohol. Small pieces of infected tissues were soaked in saline solution (0.85% NaCl) for 20 min to emigrate the bacterial cells into the solution. Standard protocol using eosin methylene blue (EMB) and nutrient agar (NA) media were used for isolation of bacteria from plant samples (Schaad et al., 2001). The cultures were incubated at 27°C for two days and colonies that were emerald green on EMB or white to gray color on NA were selected and streaked on to Luria-Bertani (LB) broth medium. Cultures were stored in 20% glycerol at -20°C until required.

**Biochemical and physiological tests**

Isolates that were described as positive to pectolytic activity were selected for the conventional biochemical, physiological and phenotypical tests including: Gram staining, facultative anaerobic activity, Pectinase production (Janse, 2006) catalase and oxidase activity (Dickey and Kelman, 1988), hypersensitivity reaction on tobacco leaf, sucrose reduction, indole production from tryptophan, phosphatase production and sensitivity to erythromycin (Schaad et al., 2001), ability to grow at 37°C in nutrient broth. Assimilation of carbon sources substances on the basal medium (Ayer et al., 1919) added with 1% carbohydrates were tested for keto-methyl glucoside, sorbitol, arabinol, melibiose, citrate, raffinose, maltose and cellulbose. Positive and negative control strains were included in each test. **Pectobacterium carotovorum** (SCRI1949), **P. atrosepticum** (SCRI11043) and **Dickeya chrisanthemi** (DSM4610) were used as reference strains in each test.

**Pathogenicity test**

Three bacterial strains isolated from **Zantedeschia aethiopica** selected for pathogenicity assay. Stems and tubers of white calla lily (cv. treasure) were disinfected by 70% ethyl alcohol for 30 sec, rinsed with sterile distilled water and inoculated by placing of 100µl bacterial suspension with the final concentration of 10^6 CFU/ml. Three replications for each strain were examined. Inoculated tubers and stems were incubated in moist chamber at 80-90% relative humidity at 30°C and disease incidence was evaluated in comparison with negative control treated with sterile distilled water after 1-5 days. The bacteria re-isolated from plant parts which inoculated in pathogenicity test, confirming Koch’s postulates.

**Bacterial DNA extraction**

One milliliter of 24h old bacterial culture grown at 27°C in Luria-bertani (LB) broth medium was micro centrifuged and the DNA was extracted by phenol-chloroform-isooamyl alcohol (Sambrook et al., 1989) method.

**Molecular detection**

PCR assay was carried out using Y1 (5’ TTACCGGACCGAGCTGTGGCGT 3’) and Y2 (5’ CAGGAAGATGTCGTTATCGCGAGT 3’) primers, selected for amplifying a pectatelyase-encoding gene of
Figure 1. Natural infection of Z. ethiopica caused by P. carotovorum subsp. carotovorum, (a: Symptoms on leaves due to petiole infection, b: symptoms on basal stem).

the Y family to detect Pectobacterium sp. (Darrasse et al., 1994). For PCR we used Maxime PCR Premix Kit (i-taq) in total volume of 20µl by adding 17 µl sterile distilled water, 1µl (10 pmol /µl) each primer and 1µl (50 ng) of template DNA. The amplified products were electrophoresed on 1 % agarose gels and stained with ethidium bromide. DNA standards ladder (100 bp DNA ladder, fermentaz) were used in each electrophoresis (Maniatis et al., 1982).

The 16S-23S rRNA intregenic transcribed spacer (ITS) region was amplified using the universal primers G1 and L1, according to Toth et al., (2001). For ITS-PCR we used Maxim PCR premix kit (i.tag) in total volume of 20µl as described above. Amplified DNA fragments were electrophoresed on 2 % agarose gel and stained with ethidium bromide and photographed under uv light. The reference strain of Pectobacterium carotovorum (SCRI 1949) and P. atrosepticum (SCRI 1043) obtained from the Scottish Crop Research Institute (SCRI) were included in each test.

Restriction fragment length polymorphism

Ten µl of each PCR product were digested with Rsal restriction enzyme. Digestion occurred for 3h in a 30 µl volume at 37°C according to supplier’s recommendations. Digested samples were electrophoresed on a 2% agarose gel in 0.5% TAE buffer at 71v for 3h, stained with ethidium bromide and photographed under uv light. The banding patterns were compared with the reference strains Pectobacterium carotovorum (SCRI 1949) and P. atrosepticum (SCRI 1043).

RESULTS AND DISCUSSION

Several samples of chlorotic and necrotic lesions from white calla lily with yellow to light brown color leaves (Figure 1: a) and extensive water soaked area on stems (Figure 1: b) were suspected to be infected by Pectobacterium from different commercially fields and greenhouses were brought to laboratory. Biochemical and physiological tests gave the following results: Gram negative, rod shape, ability to grow at 37°C and under liquid paraffin (facultative anaerobe); oxidase negative; phosphatase negative; catalase positive; positive for degradation of pectate; sensitive to erythromycin; negative to Keto-methyl glucoside utilization, indole production and reduction sugars from sucore were negative; acid production from sorbitol and arabitol were negative and from melibiose and citrate were positive. Hypersensitivity reaction (HR) on tobacco leaf with the infiltration of 10^8 CFU/ml of bacterial suspension for all isolates was positive. In pathogenicity test, disease symptoms occurred after 3 days. The differential characters of Pectobacterium carotovorum strains according to Schaad et al, (2001) could apparently separate the Pectobacterium carotovorum strains from other Pectobacterium spp. and Dickeya chrysanthemi. For the differentiation among P. carotovorum, P. atrosepticum and D. chrysanthemi three different temperatures (27°C, 35.5°C and 37°C) were used. D. chrysanthemi (DSM 4610) was able to grow on nutrient agar in all three temperatures, P. carotovorum did the same as D. chrysanthemi, but P. atrosepticum (SCRI 1043) was able to grow at 27°C only.

These finding implies that P. carotovorum is the main soft rotting Pectobacterium occurring on Z. ethiopica plant in Markazi province, Iran. Figure 1.

Using of the Y1/Y2 primers which caused the amplification of the expected bands (434 bp) in isolates, was in concord with its result of classification based on the physiological and biochemical features in this research, and the P. carotovorum was generally isolated from the infected plant tissues.

PCR amplification of the 16S-23S rRNA intergenic transcribed spacer region (ITS) using G1 and L1 primers (Toth et al., 2001) produced two main bands about 535 and 580bp and one faint band at about 740bp when...
Figure 2. (a) Electrophoresis banding pattern of the ITS on 2% agarose gel; M- Molecular marker 1 kb, Lane 1-8: *Pectobacterium carotovorum* from *Z. ethiopica*, Lane 9: *Pectobacterium carotovorum* (reference strain; SCRI 1949) Lane 10: *P. atrosepticum* (SCRI 1043) and (b) ITS-RFLP, digested with *Rsa*I restriction enzyme. M; Molecular marker 100 bp, Lane 1-8: *P. carotovorum* from *Z. ethiopica*. Lane 9: Negative control. Lane 10: standard isolates of *Pectobacterium carotovorum* SCRI 1949, Lane 11- *Pectobacterium atrosepticum* SCRI 1043.

electrophoresed through a 2% agarose gel (Figure 2: a). For discrimination between *P. carotovorum* and *P. odoriferum*, all strains were subjected to α-methyl glucoside test. The ITS-PCR products then digested with *Rsa*I restriction enzyme and gave three banding pattern at about 195, 355, 520bp for all strain (Figure 2: b) Based on biochemical and physiological characteristics, PCR based pel gene and analysis by ITS-PCR, and the ITS-PCR digestion with *Rsa*I, all isolates were identified as *P. carotovorum* subsp. *carotovorum*. Since the symptoms of *P. carotovorum* are very similar to soft rot that originating from other soft rotting bacteria, the exact and fast diagnosis of soft rot disease is so noteworthy from the aspect of import and export regulations for farm and greenhouse products. Use of the Y<sub>1</sub>/Y<sub>2</sub> primers, which caused the amplification of the expected bands in isolates, was in concordance with the results of classification based on the phenotypic features and *P. carotovorum* subsp. *carotovorum* was generally isolated from the infected plant tissues. Due to high humidity and temperature, being dominant for *P. carotovorum* in greenhouses, is unexpected. The primers used in this research were notable in trace and diagnose of other *Pectobacterium*. Comparisons using biochemical, physiological tests, and PCR methods using suitable primers are preferred because of their easy to use. Figure 2.

The use of G<sub>1</sub>/L<sub>1</sub> primers that are able to amplify the 16S-23S rRNA gene of *Pectobacterium* species and some other bacterial genus in PCR reaction, have not proven the existence of *D. chrysanthemi* and other *Pectobacterium* spp. in collected isolates but because of suitable climate, the existence of other soft rot bacteria like *D. chrysanthemi* is not cancelled, and need to be more researched. By using G<sub>1</sub>/L<sub>1</sub> primers that were able to produce specified banding patterns in PCR reaction for *P. atrosepticum*, *P. carotovorum*, it would be possible to separate these two species from each other (Toth et al., 2001). The banding patterns using G<sub>1</sub>/L<sub>1</sub> primers were about 535 and 570, due to the similarity in producing banding patterns in *P. odoriferum* and *P. carotovorum*, for separation of these two from each other, the use of Keto-methyl glucoside test is suggested. The results obtained from using G<sub>1</sub>/L<sub>1</sub> primers and doing RFLP test on ITS-PCR products were able to recognized *P. carotovorum*. The results were in parallel with biochemical tests and the one obtained from the sequencing of Pel gene.


