ISOLATION AND IDENTIFICATION OF *XANTHOMONAS EUVESICATORIA* CAUSING BACTERIAL SPOT OF EGGPLANT IN CHINA

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Abstract. Eggplant (*Solanum melongena* L.) is an important solanaceous vegetable and economic crop in the world. It is a very popular and nutrient rich vegetable, and it can be grown round the year in China. In September 2023, a suspected bacterial disease of the eggplant was found in the Greenhouse Eggplant Seedling Production Base in Tieling, Liaoning Province, China. The disease symptom was water-soaked tawny or light brown spots on eggplant seedling leaves, that gradually became black. Pathogenic bacteria were isolated from the infected leaf spots. Based on the phenotypic characteristics, bacteriological characteristics, physiological and biochemical tests and molecular biological identification, the bacteria isolates obtained in this study were identified as *Xanthomonas euvesicatoria*. Pathogenicity of these isolates was confirmed on ground tomato and pepper seedlings by artificial inoculation. To our knowledge, this is the first report of *X. euvesicatoria* causing bacterial spot of eggplant in China.
Keywords: *Solanum melongena L., Xanthomonas euvesicatoria, bacterial spot disease, pathogenicity, pathogen identification*

Introduction

Eggplant (*Solanum melongena* L.) is one of the important vegetable crops in the Solanaceae family, widely cultivated on large scale in many parts of the world particularly, China, USA, Japan, Russia, India, Pakistan, Philippines, Bangladesh, Egypt, France and Italy (Chadha, 1993; Han et al., 2021). Eggplant is the most important protein and vitamin producing crop and occupies an important place in international trade. Eggplant is one of the five most important vegetables in the world in production (FAO, 2021). The domestication of eggplant occurred in the region between northeastern India and southwestern China (Frary et al., 2007). China is the largest eggplant producer, with a long history of cultivation and abundant variety resources. However, eggplant production is challenged by various pathogens, such as bacteria, viruses, fungi, and nematodes. The bacteria cause the most severe economic loss to eggplant farmers due to their widespread incidence and devastating impact on crop yield.

The genus *Xanthomonas* is a widely studied plant-associated Gram-negative bacterium that belong to the family *Xanthomonadaceae* subclass Gammaproteobacteria (Jun et al., 2010). Most of the species in the *Xanthomonas* genus are plant-pathogenic and infect approximately 400 different host plants, including important food crops such

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as rice, tomato and pepper (Hayward, 1993; Kebede et al., 2014; Potnis et al., 2015). Four different *Xanthomonas* species are known to cause bacterial spot on tomato and pepper: *X. euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri* (Martiny et al., 2006; Jones et al., 2000; Jones et al., 2004). Phenotypic and phylogenetic analyses indicated extensive differences between *Xanthomonas* strains causing bacterial spots on tomato and pepper (Stall et al., 1994; Almeida et al., 2010). Both *X. euvesicatoria* and *X. gardneri* strains are the pathogens of tomato and pepper, and *X. perforans* strains derived only from tomato, and *X. vesicatoria* strains mainly infect tomato.

Xanthomonas euvesicatoria is a group of important Gram-negative bacteria belonging to the genus *Xanthomonas* (Leyns et al., 1984), that can cause bacterial spot disease in the *Solanaceae* family. *X. euvesicatoria* has a worldwide distribution (Astua-Monge et al., 2000; Jones et al., 2004). The disease can cause fruit quality reduction and severe yield loss in tomato and pepper (Hamza et al., 2010; Wang et al., 2018; Adhikari et al., 2020). Although the use of resistant varieties is the most effective way to control the disease, the presence of multiple *Xanthomonas* species and the rapid transfer of species/race in the same region are one of the most important reasons for the failed management of the disease (Wang et al., 2018; Li et al., 2019; Adhikari et al., 2020). Therefore, sources with more durable and broad-spectrum resistance are suitable for the development of new varieties. Although the species of *Xanthomonas* have been well studied, the genus is still responsible for many crop diseases, resulting in yield loss of economically important crops around the world (Ryan et al., 2011; An et al., 2020). But no information has so far been available about their infecting eggplant.

In September 2023, the eggplant with suspected bacterial disease symptoms were observed in the Greenhouse Eggplant Seedling Production Base in Tieling, Liaoning Province, China. The disease symptom was water-soaked tawny or light brown spots on eggplant seedling leaves, and the spots became black brown gradually. The annual incidence of this disease in the seedling base is 10-20%. In this study, the pathogen of this suspected bacterial disease of the eggplant was identified and provided a theoretical basis for the diagnosis of the disease using physiological and molecular means.

Materials and methods

Pathogen isolation

Diseased leaves of eggplant were taken as samples. Margins between symptomatic and healthy tissues of the diseased leaves were sampled by sterilized blade, disinfected with 75% ethanol for 20 s and 0.1% mercuric chloride for 1 min sequently, cleaned with sterile water for 3 times. Then they were mixed with 1 ml sterile water, ground with sterile mortar and glass rod. Bacterial solution was transplanted to nutrient agar (NA) medium plate by sterilized transplantation ring in order to isolate pathogenic bacteria. Single colony was isolated after culturing at 28℃ for 48 h. They were purified and stored in a -80℃ refrigerator for subsequent identification experiments.

Pathogenicity test

Seeds of eggplant and pepper were sown in separate 12 cm pots using sterile soil and cultivated in a growth chamber at 27°C with a 12 h light period and at 70% humidity. The obtained pathogens were grown in pure culture on NA plate medium and incubated at 28℃ at constant temperature for 36–48 h. A certain amount of single bacterial

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colonies were selected from the plate into sterile distilled with a sterilized graft ring, and then oscillated evenly. The bacterial suspension of XeuTY-1 was adjusted to an OD 600 = 0.1, further diluted to 3 × 10^6 cfu/mL and sprayed on eggplant and pepper seedlings (Kyeon et al., 2016). Each piece of material was repeated in triplicate with ten seedlings. Meanwhile, sterile water used as negative control. All treated seedlings were covered with transparent polythene bags for 24 h to keep high relative humidity and then cultivated in growth chamber with the same conditions above. Symptoms were observed every 1 to 2 d. After disease onset, the pathogen was again isolated from the diseased spot and compared with the original inoculum.

Pathogen identification

Biochemical tests were carried out in duplicate for the isolate as described by Schaad et al. (2001). For molecular identification, the pathogens were grown to log phase and total genome DNA was extracted using the Rapid Bacterial Genomic DNA Isolation Kit (Tiangen Biotech, Beijing, China) according to the manufacturers' instructions. The extracted DNA was amplified by PCR using 16S universal primer (Lane, 1991) and the species-specific primer pairs, BS-XeF/BS-XeR, BSXvF/BS-XvR, BS-XgF/BS-XgR and BS-XpF/BS-XpR (Jones et al., 2004) (*Table 1*). PCR amplification was carried out in a 50 μL reaction containing 1.0 μL template DNA, 1.0 μM of each forward and reverse primers and 25 μL 2 × Taq MasterMix. The cycling conditions were 5 min at 94°C, 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, elongation at 72°C for 1 min, and a final elongation at 72°C for 10 min. Products were purified from agarose gel and sequenced by Sangon Biotech (Shanghai) Co., Ltd. were used for homology comparison with the data in GenBank using BLAST software, and to construct phylogenetic trees by means of Maximum-likelihood method with 1000 bootstrap replicates using MEGA v.7.0 software.

Table 1. *Primers for the test*

	Primer	Sequence	Amplicon size
16S rDNA	F	5'-AGA GTT TGA TCM TGG CTC AG-3'	1465 bp
	R	5'- TAC GGY TAC CTT GTT ACG ACT T-3'	
Xanthomonas euvesicatoria (Xe)	Bs-XeF	5'-CATGAAGAACTCGGCGTATCG-3'	173 bp
	Bs-XeR	5'-GTCGGACATAGTGGACACATAC-3'	
Xanthomonas vesicatoria (Xv)	Bs-XvF	5'-CCATGTGCCGTTGAAATACTTG-3'	138 bp
	Bs-XvR	5'-ACAAGAGATGTTGCTATGATTTGC-3'	
Xanthomonas gardneri (Xg)	Bs-XgF	5'-TCAGTGCTTAGTTCCTCATTGTC-3'	154 bp
	Bs-XgR	5'-TGACCGATAAAGACTGCGAAAG-3'	
Xanthomonas perforans (Xp)	Bs-XpF	5'-GTCGTGTTGATGGAGCGTTC-3'	197 bp
	Bs-XpR	5'-GTGCGAGTCAATTATCAGAATGTGG*-3'	

Results

The disease symptoms

In the early stage of the disease, the eggplant leaves will produce yellow brown or light brown water spots, the disease gradually become black brown, and the late disease spot perforated necrosis (*Fig. 1*). This disease can lead to the incidence of

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20%–40%. The bacterium can be spread by seeds, seedlings, grafting tools, manual pruning tool or hand contact.

Figure 1. *Symptoms caused by Xanthomonas euvesicatoria on eggplant*

Pathogenicity test

The inoculated eggplant and pepper plants exhibited light brown spots with yellow halo around on the leaves after 10 days post inoculation, and gradually become black brown after 20 days post inoculation, which were similar with the symptoms in the field. No disease symptom was observed on any of the control plants. Bacterial colonies which showed the same phenotypic characteristics as originally described were re-isolated from the artificially inoculated plants.

Pathogen identification

The colony was Pale-yellow, smooth, mucoid, domed circular colonies of 1-2 mm in diameter, which are characteristic for *Xanthomonas*, were present after 48 h (*Fig. 2*). The cell morphology of the pathogen was rod-shaped. The pathogen was gram negative bacterium with negative reaction in oxidase, urease and arginine dihydrolase tests, as well as in production of indole and nitrate reduction tests. And the pathogen gave positive reaction in contact enzyme, B-galactosidase, gelatin hydrolyzation and aesculin hydrolyzation tests. Results of growth test on carbon source utilization showed that the pathogen could utilize D-cellobiose, D-glucuronic acid, α- D-glucose, D-mannose, D-galactose, gelatin, D-trehalose, pectin and tween 40.

Figure 2. *Colony morphology of the pathogen on NA medium*

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16S rDNA universal primers (27F/1492R) were used to amplify the tested strain XeuTY-1, the partial sequence deposited in GenBank (accession No. MZ087937.1) showed 99% identity (1426 bp out of 1425 bp) to the sequence of *Xanthomonas euvesicatoria* strain PhXeu-1 (accession No. MG654642.1) (*Fig. 3*).

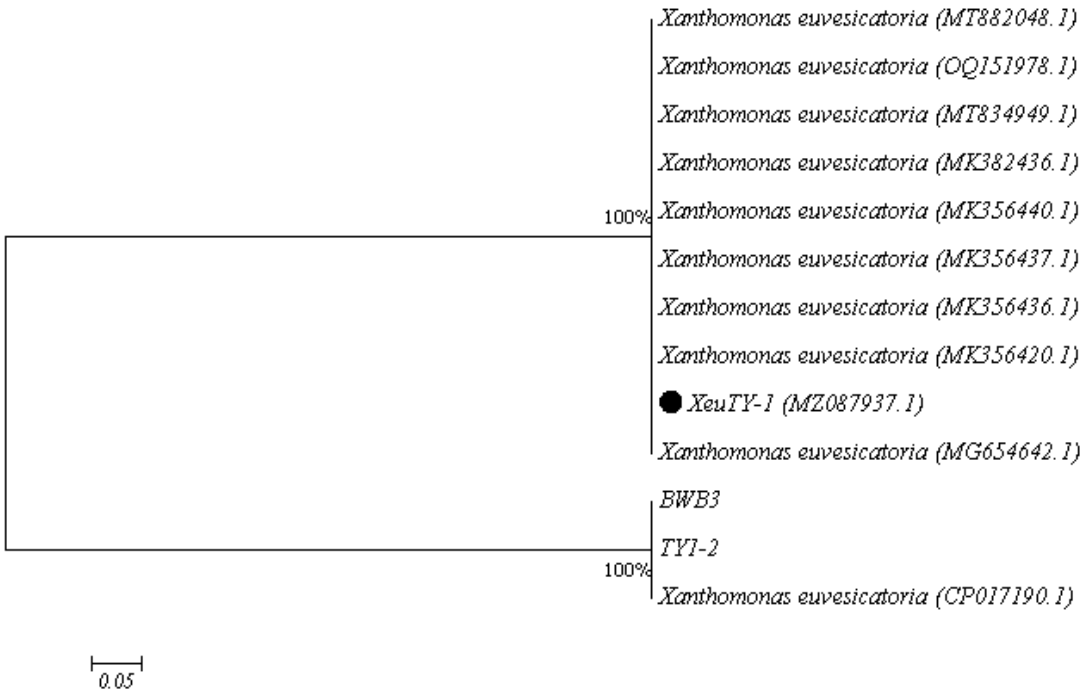

Figure 3. *The phylogenetic evolutionary tree of the test strains based on the 16S rDNA sequence*

The genome of the test strains was amplified using specific primers and detected by agarose gel electrophoresis. The results showed that the *X. euvesicatoria* specific 173 bp amplicons with the primers BS-XeF/BS-XeR were obtained from the bacterial strain tested and no amplicons with other *Xanthomonas vesicatoria (Xv)*, *Xanthomonas gardneri (Xg) and Xanthomonas perforans (Xp)* primer sets (*Fig. 4*).

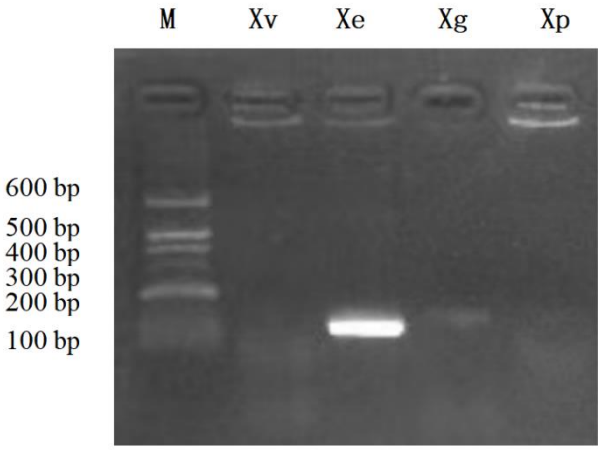

Figure 4. *Results of specific primers for the test strain XeuTY-1*

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BLST analysis of the Xe gene sequence of the test strain on the NCBI database showed that the test strain was 100% similar to *Xanthomonas euvesicatoria* (GenBank Accession No. CP018467.1), indicating that the strain was *Xanthomonas euvesicatoria*. Therefore, it can be concluded that the isolates XeuTY-1 in this study was finally identified as *Xanthomonas euvesicatoria*.

Discussion

Eggplant is an important vegetable crop that is prone to numerous bacterial diseases that devastate crop production. This study reports the bacterial spot of eggplant caused by *Xanthomonas euvesicatoria* in China for the first time to our knowledge. *X. euvesicatoria* has not been reported to cause disease in eggplant in the world previously (Clarke et al., 2014; Osdaghi et al., 2016).

X. euvesicatoria (Hamza et al., 2010), and three other *xanthomonad* species, *Xanthomonas vesicatoria*, *Xanthomonas perfora* (Jones et al., 2004), *Xanthomonas gardneri* (Ma et al., 2011) have been reported to cause bacterial spot of pepper and tomato, and have a worldwide distribution (Potnis et al., 2015; Osdaghi et al., 2016). With the rapid development of immunology and molecular biology, we have greatly improved the identification methods of pathogenic bacteria. The pathogen of bacterial spot disease is relatively complex in classification. Due to the high conservation of 16S rDNA, it cannot distinguish closely related species, so it is particularly important to study the biological characteristics and differential genes of closely related species. Due to the sequence similarity, homologous recombination is more likely to occur between closely related strains (Robertset al., 1993). However, recombination is difficult to detect when recombination occurs between highly similar sequences; therefore, some sequence differences are required to identify the recombinant sequences (Posada et al., 2002). Jones et al. (2004) renamed the four phenotype groups A, B, C, and D into four species: *Xanthomonas euvesicatoria* (Xe), *Xanthomonas vesicatoria* (Xv), *Xanthomonas perforans* (Xp), and *Xanthomonas gardneri* (Xg). All these four phenotypes have been existed in China (Chen et al., 2011). Studies have designed a pair of specific primers based on the sequence fimA in Xcv bacteria, but could only detect group B bacteria (*X. vesicatoria*) (Van et al., 2001).

In recent years, it has been suggested that *Xanthomonas* isolated from each of the aforementioned host plants are sufficiently distant in their physiological and pathological properties to require formal taxonomic identification as separate species, specialized forms or variants. To identify *X. vesicatoria*, *X. gardneri* and *X. perforans* in symptomatic eggplant leaves, we designed four specific primers to amplify the test strain. At the same time, these species can be easily differentiated using specific primers. PCR showed that only group A bacteria (Xe) were detected, indicating that the test strain was *Xanthomonas euvesicatoria.* The results showed that homologous specific 173 bp amplicons were obtained from the sample tested by BS-XeF/BSXeR primer, and no amplicons were obtained from other primers, this is consistent with the previous report (Song et al., 2019).

Conclusion

In conclusion, the bacterial spot disease of eggplant caused by *Xanthomonas vesicatoria* hitherto has not been recorded from China. In this study, *X. euvesicatoria*

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was identified as the only causal agent of bacterial spot disease in eggplant on the result of a pathogenicity test and the basis of biochemical and genetic characterizations. This research will provide a scientific basis for the identification, prevention and breeding of eggplant bacterial spot disease.

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