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Detection of bacterial soft-rot of crown imperial caused by *Pectobacterium carotovorum* subsp. *carotovorum* using specific PCR primers

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Summary. Pectobacterium is one of the major destructive causal agent in most crop plants throughout the world. During a survey in spring of 2005 in the rangeland of Kermanshah and Isfahan, provinces of Iran, samples of bulbs and stems of crown imperial with brown spot and soft rot were collected. Eight strains of pectolytic Erwinia were isolated and purified from these samples. Phenotypic tests indicated that the strains were gram-negative, facultative anaerobic, rod shaped, motile with peritrichous flagella. They were oxidase negative, catalase positive and also able to macerate potato slices. Pathogenicity of all the strains were confirmed on corn, philodendron and crown imperial by inoculation of these crops with a bacterial suspension and reisolation of the strain from symptomatic tissues. A pair of specific PCR primers was used to detect these bacterial strains. The primer set (EXPCCF/EXPCCR) amplified a single fragment of the expected size (0.55 kb) from genomic DNA of all strains used in this study. In nested PCR, the primer set (INPCCR/INPCCF) amplified the expected single fragment (0.4 kb) from the PCR product of first PCR amplification. On the basis of the biochemical and phenotypic characteristics and PCR amplification by the specific PCR primers, these strains were identified as *Pectobacterium carotovorum* subsp. *carotovorum*. This is the first report of occurrence of crown imperial bacterial soft-rot in Iran.

Keywords: *Fritillaria imperialis,* nested PCR.

Introduction

Pectolytic Erwinias which cause soft rot disease have a wide host range including potato, cabbage, cucumber, tomato and ornamental plants (Dhanvantari and Dirks, 1987; Dickey and Kelman, 1988; Schuerger and Batzer, 1993; Harward *et al.,* 1994). Of the pectolytic Erwinias, *Erwinia carotovora* and *E. chrysanthemi* are the most important pectolytic bacteria. Previously, Hauben *et al*., (1998) reviewed the suggestion that the pectolytic Erwinia be placed in a separate genus *Pectobacterium*, on the basis of the 16s rDNA sequences, but the generic epithet, *Erwinia*, remains the preferred designation in the literature (Gardan *et al.,* 2003). The strains of *Erwinia* associated with soft rot of potato have been studied extensively because of their economic importance (Perombelon and Kelman, 1980; Kritzman, 1989). In contrast, *Erwinia* spp. isolated from other plants have been less studied.

Erwinia carotovora subsp. *carotovora* (Jones, 1901) Bergey *et al.,* 1923 has been described as a

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pathogen causing soft rot of several ornamental bulbous crops including crocus, freesia, gladiolus, hyacinth and iris (Moore, 1979). There are few records of soft rot of crown imperial in the literature. Babadoost (1990) reported that *E. carotovora* and *E. chrysanthemi* can cause crown imperial soft rot. He stated that these bacteria cause soft rot of many fleshy fruits, vegetables and ornamental plant such as cabbage, calla, chicory, dahlia and fritillaria. Several methods have been employed to distinguish between pectolytic Erwinias. The most commonly used methods are biochemical tests (Dickey and Kelman, 1988) and pathogenicity tests (Smith and Bartz, 1990). Serological assays involving monoclonal and polyclonal antibodies are used for detection of *P. carotovorum* strains (De Boer *et al*., 1987; Klopmeyer and Kelman, 1988; Vander Wolf *et al*., 1996), but identification of *P. carotovorum* at the subspecies level has been problematic due to the complexity of the closely linked serogroups within the subspecies. As an alternative method, DNA markers useful in DNA hybridization and PCR techniques have been employed for sensitive and rapid detection and identification of *P. carotovorum* strains. In particular, PCR primers and DNA-probes have been used for the specific detection of *P. atrosepticum* among other subspecies and genera of bacteria (Darrasse *et al*., 1994a; Ward and De Boer, 1994). A set of specific PCR primers was recently designed to detect *P. carotovorum* subsp. *carotovorum* strains (Kang *et al*., 2003). This paper describes a PCR protocol useful for the sensitive and specific detection of *P. carotovorum* subsp. *carotovorum* strains causing bacterial soft rot of crown imperial collected from mountainous areas of Iran.

Materials and methods

Isolation

Infected crown imperial bulbs were surface-sterilized with a 0.5% sodium hypochlorite solution and were cut in half longitudinally. Small portions (1–2 mm^3) from the border of healthy and macerated tissue were removed and comminuted with a sterile glass rod in 1 ml of sterile water. The resulting suspensions were streaked on eosin methylene blue agar (EMB). After incubation at 25°C for 48 h, representative single bacterial colonies with a green metallic color were subcultured on nutrient agar (NA), incubated at 25ºC for 48 h, and maintained at 4ºC in the dark. A total 8 strains were selected and maintained for further studies.

Physiological and biochemical tests

The determinative tests (Table 1) were conducted as described previously: Gram staining and flagella staining (Schaad *et al.,* 2001), motility under phase-contrast microscope, colony morphology on nutrient agar (NA), pectate degradation (Cuppels and Kelman, 1974), potato soft rot (Lelliot *et al.,* 1966), gelatin liquefaction (Dye, 1968), acetoin production (Schaad *et al.,* 2001), gas production from glucose (Dye, 1968), sensitivity to erythromycin with 15 μg incorporated into Mueller-Hinton agar plates (De Boer and Kelman, 1978), phosphatase (Gerhardt *et al.,* 1981), glucose oxidation or fermentation (Hugh and Leifson, 1953), lecithinase (Gerhardt *et al.,* 1981), indole production with Kovac's reagent (Gerhardt *et al.,* 1981), hydrolysis of casein (Dye, 1968), pigment production (King *et al.,* 1954), starch hydrolysis (Lelliot and Stead, 1987), reducing substances from sucrose and blue pigment on YDC (Schaad *et al.,* 2001), growth at 37°C in yeast salts broth in a rotary shaker (Schaad *et al.,* 2001), growth in 3, 5 and 7% NaCl (Schaad *et al.,* 2001), catalase, arginine dihydrolase (Schaad *et al.,* 2001), H_2S production from peptone, L-cysteine, methionin and sodium thiosulfate (Dye, 1968), urease (Gerhardt *et al.,* 1981), phenylalanine deaminase (Dye, 1968), oxidase test (Lelliot *et al.,* 1966), nitrate reduction (Gerhardt *et al.,* 1981), susceptibility to antibiotics (Table 2) (Klement, 1990), organic acid and amino acid utilization (Schaad *et al.,* 2001). All carbohydrates used in the tests for acid production (Table 3) were filter-sterilized before being added to Ayers, Rupp, and Johnson basal agar medium (Gerhardt *et al.,* 1981).

Pathogenicity test

***Fritillaria imperialis*.** For each of the 8 isolates, 8 intact bulbs of crown imperial were dipped in 95% ethanol to disinfect their surfaces. A sterile cork borer (3 mm diam.) was used to make a 10 mm deep puncture wound midway between the top and bottom of each bulb. A bacterial suspension (5 μl) containing 1×10^8 colony-forming units per ml (CFU ml^{-1}) was inoculated into the wounds. The control bulbs were inoculated with sterile water. Bulbs were then placed in polyethylene bags, which

Table 1. Physiological tests for identification of soft rot *Pectobacterium* isolated from *Fritillaria*.

Characteristic	Bacterial strain[a]							
	M1	M2	M3	M4	M5	M6	M7	M8
Gram stain	-	-	-	-	-	-	-	-
Pectate degradation	+	+	+	+	+	+	+	+
Potato soft rot	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-	-	-	-
Casein hydrolysis	+	+	+	+	-	+	-	+
Hydrolysis of Tween 80	-	-	-	-	-	-	-	-
Oxidation/ fermentation of glucose	+	+	+	+	+	+	+	+
Acetoin production	+	+	+	+	+	+	+	+
Sensitivity to erythromycin ($15\ \mu g\ disc^{-1-1}$)	-	-	-	-	-	-	-	-
Litmus milk	RCPD*	RCPD	RCPD	RCPD	RCPD	RCPD	RCPD	RCPD
Phosphatase	-	-	-	-	-	-	-	-
Lecithinase	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	+	+	-	-
Pigment production	-	-	-	-	-	-	-	-
Reducing substance from sucrose	-	-	-	-	-	-	+	+
Gas from glucose	-	-	-	-	-	-	-	-
Blue pigment on YDC*	-	-	-	-	-	-	-	-
Growth at								
37°C	+	+	+	+	+	+	+	+
39°C	-	-	-	-	-	-	-	-
Growth in								
3% (w:v) NaCl	+	+	+	+	+	+	+	+
5% (w:v) NaCl	+	+	+	+	+	+	+	+
7% (w:v) NaCl	+	+	+	+	+	+	+	+
Urease production	-	-	-	-	-	-	-	-
Phenylalanine deaminase	-	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-	-
Tobacco hypersensitivity	-	-	-	+	+	-	-	-
H_2S production source:								
Peptone	+	+	+	+	+	+	+	+
Sodium thiosulfate	+	+	+	+	+	+	+	+
Cystein	+	+	+	+	+	+	+	+
Methionin	+	+	+	+	-	-	+	+
Nitrate reduction	+	+	+	+	+	+	+	+

[a] +, positive; -, negative; RCPD, red, cured formation, peptonized and discolored; YDC, yeast extract dextrose calcium carbonate agar.

were tightly sealed and incubated in the dark at 25°C for 7–14 days. After incubation, the bulbs were cut in half longitudinally through the inoculation site with a sterile knife and examined for the extent of soft rot. To confirm Koch's postulates, bacteria reisolated from diseased bulbs were streaked on NA and reidentified using the methods outlined by Lelliott and Stead (1987) and Sands (1990).

***Philodendron* sp**. Mature plants with 3–4 expanded leaves were kept on the green house bench until inoculated. Two leaves immediately below an expanding or recently expanded leaf were inoculated. The plants were inoculated by removing a small loopful of bacterial cells from a 24 h slant culture and placing them on the leaf surface. A sterile dissecting needle was punctured through the

Table 2. Susceptibility of *Pectobacterium* strains isolated from *Fritillaria* to antibiotics.

Antibiotic	Bacterial strain[a]							
	M1	M2	M3	M4	M5	M6	M7	M8
Amikacin (30 μg disc^{-1})	S	R	R	S	S	S	S	S
Ampicilin (10 μg disc^{-1})	R	S	R	R	R	S	R	R
Cefalexin (30 μg disc^{-1})	R	R	S	R	R	S	R	R
Cephalotin (30 μg disc^{-1})	R	S	S	R	R	S	R	R
Chloramphenicol (30 μg disc^{-1})	S	S	S	S	S	S	S	S
Doxycycline (30 μg disc^{-1})	S	S	S	S	S	R	R	S
Erythromycin (15 μg disc^{-1})	R	R	R	R	R	R	R	R
Gentamycin (10 μg disc^{-1})	R	R	R	R	R	R	R	R
Kanamycin (30 μg disc^{-1})	R	R	R	R	R	R	R	R
Nalidixic Acid (30 μg disc^{-1})	S	S	R	R	S	S	S	R
Neomycin (30 μg disc^{-1})	R	R	R	R	R	R	R	R
Penicilin (30 μg disc^{-1})	R	R	R	R	R	R	R	R
Sterptomycin (10 μg disc^{-1})	R	S	S	R	R	S	R	R
Tetracyclin (30 μg disc^{-1})	S	S	S	S	S	S	S	S
Tobramycin (10 μg disc^{-1})	R	R	R	R	R	R	R	R

[a] R, Resistant; S, Susceptible.

cells and leaf, and the bacterial cells were gently teased into the wound by needle to assure intimate contact of the cells with the leaf tissues beneath the epidermis. Each leaf was inoculated with four strains and a sterile needle puncture as a plus check. The plants were immediately placed in greenhouse with 27±2°C and 85–90% relative humidity. Plant reactions were recorded 4, 5 and 7 days after inoculation. Leaf reactions were usually visible after 2 days. The spreading water-soaked lesions were rated as positive, while the dry, negligible to small lesions were considered negative (Dickey, 1981).

Zea mays. The test was a modification of the leaf puncture inoculation method developed by Victoria (Victoria, 1977). The two or three top leaves of corn cv. Single cross 704, were cut from 4–5-week-old plants. The center portions (about 10 cm long) of the leaves were excised and placed on sterile Petri dishes in a moist chamber. An aqueous suspension of bacterial cells from 24-h-old nutrient agar culture was prepared which contained approximately 10^8 CFU ml^{-1}. The surface was inoculated at 4 sites (one site/strain) by gently pressing the tip of an Eppendorf micropipette against the epidermal tissue to make a slight depression or wound and then depositing 5 μl of the suspension onto the depression. The chambers were tightly covered with Parafilm and incubated in the dark at 32–33°C for 48–72 h. Leaves inoculated with distilled water were used as control. A water-soaked lesion > 5 mm in length was considered positive, a length of 3–4 mm was slightly positive, and 2> mm was negative (Dickey, 1981).

DNA extraction

The SDS-boiling method was used for DNA extraction. Total DNA was extracted from bacterial culture in Luria Bertani (LB) medium (0.5% yeast extract, 1% tryptone, 1% NaCl). After 20 h, 3 ml of culture was removed and centrifuged at 14,000 ×*g* for 3 min. The pellet was resuspended in 500 μl extraction buffer (0.2 M Tris-HCl, pH 8.0; 0.2 M EDTA; 2% SDS) and boiled at 100ºC for 8 min. Then 250 μl phenol and 250 μl chloroform-isoamyl alcohol (24:1) were added to the tube and centrifuged at 13,000 ×*g* for 5 min and the supernatant was transferred to a new tube. For elimination RNA, 3 μl of RNase-A (Sigma R.4857, Sigma Chemical Co., St. Louis, MO, USA) was added to the tube and the nucleic acid solution was incubated at 37ºC for 1 h. and then extracted with chloroform- isoamyl alcohol (24:1; v:v). The mixture was centrifuged at 13,000 ×g for 5 min., after which the upper phase was transferred to a new tube and the DNA was precipitated with an equal volume of cool isopropanol, washed with 70% (v:v) ethanol, dried and

Table 3. Acid production from carbohydrates used for the identification of soft rot *Pectobacterium* isolated from *Fritillaria*.

Carbohydrate source	Bacterial strain							
	M1	M2	M3	M4	M5	M6	M7	M8
D- Lactose	+	+	+	+	+	+	+	+
D- Trehalose	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+
D- Mannitol	+	+	+	+	+	+	+	+
D- Rhamnose	+	+	+	+	+	+	+	+
D- Ribose	+	+	+	+	+	+	+	+
D- Mannose	+	+	+	+	+	+	+	+
L- Arabinose	+	+	+	+	+	+	+	+
D- Galactose	+	+	+	+	+	+	+	+
D- Dextrose	+	+	+	+	+	+	+	+
D- Fructose	+	+	+	+	+	+	+	+
D- Glucose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	-
D- Rafinose	+	+	+	+	+	+	+	+
Maltose	-	-	-	-	-	-	-	-
D- Melizitose	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	+	+	-
Dulcitose	-	-	-	-	-	-	-	-
L- Inositol	+	+	+	+	+	+	+	+
L- Prolin	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-
Asparagine	+	+	+	+	+	+	+	+
Ethanol	-	-	-	-	-	-	-	-
Glycerol	+	+	-	-	+	-	+	+
Utilization of:								
Malonate	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	+
Lactate	+	+	+	+	+	+	+	+
Malate	+	+	+	+	+	+	+	+
Glutamic acid	+	+	+	+	+	+	+	+
Oxalic acid	-	-	-	-	-	-	-	-
L-Tartaric acid	-	-	-	-	-	-	-	-

Note, (+) positive reaction, (-) negative reaction.

resuspended in 50 μl of TE buffer. Five microlitres were used for PCR amplification.

PCR amplification

Oligonucleotide primers EXPCCR (5-GCCG-TAATTGCCTACCTGCTTAAG-3') and EXPCCF (5'-GAACTTCGCACCGCCGACCTTCTA-3') were used in a standard PCR assay (Kang *et al*,. 2003). The PCR reactions were performed in a 50-μl PCR mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w:v) gelatin, 200 μM each of dNTP, 100 ng primer, 2.5 unit of *Taq* polymerase. PCR amplification was carried out as follows: one cycle of 4 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C; and a final extension for 7 min at 72°C. PCR products were separated on a 1.5% agarose gel in tris-acetate-EDTA (TAE) buffer and visualized by staining with ethidium bromide (1 mg l^{-1}). For nested PCR, primers IN-PCCR (5' GGCCAAGCAGTGCCTGTATATCC-3')

and INPCCF (5'-TTCGATCACGCAACCTGCATTACT-3') were selected from the sequence bases downstream of the 3'-ends of primers EXPCCF and EXPCCR for amplification of an expected 0.4 kb (Kang *et al*, 2003). Two microlitres of amplified template from the first PCR solution was used in the nested PCR reaction under the same PCR conditions as stated above.

Results and discussion

Disease symptoms

The first symptoms of soft rot disease in crown imperial (*Fritillaria imperialis*) plants are small, water-soaked lesions that appear on the bulbs and stem bases. In warm, wet conditions, which are favorable for disease development, the lesions rapidly enlarge in both diameter and depth, resulting in a soft and mushy decay with a gray, brown and black color. In the final stage, the infected plants are stunted, weak and chlorotic and the bulbs rot completely; eventually the infected plants collapse and die (Fig. 1).

Bacterial identification

The bacterial cultures isolated from crown imperial plants were all identical in their morphological, cultural and biochemical properties. Morphological examination revealed that the bacterial strains were Gram- negative, rod-shaped, occurred singly, in pairs and rarely in short chains, and had peritrichous flagella (Fig. 1H). On NA, the colonies were transparent, round, convex and cream colored after 48 h incubation at 28ºC.

Bacteria from purified colonies were facultatively anaerobic, and negative for oxidase, arginine dihydrolase, sensitivity to erythromycin, phosphatase, lecithinase, pigment production, and urease production. All strains were positive for catalase, gelatin liquefaction, casein hydrolysis, nitrate reduction, production H_2S from peptone, L-cystein, sodium thiosulfate and methionine as well as pectate degradation, potato soft rot development within 24h, acetoin production and growth at 37ºC. All isolates were negative for production of indole and reducing substance from sucrose, and were positive for production of acid from D-lactose, L-arabinose, D-ribose, D-rhamnose, D-galactose, cellobiose, manitol and trehalose. None of the strains produced acid form D-melezitose, dulcitul and maltose. Other characteristics are listed in Tables 1, 2 and 3. The isolates showed homogeneity in their physiological, biochemical and nutritional characteristics, but some of the isolates exhibited variation in a few characteristics such as indole production, reducing substance from sucrose and utilization of a few carbon sources.

The resistance of the strains to erythromycin, lack of pigment production, ability to grow in medium with high salt concentrations support the identification of these strains as *Pectobacterium* rather than the closely related pectolytic *Dickeya*. Growth of these strains at 37°C supports the identification of these strains as *P. carotovorum* rather than *P. atrosepticum*.

Pathogenicity tests

All strains when inoculated into healthy crown imperial bulbs induced severe rotting identical to that observed under natural conditions. Approximately 1×10^8 bacterial cells were inserted into each bulb wound, the symptoms began to appear as a small, water-soaked lesion around the site of inoculation, which enlarged rapidly, then extended to the whole bulb that became soft and mushy within 24 h. The outer surface remained intact whereas the entire contents changed into a slimy disintegrating mass of tissue (Fig. 1E). None of the control bulbs developed soft rot symptoms. All the strains produced water-soaked lesions followed by necrosis on the leaves of *Phylodendron* sp. as a positive reaction to pathogenicity (Fig. 1G), which developed after 2–5 days of inoculation. Five of the 8 strains of *P. carotovorum* subsp. *carotovorum* isolated from crown imperial caused a positive or slightly positive reaction in leaves of corn cv. Sc 704, in the *in-vitro* assay (Fig. 1F).

PCR assays

The oligonucleotide primers (EXPCCF/EXPCCR) were used to detect isolated bacteria from crown imperial in a PCR test. All bacterial strains produced the expected 0.55-kb product following PCR with these primers (Fig. 2A). Based on the first PCR amplification, the primer set (INPCCR/INPCCF) that used in nested PCR an expected 0.4-kb fragment amplified in all strains (Fig. 2B). These observations con-

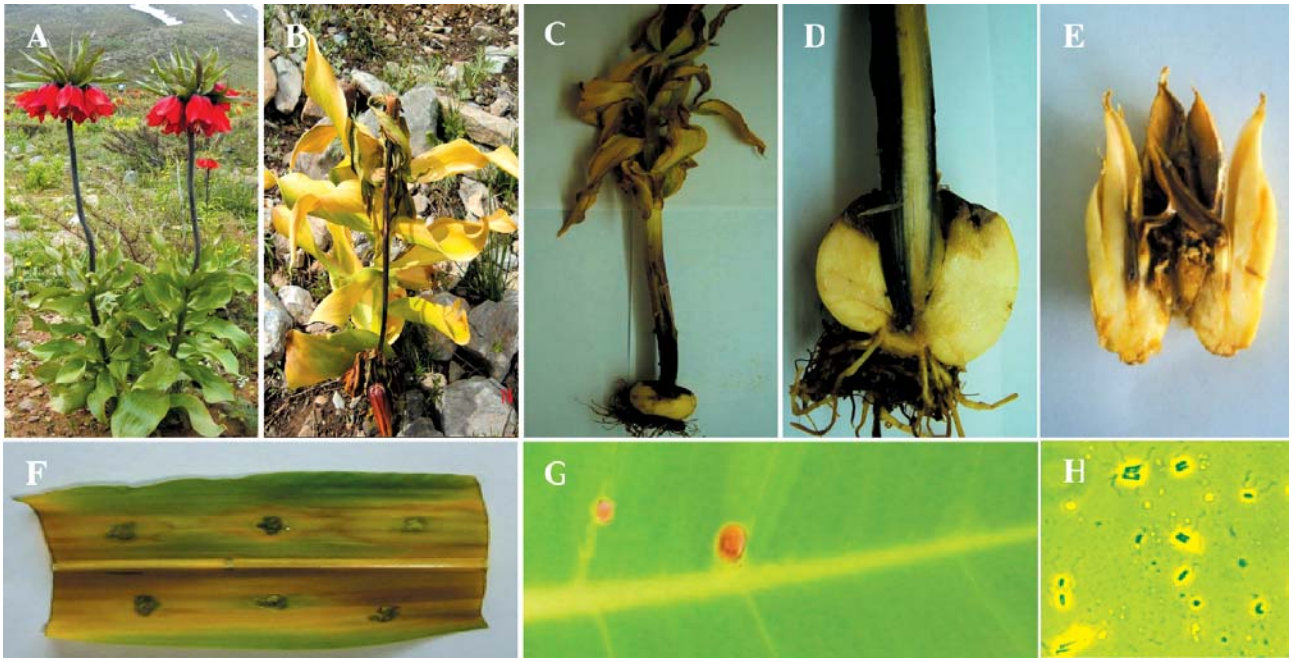

Fig. 1. A, Healthy plant; B, Infectious plant in the field; C, D, symptoms of black and soft rot on bulb; E, first stage of *Pectobacterium carotovorum* subsp. *carotovorum* (pcc) soft rot on bulb; F, water-soaked and necrosis lesion of pcc on corn leaf; G, effect of pcc on philodendron; H, peritrichous flagella of pcc.

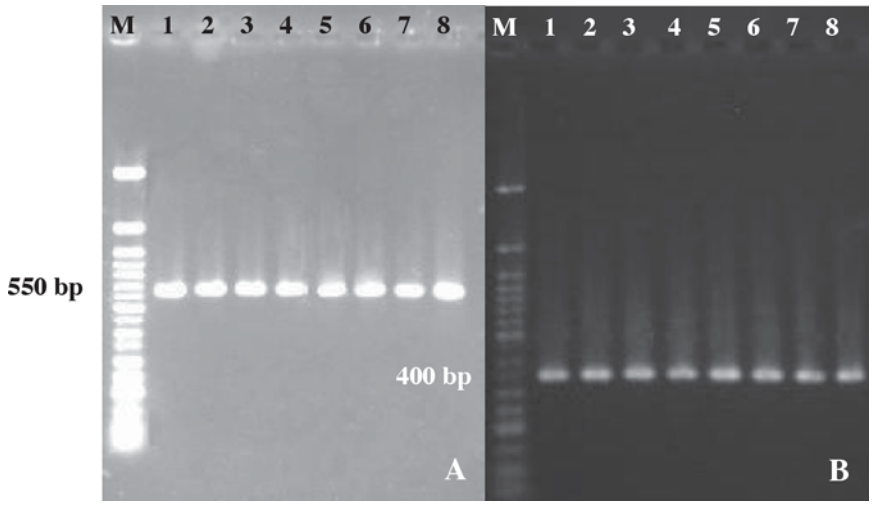

Fig. 2. PCR detection of *Pectobacterium carotovorum* subsp. *carotovorum* with specific primer pairs. Primer sets EXPCCF/EXPCCR and INPCCF/INPCCR were used for standard PCR (A) and nested PCR (B), respectively. Aliquots of 15 μl from each amplified PCR mixture were separated by electrophoresis on 1·5% agarose gel. Lane M, 50 bp DNA ladder. Lane 1–8, bacterial strains

firmed the results of phenotypical and biochemical tests used to identify *P. carotovorum* subsp. *carotovorum* from these host plants. Specific DNA markers for the detection of phytopathogenic bacteria at subspecies or pathotype level are well documented in many reports (Minsavage *et al*., 1994; Mills *et al*., 1997). There have also been some reports for the specific detection of *P. carotovorum* using serological and DNA hybridization methods (De Boer *et al*., 1987; Klopmeyer and Kelman, 1988; Ward and De Boer, 1990). However, DNA detection of *P. carotovorum* at subspecies level has largely focused on *P. atrosepticum* (Darrasse *et al*., 1994a; Ward and De Boer, 1994). PCR methods have also recently been used for the detection and identification of *P. carotovorum* at subspecies and strain level (Toth *et al*., 2001). Recently, the internal transcribed spacer (ITS) region of soft rot Erwinias were analysed to identify them (Toth *et al*., 2001). PCR techniques such as nested PCR, PCR-dot blot and reverse-blot hybridization have been employed for their high sensitivity of detection (McManus and Jones, 1995). Individual cells have been detected using these techniques. Such sensitivity permits early diagnosis of the causal pathogen at low density and the diagnostic result can potentially be used to provide basic information for effective control.

In the present study, the bacteria isolated from crown imperial were identified as *P. carotovorum* subsp. *carotovorum* on the basis of morphological, biochemical and pathogenicity tests (DeBoer and Kelman, 2001) and specific PCR primers. It appears therefore that crown imperial is a natural host of *P. carotovora* subsp. *carotovora*. This is the first report of bacterial soft rot on crown imperial caused by *P. carotovorum* subsp. *carotovorum*.

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