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Short communication

Characterization of the pathogen causing a new bacterial vein rot disease in tobacco in China

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ABSTRACT

In 2015, red or black-brown necrotic lesions on main or lateral veins associated with the wilting of leaves and dry rot caused by plant pathogenic bacteria were found in tobacco plants in different fields in Shaowu city, Fujian province, China. Two bacteria (FM3 and FM4) were isolated and selected for further tests. Pathogenicity test results showed that all the tested isolates developed typical vein rot symptoms in tobacco 4 days after inoculation and soft rot symptoms in potato tuber, okra, pepper, capsicum, celery, and Chinese cabbage 2 days after inoculation, with the bacterium re-isolated from all the inoculated plants. On the basis of phenotypic properties, maceration characteristics, biochemical tests, Biolog system, and 16S rRNA sequences analysis, isolates FM3 and FM4 were identified as *Pectobacterium carotovorum* subsp. *carotovorum*. To our knowledge, this is the first report that the new bacterial vein rot disease in tobacco in Shaowu city, China, was caused by *P. carotovorum* subsp. *carotovorum*.

As one of the first provinces that introduced tobacco plantation, Fujian province is listed as “the most appropriate tobacco District” and one of the key areas of high-quality tobacco leaf production in China. The subtropical monsoon climate and the relatively moderate weather of Fujian, which lies in southeast China in Asia, are suitable for growing tobacco. Because of the favorable climate and long-time plantation, tobacco has already become one of the major economic crops of the west and north districts in Fujian (including Longyan, Sanming, and Nanping city). Nanping city is one of the major tobacco-producing districts of Fujian, with a total production of approximately 4.3×10^7 kg every year (Li et al., 2015).

Tobacco production is threatened by various constraints such as declining soil fertility, socioeconomic problems, pests, and diseases. Warm and humid climate is conducive for the epidemic development of diseases and pests. During June and July 2015, a severe outbreak of leaf vein rot in tobacco was observed in Shaowu city, which is one of the major tobacco production areas of the Nanping region. This disease was observed to occur in different fields with varying degrees of intensity. The disease incidence varied from 10% to 30%, and the average leaf mortality in the severely infected plots was above 90%. The disease was more prevalent in the mature leaf. At first, brown-green lesions with a water-soaked margin were observed on the leaf vein. As the lesions enlarged, they turned red-brown or black-brown and spread rapidly toward the leaf tip, and the infected vein became brittle and easily broke. The entire leaf showed terminal wilting and die-back. Tobacco plant exhibited different symptoms from previously reported diseases. Therefore, the objective of this study was to isolate and identify the plant pathogenic bacteria causing this new vein rot disease of tobacco in Shaowu city and identify them through pathogenicity, physiological and biochemical characteristics, and molecular analysis.

Tobacco leaves with typical symptoms of vein rot infection were collected during the tobacco-growing season in July 2015 from three tobacco fields where vein rot disease outbreaks occurred in Shaowu city. The isolation from the infected leaves was performed after washing the leaves under running tap water and sterilizing each leaf vein surface with 1% sodium hypochlorite solution. Pieces of the tissue sections were obtained from the transition zone between the diseased and healthy tissues using a sterilized knife,

Abbreviations: Pcc, *Pectobacterium carotovorum* subsp. *carotovorum*; SDW, sterilized distilled water.

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macerated in 75% alcohol for 4 min, washed in sterilized distilled water (SDW) thrice, then transferred to a mortar, and ground to tissue juices after adding 1 mL of SDW. Fifty microliters of tissue juice was cultured on NA medium (0.5% peptone, 0.3% yeast extract, 0.5% sodium chloride, 1.5% agar, and distilled water) plates using the streaking plate method. All sealed plates were incubated at 28 °C in the dark until the bacteria completely grew. Two dominating bacterial colonies (designated as FM3 and FM4) were isolated and cultured on NA. All strains were routinely maintained on NA and stored in 20% glycerol medium at −80 °C for further use. In addition, the two isolates have been deposited at the China General Microbiological Culture Collection Center (CGMCC No. 7.226 and No. 7.227).

Strains FM3 and FM4 were tested for pathogenicity in tobacco leaf veins (*Nicotania tabacum* cultivar K326). The pathogenicity of the two isolates was confirmed by stab inoculation of tobacco veins. All tissues were surface sterilized with 75% ethanol. Two veins per plant, a total of three seven-week-old tobacco seedlings, 3 cm from the base of the petiole, were stab inoculated using a sterile toothpick with 5 μl of a bacterial suspension (approx. 2×10^6 cfu/ml) at the hole of inoculation. An equal number of veins were inoculated with SDW as controls. All inoculated seedlings were maintained under greenhouse conditions (at 28 °C, 80% humidity, in the dark for 12 h) and periodically inspected until the appearance of the symptoms and necrosis of leaves. Re-isolation of the pathogen was performed from the inoculated plants by using the same bacterial isolation method.

Pathogenicity was also tested on other different vegetables, including celery (*Apium graveolens*), potato tuber (*Solanum tuberosum*), chili and green pepper (*Capsicum annuum*), okra (*Abelmoschus esculentus*), and Chinese cabbage (*Brassica oleracea*), which were obtained from the market, per tested material per strain, using the same inoculation method described above. All inoculated tissues were placed in sealed plastic bags under identical conditions. After 48 h, all tissues were evaluated for the presence of symptoms.

Single colonies of FM3 and FM4 were used for biochemical tests. At first, two isolates were performed on the selective medium, crystal violet pectate (CVP) medium, for pectolytic activity (Hyman et al., 2001). The plates were incubated at 28 °C for 48 h to allow bacterial colonies to grow. Biochemical characterization tests also included the determination of auxin from β-methyol-D-glucoside and uridine; reducing substances from dextrin, palatinose, *N*-acetyl-D-glucosamine, glycerol, and D-sorbitol; organic acid production from malonic acid, acetic acid, and L-lactic acid; growth at 37 °C; and decomposition of Tween 80 and 40 (Duarte et al., 2004; Schaad et al., 2001). The two strains were also identified using Biolog GEN III Microplate. The experimental procedure was conducted according to the operation guide of Biolog GEN III system (June et al., 2006; Wang et al., 2012).

Total genomic DNA was extracted from 48-h-old individual colonies of strains FM3 and FM4 using EasyPure®Bacteria Genomic DNA purification kit (Transgen, Beijing, China). In accordance with the manufacturer's instructions, the extracted DNA was stored at −20 °C until required. To identify the two isolates, the 16S rRNA genes were amplified using the universal primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Frank et al., 2008). Each amplification was performed in a total volume of 50 μl containing 10 μl PCR buffer (5 ×), 2.50 μl $MgCl_2$ (25 mM), 0.75 μl dNTPs (10 mM each), 0.75 μl each forward and reverse primer (10 μM), 0.25 μl Taq polymerase (5 U/μl Promega Corporation) and 1 μl DNA (50 ng/μl), and 34 μl double-distilled H_2O. PCR reaction conditions were 94 °C for 5 min and 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s, followed by a final extension at 72 °C for 10 min. Six microliters of each amplification mixture was electrophoresed on 1% agarose gel. Each DNA was stained with ethidium bromide and then visualized under short-wavelength UV light. Two purified products were sent to BGI Tech. (Beijing, China) for sequencing. BLAST analysis of these amplicons was conducted in GenBank.

Under natural conditions, serious symptoms of this unidentified pathogen can be observed on the veins and branches of tobacco leaves. The symptom consists of brown necrotic lesions on the main and lateral veins and a number of leaves showing dry rot, terminal withering, and death (Fig. 1a and b). The results of pathogenicity assays showed that small, light water-soaking spots were initially observed around the inoculation spots at 28 °C within 12 h of inoculation. With the development of the disease, necrotic lesions appeared and became brown or black-brown; then large necrosis could be observed 4 days after inoculation (Fig. 2a, c). Ten days after inoculation, necrotic red-brown leaf spots expanded to the leaf tips (Fig. 2b, d). These symptoms were consistent with those caused by natural infections. In comparison, no disease symptoms developed on the control plants. Moreover, the re-isolated strains were identified as the tested species with 100% similarity by pectolytic activity and 16S rRNA sequence analysis, fulfilling Koch's postulates and demonstrating that the two isolates were the pathogens of this disease.

Moreover, artificial inoculation results also showed that FM3 and FM4 strains developed typical soft rot symptoms at the inoculation points in potato tuber, okra, green pepper, capsicum, celery, and Chinese cabbage within 24–48 h after inoculation; the control tissues treated with SDW did not develop any symptoms (Fig. 3).

The colonies of the two strains isolated from diseased veins were shiny, grey, round, and convex on NA. The results of biochemical and phenotypical tests were as follows: gram negative; ability to grow at 37 °C; positive for production of fluorescent pigment on King's medium; positive for uridine test and negative for β-methyol-D-glucoside; positive for degradation of pectate; positive for reducing substance from galactose and glucose; positive for Tween 40 and Tween 80 hydrolysis but FM4 was weakly positive; negative for reducting substance from dextrin, palatinose, *N*-acetyl-D-glucosamine, glycerol, and D-sorbitol; and negative for acid production from L-lactic acid (Table 1). Furthermore, FM3 produced acid from malonic acid and acetic acid, but FM4 did not. Most characteristics were consistent with *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) used by Duarte et al. (2004). Gen III Microplate results showed that the SIM values of FM3 and FM4 were 0.852 and 0.654, respectively. They were identified as *Pcc*

Fig. 1. Symptoms showing necrotic lesions on lateral veins (a) and main veins (b) in the field in mid-July 2015.