*Plant Pathol. J. 22(2) : 119-124 (2006)*

**The Plant Pathology Journal**


# Halo Blight of Kudzu Vine Caused by *Pseudomonas syringae* pv. *phaseolicola* in Korea

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*(Received on February 23, 2006; Accepted on May 19, 2006)*

**Kudzu vine (*Pueraria montana* var. *lobata*) is an invasive climbing woody vine that envelops trees and shrubs, pressing physically and shutting out sunlight, which needs to be controlled. Kudzu vine pathogens were surveyed as a way to seek its biocontrol agents in 2002. Occurrence of a bacterial halo blight disease of kudzu vine was observed at several localities in Korea including Euiwang and Suwon in Gyeonggi Province, Daejon, and Gochang and Buan in Jeonbuk Province. Symptoms of brown to black spots with a surrounding yellowish halo appeared from June and lasted till the rainy season without much expansion, but accompanying often leaf blight and defoliation. Isolated bacteria were identified as *Pseudomonas syringae* pv. *phaseolicola* based on physiological and cultural characteristics, Biolog, fatty acid and 16S rDNA sequencing analyses. In artificial inoculation test, these bacteria produced the same halo spot symptoms on kudzu vine and bean plants. They also induced hypersensitive responses (HR) on tobacco, tomato, and chili pepper leaves. This is the first report of a bacterial disease of kudzu vine in Korea, and the bacterial pathogen can be used as a biocontrol agent against the pest plant.**

***Keywords*** : bean, halo blight, hypersensitive responses, kudzu vine, *Pseudomonas syringae* pv. *phaseolicola*, 16S rDNA sequencing

Kudzu vine (*Pueraria montana* var. *lobata*) is an invasive climbing plant. It is a perennial leguminous deciduous woody vine that climbs up trees and shrubs by rapid-growing rope-like stems and inhibits their aboveground growths (Agrios, 2005). In severe cases, it grows over wooded areas, enveloping other plants completely and forming impermeable masses to kill them all by shutting out all sunlight. It has been distributed and recently much populated in Korean forest, which is one of the most important forest problems to be controlled. However, controlling kudzu vine by conventional methods such as use of herbicides and removal by mechanical cutting is hardly successful because of its rapid growth and weedy habitat in forest. Biological control by pathogens infecting kudzu vine should be considered for practical purposes.

Several kudzu vine diseases have been reported in Korea, including phythophthora blight caused by *Phytophthora erythrotheptica* (Jee et al., 2000; Kim and Kim, 1993), and leaf spot caused by *Pseudocercospora puerariicola* (Shin, 1997) and *Alternaria tenuissima* (Kim et al., 2004). These pathogens have not been extensively related for the use of biocontrol agents yet. More kudzu vine pathogens should be examined to secure proper candidates used as control agents. A bacterial pathogen for causing halo blight of kudzu vine was isolated during the survey of kudzu vine diseases. The bacterium was characterized, identified, and reported in this paper.

## Materials and Methods

**Pathogen isolation.** Kudzu vine leaves having brown spots with a surrounding halo were collected from Euiwang and Suwon in Gyeonggi Province, Daejeon, and Gochang and Buan in Jeonbuk Province, Korea, in summer, 2002 during the survey of kudzu vine diseases. Leaf tissues of the border between the diseased and healthy areas were cut and surface-sterilized with 1% sodium hypochlorite for 1 min and 70% ethanol for 1 min. After washing the leaf tissues with sterilized water two times, they were placed on water agar and incubated at 25°C in an incubation chamber. Bacterial colonies formed on and around the leaf tissues were streaked on nutrient agar and King's B agar to isolate single bacterial colonies. As the symptoms were similar to those of halo blight of bean caused by *Pseudomonas syringae* pv. *phaseolicola* (Hall, 1991), bacteria with fluore-

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scence on King's B agar, named Kuz isolates (Kuz-1, Kuz-2, and Kuz-3), were isolated and tested for further studies. The isolated bacteria were suspended in 20% glycerol and stored at −70°C before use.

**Physiological and culture properties.** Characterization of the physiological and culture properties of the Kuz isolates was conducted. To examine the morphology of the bacterial cells, a colony (Kuz-1) grown on King's B agar was picked with a spatula, placed in distilled water on a Formvar-coated copper grid, dried and stained with 2% uranyl acetate for negative staining. The preparation was examined under a JEM 1010 electron microscope (JOEL, Japan). The average bacterial size was calculated from measurements of twenty bacteria from each of three preparations examined by electron microscopy. Gram staining, growth at 41°C, oxidase reaction, levan formation, pigment production on yeast extract-dextrose-calcium carbonate agar (YDCA), and starch hydrolysis were evaluated by the usual methods (Schaad et al., 2001).

All three Kuz isolates tested for utilization of 95 carbon sources using the Biolog program following standard methods. Briefly, bacterial cells cultured on Biolog Universe Growth (BUG) agar at 28-30°C for 48 h. Bacterial colonies were suspended in GN/GP-IF aqueous solution (0.40% sodium chloride, 0.03% pluronic F-68, 0.01% gellan gum), and inoculated onto microplates (Biolog GN2 MicroPlate™) 150 microliter per each well and incubated at 28-30°C. After 24 or 48 h of incubation, the plates were read with a MicroLog™ 3-Automated Microstation system. The bacteria were identified based on the MicroLog Gram-positive database (version 4.02).

**Fatty acid analysis.** Gas chromatography of fatty acid methyl esters (GC-FAME) of Kuz isolates was conducted to confirm the bacterial identification. The bacteria were cultured on tryptic soy agar (TSA) at 28°C for 48 h. The colonies were harvested and placed in a screw-cap culture tube, and 1 ml of saponification reagent (NaOH aqueous methanol) was added and mixed thoroughly with a vortex before allowing the reaction for 10 min at 80°C. After cooling, 1.25 extraction solvent (hexane/MTBE) was added and mixed for 10 min with an orbital shaker, and then mild base (10.8 g NaOH in 900 ml) and saturated NaOH solution were added and mixed to separate fatty acids. The fatty acid composition was analyzed with the Sherlock system, followed by the generation of a similarity index for isolates that corresponded to a microorganism in the database (MIDI Library version TSBA 5.0, Library Generation system software version 5.0).

**Sequencing of 16S ribosomal RNA gene (16S rDNA).** Genomic DNA of Kuz-1 was used for sequencing of 16S rDNA. Using the 27mF and 1492mR primers, as universe primer (Brosius et al., 1978), 16S rDNA was amplified by PCR. Template DNA for PCR was isolated using IntraGene Matrix (Bio-Rad, UK) by manufacture's method. PCR reaction solution was composed of 1 μl DNA, 2 U *Taq* DNA polymerase (Takara Co.), 5 μl 10 x buffer (100 mM Tris-HCl), 25 mM $MgCl_2$, deoxyribonucleoside triphophates, and distilled water, which was made up to 50 μl final reaction solution. PCR amplification condition was composed of pretreatment at 94°C for 5 min, 30 cycles of reactions at 94°C for 30 sec for denaturation, 55°C for 30 sec for regeneration, and 72°C for 20 sec for extension, and the final extension reaction at 72°C for 7 min. Amplified DNA fragments were subjected to an electrophoresis on an 1.0% agarose gel, and the target 16S rDNA band was purified and sequenced on an Applied Biosystems DNA sequencer (model ABI 3700). The resulting sequence was compared to the GenBank database using the NCBI BLAST search program.

**Pathogenicity test.** A bacterial isolate (Kuz-1) with fluorescence on King's B agar was cultured on the agar medium for 48 h at 28°C, and suspended in sterilized distilled water to the concentration of $1\times10^8$ cells/ml. For inoculation, leaves of healthy kudzu vine plants, which were transferred from the wild into a greenhouse, were pricked about 20 spots/leaf with a pin, and sprayed with the bacterial suspension. The inoculated plants were placed in the greenhouse after storing in a humid moisture chamber for 12 h. Disease development was observed daily. As the bacterium was identified *Pseudomonas syringae* pv. *phaseolicola*, bean plants (*Phaseolus vulgaris*) at the 10 true leaf stage were also inoculated as above for pathogenicity test. For hypersensitive reaction (HR) test, the same bacterial suspension was injected into leaves of tobacco (*Nicotiana tabacum* cv. Burley 21), tomato (*Lycopersicon esculentum* cv. Seogwang), and chili pepper (*Capsicum annuum* cv. Bugang), all of which were grown in the greenhouse and at about 10 true leaf stage. HR was determined with naked eyes if HR lesions were formed within 16 h after the bacterial injection. Three replications were used.

## Results

**Disease occurrence and symptoms.** The halo blight disease of kudzu vine was observed in several localities in Euiwang and Sueon in Geonggi Province, Daejeon, and Gochang and Buan in Jeonbuk province during the disease

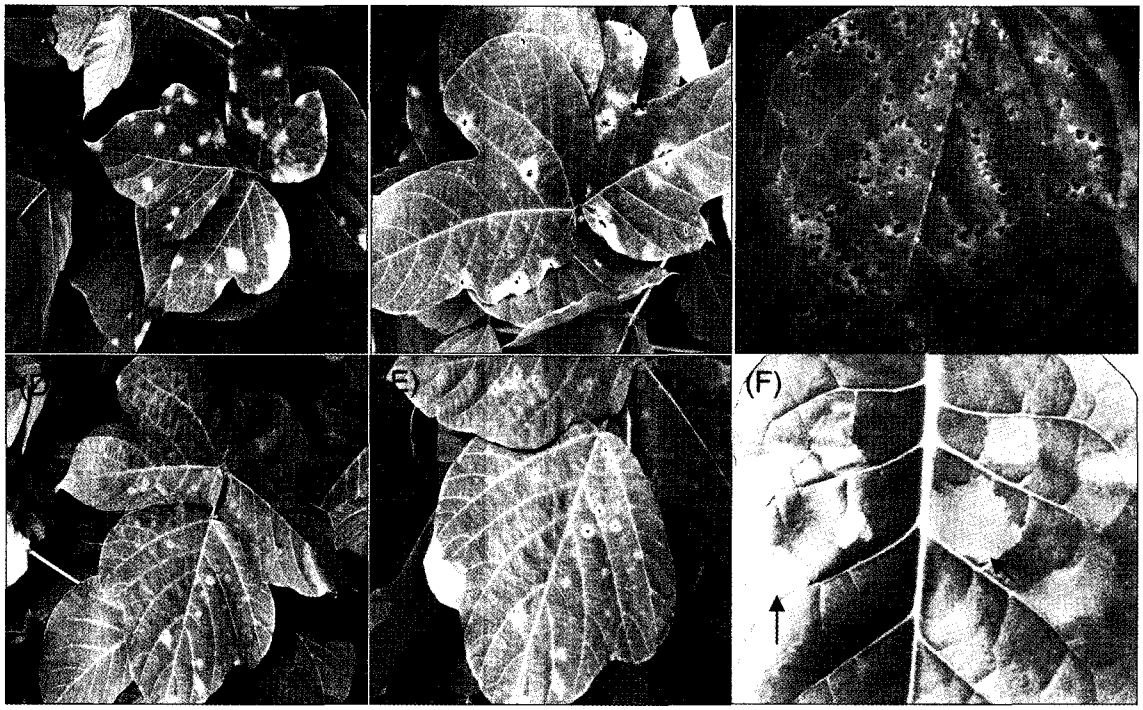


**Fig. 1.** Symptoms of kudzu vine halo blight appeared initially as chlorotic leaf spot with a surrounding yellowish halo (A), and the centers became brown and more necrotic with time (B). (C) Symptoms of bean leaves produced by the present isolate Kuz-1 on 10 days after artificial inoculation. (D and E) Bacterial halo blight on leaves of kudzu produced by the present isolate Kuz-1 at 5 and 14 days after artificial inoculation, corresponding to initial (A) and advanced (B) natural symptoms, respectively. (F) Hypersensitive responses (arrows) formed on a tobacco leaf (cv. Burly 21) after injection of Kuz-1.

survey in summer of 2002. The disease was not widely distributed, but localized in certain areas in a location. In Euiwang, where the disease observation had been made from 1999 through 2003, a severe disease occurred at the same areas every year. Brown or blackish small spots surrounded by a broad yellow halo appeared on kudzu vine leaves from June and enlarged gradually until the rainy season. The individual spots became enlarged and coalesced with other neighboring ones to form large lesions, of which the centers were sometimes necrotized and holed (Fig. 1A, B). These symptoms did not expanded further more during the hot summer season from July through August after the rainy season, and continued until mid October. Infected leaves often become blighted and defoliated. Symptoms were sometimes masked probably by high temperature in mid summer.

**Pathogenicity.** When the bacterial suspension was sprayed on kudzu leaves, yellow specks appeared at 3 days after inoculation, and the center spot became thicker and brown and enlarged from 5 days later, accompanying the enlargement of the surrounding halo (Fig. 1, panels D and E). Also in bean, these symptoms began from 4 days after inoculation, and typical spot symptoms with a necrotic center formed after 10 days of inoculation (Fig. 1C). For HR test using the isolated bacterium to tobacco, tomato, and chili pepper, all leaf tissues injected with the bacterium showed HR within 16 h (Fig. 1F).

**Physiological and culture properties.** All three Kuz isolates were Gram-negative and produced fluorescent

**Table 1.** Species identification of the present isolates from kudzu vine

| Characteristics | Kuz isolates | *P. syringae* pathovars[a] |
|---|---|---|
| Cell diameter, μm | 0.7~0.8 | 0.7~1.2 |
| Cell length, μm | 1.5 | 1.5 |
| Flagella number | 2 | >1 |
| Yellow or orange cellular pigment | – | – |
| Oxydase reaction | – | – |
| Levan formation | – | V |
| Oxidase reaction | – | – |
| Growth at 41°C | – | – |
| Potato rot | – | – |
| Starch hydrolysis | – | – |

[a]Data from the Laboratory Guide for Identification of Plant Pathogenic Bacteria by Schaad et al. (2001). +, positive; –, negative; V, variable.

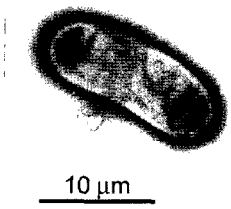


**Fig. 2.** Electron microscopy of Kuz-1 bacterial cell negatively stained with 2% uranyl acetate. It is a rod-shaped bacterium having thread-like lophotrichous flagella (typical morphological characters of plant bacteria including *Pseudomonas*.)

pigment on King's B agar. They showed negative responses in levan formation, potato soft rot, starch hydrolysis, and oxidase activity (Table 1). The bacterium was rod-shaped with 2 flagella and 0.7~0.8×1.5 μm in size (Fig. 2). In Biolog program, they ulilized the following carbon sources like *P. syringae* pv. *phaseolicola*: L-arabinose, D-arabitol, D-fructose, D-galactose, α-D-glucose, D-mannose, D-raffinose, sucrose, methyl pyruvate, cis-aconitic acid, citric acid, D-galacturonic acid lactone, propionic acid, quinic acid, D-saccharic acid, succinic acid, bromo succinic acid, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid, L-proline, γ-amino butyric acid, inosine, and glycerol. Tween 40 was utilized by *P. syringae* pv. *phaeolicola* NPS3121, but not by the Kuz isolates. Other 62 carbon sources including α-cyclodextrin were not utilized by the Kuz isolates.

**Table 2.** Comparison of utilization of carbohydrates by the kudzu isolates and *Pseudomonas syringae* pathovars

| Carbon source | Kuz isolates | *P. syringae* pathovars | | | |
|---|---|---|---|---|---|
| | | *syringae* | *aptata* | *glycinea* | *phaseolicola* |
| Mannitol | + | + | + | V | – |
| Inositol | – | + | + | + | – |
| Sorbitol | – | + | + | – | – |
| Erythritol | – | + | + | – | – |
| Cellobiose | – | – | – | – | – |
| Trehalose | – | – | – | – | – |
| L-Rhamnose | – | – | – | – | – |
| Adonitol | – | – | – | – | – |
| Quinate | + | + | + | + | + |
| L-Lactate | – | + | + | – | – |
| Glucose | + | + | + | + | + |
| L-Alanine | + | + | + | + | + |
| L-Leucine | – | – | – | – | – |
| L-Asparagine | + | + | + | + | + |
| D-Raffinose | + | – | – | – | + |

[a]Data from the Laboratory Guide for Identification of Plant Pathogenic Bacteria by Schaad et al. (2001). +, positive; –, negative; V, variable.

**Table 3.** Cellular fatty acid profiles of the bacterial isolates (Kuz-1, Kuz-2, and Kuz-3) from Kudzu vine halo blight

| Shorthand name | Percent of fatty acid (%) | | |
|---|---|---|---|
| | Kuz-1 | Kuz-2 | Kuz-3 |
| 10:0 | 0.13 | 0.23 | 0.22 |
| 10:0 3OH | 4.06 | 5.24 | 5.43 |
| 12:0 | 5.60 | 6.15 | 6.43 |
| 11:0 ISO 3OH | – | – | 0.17 |
| Unknown 12.484 | 0.05 | 0.07 | 0.06 |
| 12:0 2OH | 4.19 | 4.48 | 5.29 |
| 12:0 3OH | 5.40 | 6.06 | 6.84 |
| 14:0 | 0.33 | 0.25 | 0.27 |
| 16:0 | 25.68 | 24.56 | 23.26 |
| 17:0 ISO | 0.09 | – | 0.35 |
| 17:0 CYCLO | 0.61 | 0.21 | 0.81 |
| 16:0 2OH | 0.70 | 0.18 | 0.32 |
| 16:0 3OH | – | 0.17 | 0.18 |
| 18:1 w7c | 15.39 | 14.70 | 13.27 |
| 18:1 w5c | 0.10 | – | – |
| 18:0 | 0.71 | 0.43 | 0.37 |
| 11 methyl 18:1 w7c | 0.23 | 0.38 | 0.30 |
| 19:0 10 methyl | – | 0.14 | 0.14 |

**Fatty acid composition.** The three Kuz isolates had 15~17 fatty acids (Table 3), among which they contained hexadecanoic acid (16:0) had the highest (23~26%) and in order of cis-11-octadecanoic acid (13~15%), dodecanoic acid (6%), 3-hydroxydodecanoic acid (5~7%), and 3-hydroxydecanoic acid (5~6%). There was no significant difference in fatty acid composition between the three Kuz isolates. Fatty acid composition analysis of Kuz-1, Kuz-2, and Kuz-3 using MIDI Library version TSBA 5.0 and Library Generation system software version 5.0 provided similarity of 83.6, 55.3, and 74.2% to *Pseudomonas syringae*, respectively.

**16S rDNA sequencing.** A PCR product of 16S rDNA amplified from Kuz-1 was sequenced. A total of 1,447 nucleotides determined from the 16S rDNA were 100% identical to those of *P. syringae* pv. *phaseolicola* (accession no. AB001448) (Fig. 3).

## Discussion

Physiological and cultural characters of the bacterial

CCCATGCTGGATACACACCGTGGTAACCGTCCCCCCGGAGGTTAGACTAGC
TACTTCTGGTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGC
CCGGGAACGTATTCACCGCGACATTCTGATTCGCGATTACTAGCGATTCCG
ACTTCACGCAGTCGAGTTGCAGACTGCGATCCGGACTACGATCGGTTTTG
TGAGATTAGCTCCACCTCGCGGCTTGGCAACCCTCTGTACCGACCATTGTA
GCACGTGTGTAGCCCAGGCCGTAAGGGCCATGATGACTTGACGTCATCCCC
ACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCATAACG
TGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAAC
ATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAATGTTCC
CGAAGGCACCAATCCATCTCTGGAAAGTTCATTGGATGTCAAGGCCTGGT
AAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCG
GGCCCCCGTCAATTCATTTGAGTTTTaACCTTGCGGCCGTACTCCCCAgGC
GGTCAACTTAATGCGTTAGCTGCGCCACTAAGAGCTCAAGGCTCCCAACGG
CTAGTTGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTT
GCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCT
TCGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAA
ATTCCACCACCCTCTACCATACTCTAGCTTGCCAGTTTTGGATGCAGTTCC
CAGGTTGAGCCCGGGGATTTCACATCCAACTTAACAAACCACCTACGCGCG
CTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCTGTATTACCGCGG
CTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTCGGTAACGTCAAAACA
ATCACGTATTAGGTAACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAAT
CCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCAT
TGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCA
GTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTG
GTGAGCCATTACCTCACCAACTAGCTAATCCGACCTAGGCTCATCTGATAG
CGCAAGGCCCGAAGGTCCCCTGCTTTCTCCCGTAGGACGTATGCGGTATTA
GCGTCCGTTTCCGAGCGTTATCCCCCACTACCAGGCAGATTCCTAGGCATT
ACTCACCCGTCCGCCGCTCGCCACCAGGTACAAGTACCCGTGCT
GCCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCT

**Fig. 3.** The nucleotide sequence of 16S rDNA from Kuz-1. The isolate was identified as *P. syringae* pv. *phaseolicora* with 100% similarity.

pathogen isolated from kudzu vine showing halo blight symptoms in Korea, and its 16S rDNA sequencing revealed that the disease is caused by *P. syringae* pv. *phaseolicola*, known as the causal agent of halo blight of bean. Pathogenicity test also showed typical symptoms of the disease both on kudzu vine and bean. In Korea, no bacterial disease of kudzu vine has ever been reported yet (The Korean Society of Plant Pathology, 2004). Thus, this is the first report of bacterial disease in kudzu vine in Korea. Fatty acid compositions of the Kuz isolates were somewhat deviated from that of *P. syringae,* but the similarity to *P. syringae* was higher than to any other bacterial species.

For biological control of kudzu vine, fungal pathogens such as *Myrothecium verrucaria* (Yang and Jong, 1995; Boyette et al., 2000) and *Colletotrichum gloeosporioides* (Farris and Reilly, 2000) have been utilized. Also *P. syringae* pv. *phaseolicola* has been tried as a biocontrol agent (Zidak and Backman, 1996). The bacterial pathogen may be more useful than fungi for biological control of noxious plants because generally bacteria are easy to culture and handle. The disease progression of the bacterial disease is more rapid than that of the fungal diseases. However, the bacterium produces few, if any, secondary infections under fairly dry conditions in the field (Zidak and Backman, 1996), which may be overcome by the continuous application of the bacterial culture as inoculum. Especially, *P. syringae* pv. *phaseolicola* produces a extracellular phytotoxin phaseolotoxin (Rudolph and Stahmann, 1966), which can be developed as a herbicide for coppicing the densely growing pest plants.

## Acknowledgements

This study was supported in part by the Korean Science and Engineering Foundation (KOSEF) through the Center for Plant Molecular Genetics and Breeding Research. The authors thank Young Soon Kim in National Instrumentation Center for Ecological Management for technical assistance for electron microscopy.

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