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- Note -

Identifications of a Sprout-Rot Pathogen *Pseudomonas* Species SN239 and Selection Resistant Soybean Line

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Control microbial contamination in pathogens to soy sprouts has always been highly concerned in soybean sprout industries because the soybean sprouts are consumed largely as a nutritious fresh vegetable around the world. However, pathogens in soy sprouts are little known. Here, we isolated a strain of *Pseudomonas* sp. SN239 that caused severer symptoms in sprouts of many soybean cultivars. In phylogenetic relationships using 16S ribosomal RNA sequences of the *Pseudomonas* species, the identified *Pseudomonas* sp. SN239 was grouped with *P. putita, P. plecoglossicida, P. monteilii* and *P. mevalonii*. Thus, the bacterial strain SN239 might be a newly identified *Pseudomonas* species which closely related to *P. putida*. Furthermore, we found that a Korean indigenous soybean (*Glycine max*) cultivar YNPCSS3-19 has strong resistance against the *Pseudomonas* sp. SN239.

Key words : *Pseudomonas, Glycine max*, disease resistance soybean, 16S rRNA

Introduction

People throughout the Asia are consuming soybeans as a variety of traditional soy food products for more than 1,000 years. Soy foods are typically consumed as non-fermented and fermented conditions. Non-fermented soy foods include fresh green soybeans, whole dry soybeans, soy nuts, soy sprouts, whole-fat soy flour, soymilk and tofu. Fermented soy foods include tempeh, miso, soy sauces, natto, soybean paste, and fermented tofu and soymilk products [4]. Among soy foods, soybean sprouts contain high concentration of isoflavone and vitamins. Because of this reason, soybean sprouts are largely consumed and supplying the vitamins and isoflavones in eastern Asia [8]. In soybean sprout industry, control microbial contamination by pathogens has always been tightly concerned. Gram-negative bacteria *Pseudomonas* strains and *Bacillus* are the major pathogens for soybean sprouts [7]. Up to date, many studies on the resistance of soybean plants against plant pathogenic bacteria have been investigated for understanding the mechanism of host-pathogene interactions [6,10,12]. However, little is known about the resistance of soybean sprouts against pathogens. Here, we isolated a bacteria strain, named SN239, that caused severer symptoms of the sprouts of many soybean cultivars. We concluded that the identified bacteria SN239 is one of *Pseudomonas* species by using analysis of phylogenetic relationships based on 16S ribosomal RNA sequences. Furthermore, we found a soybean cultivar that showed strong resistance against bacteria pathogen.

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Materials and Methods

Plant materials and plant infection

A *Pseudomonas* sp. SN239 was isolated from rotten soybean sprouts collected from local market in Gyeongsan city, Korea and cultured in NB broth (3.0 g/l beef extracts and 5.0 g/l pepton) (Becton Dickinson Co., Sparks, MD, USA) at 28°C. For inoculation to soybean sprouts, bacteria were cultured until 0.5 of OD number at 600 nm using spectrophotometer. Soybean (*Glycine max*) cultivars including YNPCSS3-19, YNPCSS1-11 and Eunha were inoculated with *Pseudomonas* sp. SN239. Seeds were surface-sterilized with 3% sodium hypochlorite for 30 min and germinated in petridish at 26°C in the dark for 2 days. Seedlings were imbibed for 2 hours in NB medium with $5x10^8$ cfu/mL of *Pseudomonas* sp. SN239 infection and then were washed with sterilized water. Seedlings with/without pathogen treatments continued to grow at 26°C in the dark and harvested every 12 hours up to 72 hours. Upon harvesting, samples were frozen in liquid nitrogen and stored at -80°C. In order to determine the survivability upon *Pseudomonas* infection, 48 hours later of treatment some of the seedlings were placed

under light for further 48 hours to develop chloroplasts.

DNA sequencing and molecular genetics analysis

Nucleotide sequences of 16S ribosomal RNA of *Pseudomonas* sp. SN239 were amplified using polymerase chain reaction (PCR) with a set of primers, which are a forward primer (5′-TGACGCTGGCGGCGGCCTAAC-3′) and a reverse primer (5′-CCCGTCTGAATCCACCGTGGT-3′). PCR amplification was performed with a DNA thermal cycler by using a PCR reaction buffer containing each of the deoxynucleotide triphosphates at a concentration of 200 μM, each of the primers at a concentration of 1 μM, 10 ng of DNA and 2.5 U of Taq DNA polymerase in a total volume of 50 μl reaction. A total of 50 amplification cycles were performed following denaturation at 94°C for 1 min, primer annealing at 59°C for 45 s, and primer extension at 72°C for 2 min. By using designed PCR primers, about 1.4 kb DNA fragments were amplified from the strain SN239. The amplified DNA fragments were purified by gel electrophoresis in 1.4% agarose. The 1.4 kb of purified DNA fragment was cloned in to pGEM-T vector (Promega co. Madison, WI, USA). DNA sequencing for the ribosomal RNA in pGEM-T vector was performed with the BigDye Terminator Cycle Sequencing kits (PE Biosystems, Foster City, CA, USA) using an automated DNA sequencing machine (ABI 3100, Applied Biosystems, Rockville, MD, USA). Primers used for DNA sequencing were SP6 (5′-TATTTAGGTGACACTATAG-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′). Neucleotide and deduced amino acid sequences were analyzed using the programs in DNAsis (Hitachi, Japan). The nucleotide sequences were compared with sequences deposited in public databases of NCBI using the BLAST algorithm [1].

Data analysis

The nucleotide sequences of 16S rRNA were aligned by using the CLUSTALW computer program [11]. The 16S rRNA sequences were aligned based on their secondary structures. Evolutionary trees were constructed with the phylip program package [3], using the neighbour-joining method [9] with genetic distances computed by using the Jukes-Cantor model [5]. Phylogenetic trees were constructed from the 16S rRNA sequences.

Results and Discussion

To compare the homology of the *Pseudomonas* sp. SN239 with that of other *Pseudomonas* species, 16S rRNA sequences were analyzed. We amplified 1,452 nucleotides of 16S rRNA sequences of *Pseudomonas* sp. SN239 (FJ529815) (Fig. 1) using rRNA specific primers. The 16S rRNA sequences of *Pseudomonas* sp. SN239 was showed 99 % homology with that of *Pseudomonas putida* (ATCC17390) [2] and *Pseudomonas plecoglossicida* (AB009457.1) (Table 2). However, the 16S rRNA sequences of *P.* sp. SN239 was showed less than 99 % homology with that of *Pseudomonas fuscovaginae* (MAFF301177T), *P. asplenii* (ATCC23835T), *P. siderocapsulatus* (AF226713.1), *P. monteilii* (CIP104883), *P. mevalonii* (AJ299216.1), *P. parafulva* (AB060133.1), *P. fulva* (AB060136.1), *P. alcaligenes* (AF511436.1), *P. cermoricoloranta* (AB060137.1), *P. jessenii* (AF068259.1) and *P. syringae* (AF130950.1) (Table 2). Analysis based on phylogenetic relationship showed that the *Pseudomonas* sp. SN239 strain might be grouped with *Pseudomonas putida, Pseudomonas plecoglossicida, Pseudomonas monteilii, Pseudomonas mevalonii* (Fig. 2), but not perfectly identical. Thus, the strain *Pseudomonas* sp. SN239, a soybean sprout pathogen, is a newly identified *Pseudomonas* species having sequence characteristics similar with *P. putida*.

Hundreds of soybean cultivars were germinated and examined for resistance against *Pseudomonas* sp. SN239. Sprouts of the most soybean cultivars were susceptible to this *Pseudomonas* strain. However, few soybean cultivars

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   1 TGACGCTGGC  GGCGGCCTAA  CACATGCAAG  TCGAGCGGAT  GACGGGAGCT
  51 TGCTCCTTGA  TTCAGCGGCG  GACGGGTGAG  TAATGCCTAG  GAATCTGCCT
 101 GGTAGTGGGG  GACAACGTTT  CGAAAGGAAC  GCTAATACCG  CATACGTCCT
 151 ACGGGAGAAA  GCAGGGGACC  TTCGGGCCTT  GCGCTATCAG  ATGAGCCTAG
 201 GTCGGATTAG  CTAGTAGGTG  AGGTAATGGC  TCACCTAGGC  GACGATCCGT
 251 AACTGGTCTG  AGAGGATGAT  CAGTCACACT  GGAACTGAGA  CACGGTCCAG
 301 ACTCCTACGG  GAGGCAGCAG  TGGGGAATAT  TGGACAATGG  GCGAAAGCCT
 351 GATCCAGCCA  TGCCGCGTGT  GTGAAGAAGG  TCTTCGGATT  GTAAAGCACT
 401 TTAAGTTGGG  AGGAAGGGCA  GTAAGTTAAT  ACCTTGCTGT  TTTGACGTTA
 451 CCGACAGAAT  AAGCACCGGC  TAACTCTGTG  CCAGCAGCCG  CGGTAATACA
 501 GAGGGTGCAA  GCGTTAATCG  GAATTACTGG  GCGTAAAGCG  CGCGTAGGTG
 551 GTTCGTTAAG  TTGGATGTGA  AAGCCCCGGG  CTCAACCTGG  GAACTGCATC
 601 CAAAACTGGC  GAGCTAGAGT  ATGGTAAAAG  GGTGGTGGAA  TTTCCTGGTG
 651 TAGCGGTGAA  ATGCGTAGAT  ATAGGAAGGA  ACACCAGTGG  CGAAGGCGAC
 701 CACCTGGACT  GATACTGACA  CTGAGGTGCG  AAAGCGTGGG  GAGCAAACAG
 751 GATTAGATAC  CCTGGTACTG  CACGCCGTAA  ACGATGTCAA  CTAGCCGTTG
 801 GAATCCTTGA  GATTTTAGTG  GCGCAGCTAA  CGCATTAAGT  TGACCGCCTG
 851 GGGAGTACGG  CCGCAAGGTT  AAAACTCAAA  TGAATTGACG  GGGGCCCGCA
 901 CAAGCGGTGG  AGCATGTGGT  TTAATTCGAA  GCAACGCGAA  GAACCTTACC
 951 AGGCCTTGAC  ATGCAGAGAA  CTTTCCAGAG  ATGGATTGGT  GCCTTCGGGA
1001 ACTCTGACAC  AGGTGCTGCA  TGGCTGTCGT  CAGCTCGTGT  CGTGAGATGT
1051 TGGGTTAAGT  CCCGTAACGA  GCGCAACCCT  TGTCCTTAGT  TACCAGCACG
1101 TTATGGTGGG  CACTCTAAGG  AGACTGCCGG  TGACAAACCG  GAGGAAGGTG
1151 GGGATGACGT  CAAGTCATCA  TGGCCCTTAC  GGCCTGGGCT  ACACACGTGC
1201 TACAATGGTC  GGTACAGAGG  GTTGCCAAGC  CGCGAGGTGG  AGCTAATCTC
1251 ACAAAACCGA  TCGTAGTCCG  GATCGCAGTC  TGCAACTCGA  CTGCGTGAAG
1301 TCGGAATCGC  TAGTAATCGC  GAATCAGAAT  GTCGCGGTGA  ATACGTTCCC
1351 GGGCCTTGTA  CACACCGCCC  GTCACACCAT  GGGAGTGGGT  TGCACCAGAA
1401 GTAGCTAGTC  TAACCTTCGG  GAGGACGGTT  ACCACGGTGG  ATTCAGACGG
1451 GG
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Fig. 1. Nucleotide sequences of 16S ribosomal RNA of *Pseudomonas* sp. SN239. GenBank accession number is FJ529815.

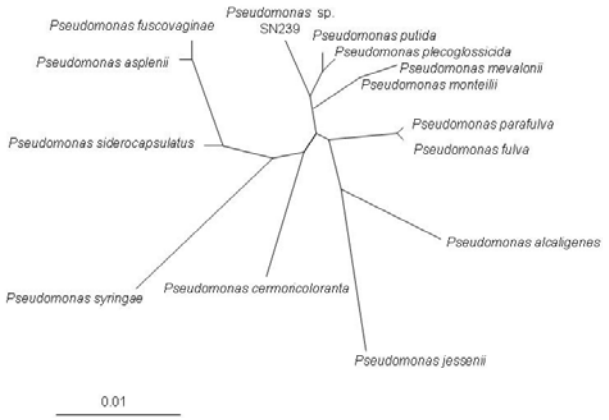

Fig. 2. Phylognetic tree derived from the analysis of the 16S rRNA sequences of the *Pseudomonas* species SN239 (GenBank accession FJ529815). The bar represents a 1% sequence differences. Accession numbers of compared bacterial sequences are: *Pseudomonas putida* (ATCC17390), *P. plecoglossicida* (AB009457.1), *P. fuscovaginae* (MAFF301177T), *P. asplenii* (ATCC23835T), *P. siderocapsulatus* (AF226713.1), *P. monteilii* (CIP104883), *P. mevalonii* (AJ299216.1), *P. parafulva* (AB060133.1), *P. fulva* (AB060136.1), *P. alcaligenes* (AF511436.1), *P. cermoricoloranta* (AB060137.1), *P. jessenii* (AF068259.1) and *P. syringae* (AF130950.1).

Table 1. Average seedling length of three soybean genotypes after inoculation with sprout rot disease strain SN239

	Strain SN239 Inoculation	Average seedling length (cm) after hours (hr) of treatments		
		24 hr	48 hr	72 hr
YNPCSS3-19	Non-inoculated	3.6	5.6	7.3
	Inoculated	3.5	5.6	6.2
YNPCSS1-11	Non-inoculated	3.9	6.0	7.4
	Inoculated	2.5	2.8	3.2
Eunhakong	Non-inoculated	3.9	6.3	7.5
	Inoculated	2.3	2.3	2.6

showed relatively resistant to *Pseudomonas* sp. SN239. Among them, YNPCS3-19, which is a Korean endogenous soybean cultivar, showed stronger resistant. Therefore, we performed detailed resistant analysis with YNPCS3-19 as a resistant cultivars, YNPCSS1-11 as a susceptible cultivar, and Eunha as a medium resistant cultivars against *Pseudomonas* sp. SN239 strain. When the seedlings were grown after 24 hours at 28°C after inoculation of *Pseudomonas* sp. SN239, both cultivars YNPCSS1-11 and Eunha showed brown rotten symptoms in their hypocotyls (Fig. 3). However, sprouts of YNPCS3-19 were not only showed symptoms up to 72 hours after the *Pesudomonas* inoculation (Fig. 3) but also kept growing in hypocotyls (Table 1), indicating that soybean cultivar

Table 2. Sequence identities of 16S ribosomal RNA of *Pseudomonas* sp. SN239 with that of other *Pseudomonas* species

Pseudomonas species	Accession number	Sequence identity to *Pseudomonas sp.* SN239
P. putida	ATCC17390	0.990
	AB009457.1	0.990
P. monteilii	CIP104883	0.989
P. mevalonii	AJ299216.1	0.986
P. parafulva	AB060133.1	0.984
P. fulva	AB060136.1	0.982
P. cermoricoloranta	AB060137.1	0.979
P. alcaligenes	AF511436.1	0.977
P. asplenii	ATCC23835T	0.977
P. fuscovaginae	MAFF301177T	0.977
P. syringae	AF130950.1	0.975
P. jessenii	AF068259.1	0.973
P. siderocapsulatus	AF226713.1	0.962

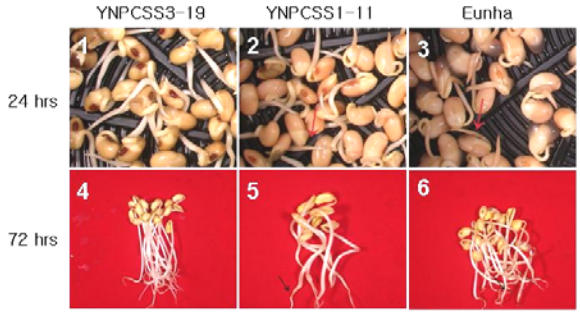

Fig. 3. Soybean sprouts inoculated with *Pseudomonas* sp. SN239. Dark grown seedlings of soybean cultivars were inoculated for 2 hours in NB media with *Pseudomonas* sp. SN239 and then were washed with sterilized water. The seedlings were grown up to 72 hours at 28°C. Photograph were taken after 24 hours (upper panel) and 72 hours (lower panel) of inoculation. Soybean cultivars were YNPCS3-19 (panels1 and 4), YNPCSS1-11 (panel 2 and 5) and Eunha (panel 3 and 6). Arrows indicate rotten roots.

YNPCS3-19 has resistance against *Pseudomonas* sp. SN239. Therefore, this Korean indigenous soybean cultivar YNPCS3-19 may be useful in breeding soybean cultivar resistant to soybean sprout disease.

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초록 : 콩나물 부패균 *Pseudomonas* sp. SN239 동정과 콩나물 부패병 내병성 계통 선발

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콩나물은 우리나라에서 오래 전부터 재배하여 온 채소로서 그 기호성이 매우 높으며 영양학적으로 우수하나 일부 열악한 재배환경으로 콩나물의 부패문제가 자주 발생해 왔다. 따라서 본 연구는 시중의 부패된 콩나물로부터 다양한 병원균을 분리함과 동시에 재래콩 유전자원으로부터 시중의 콩나물 부패병에 강한 품종을 탐색하고 선발된 내병성 계통의 생육특성을 조사하였다. 분리된 콩나물 부패균들 중 병원성이 강한 콩나물 부패균인 *Pseudomonas* sp. SN239을 분리하고 16S rRNA 염기서열을 동정한 결과 *P. putita, P. plecoglossicida, P. monteilii* 및 *P. mevalonii*와 근연관계를 보였으나 완전히 일치하지는 않았으므로 *Pseudomonas sp.* SN239는 새로이 동정된 콩나물 부패균으로 여겨진다. 또한 재래콩 194계통에 콩나물 부패병균 *Pseudomonas sp.* SN239을 접종하여 저항성을 검정한 결과, 이병성 계통은 심하게 부패되었으나 한국 고유계통 YNPCS3-19는 병원성이 없었으며 또한 지속적으로 생육하였다. 그러므로 부패균 저항성 계통 YNPCS3-19는 부패균 저항성 품종 육성에도 활용 가치가 크다고 판단된다.