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Characterization of *Pectobacterium carotovorum* subsp. *carotovorum* as a new disease on Lettuce in Malaysia

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Abstract Malaysia produces many varieties of vegetables. The Cameron Highland in the state of Pahang is the main area for lettuce (*Lactuca sativa* var. *romana*) production. A survey recently identified *Pectobacterium* spp. was responsible for more than 15 % damage to lettuce both in the field (including greenhouses) and storage. Isolates of *Pectobacterium carotovorum* were collected from different greenhouses and fields and analysed in the laboratory during 2010. Nine isolates were purified and charcterised by morphological, biochemical and molecular methods. Pathogenicity studies on lettuce using 4 strains showed there was a high susceptibility to the *P. carotovorum*. PCR amplification with Y_1 and Y_2 primers (specific for *P. carotovorum* subspecies) produced the expected band of 434 bp. PCR amplification of the intergenic transcribed spacer region (ITS) using G_1 and L_1 primers also resulted in the expected two bands (535 and 580 bp). Based on these biochemical and physiological characteristics, PCR based *pel* gene, characterisation of the ITS region and digestion of the ITS-PCR products with *Rsa*I restriction enzyme, all isolates were identified as *P. carotovorum* subsp. *carotovorum*. This is the first record of the bacterial pathogen causing disease on lettuce in Malaysia.

Keywords Vegetables · Detection · Identification · Bacterial disease

Bacterial soft rot diseases of plants are generally caused by bacteria in the genus *Pectobacterium*. The pathogen, *P. carotovorum* is one of the most important bacterial diseases of vegetables and causes rots in the field, during storage, and in transportation.

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Originally described as *Erwinia carotovora* by Jones in 1901, taxonomic and molecular characterization of the genus *Erwinia* (Kwon et al. 1997; Hauben et al. 1998; Avrova et *al*. 2002; Gardan et al. 2003) has placed this bacterium into the genus *Pectobacterium*. Infections caused by *Pectobacterium* result in extensive maceration of the paranchymatus cells due to the production of enzymes such as proteases and pectinases (Kotoujansky 1987), resulting in cell deaths (Garibaldi and Battman 1971). Maceration depends on factors such as temperature and moisture (Perombelon 1980). Optimal temperatures for disease development are between 25 and 30 °C. Control of soft rot is difficult as there are currently no effective chemical control measures available. However, cultural conditions which encourage plant growth, weed control, disease-free planting material, and the use of tolerant or resistant varieties offer the most effective control measures. Biochemical tests (Lelliot and Dickey 1984) were developed for the identification and characterization of *P. carotovorum* but do not discriminate between all *Pectobacterium* species. A PCR test based on the amplification of the pectatelyase-encoding gene (*Pel* gene) however, does separate all *Pectobacterium* spp. except *P. carotovorum* subsp. *betavascularum* (Darrasse et al. 1994). Amplification of 16S-23S intergenic transcribed spacer (ITS, rRNA gene) is capable of separating *P. carotovorum* strains especially when combined with Restriction Fragment Length Polymorphism (RFLP) (Toth et al. 2001). The aims of this study were to detect, identify and characterize *P. carotovorum* isolated from soft rot infected lettuce by biochemical and molecular methods.

The objective of this study was to investigate the phenotypic and molecular characterization of the bacterial phytopathogen *Pectobacterium carotovorum* subsp. *carotovorum*, the causal agent of bacterial soft-rot on lettuce in Malaysia. Previously, *P. carotovorum* subsp. *carotovorum* was not determined as the causal agent of soft-rot on lettuce in Malaysia. In the present work the bacterium under study

has been observed to play a very important role in soft rot disease incidence on lettuce in Malaysia.

The present study was therefore designed to identify the causal agent of soft rot in order to comprehend the etiological and disease management of *P. carotovorum* subsp. *carotovorum* in lettuce. The study was conducted with a survey of commercial lettuce production areas, and suspected samples were collected, analyzed and characterized by both biochemical and molecular methods

Samples of chlorotic or necrotic leaves with a light yellow color and exhibiting extensive water soaked lesions consistent with infection by *Pectobacterium* spp. were collected from a range of field sites and brought into the laboratory during 2010 for analysis. Small pieces of infected tissues were soaked in saline solution (0.85 % NaCl) for 20 min to allow bacterial cells to diffuse into the solution. A standard protocol using raffinose (RAF) and crystal violet pectate (CVP) media were used for isolation of the bacteria from plant samples (Segal 1971; Cuppels and Kelman 1974). Inoculated plates were incubated at 30 °C for 3 days and colonies that formed pits on CVP or were red on RAF were selected and sub-cultured at least twice to Luria-Bertani (LB) medium to obtain pure cultures for further testing. Cultures were stored in 20 % glycerol at −80 °C until required. Hypersensitivity reaction (HR) to *Nicotiana tabacum* cv. *xanthi* was tested by injecting a suspension of 10^8 CFU/ml of bacterial under the leaf epidermis. All strains gave a positive reaction. Strains were also subjected to biochemical (Schaad et al. 2001), morphological and molecular assessments. Results of biochemical and morphological tests were as followings: bacteria were Gram negative, facultative anaerobic, rod shaped, oxidase negative, phosphatase negative, able to degrade pectate, sensitive to erythromycin, negative for utilization of α-methyl glucoside and indole production, negative for reduction of sugars from sucrose, negative for acid production from arabitol and sorbitol, and positive from citrate, raffinose, and melibiose.

To test pathogenicity of the isolates leaves were surface sterilised with 70 % ethyl alcohol, washed with sterilized distilled water, cut into small pieces, and inoculated with 20 μl aliquots of a bacterial suspension (108 CFU/ml) (Luzzatto et al. 2007). Five representative strains were tested. Inoculated and uninoculated (control) leaves were placed in a growth chamber with 80-90 % relative humidity at 27 °C. Symptoms occurred 24 h after inoculation and were consistent with naturally occurring infections, whereas leaves inoculated with water remained healthy (Fig. 1). Koch´s postulate was fulfilled with re-isolation of P. carotovorum which had the same characteristics as described earlier. PCR conditions for amplification of the pel gene followed the method of Darrasse et al. (1994). The two oligonucleotides Y1 and Y2 were used as primers. The amplified products were visualized electrophoresis on 1 % agarose gels w/v run in TG buffer (3 gr/li Tris-Base MW = 121.10, 28.8 gr/li glycine MW = 75.07), stained with 1.0 % ethidium bromide, visualized under uv light and photographed. The expected amplified fragment of approximately 434 bp was obtained from all isolates (Fig. 2). To discriminate between P. carotovorum subsp. carotovorum and P. carotovorum subsp. odoriferum, all isolates were subjected to an α-methyl glucoside test. ITSPCR was performed as described by Toth et al. (2001) using the universal primers G1 and L1. The product was visualized as described above, except on 2 % agarose. As expected, two main bands were observed (Fig. 3). The ITS-PCR products were digestion with RsaI restriction enzyme. RFLP pattern analysis showed that all isolates from the lettuce samples gave the expected three banding pattern of 200, 355, 520 bp. Based on biochemical and physiological characteristics, PCR based pel gene and analysis by ITS-PCR, all isolates were identified as P. carotovorum subsp. carotovorum. This is the first report of this bacterium causing a soft rot disease of lettuce in Malaysia. Because the symptoms caused by P. carotovorum resemble those of soft rot caused by other soft rot bacteria, The development of a specific, rapid diagnostic method for soft rot bacteria is noteworthy with regard to import and export

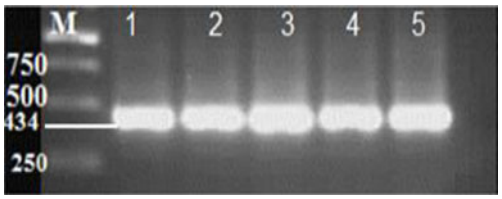

Fig. 2 Pectate lyase (*pel*) PCR amplification of five isolates of *P. carotovorum* subsp. *carotovorum:* M-molecular marker 1 Kb, Lanes 1–5 *P. carotovorum* strains isolated from lettuce. All five strains characterized in this study were positive for *pel* gene amplification

Fig. 1 Lettuce leaf slices inoculated with 20 μL of 10^8 CFU/ml of bacterial suspension. Photographed 36 h after inoculation

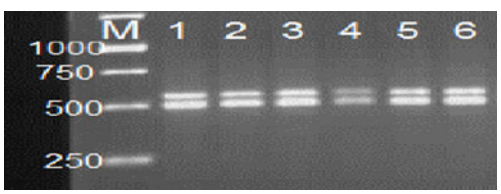

Fig. 3 ITS-PCR banding pattern of *P. carotovorum* subsp. *carotovorum* isolated from Lettuce: M- molecular marker 1 kb, lanes 1–5 *P. ccarotovorum* subsp. *carotovorum*, lane 6- standard strain of *P. ccarotovorum* subsp. *carotovorum* (SCRI 1949)

regulations for farm and greenhouse products. Results using primers Y1/Y2, which amplified the expected bands from the isolates, were in accord with the classification based on physiological and biochemical features, and P. carotovorum was generally isolated from the infected lettuce. Due to the similarity in banding patterns between P. carotovorum and P. odoriferum in ITS-RFLP and the separation of these two from each other, the use of the α- methyl-D-glucoside test is suggested. Because of the high humidity and temperature, and prevalence of P. carotovorum in Malaysian fields and greenhouses, the disease is not unexpected. Therefore, using observations to determine the percentage of plants with soft rot caused by P. carotovorum in Malaysian farms and greenhouses is not conclusive, and complementary tests are needed to confirm the identity of the pathogen. Compared with biochemical and physiological tests, the PCR method with the use of suitable primers is preferred because it is cheaper and easier.

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