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Causal Agents of Blossom Blight of Kiwifruit in Korea

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The causal agents of bacterial blossom blight in kiwifruit were isolated from flowers displaying symptoms in Korea. The pathogens were characterized by biochemical and physiological tests, and identified on the basis of 16S rDNA and 16S-23S internal transcribed spacer (ITS) sequences. Pathogenicity tests demonstrated that the blossom blight of kiwifruit in Korea is caused by two pathogens, *Pseudomonas syringae* pv. *syringae* and *P. fluorescens*. Carbon source utilization and DNA-DNA hybridization experiments confirmed *P. fluorescens* as one of the causal agents of blossom blight of kiwifruit. *P. syringae* pv. *syringae* and *P. fluorescens* can be distinguished from each other by the symptoms they produce in flowers. *P. syringae* pv. *syringae* primarily affected the stamen, while *P. fluorescens* caused rotting of all internal tissues of buds or flowers.

Keywords : blossom blight, kiwifruit, *Pseudomonas fluorescens*, *Pseudomonas syringae* pv. *syringae*

Blossom blight or rot, along with bacterial canker, is a major bacterial disease of kiwifruit (*Actinidia deliciosa* [A. Chev] C. F. Liang et A. R. Ferguson) in Korea. One of the main causes of crop loss of kiwifruit is bacterial infection of flowers. This effect was most prominent during the rainfall in the flowering season, where disease outbreaks reached up to 50% of flowers causing a severe reduction in kiwifruit production (Koh, 1995; Koh et al., 2001). The disease affects flower buds of kiwifruit by rotting the inner tissues, which become brown in color. This affection is most apparent when the bud opens into a flower and the general browning can be observed.

The bacterial blossom blight of kiwifruit was first described in New Zealand and its causal agent was identified as *Pseudomonas viridiflava* on the basis of LOPAT II characteristics (Wilkie et al., 1973). However, subsequent investigations using phenotypic and genotypic methods, and DNA-DNA hybridization technique have revealed that the pathogen identified in New Zealand kiwifruit is unrelated to *P. viridiflava* but rather is a hitherto unreported pathogen in the genomic species *P. savastanoi* (Young et al., 1997; Hu et al., 1999). In addition to *P. viridiflava*, *P. syringae* pv. *syringae* was also reported to adversely effect the reproductive and vegetative tissue of kiwifruit in France and Italy (Luisetti and Gaignard, 1987; Balestra and Varvaro, 1997; Balestra et al., 2001). Furthermore, the bacterial pathogens *P. syringae* and *P. viridiflava* were also found together in symptomatic vines in California, USA. *P. viridiflava*, however, was shown to cause significantly more flower bud rot and blossom blight than *P. syringae* (Conn et al., 1993). In Korea, *P. syringae* was first reported to cause blossom blight of kiwifruit (Koh et al., 1993) and the causal agent was identified as *P. s.* pv. *syringae* lately (Shin et al., 2004).

Based on these previous finding, the objective of this study was to identify the etiological agents of blossom blight of kiwifruit in Korea.

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Materials and Methods

Isolation of pathogens. Isolation of bacterial strains was made from affected female buds and flowers collected from kiwifruit orchards of 16 cultivation areas in Korea. During blossom, when disease symptoms became evident, infected buds and flowers were collected from kiwifruit plants cv. Hayward. Homogenized samples, suspended in sterile distilled water, were vigorously shaken for 5 min. Aliquots of 0.1 ml from fivefold serial dilution were streaked on peptone-sucrose (PS) plates (Young et al., 1988), which were incubated at 27°C for 48 h. One-hundred colonies were randomly selected for the biochemical and physiological tests.

Biochemical and physiological tests. Cultures were incubated at 27°C unless otherwise stated. Isolates were tested for gram staining. Fluorescence on KB medium was checked under UV light after 24-48 hr. The levan production, oxidase activity, pectolytic activity on potato, and arginine dihydrolase activity tests were performed according to the standard methods (Lelliott et al., 1966; Braun-

Kiewnick and Sands, 2001). The gelatin liquefaction, aesculin hydrolysis, tyrosinase activity, and utilization of tartrate (GATTa) tests are used to identify pathovar *syringae* from other pathovars of *P. syringae* (Latorre and Jones, 1979). Type strains of *P. s.* pv. *syringae* DSM10604 and *P. fluorescens* LMG1794 were used as a control in biochemical and physiological tests.

Carbon source utilization. Utilization of sole carbon source was tested for sorbitol, trehalos, sucrose, and arabinose. Carbon sources were filter sterilized and added at 0.1% (w/v) final concentration to autoclaved and cooled Ayers's medium (Braun-Kiewnick and Sands, 2001). Bacteria were streaked onto the medium and incubated at 27°C for 7 days. Growth was compared to plates containing no added carbon source.

Pathogenicity tests. Healthy canes were collected during April from an orchard, and immature buds were detached. Bud inoculation was done by injecting 0.5 ml (1×10^6 cfu/ml) of inoculum into the side of unopened buds using a syringe. Sterile distilled water was injected as controls. The inoculated buds were placed in moist chambers and incubated 5 days at 25°C before evaluating. In the open field experiments, test strains were inoculated into the side of unopened buds using syringe by injecting 0.5 ml of 1×10^6 cfu/ml.

Amplification of the 16S rRNA gene and the 16S-23S rDNA spacer region. The sequences of the 16S rDNA and 16S-23S rDNA spacer regions, referred to as the internal transcribed spacer (ITS) region, were amplified for sequence analysis. Amplification of the 16S rRNA gene was performed by using 16S rDNA universal primers, corresponding to the following position of *Escherichia coli* 16S rRNA gene sequence (Neefs et al., 1990): position 9-27 (29f, 5'-AGAGTTTGATCCTGGCTCAG-3') and 1474-1492 (1492r, 5'-GGTTACCTTGTTACGACTT-3'). The 16S-23S ITS sequences were amplified using the PCR primers designed from the sequences of the 16S and 23S rRNA genes adjacent to the spacer region. The sequence of primer D21 at the 3' end of 16S rRNA was 5'-AGCCGTAGGGGAACCTGCGG-3'. The primer D22 designed from the sequence at the 5' end of 23S rRNA was 5'-TGACTGCCAAGGCATCCACC-3' (Manceau and Horvais, 1997). All bacteria to be tested by PCR were grown in 3 ml PS broth for 24 h. Genomic DNA was extracted using a genomic DNA extraction kit (Bioneer).

PCR amplification. PCR amplifications were performed in a total of 25 microliter. Reaction mixtures consisted of 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 uM each primer, 200 umol each dNTP, 1.25 U *Taq* DNA polymerase (Takara), and 40 ng of bacterial DNA. The reactions were performed in a Takara PCR Thermal Cycler using the following program; initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30s, and an extension at 72°C for 30 s. Reactions were terminated after a final 5 min elongation at 72°C. The PCR products were separated by 1.0% agarose gel electrophoresis, stained with ethidium bromide, and visualized with UV light.

DNA-DNA hybridization. DNA-DNA hybridization was done by the membrane filter technique using DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Molecular Biochemicals). Genomic DNA of KDK8 was denatured by the alkaline method, then immobilized on a nylon membrane (Hybond-N^+; Amersham) by applying a small vacuum. The denatured DNA of *P. fluorescens* LMG1794 was labelled according to the manufacturer's protocol. The membranes were prehybridized in a hybridization solution at 45°C for 30 min. The actual hybridization was carried out in a hybridization solution containing labelled DNA at 45°C for 2 h. After hybridization, the membranes were washed twice in washing solution at 65°C. Detection reagents were added to the membranes for 5 min at room temperature, and then the excess liquid was squeezed out. The membranes were exposed to autoradiography film (Hyperfilm-ECL; Amersham) for 15 min, and the signal intensities were determined using the TINA 2.0 program. The signal produced by self-hybridization was taken as 100%, and percentage homology values were calculated from duplicate samples.

Sequence analysis. After amplification, PCR products were cloned into a pGEM-T Easy vector (Takara) following the manufacturer's instructions. Plasmid DNA containing the PCR-amplified fragment was verified by *Eco*RI restriction digestion. The nucleotide sequence of the inserted DNA was determined using a model 3730*xl* DNA analyzer (Applied Biosystems). Nucleotide sequences were adjusted manually and aligned with sequences obtained from GenBank by using the PHYDIT program (available at http://plaza.snu.ac.kr/~jchun/phydit).

Nucleotide sequence accession numbers. The 16S rRNA gene and 16S-23S rRNA ITS sequences of *P. s.* pv. *syringae* BBK3 and *P. fluorescens* KDK8 were deposited in the GenBank nucleotide sequence database under the accession numbers EU708321, EU708322, EU708323, and EU708324, respectively.

Results and Discussion

Isolation and characterization of the pathogens. Most of the kiwifruit orchards surveyed in Korea showed symptoms of bacterial blossom blight. One hundred isolates, which contained pale cream-colored colonies, were tested for 10 biological and physiological characteristics. Even though all isolates belonged to Gram-negative bacteria, they were divided into seven groups based on the nine characteristics listed in Table 1. While the most predominant group, Group I, was composed of 41 isolates, Group VII consisted of only two isolates. Pathogenicity tests for the seven groups of bacteria were performed on 14 isolates, two randomly selected strains from each group. Among the seven groups tested, only bacteria belonging to Group I and VII caused blossom blight symptoms in the detached flowers (Fig. 1). However, disease symptoms on the floral buds caused by Group I and VII bacteria were distinctive from each other. While isolates belonging to Group I primarily resulted in rotting stamens, Group VII isolates caused rotting of pistils and stamens simultaneously and the entire floral buds became a brown color. Bacterial colonies identical to those used for artificial inoculation were then re-isolated from the affected buds and flowers.

The results from the biochemical and physiological tests conducted to identify the isolates of Group I and VII were as follows: Group I strains fluoresced blue-green under UV light and were positive for levan production, gelatin liquefaction, and aesculin hydrolysis. However, they were negative for oxidase activity, potato soft rot, arginine dihydrolase, tyrosinase activity and tartrate utilization ($G^+A^+T^-Ta^-$). Strains belonging to Group VII, fluoresced green under UV light, were positive for levan production, oxidase activity, arginine dihydrolase activity and gelatin liquefaction but were negative for potato soft rot, aesculin hydrolysis, tyrosinase activity and tartrate utilization ($G^+A^-T^-Ta^-$). Thus, Group I and VII isolates were identified as *P. s.* pv. *syringae* and *P. fluorescens*, respectively since the results from these biochemical and physiological tests were consistent with the characteristics of reference strains of each bacterium (Table 1).

Identification of the strains by 16S rDNA and ITS sequences. It is known that the nucleotide sequences of the 16S and 16S-23S rDNA ITS regions can allow for the accurate identification of bacteria at the species and intra-species level. Thus, to confirm the results of biochemical tests, the 16S rDNA and ITS sequences of the isolates were determined. As shown in Table 2, three Group I strains (BBK3, TDS2 and SSB2) showed a high level of 16S rDNA similarity of 99.1% with *P. s.* pv. *syringae*. The similarity values of the ITS sequences of the isolates of

Table 1. Characteristics of bacterial strains isolated from blossom blight of kiwifruit in Korea

Group	G^a	K	L	O	P	A^a	G^b	A^b	T	T^a	No. of Isolates
I	−	+	+	−	−	−	+	+	−	−	41
II	−	−	+	−	−	−	−	+	−	+	8
III	−	+	+	−	−	−	+	−	−	−	9
IV	−	−	−	−	−	+	−	−	+	−	21
V	−	+	−	+	−	−	−	−	−	−	15
VI	−	+	−	+	−	+	−	−	−	+	4
VII	−	+	+	+	−	+	+	−	−	−	2
P.s. pv. *syringae*	−	+	+	−	−	−	+	+	−	−	
P. fluorescens	−	+	+	+	−	+	+	−	−	−	

G^a, gram stain; K, fluoresced green under UV light on KB; L, levan formation test; O, oxidase test; P, potato soft rot test; A^a; arginine dihydrolase test; G^b, gelatin liquefaction test; A^b, aesculin hydrolysis test; T, tyrosinase activity test; T^a, tartrate utilization test.

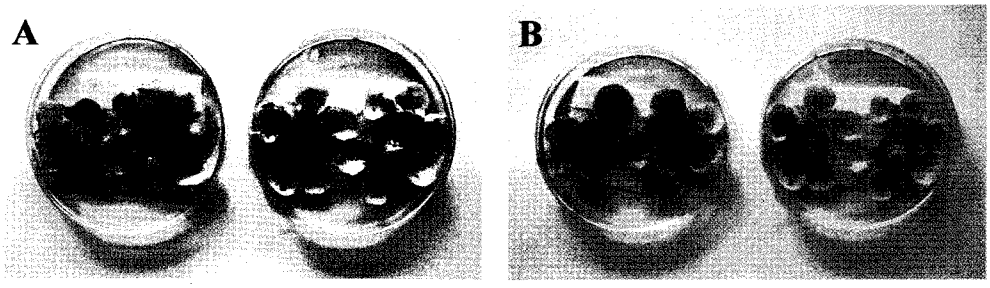

Fig. 1. Symptoms on buds inoculated with Group I (A) and VII isolates (B). Left plate was infected flowers and right plate was uninfected flower.

Table 2. Sequence similarity of 16S rDNA and ITS sequences

Strains	% similarity with BBK3		Strains	% similarity with KDK8	
	16S rDNA	ITS		16S rDNA	ITS
TDS2	100.0(1462/1462)	100.0(0/610)	SSB8	99.8(1459/1462)	100.0(610/610)
SSB2	100.0(1462/1462)	100.0(0/610)	*Pfl*	99.5(1456/1463)	99.2(507/511)
Pss	99.1(1448/1461)	99.1(466/470)			

Pss, *P. s.* pv. *syringae*; *Pfl*, *P. fluorescens*

Table 3. Utilization of carbon sources

Strains / Carbon	KDK8	SSB8	*Pfl*[a]	*Pvi*[b]	*Pma*[c]	*Pss*[d]
sorbitol	+	+	+	+	+	+
trehalose	+	+	+	−	+	−
sucrose	+	+	+	−	+	+
arabinose	+	+	+	−	−	−

Pfl[a], *P. fluorescens*; *Pvi*[b], *P. viridiflava*; *Pma*[c], *P. marginalis*; *Pss*[d], *P. s.* pv. *syringae*

group I and *P. s.* pv. *syringae* were also high, showing 99.1% homology. Likewise, the similarity values of 16S rDNA and 16S-23S ITS sequences for Group VII isolates (KDK8 and SSB8) and *P. fluorescens* were >99.5% and 99.2%, respectively, indicating that these organisms are the same species.

Confirmation of *P. fluorescens*. *P. fluorescens* is a non-specific and epiphytic bacterium that has been isolated from both symptomatic and asymptomatic vines (Conn et al., 1993). However, to date there has been no report that *P. fluorescens* is the causal agent of disease in kiwifruit flower. Therefore, two additional experiments have been conducted to determine Group VII isolates are *P. fluorescens*. One of the classical means of discriminating bacterial species has been a sugar utilization ability assay. The same carbon source utilization profiles were obtained for bacterial isolates designated as Group VII, KDK8 and SSB8, and a reference strain of *P. fluorescens* (Table 3). Differences in trehalose, sucrose and arabinose utilization between Group VII isolates and a strain of *P. viridiflava*, which was reported as pathogen in kiwifruit bud and blossom in New Zealand (Everett and Henshall, 1994; Young et al., 1988), France (Luisetti et al., 1987), and California (Conn et al., 1993), were found. DNA-DNA hybridization showed the same results. Strain KDK-8 showed 97% similarity to the type strain of *P. fluorescens* LMG1794 in DNA-DNA hybridization test. These results further confirm that the Group VII isolates are *P. fluorescens*.

Symptoms of blossom blight. Typical symptoms of blossom blight caused by these two groups of isolates on removed buds were also obtained in field trials where intact buds of kiwifruit plant were inoculated with the pathogens

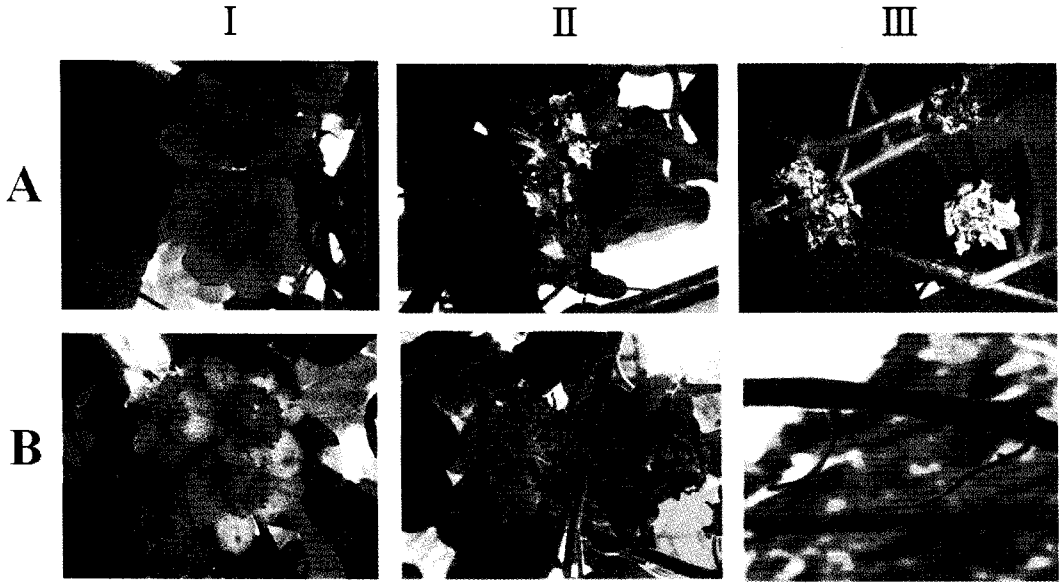

Fig. 2. Symptoms on flowers caused by *P. s.* pv. *syringae* TDS2 (A) and *P. fluorescens* KDK8 (B). The pictures show typical symptoms of blossom blight of 3 (I), 7 (II) and 14 days (III) after artificial inoculation.

(Fig. 2). *P. s.* pv. *syringae* and *P. fluorescens* can be distinguished from each other by the symptoms they produce in flowers. *P. s.* pv. *syringae* affected the floral buds of kiwifruit by rotting the stamen, which became a chocolate-brown color. In the case of severe infection, most of the flowers fell to the ground and did not develop any kiwifruit. Flowers with slight infection developed only small or distorted kiwifruit. However, *P. fluorescens* caused rotting of all internal tissues, stamen and pistil, in the bud, resulting in general browning. Such buds invariably fell before reaching maturity.

In conclusion, these results clearly show that the causal agents of blossom blight of kiwifruit in Korea are *P. s.* pv. *syringae* and *P. fluorescens*. However, it is believed that *P. syringae* pv. *s.* is the dominant pathogen causing blossom blight of kiwifruit in Korea, since it is found in 41 out of 43 pathogenic isolates collected from orchards at 16 different areas.

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