Biocontrol of Tomato Fusarium Wilt by a Novel Genotype of 2,4-Diacetylphloroglucinol-producing *Pseudomonas* sp. NJ134

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The rhizobacterium NJ134, showing strong *in vitro* antifungal activity against *Fusarium oxysporum*, was isolated from field grown tomato plants and identified as *Pseudomonas* sp. based on 16S ribosomal DNA sequence and biochemical analyses. The antifungal compound purified by gas chromatography-mass spectrometry, infrared, and nuclear magnetic resonance analyses from NJ134 cultures was polyketide 2,4-diacetylphloroglucinol (DAPG). Analysis of the sequence of part of one of the genes associated with DAPG synthesis, *phlD*, indicated that the DAPG producer NJ134 was a novel genotype or variant of existing genotype termed O that have been categorized based on isolates from Europe and North America. A greenhouse study indicated that about 10⁸ CFU/g of soil NJ134 culture application was required for effective biocontrol of Fusarium wilt in tomato. These results suggest that a new variant genotype of a DAPG-producing strain of *Pseudomonas* has the potential to control Fusarium wilt under the low disease pressure conditions.

Keywords : antifungal compound, biological control, genotype

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* results in severe losses in tomatoes (Larkin and Fravel, 1998). The first symptom of Fusarium wilt is chlorosis of older leaves, and a browning of vascular tissue that extends up the stem, although the pith remains healthy. Yellowing gradually affects the entire plant, wilting occurs in hot temperatures, and the plant eventually dies (Jones et al., 1991). Nonpathogenic fungal of *F. oxysporum* and *F. solani* collected from a Fusarium wilt-suppressive soil were significantly reduced Fusarium wilt of tomato (Larkin and Fravel, 1998). Antagonists recovered from Fusarium suppressive soils, particularly nonpathogenic *F. oxysporum* reduces Fusarium wilt (Jarvis, 1988). Rhizobacterial com-

petent isolates of *Pseudomonas* and *Bacillus* spp. also provide disease protection (Larkin and Fravel, 1998). However, a commercially acceptable biocontrol strain is not available. Microorganisms isolated from the root or rhizosphere of a specific crop may be better adapted to that crop and may provide better disease control than organisms originally isolated from other plant species (Cook, 1993).

The antibiotic DAPG is produced by pseudomonads of worldwide origin, and its biosynthetic locus is conserved in pseudomonads obtained from diverse geographic locations. In genetic diversity studies, most of the distinct, DAPG producer groups have been identified from European and US collections (Keel et al., 1996; Landa et al., 2002a; Mavrodi et al., 2001; McSpadden Gardener et al., 2000). Seventeen different *phlD* genotypes (designated A–Q) have been recognized by BOX-PCR analysis, RFLP-*phlD* analysis (Landa et al., 2002a; Mavrodi et al., 2001; McSpadden-Gardener et al., 2000, 2001), and the clusters defined by different genomic fingerprinting methods or by phylogenetic analysis of *phlD* correlate well. However, the actual number of genotypes in soils worldwide and the basis for their biogeography is unknown, and most of the bacteria show a substantial degree of endemism (McSpadden-Gardener et al., 2001). For example, genotype A has been isolated from birdsfoot trefoil, corn, cotton, cucumber, tobacco, pea, and soybeans from soils in the Czech Republic, Ghana, Italy, Switzerland, Uruguay and various states in the USA (De La Fuente et al., 2006; Landa et al., 2002b; McSpadden-Gardener et al., 2000, 2005), whereas genotype D has been isolated from a variety of crops in France, Germany, Spain, Switzerland, and various states in the USA (McSpadden-Gardener et al., 2000, 2005). In addition, all genotypes (A, B, D, E, F, G, I, J, L, O, P, T, and Q) present at very low densities have been isolated in soil from pea, wheat, and take-all decline fields throughout the US (McSpadden-Gardener et al., 2000). In biogeography and endemism of *Pseudomonas* sp. genotypes, soil heterotrophic bacteria in undisturbed sites are not globally mixed (Cho and Tiedje, 2000).

In this study, bacterial strains that were antagonistic to Fusarium growth were isolated from soils of the tomato cultivated field in Jeollanamdo Province, Korea. Strain

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Table 1. Characterization of *Pseudomonas* sp. NJ134

Test	NJ134		<i>P. fluorescens</i>	
	24 hour	48 hour	24 hour	48 hour
Denitrification (Nitrate reduction)	—	—	—	—
Indole production	—	—	—	—
Fermentation	—	—	—	—
Arginine dihydrolase	+	+	+	+
Urease	—	—	—	—
Hydrolysis (β -glucosidase)	—	—	—	—
Hydrolysis (protease)	+	+	+	+
Nitrophenyl- β D-galactopyranosidase	—	—	—	—
D-Glucose	+	+	+	+
L-arabinose	+	+	+	+
D-Mannose	+	+	+	+
D-Mannitol	+	+	+	+
N-acetyl-glucosamine	—	—	—	—
D-Maltose	—	—	—	—
Potassium Gluconate	+	+	+	+
Oxidase	+	+	+	+
Cell form	Rod			
Gram staining	—			
Number of flagella	(polar) > 1			
Endospore production	—			
Fluorescens pigment	+			

*API20 NE identification system (BioMérieux, Marcy l'Etoile, France)

NJ134 is a Gram-negative, short rod, motile bacterium with lophotrichous flagellar as observed under transmission electron microscopy (Table 1). It produced a brownish-yellow

fluorescent pigment on KB medium. Based on 98% similarity, the API20 NE identification system (BioMérieux, Marcy l'Etoile, France), which exploits the bacterial utili-

Fig. 1. Neighbor-joining tree showing the phylogenetic relationships of strain NJ134 to other related species based on 16S rDNA gene sequences. Bootstrap values are given in percentages of 1000 replicates.

zation patterns of various carbon sources, identified the NJ134 as *Pseudomonas fluorescens*.

NJ134 was biochemically identified by 16S rDNA sequence analysis. 16S rDNA was amplified using the fD1 and rP2 universal primers (Weisburg et al., 1991), and their nucleotide sequences were determined. A comparative 16S rDNA sequence analysis of the 1.5-kb PCR product from the NJ134 strain revealed that it formed a distinct lineage in the family Pseudomonadaceae and showed highest nucleotide sequence identity with *Pseudomonas*.

An almost complete strain NJ134 16S rDNA sequence (1514 nt) was obtained and used for initial BLAST searches in GenBank and for phylogenetic analyses. Strain NJ134 was closely related to recognized species such as *Pseudomonas lini* and *P. fluorescens* (Fig. 1).

In vitro antifungal activities of *Pseudomonas* sp. NJ134, in dual growth with various fungal pathogenic fungi, were determined on PDA (Difco Lab., Detroit, MI, USA) plates.

Strain NJ134 showed strong growth inhibition against *F. oxysporum* and *B. cinerea* (KACC 40574), causing Fusarium wilt and gray mold on tomato plants, respectively. To determine the major antibiotic compound involved, *in vitro* inhibition of *F. oxysporum*, *Colletotrichum acutatum* (KACC 40042) causing anthracnose, *Rhizoctonia solani* (KACC 40101) causing “rice” sheath blight, *Phytophthora infestans* (KACC 40718) and *Pyricularia grisea* (KACC 40417) was determined (data not shown).

To determine the major antifungal compound of *Pseudomonas* sp. NJ134, strain NJ134 was grown on modified KB (proteose peptone #3 20 g, K₂HPO₄ 1.5 g, MgSO₄·7H₂O 0.4 g per 1 L) containing 1% (w/v) mannitol at 26°C with agitation for 7 days. Our previous work showed that NJ134 cultures showed their highest *in vitro* antifungal activity when cells were grown in modified King’s B medium with mannitol (unpublished result). The crude NJ134 metabolites were extracted by partitioning (1:1 ratio) using different organic

Fig. 2. Identification of the major antifungal metabolite from *Pseudomonas* strain NJ134. Thin layer chromatography profiles of antifungal metabolites produced by the antagonistic strain NJ134 and inhibition of *F. oxysporum* mycelia growth with an ethyl acetate (EA) extract (A). 1–6, EA extract; 7, Methanol (Control). Gas chromatography-mass spectrometry spectra of an antifungal antibiotic (B) and the chemical structure of authentic 2,4-diacetylphlogucinol (C).

solvents. The extracted metabolites were tested (without concentration) for their efficacy against pathogens. Among the metabolites tested against *F. oxysporum*, the ethyl acetate-extracted metabolite (R_f 0.62) effectively inhibited 81% of growth at 5 mg/ml (Fig. 2A). The active antifungal metabolite present in the ethyl acetate extract of the cell-free culture supernatant was purified by preparative thin-layer chromatography (Merck, Darmstadt, Germany). The purified active compound was obtained from a single gas chromatograph-mass spectrometry (GC-MS) peak with a retention time of 21 min (Shimadzu Prominence, Tokyo, Japan) (Fig. 2B). The structure of the purified antifungal compound was determined by nuclear magnetic resonance (NMR) and mass spectral analysis (Bruker Company, Bad Herrenalb, Germany) (Fig. 3C). The mass spectrum of the isolated compound was matched to that of ethanone 1,1-(2,4,6-trihydroxy-1,3-phenylene) bis, known by the name 2,4-diacetylphloroglucinol

(DAPG). Based on these data in conjunction with ^1H and ^{13}C NMR, infrared spectra, melting point, and elemental analyses, the structure was confirmed by the mass spectrum data.

To date, a number of these disease-suppressing antibiotic compounds have been characterized chemically, including nitrogen containing heterocyclic such as phenazines (Brisbane et al., 1987; Gerber, 1969; Thomashow et al., 1990), pyrrol-type antibiotics (Hashimoto and Hattori, 1966ab; Imanaka et al., 1965), pyro-compounds (Hays et al., 1945), and indole derivatives (Wratten et al., 1977). A small number of antibiotic-like compounds that do not contain nitrogen have also been isolated from fluorescent pseudomonads (Broadbent et al., 1976). Among these is DAPG, which has been used to control plant root diseases (Haas et al., 1991; Keel et al., 1990).

The importance of 2,4-DAPG production by strain NJ134 is to suppress soilborne plant pathogens in the rhizosphere.

Fig. 3. Comparison of the genotype diversity of *phlD*-containing *Pseudomonas fluorescens* strain Pf-5 and strain NJ134 defined by *phlD* gene with *Hae* III, *Msp* I, or *Taq* I (A). Portion of BLAST tree indicating that NJ134 is most closely related to 7MA12 and 7MA20 (genotype O) (B). The next nearest neighbors were those of MVW1-1 (P genotype), which were both detected on pea. Further afield were the FTD1R36 and FFL1R8 groups (genotypes GHII).

GC-MS analysis analyses provided further evidence for its production. To confirm that *phlD* gene expression leads to an essential phloroglucinol biosynthetic pathway gene, we performed a rapid PCR-based assay targeting *phlD* (McSpadden-Gardener et al., 2001). To assess the NJ134 genotype based on *phlD* gene diversity, 16S ribosomal DNA gene and phylogenetic analyses of the partial *phlD* gene sequence analysis were conducted, as described previously (McSpadden-Gardener et al., 2001). Restriction fragment length polymorphism (RFLP) analysis of the amplified *phlD* sequence was used to identify the DAPG genotype (Fig. 3A). The NJ134 strain *phlD* gene, amplified by PCR-based assay, confirmed the presence of a 626-bp DNA product in the KB culture reaction used as the whole cell template for PCR amplification. The NJ134 product size detected by a *phlD*-specific primer was quite similar to that determined by producing *P. fluorescens* Pf-5. No RLFPs were found among the 16S rDNA gene sequences from *phlD*⁺ strain NJ134 upon digestion with *Hae* III, *Msp* I, or *Taq* I (Fig. 3A). The *phlD* gene is predicted to encode a polyketide synthase that synthesizes monoacetylphloroglucinol, the immediate precursor to 2,4-DAPG (Dwivedi and Johri, 2003). A conserved portion of the *phlD* open reading frame has been cloned and sequenced from NJ134 and compared to five genotypically distinct 2,4-DAPG-producing *Pseudomonas* spp. strains such as CHA0, Pf-5, Q8r1-96, 1M1-96, and Q2-87 (McSpadden-Gardener et al., 2001).

Our studies with the NJ134 *phlD* gene restriction fragment showed a new portion of the DAPG genotype that was used for initial BLAST searches in GenBank. A 626 bp *phlD* gene fragment from strain NJ134 has been amplified by PCR using the Phl2a and Phl2b primers (McSpadden Gardener et al., 2001). Consensus sequence quality was checked using the translating BLAST function BLASTX (National Center for Biotechnology Information). *phlD* sequences were aligned using the CLUSTALW service (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). Phylogenetic analyses were performed with MEGA 4.0 software (Tamura et al., 2007) using the neighbor-joining method (Fig. 3B). The BLAST tree of strain NJ134 was most closely related to *P. fluorescens* strains 7MA20 (94%) and 7MA12 (93%) (O genotype) (Landa et al., 2002b) and MVW1 (P genotype), which were both detected on pea (De La Fuente et al., 2006). The next nearest neighbors were those of MVW1 (P genotype), both detected on pea. Further afield were the FTD1R36 and FFL1R8 groups (genotypes GHJ). Fluorescent *Pseudomonas* spp. strains producing 2,4-DAPG exhibit considerable genetic and phenotypic diversity (McSpadden-Gardener et al., 2000). Our study is the first to isolate a novel *phlD* genotype from *Pseudomonas* spp. NJ134, indicating that geological distribution of *phlD*⁺ may be a major factor for isolating a novel genotype in Korea.

DAPG-producing bacteria of different genotypes play a

Fig. 4. A, Relationship between the rhizosphere population density of *Fusarium oxysporum* and tomato Fusarium wilt on roots. Tomato seedlings were treated with *F. oxysporum* at densities of approximately 10^1 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , and 10^7 spore per g of soil to achieve a range of population densities in the rhizosphere. B, Relationship between the rhizosphere population density of *Pseudomonas* strain NJ134 and that of *F. oxysporum* per population unit (10^5 CFU). Tomato seedlings were treated with strain NJ134 at densities of approximately 10^1 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , and 10^{10} CFU per g of soil to achieve a range of population densities in the rhizosphere. The disease index was as follows: 0 = healthy plant, all leaves were green; 1 = the first yellow leaf; 2 = lower leaves yellow; 3 = lower leaves dead and some upper leaves yellow; 4 = upper leaves wilted, lower leaves dead; 5 = dead plant. The experiment was conducted two times with 30 plants per treatment.

key role in agricultural environments, and their potential for use in sustainable agriculture is shown by *in situ* biological activities such as root colonization. In general, biocontrol approaches are highly effective under low disease pressure, but biocontrol efficacy of biopesticides is not satisfied under high disease pressure (Kim et al., 2010). We investigated the severity of tomato Fusarium wilt with inoculum doses of conidial spores from the pathogen (Fig. 4A). The pathogen was inoculated with infested soil (De Boer et al., 2003). The infested Fusarium wilt soil was used for *F. oxysporum*. The pathogen was cultured in aerated 10% PDB (Difco Lab) medium at 25°C. After 21 days of growth, cultures were filtered through sterile glass wool to remove mycelia mats. Microconidia were harvested by centrifugation at $8,000 \times g$ for 20 min, resuspended in 10 mM $MgSO_4$, and mixed with a potting soil-sand mixture (12:5, v/v) to different densities.

Inoculated soil was incubated in polyethylene bags for 3–5 days at 25 °C before use in the potting soil bioassays and then stored at 4 °C. Inoculum viability of each fungi dosage was determined by serial dilution on a peptone pentachloronitrobenzene (PCNB) medium for pathogens (Threlfall, 1972). Inoculums were prepared by dilution with non-infested soil. The experiment was conducted two times with 30 plants per treatment. The disease index (0–5) was assayed in plants showing any wilt symptoms due to the pathogen (yellowing and dropping of leaves, vascular discoloration, or wilting). Stem sections from wilted plants were surface-disinfested in 0.5% sodium hypochlorite and plated on PCNB medium to confirm the presence of the wilt pathogen.

After 2 weeks, the shoots languished, and the roots started to rot gradually and had a dark brown appearance. Tomato plants began to show their first leaf yellowing (disease index, 1) against *F. oxysporum* infection at a concentration of 1×10^4 spore g^{-1} of soil. A pathogen density $> 1 \times 10^5$ spore g^{-1} of soil resulted in wilted upper leaves and dead lower leaves (disease index, 4) (Fig. 4A). However, $< 1 \times 10^4$ spores/g of soil did not result in any visible symptoms. We determined the biocontrol efficacy of the novel *phlD*+ NJ134 under high-disease pressure greenhouse conditions with different bacterial dosages of NJ134 grown in modified KB amended with 1% mannitol.

The new DAPG-producing NJ134 genotype was introduced at a dosage from 1×10^1 to 1×10^{10} cells g^{-1} of soil into the infected *F. oxysporum* of 1×10^5 spore g^{-1} (Fig. 4B). The leaves of infected seedlings grown without NJ134 colonization became yellow, and plants wilted 2 weeks after pathogen application. Root cross sections of these infected tomato plants were characterized by dark red to black discoloration and rotting.

Tomato seedlings with roots colonized with $> 1 \times 10^9$ cells g soil $^{-1}$ NJ134 showed a disease index of 1.3 (Fig. 4B). Biocontrol efficacy of Fusarium wilt disease by NJ134 was dependent on the cell density applied. The optimal cell density to obtain effective control efficacy of Fusarium wilt was $> 1 \times 10^{7.8}$ density (disease index, 1.6). The population densities ranged from up to 1×10^4 cells g^{-1} of soil, and the disease index ranged from 5.0 to 4.1. NJ134 did not show any phytotoxicity to tomato plants, even when they were treated with high concentrations. These results indicated that at least 1×10^8 of the NJ134 culture was required to effectively control tomato Fusarium wilt in plants. Previous studies with 2,4-DAPG-producing fluorescent *Pseudomonas* spp. showed the threshold density to be 10^5 CFU g root $^{-1}$ for take-all decline in Washington State and the Netherlands (Weller et al., 2002).

Biocontrol efficacy was evaluated with the 10^8 CFU/ml NJ134 batch cultures (Fig. 5). Field assays were run under greenhouse soil, located in Naju (Jeonnam Province, Korea), using 18–20 leaf stage tomato plants (Unicorn, Hungnong

Fig. 5. Efficacy of a bacterial suspension on tomato wilt disease under field conditions. Bacteria were grown for 7 days in KB. A bacterial suspension containing 1×10^8 CFU/ml was drenched four times for 60 days after transplanting. Plants raised from bacterial-treated and untreated seedling were wound-inoculated with *F. oxysporum* at 10^6 CFU/mL when tomato plants were 70 days old in the greenhouse. The disease index was as follows: 0 = healthy plant, all leaves were green; 1 = first leaf yellow; 2 = lower leaves yellow; 3 = lower leaves dead and some upper leaves yellow; 4 = upper leaves wilted and lower leaves dead; 5 = dead plant. ** significant at 1% level by *t*-test.

Seeds, Seoul, South Korea), during spring and summer. The introduced NJ134 suspensions of 10^8 CFU/ml were drenched four times for 60 days after transplanting. Plants raised from bacteria-treated and untreated seedling were wound-inoculated with *F. oxysporum* at 10^6 spore/g when tomato plants were 70 days old. Disease was monitored for 6–8 weeks and assayed as the index (0–5) of plants showing any wilt symptoms due to the pathogen (yellowing and dropping of leaves, vascular discoloration, and wilting). Stem sections of wilted plants were surface disinfested with 0.5% sodium hypochlorite and plated on PCNB medium to confirm the presence of the wilt pathogen. The plants treated with NJ134 showed the disease index ranged from 0.7 to 1.0, whereas it averaged 2.5 in untreated plants. The untreated plants showed severe disease symptoms; the first or lower leaves died and some upper leaves turned yellow, whereas the plants treated with the NJ134 DAPG producer were relatively healthy or only weakly yellow.

In summary we demonstrate that a pseudomonad isolated from Korean soils was of a new genotype different from those classified previously from Europe and North America. The production of DAPG correlated with the presence of the *phlD* gene. Isolate NJ134 had potent *in vitro* and *in vivo* antifungal activity against fungal plant pathogens that was demonstrated in commercial soils for Fusarium wilt of tomato. Maximization of biocontrol efficacy of this strain will require further evaluation of the role played by DAPG and the formulations for growth and application under field conditions.

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