Short Communication

PECTOBACTERIUM CAROTOVORUM subsp. *BRASILIENSE* CAUSING PEPPER BLACK SPOT DISEASE IN CHINA

X.M. She[1,2], G.B. Lan[1], Y.F. Tang[1] and Z.F. He[1,2]

[1] *Plant Protection Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, 501640, China*
[2] *Guangdong Provincial Key Laboratory of High Technology for Plant Protection, Guangzhou, 501640, China*

SUMMARY

In 2015, pepper black spot disease became seriously prevalent in Guangdong Province, China. The stem and petioles of diseased plants were strewn with black, circular to oval and bulged spots. A bacterium was isolated from black spots on stem. The bacterium could cause same symptoms on pepper plants by sprinkling inoculation. 16S rDNA sequences of bacterium shared 99-100% identity with *Pectobacterium carotovorum* subsp. *brasiliense* strain 1033. The 322 bp specific fragment could be amplified from the five bacterial isolates using primers Br1f/L1r specific for *P. carotovorum* subsp. *brasiliense*. Characteristics of the bacterium were consistent with those of *P. carotovorum* subsp. *brasiliense* reported. Thus, the causal bacterium identified was *P. carotovorum* subsp. *brasiliense*. To the best of our knowledge, this is the first report of pepper black spot disease caused by *P. carotovorum* subsp. *brasiliense* in China.

Keywords: pepper black spot disease, *Pectobacterium carotovorum* subsp. *brasiliense*, 16S rDNA, bacteriological characteristic.

Pepper [*Capsicum* spp.] is an important economic crop planted with more than 50000 ha every year in winter in Guangdong province, China. In December 2015, a black spot disease seriously occurred on pepper (*Capsicum annuum* L. var. *grossum* cv. Zhongjiao No.105) plants in Zhanjiang city, Guangdong province, China, after the typhoon Rainbow. The stem and petioles of diseased plants were strewn with black, circular to oval and bulged spots (Fig. 1A), occasionally accompanied by canker on branching node (Fig. 1B). More than 75% pepper plants in the fields had symptoms. Under light microscopy, bacterial ooze could be observed from diseased spot tissues. No fungal hyphae or spores were found in black spots. We considered that the pepper black spot disease was likely to be a previously unreported bacterial disease in China. In this study, we identified the causal agent of the pepper black spot disease.

To isolate the putative causative agent from diseased plants, thirty-seven diseased plants with typical symptom were collected from fields. Diseased tissues with spot (1 mm length and 0.5 mm width) were excised and surface-sterilized with 70% (v/v) ethanol for 30 s and 0.5% (v/v) sodium hypochlorite for 30 s, then rinsed with sterile water four times. The tissue was mashed and macerated in 100 µl sterile distilled water and left to stand for three min. The suspensions were then streaked on nutrient agar (NA) medium (Schaad *et al*., 2011) and incubated at 28°C for two days. White colonies predominated in the NA medium plates (Fig. 1C). One isolate was obtained from each plate after twice single-colony isolation. All isolates were stored in 15% (w/v) glycerol at –80°C and five representative isolates, named Pcb-zj-1, Pcb-zj-2, Pcb-zj-3, Pcb-zj-4 and Pcb-zj-5, were used for further experiments.

DNA was extracted from each isolate using an EasyPure® Genomic DNA kit (TransBionovo Co., Ltd., China) according to the manufacturer's instructions. The DNA samples were used as templates for PCR amplification of the 16S rRNA gene using the universal primers 27f/1491r (Woese *et al*., 1983). Approximately 50 ng of DNA template was added to 25 µl Premix Taq™ (0.625 U *Taq*, 0.2 mM dNTP mixture, PCR buffer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) (TaKaRa Biotechnology Co., Ltd, China), 10 pmoles of the primers 27f and 1491r (Woese *et al*., 1983) and ddH_2O to a final volume of 50 µl. The PCR program consisted of an initial denaturation at 96°C for 5 min, followed by 35 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 3 min, and a final extension at 72°C for 10 min in a Mastercycler® Gradient Thermal Cycler (Eppendorf, Germany). Each resulting amplicon was ligated into the vector pMD-20T (TaKaRa Biotechnology). The 16S rRNA fragment-containing recombinant plasmids were transformed into *Escherichia coli* JM109. Three clones were randomly picked up from each transformation and sequenced in both directions using primer walking (Invitrogen Life Technologies China Co., Ltd, China). The

Corresponding author: Z.F. He
Fax: + 86.20.85201471
E-mail: hezf@gdppri.com

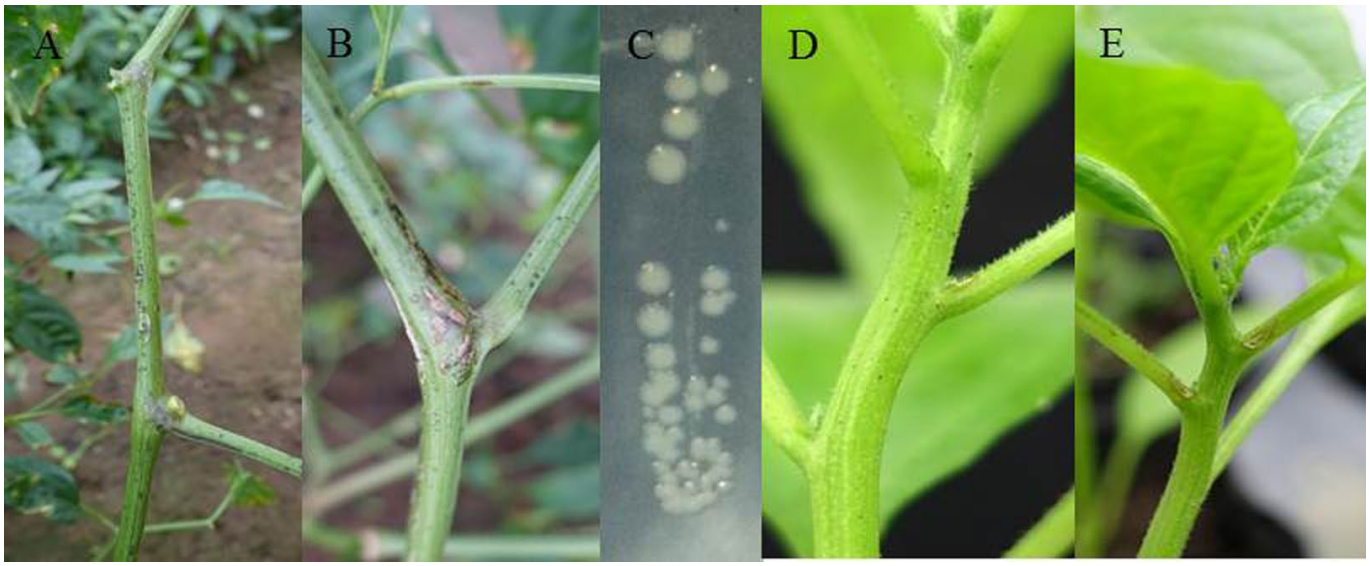

Fig. 1. Black spots on stems and colonies on NA. A: black spots on stem in the field; B: canker on branch node in the fields; C: colonies of Pcb-zj-1 on NA plate; D: black spots on inoculated pepper; E: cankers on some sprinkling-inoculated plants.

similarity of 16S rRNA gene sequences were searched using BLAST program in NCBI database. A phylogenetic tree of 16S rDNA of five isolates and other seven strains of *Pectobacterium carotovorum* subsp. *brasiliense* (GenBank Accession Nos. JF926718, JF926720, JF926721, KP187505, KP187510, KP187523, KJ343633) was constructed using neighbor-joining (NJ) method (MEGA software version 6.0) (Tamura *et al*., 2013). Further, DNA samples of the five bacterial isolates were detected by PCR using the specific primers Br1f/L1r for *P. carotovorum* subsp. *brasiliense* (Duarte *et al*., 2004). Approximately 20 ng of DNA template was supplemented with 10 µl Premix Taq™ (TaKaRa), 10 pmoles of the primers Br1f and L1r (Duarte *et al.* 2004) and ddH_2O to a final volume of 20 µl. The PCR program consisted of an initial denaturation at 95°C for 4 min, followed by 35 cycles of 94°C for 30 s, 52°C for 45 s and 72°C for 90 s, and a final extension at 72°C for 7 min. Eight µl PCR product for each strain was subjected to electrophoresis on 1.5% agarose gel at 120 V/cm for 25 min, stained with DNA Green (Tiandz, Inc., China) and visualized using a UV-transilluminator.

Virulence tests were performed on six- to eight-leaf stage pepper plants (cv. Zhongjiao No.105). Before inoculation, all plants were kept at 22-26°C, 85-95% humidity for 24 h, 12 h light/12 h dark. Pepper plants were inoculated by sprinkling stems with bacterial suspension (3×10^8 CFU/ml). Five potted plants were inoculated with each isolate. The negative control was sprinkling-inoculated with liquid nutrient medium. The experiment was repeated three times. Inoculated pepper plants were first incubated at 22-26°C, 85-95% humidity for 48 h, 12 h light/12 h dark, then at 22-26°C, 60-75% humidity for three days. The bacterium was re-isolated from black spots of inoculated pepper plants.

The five representative isolates were streaked on semi-selective crystal violet pectate (CVP) agar medium (Cupples and Kelman, 1974; Schaad *et al*., 2011) and incubated at 28°C for 48 h to confirm their pectolytic activity. All isolates were also subjected to further biochemical and physiological assays according to Schaad *et al*. (2011).

All the five isolates showed circular and translucent colonies on NA medium. PCR analysis using the universal PCR primers 27f and 1491r (Woese *et al*., 1983) showed that each of the five isolates generated a 1436 bp fragment of 16S rRNA gene. BLAST analysis of these 16S rDNA sequences revealed that 16S rDNA sequences of five isolates, which had 99.9-100% identities with each other, were highly similar to *P. carotovorum* subsp. *brasiliense* strain 1033 (JF926720) with 99-100% identities. The 16S rDNA sequences of the five isolates were deposited in GenBank under accession Nos. KX377593 to KX377597. Phylogenetic analysis showed that the five isolates formed a monophyletic clade with other seven strains of *P. carotovorum* subsp. *brasiliense* (Fig. 2). Using the specific PCR primers Br1f and L1r (Duarte *et al*., 2004) for *P. carotovorum* subsp. *brasiliense*, the 322 bp specific fragment was amplified from the five isolates. All together, these results strongly indicated that the isolates from the diseased pepper plants exhibiting black spot in Guangdong of China were *P. carotovorum* subsp. *brasiliense*.

The five isolates caused water-soaked lesions on pepper plants stem and branch at 48 h after inoculation, and the lesions changed into black spots at three days post inoculation (dpi) in low humidity (Fig. 1D). Canker-like lesions were also observed on some stem nodes of the inoculated plants (Fig. 1E). The bacterium was re-isolated from the black spots of the inoculated pepper plants. The 16S rRNA gene sequences of three re-isolated bacterial isolates had 99.9-100%

Table 1. Bacteriological characteristic of the five isolates.

Test	Five isolates in this paper	*P. carotovorum* subsp. *brasiliense* (Okhee and Jinwoo, 2013; Merwe *et al*., 2010)
Acid from a-methyl glucoside	+	+
Reducing substances from sucrose	+	+
Growth at 37°C	+	+
α-cyclodextrin	–	–
D,L-lactic acid	–	–
D-arabitol	–	–
L-glutamic acid	–	–
L-lactic acid	–	–
N-acetyl-D-glucosamine	–	–
Malonic acid	–	–
D-trehalose	+	+
Inosine	–	–
Gentiobiose	+	+
Maltose	–	–
D-melibiose	+	+
D-raffinose	+	+
D-malic acid	–	–
Sorbitol	–	–
Tween-80	–	–
Cellobiose	+	+
Acetic acid	–	–
Erythromycin		
D-arabinose	–	/
Gelatin liquefaction	–	/
D-glucose(fermentation)	+	/
Catalase	+	/
Oxidase	–	/
Nitrate reduction	+	/
Indole production	+	/
Citrate trisodium	+	/
Malonate	–	/

identity with Pcb-zj-1, Pcb-zj-2, Pcb-zj-3, Pcb-zj-4 and Pcb-zj-5. No symptoms were observed on control plants.

The representative five bacterial isolates showed violet, circular and typically sunken colonies on CVP medium (Fig. 3). The characteristic of the bacterium was consistent with those of *P. carotovorum* subsp. *brasiliense* in previous reports (Duarte *et al*., 2004; Merwe *et al*., 2010; Okhee and Jinwoo, 2013) (Table 1). Besides, all five bacterial isolates also can utilize citrate trisodium but not malonate and D-arabinose. The bacterium was positive for indole production, catalase, D-glucose fermentation and nitrate reduction, negative for gelatin liquefaction and oxidase.

Based on the results of 16S rDNA sequence analyses, virulence tests and bacteriological characteristic, the causal bacterium identified was *P. carotovorum* subsp. *brasiliense*. It has reported that the bacterium caused disease on potato in Brazil (Duarte *et al*., 2004), South Africa (Merwe *et al*., 2010), Kenya (Onkendi and Moleleki, 2014) and pepper in Korea (Okhee and Jinwoo, 2013). To the best of our knowledge, this is the first report of pepper black spot disease caused by *P. carotovorum* subsp. *brasiliense* in China.

P. carotovorum subsp. *brasiliense* has been first reported to cause blackleg disease on potato in Brazil (Duarte *et al*., 2004). The pathogenicity of the isolates from pepper

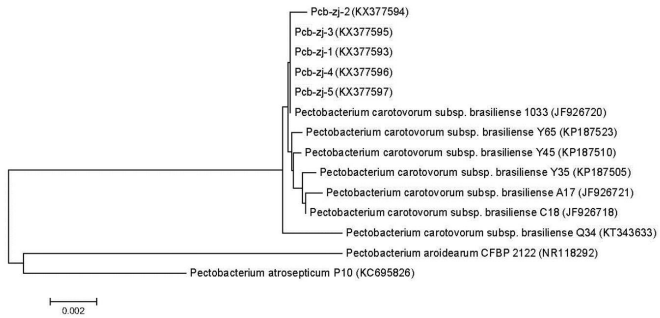

Fig. 2. Phylogenetic tree of *P. carotovorum* subsp. *brasiliense* based on nucleotide sequences of 16S rDNA sequence using neighbor-joining (NJ) method (MEGA software version 6.0). Bootstrap results after 1000 replicates are indicated at each branch node.

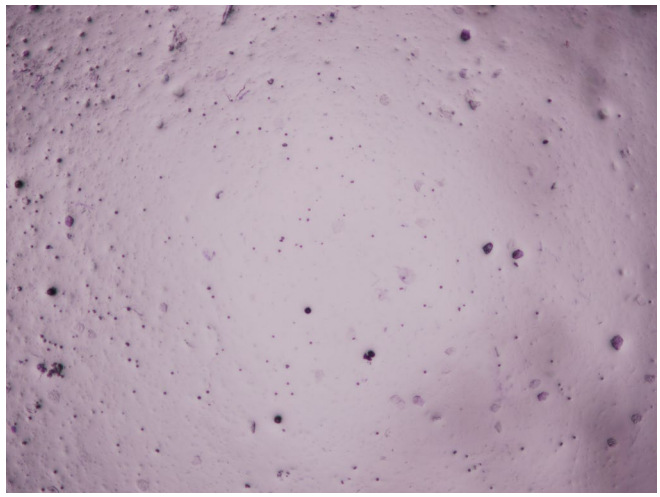

Fig. 3. Cavity formation of the isolate Pcb-zj-1 on CVP medium.

Fig. 4. Potato plants inoculated by isolate Pcb-zj-1 showing blackleg symptoms.

in Guangdong of China on potato was also tested. Five potato plants (cv. Favorita) at the five- to six-leaf stage were inoculated by injecting stem method with 500 µl bacterial suspension (3×10^8 CFU/ml) for each isolate. Five potato plants were inoculated by injecting liquid nutrient medium as negative control. The experiment was repeated three

times. All inoculated potato plants were incubated at 16-21°C, 65-85% humidity. Plants exhibited blackleg and soft rot symptoms at 3 dpi (Fig. 4), while control plants had no symptom. These results suggested that the isolates of *P. carotovorum* subsp. *brasiliense* infecting pepper could also cause blackleg disease on potato in China.

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