

Original Research Article

<http://dx.doi.org/10.20546/ijcmas.2016.512.022>

Isolation and Identification of the Pathogen of *Cardamine violifolia* Bacterial Blight Disease in China

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ABSTRACT

Keywords

Cardamine violifolia,
Bacterial blight,
Pseudomonas sp.,
16S rDNA sequences.

Article Info

Accepted:
12 November 2016
Available Online:
10 December 2016

A bacteria disease was observed on *Cardamine violifolia* in production base, Enshicity, Hubei Province, China in 2015. The disease mainly happened on *Cardamine violifolia* leaves. Four strains were isolated from the diseased *Cardamine violifolia* leaves. And the same strain was re-isolated from the inoculated leaves by the Koch's rule. The similar symptom was produced on healthy leaves after inoculating the strain. The pathogen was identified as *Pseudomonas* sp. according to the characters of cultural colonies, results of 16S rDNA sequences analysis (NCBI acc. no.1918730)

Introduction

Cardamine violifolia is found in Yutangba, Shuanghe Town (an area rich in Se) of Enshi city, Hubei province, China, has strong capacity of accumulating Se. The Se content in seedling leaves exceeds 1000 mg/kg, which is the international standard of Se-hyperaccumulator (Karol *et al.*, 2015). So *Cardamine violifolia* was artificial cultivation. A bacterial disease was found on leaves of *Cardamine violifolia* grown under greenhouse in winter of 2014 and 2015. The disease usually started from leaf edges near hydathodes and quickly enlarged to form V-shaped lesions frequently surrounded by chlorotic areas. These lesions finally coalesced to form the severe blight along the whole leaves.

The objective of this research was to identify the causal agent of *Cardamine violifolia* disease suspected to be bacterial blight disease.

Materials and Methods

Isolation of bacteria causing bacterial blight disease

Blight disease was observed on leaves of *Cardamine violifolia* (Fig.1). The pathogens were isolated by tissue segment method (Rangaswami, 1958) on nutrient agar (NA: 3 g l⁻¹ beef extract, 5 g l⁻¹ peptone, 17 g l⁻¹ agar; pH 7.2) medium. Infected *Cardamine violifolia* leaves were cut into small pieces

of 1.0~1.5 cm, surface sterilized with 0.1% mercuric chloride for 1 min, and washed in sterile distilled water thrice and blotted dry with sterilized filter paper. Then the leaf bits were placed in Petri plates containing NA. The plates were incubated at $28 \pm 2^\circ\text{C}$ for 24~48 h. Colony grew around the tissue mass, which were aseptically moved by using an inoculation loop and transferred to NA at $28\sim 30^\circ\text{C}$ for 24~48 h, discrete bacterial colonies were removed by using an inoculation loop, re-streaked on NA and incubated aerobically for 24~48 h. Individual colonies were isolated, sub-cultured twice to ensure purity and then stored in 15% sterilized glycerol at -80°C .

Pathogenicity tests

The bacterial suspension of 1×10^8 CFU $\cdot\text{ml}^{-1}$ prepared in sterile distilled water, wound-inoculated and then incubated at $28 \pm 2^\circ\text{C}$ and kept in humid conditions (Fang, 1996). Plants used as negative controls were inoculated with the sterile distilled water. Blight disease symptoms were observed every 12 hours. Each treatment repeated for three times.

Phylogenic characterization

The sequence of the 16S rDNA was obtained from the total of the pathogen by PCR amplification with sense primer 5'-AGAGTTTGATCCTGGCTCAG-3' and antisense primer 5'-CGGCTACCTTGT TACGACTTC-3' (Weisburg *et al.*, 1991). The PCR amplification conditions were as follows: one denaturation step (3min at 95°C), 35 cycles of amplification (30s at 95°C , 30s at 50°C , 1.5 min at 72°C), and a final elongation step of 10 min at 72°C . The PCR products were checked by electrophoresis on 1.0% agarose gels, and then the corresponding products were purified with an AxyPrepTM DNA gel extraction kit

(Axygen Scientific, Inc, USA). Purified products were ligated into pGEM-T vectors (Promega Co., China), and then transformed into *E. coli* DH-5 α cells. Positive colonies were selected by the blue-white screening procedure (Sambrook *et al.*, 1989). The bacteria for seeking out was cultured 12 h in liquid LB culture, 37°C , $200 \text{ r}\cdot\text{min}^{-1}$, and then amplified by using a pair of primers pUC/M13, detected by gel electrophoresis and confirmed to contain the fragment. Positive clones were sequenced (BGI Co., Beijing, China) and the obtained sequences were compared with the sequences in Genbank. The 16S rDNA sequences of other related bacteria were obtained from GenBank and BLASTN with the pathogen. Phylogenic analysis was using the Molecular Evolutionary Genetics Analysis (MEGA) Software Version 5.1.

Results and Discussion

Pathogenicity tests

Individual colonies were achieved by using an inoculation loop. The bacteria strain was round, smooth, protruding. It was Gram-negative, rod shaped, not endospore-forming. Water-soaked lesions first appeared on the wounds after 3d, the Blight disease on leaves in 7d (Fig.2). Re-isolation from inoculated leaves yielded colonies that consistent with the pathogen tester isolates. The repeated experiments confirmed the same results.

Phylogenic characterization

The partial 16S rDNA sequence containing 1,419bp nucleotides (Accession nos: 1918730) was aligned with all related sequences in the NCBI database by the BLASTN program. The sequence identity among the sample and other *Pseudomonas* sp.species was 100%. The phylogenetic tree

of the strain and the other *Pseudomonas* species in Fig.3 shows that the pathogenic

16S rDNA region and *Pseudomonas* sp. comprise the same cluster.

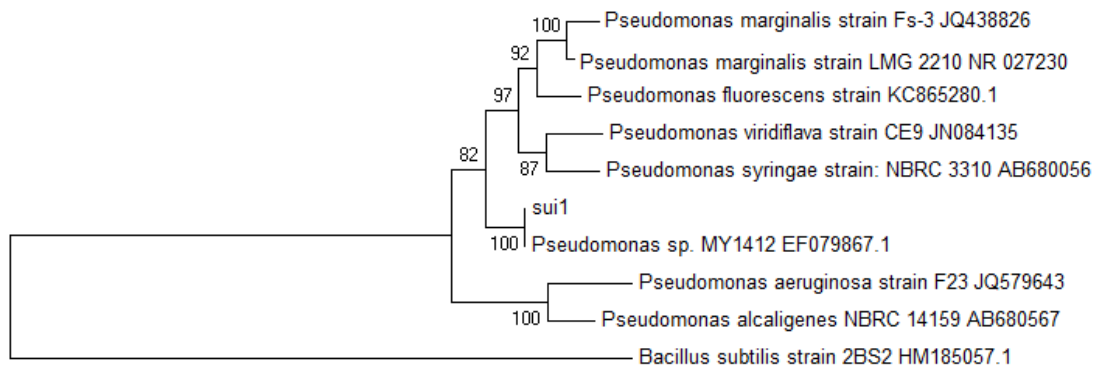
Fig.1 Natural symptoms of *Cardamine violifolia* bacterial blight disease.



Fig.2 The symptoms of *Cardamine violifolia* at 7d post-inoculation.



Fig.3 Phylogenetic tree showing the position of *Pseudomonas* sp. based on 16S rDNA sequences using neighbour-joining method. *Bacillus subtilis* 2BS2 (HM185057) was used as an outgroup



Classification standard of 16S rDNA sequence indicates that the same species share higher than 97% of sequence identity each other. So, it can preliminary predicate that the strain is *Pseudomonas* sp.

To our knowledge, this is the first report of *Pseudomonas* sp. the causal agent of *Cardamine violifolia* bacterial blight in Enshi city, Hubei province, China. To prevent the spread of the disease to other regions, firstly, characterization of potential host range of the pathogen and after then the evaluation of *Cardamine violifolia* cultivars and lines for their reaction to the pathogen strains for putative sources of resistance are recommended.

Acknowledgement

This work was financially supported by The Modern Agricultural Industry Technology System Project Supported by Ministry of Agriculture [Grant No. nycytx-35-02-06].

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How to cite this article:

Jinping Wu, Zhenbiao Jiao, Leifu Chen, Zhengming Qiu and Fengling Guo. 2016. Isolation and Identification of the Pathogen of *Cardamine violifolia* Bacterial Blight Disease in China. *Int.J.Curr.Microbiol.App.Sci*. 5(12): 203-206. doi: <http://dx.doi.org/10.20546/ijcmas.2016.512.022>