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Note

First Report on Bacterial Heart Rot of Garlic Caused by *Pseudomonas fluorescens* in China

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An unreported disease of garlic was observed in commercial fields in Jiangsu province, China. The symptoms started as water soaked lesions at the base of the leaves. Later, water-soaked areas developed on stems and spread to the internal tissues, followed by yellowing and necrosis along leaf edges and soft rot of the stems. The causal organism isolated from symptomatic plants was identified as *Pseudomonas fluorescens* based on its biochemical and physiological characteristics and confirmed by the cellular fatty acid composition and Biolog data as well as 16S rRNA gene sequence analysis. The bacterial isolates caused similar symptoms when inoculated onto garlic plants. In addition, leek and shallot were susceptible to the *P. fluorescens* pathogen. However, the *P. fluorescens* pathogen failed to cause any symptoms when it was inoculated onto 15 other plants. This is the first report of a bacterial disease of garlic caused by *P. fluorescens* in China.

Keywords : garlic, heart rot disease, host range, identification, *Pseudomonas fluorescens*

Garlic (*Allium sativum* L.) is one of the many important economic crops grown in China. Planted acreage of this crop has been increasing in recent years and, in 2007, over 700,000 hectares were grown and about 1.2 million metric tons of frozen garlic was exported with a gross value of $ 1.1 billion (Anon, 2007). Nowadays, China is the world's biggest garlic producer and more than 139 countries imported garlic from China. In addition, Shandong, Henan and Jiangsu provinces are becoming the leading producer of garlic in China.

Garlic extracts exhibit a wide spectrum of antibacterial activity (Slusarenko et al., 2008). However, garlic has been reported to be a natural host of *Erwinia herbicola*, which caused leaf tip dieback in Israel (Koch et al., 1996). Furthermore, fluorescent pseudomonads have been isolated from symptomatic garlic in France and Italy and the causal bacteria were finally identified as *Pseudomonas salomonii* (Calzolari and Bazzi, 1985; Gardan et al., 2002; Girard et al., 1994). Nowadays, *Pectobacterium carotovorum* subsp. *carotovorum* is a ubiquitous bacterium that causes bacterial rot of garlic and many other plants (Kim et al., 2007).

During the survey of garlic disease in 2002, a typical heart rot disease was found on garlic in China, which reduced the quality and marketability of garlic. When examined with a microscope, cut edges of symptomatic leaves consistently exhibited bacterial streaming. The causal agent differed from *P. carotovorum* subsp. *carotovorum* based on DAS-ELISA although the symptoms were similar to bacterial soft rot of garlic that has been reported in many regions in China (data not shown). Herewith we report the occurrence of heart rot disease of garlic, and the identification and characterization of the bacterial pathogen for the first time.

Disease occurrence and symptoms. Beginning in 2002, a previously undescribed bacterial heart rot disease of garlic was observed in commercial fields in Jiangsu province, China. Initial symptoms were water-soaked lesions starting at the base of leaf blades and extending down into the stem tissue. As these lesions expanded and elongated, chlorotic borders developed, and eventually leaves wilted and died (Fig. 1A, B). In addition, the diseased stem tissues became soft and watery and then turn slimy and foul-smelling. In severe cases, the incidence of disease was more than 40% in some fields. Severely affected plants were stunted and misshapened to be unmarketable.

Bacterial isolation. Symptomatic leaves and stems were surface-sterilized by soaking leaf pieces in 0.525% sodium hypochlorite solutions for 3 min, and rinsed in sterile distilled water. Small sections of the surface-sterilized leaf tissue were excised aseptically from leaf spot margins and macerated individually in 40 μl of sterile distilled water.

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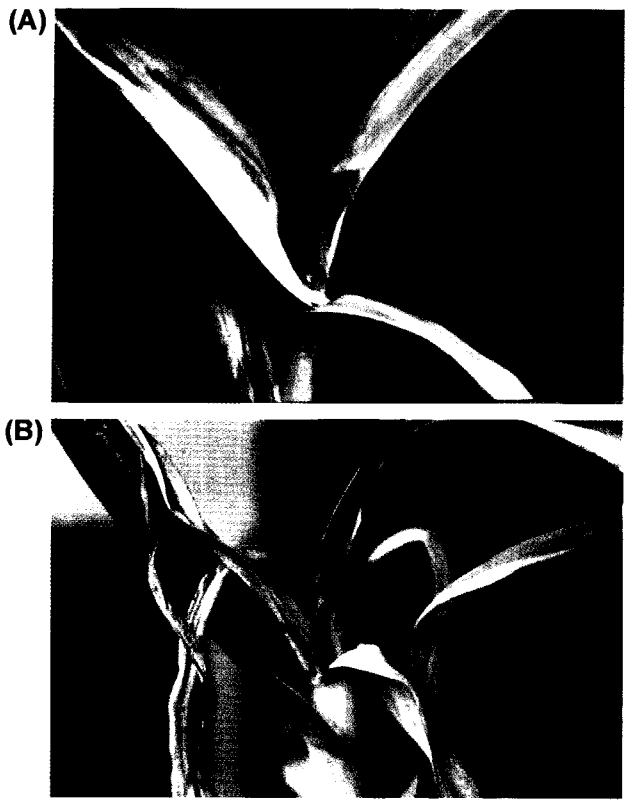

Fig. 1. Symptoms caused by *Pseudomonas fluorescens* on garlic. (A) water-soaked stem lesions; (B) leaves wilted and dead.

The resulting suspensions were streaked onto nutrient agar (NA) medium (Schaad et al., 2001). Individual bacterial colonies formed after 2-3 days of incubation at 28°C were isolated from culture plates, and stored in 15% aqueous glycerol at –70°C for further studies. Totally fifteen bacterial isolates were obtained from lesions on field-grown garlic. The bacterial isolates all produced fluorescence on King's medium B and bacterial colonies were yellow, smooth, convex, entire and round. In addition, infiltration of tobacco leaves with cell suspensions of the bacterial isolates all resulted in typical hypersensitivity reactions within 24 h (Table 1).

Pathogenicity. Artificial inoculations on potted plants of garlic (cultivars Taicangbaisuan, Xuzhoubaisuan and Hongpi-1) were made. Bacterial colonies were grown on NA medium for 48 h at 28°C and suspended in sterile distilled water. The suspensions were diluted to approximately 1.0 $\times 10^7$ colony forming units/ml. Suspension concentrations were confirmed by dilution plating on NA medium (Li et al., 2008a). Wounds were made with a 26-gauge needle in the epidermis of leaves and on the stems and then bacterial suspensions were sprayed onto leaf and stem surfaces of garlic plants until runoff with an aerosol-propelled sprayer. Negative control plants were inoculated with sterile water

Table 1. Morphological, physiological and biochemical characters of the bacterial isolate G0201 obtained from naturally infected *Allium sativum*

Test	Bacterial isolate G0201	*P. fluorescens*[a]
Cell morphology	Rod-shaped	Rod-shaped
Gram reaction	–	–
Flagellation	Polar, >1	Polar, >1
Fluorescence on King's B agar	+	+
Starch hydrolysis	–	–
Growth at 41°C	–	–
Levan from sucrose	+	d
Arginine dihydrolase	+	+
Gelatin liquefaction	+	+
Grows aerobically	+	+
Grows anaerobically	–	–
Oxidative/Fermentative	Oxidative	Oxidative
Denitrification reaction	–	d
Oxidase	+	+
Catalase	+	+
Potato soft rot	+	NA
Chinese cabbage soft rot	+	NA
Tobacco hypersensitivity	+	NA
Utilization of[b]		
Alanine	+	+
Cellobiose	–	–
Fucose	–	–
Glucose	+	+
Glycerol	+	+
Inositol	+	+
Lactose	–	–
Maltose	–	–
Mannitol	+	d
Proline	+	+
Pyruvate	+	+
Sorbitol	+	d
Trehalose	+	+

[a]Data selected from Krieg and Holt (1984).
[b]+, positive; –, negative; d, 11-89% positive; NA, no information available.

in the same way. Garlic plants were 3 to 4 leaf stage and three plants per treatment were used as replicates. All plants were then incubated in a humidity chamber maintained at 100% relative humidity for 72 h and then incubated at 25°C in a greenhouse and examined for disease symptoms. The inoculation experiments were conducted twice. After 1 week, garlic plants artificially inoculated with the 15 isolates developed symptoms similar to the commonly observed field symptoms. In addition, the same bacterium was reisolated from inoculated garlic plants. Control plants remained symptomless.

Host range. To test pathogenicity of the bacterial isolates on hosts other than garlic, one representative isolate G0201

Table 2. Pathogenicity of the bacterial isolate G0201 obtained from naturally infected *Allium sativum* to various commercial crops in Jiangsu province, China

Inoculated plants	Cultivar	Bacterial isolate G0201
Garlic (*Allium sativum*)	Taicangbaisuan	+
Garlic (*Allium sativum*)	Xuzhoubaisuan	+
Garlic (*Allium sativum*)	Hongpi-1	+
Soybean (*Glycine max*)	Sudou-4	−
Tomato (*Solanum lycopersicum*)	Jiangshu-1	−
Wheat (*Triticum aestivum*)	Sumai-3	−
Maize (*Zea mays*)	Ludan-981	−
Cantaloupe (*Cucumis melo*)	huangzhuixian	−
Cantaloupe (*Cucumis melo*)	Xinhuanghou	−
Cantaloupe (*Cucumis melo*)	Xiyu-1	−
Cucumber (*Cucumis sativus*)	Jingyan-4	−
Cucumber (*Cucumis sativus*)	Jingchun-5	−
Pumpkin (*Cucurbita moschata*)	Xitu-1	−
Towel gourd (*Luffa cylindrical*)	Jiangshu-1	−
Summer squash (*Cucurbita pepo*)	Longfa	−
Watermelon (*Citrullus lanatus*)	Sumi-5	−
Rice (*Oryza sativa*)	Shanyou-63	−
Rice (*Oryza sativa*)	Liangyoupeijiu	−
Leek (*Allium porrum*)	Tongshangzaotaijiu	+
Shallot (*Allium ascalonicum*)	Jiangsuxingcong-21	+

was selected and inoculated onto the following plants: soybean, tomato, wheat, maize, cantaloupe, cucumber, pumpkin, towel gourd, summer squash, watermelon, rice, leek and shallot. Each treatment had three replicates and the experiment was carried out twice. Plants were inoculated as described above at the two or three fully expanded leaf stage. After 2 weeks, disease symptoms were not developed on the 15 selected plants inoculated with the representative isolate except leek and shallot on which small and water soaked lesions were produced around the inoculation sites at the base of the leaf (Table 2). None of the control plants treated with sterile distilled water developed symptoms. These data suggest that the garlic pathogen might be host specific to *Allium* spp.

Identification of the representative isolate. Bacterial characteristics of the representative isolate G0201 were investigated by the methods of Krieg and Holt (1984) and Schaad et al. (2001). Classical bacteriological tests indicated that the isolate was Gram-negative, obligatorily aerobic, arginine dihydrolase-positive and oxidase-positive. Also, the isolate was negative for denitrification and unable to hydrolyze starch, while it was positive for production of levan from sucrose and ability to rot potato and Chinese cabbage. In addition, the isolate utilized glucose, sorbitol and trehalose while did not utilize cellobiose, fucose, lactose and maltose (Table 1). These results indicate that the causal agent causing the garlic disease is identified as *P. fluorescens*.

The cellular fatty acid composition of the representative isolate G0201 was determined as described by Kim et al. (2007) and Li et al. (2008b). Profiles were compared with the MIDI identification database TSBA50, version 5.00 (MIDI Inc., Newark, DE, USA). The fatty acid profiles of the representative isolate G0201 give best matches with *P. fluorescens* with 0.52 similarity indices. Also carbon source assimilation of the isolate G0201 was examined by the Biolog GN test kit (Biolog Inc., Hayward, Co.) according to the manufacturer's specifications. Experiments were performed as described by Li et al. (2006) and the profile was compared with the BIOLOG identification database GN4.01. The Biolog database identified the isolate G0201 as *P. fluorescens*, with similarity indices of 0.75 (Table 1). Thus, based on the results of the above two analyses, the bacterial isolate G0201 was confirmed to be *P. fluorescens*.

Amplification of the 16S rRNA genes was performed by PCR as described by Li et al. (2006) using the specific primers 16F27 and 16R1522. The Sequencing was performed with an ABI 3730 automatic sequencer (Bioasia, Shanghai, China). The sequence obtained from the isolate G0201 was used for sequence database searches in GenBank and sequence similarities to the known *P. fluorescens* strains were measured using the BLAST program. Sequences were assembled using the SeqMan program and analyzed with the MegAlign programs (DNASTAR, Inc., USA) as described by Jeon et al. (2006, 2008). The GenBank accession numbers for 16S rRNA gene sequence of the isolate G0201 in this study is FM203408 and BLAST analyses showed that they had 100% homology to those of other *P. fluorescens* strains (GenBank accession numbers EU169163 and EU862565) that had been deposited in the GenBank database. This data is consistent with the result of morphological, biochemical and physiological tests, as well as Biolog data and fatty acid analysis.

This is the first report in China that *P. fluorescens* is the causal pathogen of bacterial heart rot of garlic. Previous studies indicated that *P. fluorescens* has wide host range under natural field infection (Aboaba, 2007; Cui and Harling, 2006; Saygili et al., 2004). However, *P. fluorescens* strains from different sources exhibit differential pathogenicity. Tekorienë et al. (2003) reported that bacteria of *P. fluorescens* isolated from leek leaves were not pathogenic, while the same bacterial species isolated from beetroots showed pathogenicity. Indeed, fluorescent pseudomonads have been isolated from symptomatic garlic and the causal bacteria were finally identified as *P. salomonii* (Gardan et al., 2002). Therefore, it is necessary to compare the obtain-

ed *P. fluorescens* isolates in this study with *P. salomonii* strains pathogenic to garlic previously described. Interestingly, *P. fluorescens* have long been identified as biological control agents since they are widely occurring, many non-pathogenic species are available, and they are highly effective at eliminating competing microbes (Choi et al., 2006; Ipper et al., 2005; Lee et al., 2005; Manjula et al., 2004). Further studies are required to evaluate the phenotypic and genetic differences between pathogenic and non-pathogenic *P. fluorescens* isolates.

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