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Pathogenic bacteria causing rot in commercial soybean sprout cultivation

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ABSTRACT: Soybean sprout pathogenic bacteria were isolated from the large, deep containers of a commercial factory. Over a period of one year, 40 pathogenic-like bacteria were isolated among a total of 732 isolates. In addition to bacteria previously reported to be associated with rotting, such as *Pseudomonas putida* and *Erwinia carotovora*, several other genera were also identified: *Acinetobacter* spp., *Chryseobacterium* spp., *Klebsiella* sp., *Pantoea agglomerans*, *Bacillus* sp. Fatty acid methyl ester (FAME) analysis using the Microbial ID (MIDI) system, and 16s rRNA sequence analysis, yielded identical results, confirming the identities of these microorganisms. Several types of selective media were not good for identification and determination of population structure in commercial environments, as colony type was not specific to the genus. There was no dominant bacterium, and we were not able to find the main bacterium responsible for soybean spout rot. Even though we did not identify a major target for controlling rot or screening for resistant cultivars, the results of this study indicated that bacterial rot of soybean sprout is endemic. In addition, it emerged that factory epidemics in summer are not caused by the bacteria isolated in this study.

Keywords : soybean sprout, rot, bacterial identification, MIDI, 16s rRNA

Soybean sprouts have been consumed in Korea for more than 1,000 years. However, their cultivation is changing from home-based to commercial mass production systems. To increase profit, commercial containers are narrower and deeper than those traditionally used. Furthermore, there is increasing consumer demand for environmentally friendly products, produced without the use of fungicides and plant hormones during cultivation. The conditions at the center of commercial containers, *i.e.*, high temperature, humidity, and CO_2 concentration due to the high densities of soybean sprouts, are much more conducive to rot than are those of traditional systems. Rotting of soybean sprouts is, therefore, a serious problem in commercial cultivation, especially in summer. However, environmental control at the factory is the only way to control the disease because of consumer demand for organic soybean sprouts.

There have been few studies published on soybean sprout rot (Myung, 1987; Oh and Park, 1996; Park *et al.*, 1997a, b). Several fungi, such as *Fusarium moniliforme, F. oxysporum,* and *F. solani* (Oh and Park, 1996), and two bacteria, *Pseudomonas putida* (Park *et al.*, 1997a) and *Erwinia carotovora* (Park *et al.*, 1997b), have been reported to be the causal pathogens of rot. In addition, *Rhizoctonia solani, Macrophomia phascoli, Colletotricum* sp., and *P. fluorescens* Biotype II have been reported in Japan (Takao and Katao, 1986). Rot can be controlled by fungicides, such as benzimidazole or thiophanate. However, these chemical agents remain in the sprouts even when used to treat seeds before cultivation (Park and Kim, 1998). Treatment with the plant hormone auxin can produce higher yield and more rapid growth than can organic culture but, despite the legality of its use, it is not favored by consumers (Bae *et al.*, 1998).

Identification of the pathogenic bacteria responsible for serious rot in soybean sprout mass production is important not only for developing strategies of control but also for enabling screening for resistant varieties of soybean seeds. In addition to treatment with chemicals, such as fungicides and plant growth regulators, there are other methods available for decreasing bacterial populations, such as exposure to ozone or super-reductive water. Isolation and identification of the major bacterial pathogens responsible for soybean sprout rot will facilitate the development of new techniques for control *in vitro*. The causal pathogen(s) can be used to select resistant soybean cultivars. *E. carotovora* (Park *et al.*, 1998) has been used to screen for rot-resistant soybean varieties.

As the size of the rhizospheric population of bacteria in soybean sprouts is 10^6~10^7 bacteria per gram of fresh soybean spouts, there is a high probability of the presence of pathogenic strains. Although the pathogens are not highly aggressive, the environment in commercial soybean sprout containers is conducive to bacterial attack. Two bacterial pathogens, *P. putida* (Park *et al.*, 1997a) and *E. carotovora* (Park *et al.*, 1997b), were reported to have little soybean sprout penetrating ability. *P. putida* is a saprophyte and *E.*

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carotovora requires an opening to penetrate into plant tissue. Thus, other saprophytes may be pathogenic under commercial culture conditions. Investigation of the bacterial community structure over a long period will provide information on bacterial pathogen profiles. In addition, analysis of their pattern of occurrence will identify epidemic and endemic strains among the isolates. Frequent and repeated isolation of a particular bacterium over the course of a year would suggest that it could be a major cause of rot. As serious soybean sprout rot at commercial facilities occurs mainly in the summer, epidemic bacteria were expected to be isolated during this season.

Here, we report the identification and frequency of pathogenic-like bacteria in commercial soybean sprout cultivation over a period of one year. Several methods of bacterial identification were compared using pathogenic isolates to find the best method for use in a commercial environment.

MATERIALS AND METHODS

Isolation of bacteria

Diseased soybean sprout samples were obtained from a commercial factory (Umsung, Korea) just before packing. The commercial mass production containers were filled with 300 kg of fully grown soybean sprouts stacked in ten or more layers to a height of 110 cm. The rotted sprouts mainly showed a water-soaked brownish hypocotyl and cotyledon. Rotted soybean sprouts were surface-sterilized with 70% ethyl alcohol for 1 minute, chopped into small pieces on slide glasses in sterile water, and the chopped plant tissue and water were streaked on nutrient agar petri dishes. After 24 hr in culture at 30°C, at least 30-120 single bacterial colonies were pure cultured for infection. A total of 732 pure isolates were tested for pathogenicity (Table 1).

Pathogenicity test and selective media

Pathogenicity tests were conducted on germinating soybean seeds (Junjeori) in test tubes with 2% agar. Three soybeans surface-sterilized with 0.5% NaOCl were soaked in each pure-cultured bacterial suspension (10^6~10^7 cells/ml) for 1 minute.

The pathogenicity of each bacterial isolate was determined after 4 days in culture, in the dark at 25°C. The water-soaked brownish color was similar to the original color of the rotted sprouts. Only pathogenic isolates were cultured on NBY (0.8% nutrient broth, 0.2% yeast extract, 0.2% K_2HPO_4, 0.25% glucose) broth medium, and preserved with 70% glycerol at -80°C.

Colony characteristics were investigated on several solid media. Nutrient agar and Pseudomonas Agar F were purchased from Difco (Kansas City, MO). MGY agar medium (1% mannitol, 0.2% glutamic acid, 0.05% KH_2PO_4, 0.02% NaCl) was used for selective medium. Colony size and color were observed after 24 hr in culture.

Bacterial identification by fatty acid methyl ester (FAME) analysis using the microbial ID (MIDI) system

The microbial identification system (MIS; Microbial ID, Newark, DE) is a well-established fully automated gas chromatography (GS) analytical system for identification of bacteria based on their unique fatty acid profiles (Miller and Berger, 1985). Tryptic soy broth agar (Difco, Kansas City, MO) was used to culture the 40 pathogenic bacteria at 28°C for 24 hr. Purified fatty acid was obtained from the second generation cultures as described by MIDI (1995). Bacterial preparation for the FAME-MIDI identification was per-

Table 1. Isolation of pathogenic-like bacteria from rotted soybean sprouts.

Year	Month	Number of pathogenic isolates	Number of single colony isolates	% of pathogenic isolates
2000	July	4	45	8.9
	August	6	45	13.3
	September	4	33	12.1
	October	3	67	4.5
	December	1	31	3.2
2001	February	2	100	2.0
	March	10	70	14.3
	April	4	65	6.2
	June	1	102	1.0
	July	5	120	4.2
	August	0	54	0.0
Total		40	732	

Table 2. Characteristics of 40 isolates on several selective media based on the identification of seven different genera.

Name	Isolated date	Genus Name	NA medium†		PAF medium†		EW medium‡	
			Size§	Color	Size	Color	Size	Color
10407	2001. 4.	*Pseudomonas putida*	b	fluorescence	a	fluorescence	c	fluorescence
W2	2000. 10.	"	c	fluorescence	b	fluorescence	d	fluorescence
80309	2000. 8.	"	c	transparent white	b	fluorescence	c	fluorescence
1215a	2000. 12	*Pseudomonas flectens*	c	milky white	b	milky white	d	pale yellow
810j	2000. 8.	*Pseudomonas fluorescens*	c	milky white	c	milky white	e	milky white
X1	2000. 10.	"	a	white	a	transparent white	b	fluorescence
71149	2001. 7.	"	n/a	n/a	b	milky white	d	pale yellow
10416	2001. 4.	*Erwinia carotobora*	c	transparent white	a	transparent white	c	milky white
10417	2001. 4.	"	b	transparent white	b	milky white	b	milky yellow
10418	2001. 4.	"	c	transparent white	a	transparent white	n/a	n/a
907g	2000. 9.	"	c	milky yellow	b	pale yellow	f	pale yellow
72706	2000. 7.	*Chreyseobacterium sp.*	a	yellow	b	yellow	a	milky yellow
72704	2000. 7.	"	a	yellow	b	yellow	a	milky yellow
10313	2001. 3.	"	a	pale yellow	b	yellow	a	n/a
928a	2000. 9.	"	a	pale yellow	b	yellow	a	n/a
10363	2001. 3.	"	a	pale yellow	b	yellow	a	n/a
0711d	2001. 7.	"	n/a	n/a	b	yellow	a	n/a
0711h	2001. 7.	"	n/a	n/a	b	yellow	n/a	n/a
72710	2000. 7.	*Klebsiella planticola*	b	white	c	milky white	e	milky white
810i	2000. 8.	"	c	white	c	milky white	d	milky white
810f	2000. 8.	"	c	milky white	c	milky white	e	milky yellow
80302	2000. 8.	"	c	milky white	d	milky white	e	milky white
907f	2000. 9.	"	e	milky white	d	milky white	f	milky white
928i	2000. 9.	"	e	milky white	c	milky white	e	milky white
10349	2001. 3.	"	c	white	a	transparent white	b	milky white
10302	2001. 3.	"	d	milky white	c	milky white	e	milky white
20123	2001. 2.	*Acinetobacter spp.*	c	milky white	b	milky white	d	pale yellow
10341	2001. 3.	"	c	white	a	transparent white	b	milky yellow
10351	2001. 3.	"	c	milky yellow	b	milky white	d	pale yellow
10373	2001. 3.	"	c	milky white	a	transparent white	c	milky white
10372	2001. 3.	"	c	milky white	a	transparent white	c	milky white
62774	2001. 6.	"	n/a	n/a	a	transparent white	d	milky yellow
71101	2001. 6.	"	n/a	n/a	a	transparent white	b	milky white
80310	2000. 8.	*Pantoea agglomerans*	c	milky yellow	c	milky yellow	e	milky yellow
72705	2000. 7.	"	c	milky white	c	milky white	f	milky white
71106	2001. 7.	"	n/a	n/a	a	transparent white	b	milky white
W1	2000. 10.	*Bacillus sp.*	d	milky white	e	milky white	d	milky white
10312	2001. 3.	*Unknown*	b	white	b	milky white	c	milky white
20154	2001. 2.	"	n/a	n/a	n/a	n/a	n/a	n/a
10327	2001. 3.	"	d	milky white	n/a	n/a	n/a	n/a

†NA (nutrient agar) and PAF (Pseudomonas agar F) media were commercial products (Difco, USA).
‡MGY agar medium (Erwinia selective medium) was made of mannitol 1%, glutamic acid 0.2%, KH_2PO_4 0.05%, NaCl 0.02%, $MgSO_4 \cdot 7H_2O$ 0.02%, Yeast extract 0.025%.
§The colony sizes were compared after 48 hours culture; a to f indicates increasing size.
The color of some of the colonies on MGY medium could not be detected because the colonies were so small.

formed according to the procedure described in the MIS operating manual (MIDI, 1995). Briefly, each sample was saponified with sodium hydroxide in methanol, methylated with hydrochloric acid in methanol, and extracted with hexane in methyl *tert*-butyl ether, followed by base wash with sodium hydroxide. Specimens were processed on a Hewlett-Packard (Avondale, PA) GC system that included a model 5890 Series II GC with a split injector and a flame ionization detector, a model 6890 automatic sampler, a Vectra XU 5/90C computer, a model 3365 Series II Chem-Station and a fused-silica column.

Bacterial identification by 16s rRNA sequence analysis

DNA was isolated from the bacteria listed in Table 2 by boiling for 2 min to obtain templates for PCR. The PCR mixtures contained 10 to 50 ng of DNA template, 2.5 μl of each primer [forward, 5'-AGAGTTGATCMYGGCTCAG-3' (M:A or C); reverse, 5'-GGYTACCTTGTTACGACTT-3' (Y: C or T)], 5 μl of each deoxynucleoside triphosphate, 5 μl of 10 X reaction buffer (pH 8.8; Takara, Shiga, Japan), 1.5 μl of $MgCl_2$, and 5 units of *Taq* DNA polymerase in 50 μl reaction. The PCR program was as follows: initial denaturation 5 minutes at 94°C, followed by denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and polymerization at 72°C for 1 minute, for a total of 30 cycles, followed by a 5-minute extension at 72°C. The amplified 1.5 kb DNA fragments were examined by electrophoresis on 1% agarose gels.

Sequencing of the PCR products was performed using a Takara PCR kit (Shiga, Japan), and analyses were performed with a model ABI 377 automated sequencer (PE Biosystem) using the two primers described above. The DNA sequences obtained were compared to those in nucleotide databases using the BLAST search program (http://www.ncbi.nim.nih.gov). Sequencing was conducted on only 13 isolates, taken to represent different groups, based on the results of MIDI analyses and the colony characteristics on several selective media.

RESULTS

Among 732 pure cultures isolated from rotted soybean sprouts, only 40 isolates were found to be pathogenic in test tube cultures. After 4 days of growth in these tubes, the sprouts showed a brownish water-soaked appearance, which was similar to the original symptoms of rotted sprouts seen in the commercial container. The ratio of pathogenic-like bacteria among the isolates ranged from 0% to 14.3%, depending on the incubation time (Table 1). The frequency of bacteria was slightly higher in summer than in the other seasons, except for March and August, 2001. However, the seasonal differences were not significant. Serious rot occurred in the commercial factory in summer, regardless of the pattern of pathogenic bacteria during the course of the present study.

The results of bacterial identification obtained by fatty acid methyl ester (FAME) analysis using the Microbial ID (MIDI) system, and by 16s rRNA sequence analysis using web-based BLAST database searches, were compared (Table 2). Based on the FAME-MIDI results, 13 bacterial isolates were selected for 16s rRNA sequencing. The results of 16s rRNA sequencing indicated the same genera as the MIDI results. Thus, the two identification methods were complemented each other. With the exception of several results for *Acinetobacter* spp., the probabilities of MIDI results were 0.7 or more, whereas the similarities of the results of 16s rRNA sequencing were 94-99%.

The 40 pathogenic-like isolates were sorted by genus, based on the results of FAME-MIDI and 16s rRNA sequence analyses. The characteristics of the colonies with regard to size and shape on several selective media are listed in Table 3. Pseudomonas and Erwinia selective media were used to characterize the colony types of the pathogenic isolates, as these two genera have been reported as pathogens involved in rotting of soybean sprouts (Park *et al.*, 1997a, b). Three isolates were fluorescent on Pseudomonas selective medium. Examination of the colony characteristics on selective media was one of the easiest ways to identify *Pseudomonas putida*. However, *P. fluorescens* did not show fluorescence on Pseudomonas selective medium (Table 3). *Chryseobacteria* spp. colonies were always small and yellow, *Klebsiella* spp. colonies were always large and milky white, and most *Acinetobacter* spp. formed quite large, transparent white colonies. Thus, growth on Pseudomonas selective medium was not a good way to identify Pseudomonas species among the pathogenic bacteria.

Growth on Erwinia selective medium was also not a good way to identify this genus, as the four Erwinia isolates formed colonies of different size and color on this medium, and some did not show extracellular slime production. However, most *Klebsiella*, *Acinetobacter,* and *Pantoea* spp. isolates showed white colonies with slime production and grew well (Table 3).

The year-round occurrence of pathogenic-like bacteria is shown in Table 4, according to genus name. Neither *Pseudomonas* spp. nor *Erwinia* spp. were the dominant pathogenic species in commercial cultivation. Soybean sprout rot was found to be caused by 5-6 different bacterial genera throughout the year. The number of identified pathogenic-like bacterial isolates in the present study, 40, was too small

Table 3. Results of the bacterial cell wall composition (MIDI) and sequence analysis of 16s rRNA of the pathogenic isolates.

Isolates	Results of MIDI	Probability	Results of 16s rRNA (similarity)
10407	*Pseudomonas putida*	0.926	-
W2	*Pseudomonas putida*	0.627	*Pseudomonas putida* (99%)
80309	*Pseudomonas putida*	0.792	-
1215a	*Pseudomonas flectens*	0.8	-
810j	-	-	*Klebsiella sp.* (98%)/ *Pseudomonas florescence*
X1	*Pseudomonas fluorescens*	0.954	-
71149	*Pseudomonas fluorescens*	0.739	-
10416	*Erwinia chrysanthemi*	0.766	-
10417	*Erwinia carotobora*	0.98	-
10418	*Citrobacter freundii*	0.715	*Klebsiella* sp. (98%)
907g	*Pantoea ananas(Erwinia ananas)*	0.637	*Erwinia rhaponitcs* (96%)/*Erwinia amylovora*
72706	*Chryseobacterium(Flavobacterium)*	0.888	-
72704	*Chryseobacterium balustinum*	0.916	*Chreyseobacterium proteolyticum* (96%)/rhizospheric bacteria
10313	*Chryseobacterium indologenes*	0.766	*Chreyseobacterium* sp. (96%)
928a	*Chryseobacterium indologenes*	0.832	-
10363	*Chryseobacterium indologenes*	0.826	-
0711d	*Chryseobacterium balustinum*	0.872	*Chreyseobacterium proteolyticum* (94%)/rhizospheric bacteria
0711h	*Chryseobacterium balustinum*	0.874	-
72710	*Klebsiella planticola*	0.799	-
810i	*Klebsiella planticola*	0.932	-
810f	-	-	*Klebsiella* sp. (99%)
80302	*Klebsiella pneumoniae*	0.874	-
907f	*Klebsiella pneumoniae*	0.883	-
928i	*Klebsiella pneumoniae*	0.885	-
10349	-	-	-
10302	*Klebsiella pneumoniae*	0.88	-
20123	*Acinetobacter baumannii*	0.766	-
10341	*Acinetobacter radioresistens*	0.854	-
10351	-		*Acinetobacter* sp. (99%)
10373	*Acinetobacter radioresistens*	0.4	*Acinetobacter* sp. (98%)
10372	*Acinetobacter radioresistens*	0.415	-
62774	*Acinetobacter baumannii*	0.746	-
71101	*Acinetobacter radioresistens*	0.543	*Acinetobacter* sp. (97%)
80310	*Enterobacter cloacae*	0.846	*Pantoea agglomerans* (99%)
72705	*Enterobacter agglomerans(Pantoea)*	0.913	-
71106	*Enterobacter cloacae*	0.718	-
W1	*Bacillus cereus*	0.713	*Bacillus* sp. (99%)
10312	*Citrobacter freundii*	0.886	-
20154	-	-	-
10327	-	-	-

to allow us to reach any definitive conclusions regarding the seasonal pattern of bacterial disease of soybean sprouts in the commercial environment. However, it seemed that 5-6 different bacteria occurred whenever conditions were favorable for their growth, regardless of season.

DISCUSSION

Pseudomonas putida and *Erwinia carotovora* subsp. *carotovora* have been reported to be the causal organisms of bacterial rot on soybean sprouts (Park *et al.*, 1997a, b).

Table 4. Occurrence of seven genera in mass-production soybean sprout container.

Genus name of bacteria	Frequency†	Month in which isolated (+ Number‡)
Pseudomonas spp.	17.5	Apr. (1); Jul. (1); Aug. (2); Oct. (2); Dec. (1)
Erwinia sp.	10.0	Apr. (3); Sep. (1);
Chreyseobacterium sp.	17.5	Mar. (2); Jul. (4); Sep (1)
Klebsiella planticola	20.0	Mar. (2); Jul. (1); Aug. (3); Sep (2)
Acinetobacter spp.	17.5	Feb. (1); Mar. (4); Jun. (2)
Pantoea agglomerans	7.5	Jul. (2); Aug. (1)
Bacillus sp.	2.5	Oct. (1)
Unknown	7.5	Feb. (2); Mar. (1)

†Frequency is the percent of each genus among 40 pathogenic-like bacteria from the isolates.
‡The number is the number of bacterial isolates in each genus for that month.

However, in addition to these bacteria we also isolated several other genera, *i.e.*, *Acinetobacter* spp., *Chryseobacterium* spp., *Klebsiella planticola, Pantoea agglomerans,* and *Bacillus* sp., from rotting soybean sprouts. The Dept. of Crop Protection team at the Rural Development Administration Research identified *Bacillus* spp., *Pantoea* spp., and *Enterobacter* spp., as well as *P. putida* and *E. carotovora* subsp. *carotovora* in isolates from rotting soybean sprouts (1998, personal communication). Robbs *et al.* (1996) identified *Klebsiella* sp., *Pantoea agglomerans,* and *Bacillus* sp. as the organisms responsible for decay of freshly cut celery. Rotting of soybean sprouts seems to be caused by various bacterial genera depending on both time and place. Most of the bacteria identified were saprophytic, including *P. putida* and *E. carotovora* subsp. *carotovora.* Soybean sprouts are cultured in the dark, under conditions of high humidity at a temperature of 22-25°C. In addition, soybeans secrete large amounts of root exudates during sprouting. The size of the rhizospheric population of bacteria in soybean sprouts can reach 10^6~10^7 bacteria per gram, and it is possible that some of these bacteria could cause weak pathogenicity.

All of the bacteria isolated from commercial mass production containers in the present study seemed to be endemic, as bacterial pathogens were found evenly throughout the year and no particular bacterium was dominant among the pathogens isolated during the investigation. However, serious rot occurred in the summers of 2000 and 2001 in the factory. If the pathogenic bacteria are the major causal agents of soybean rot, they should be found at a higher frequency under epidemic conditions. Although we were unable to identify the epidemic pathogen responsible for soybean sprout rot, it was possible to decrease the rot problem by controlling the temperature inside the containers.

There are more than 3,000 soybean sprout factories in Korea, most of which use small and shallow homemade containers. Due to increasing consumer demand for fresh soybean sprouts with a high degree of consistency, commercial growers are attempting to mass-produce sprouts under well-controlled conditions. Thus, for industrial production, it is necessary to study breeding and cultivation of soybean sprouts with well-controlled watering, in larger and deeper containers. The bacterial genera identified in the present study were not the major causative organisms of soybean sprout rot under mass production conditions; furthermore, these microorganisms were introduced only after infection by more aggressive pathogens. Thus, control strategies should not focus on these bacteria, including *Pseudomonas* spp. and *Erwinia* spp.

Although culture on selective medium is a simple and rapid way to detect bacterial pathogens, this method may yield misleading results regarding the occurrence of pathogenic bacteria in commercial containers. Prior to the present study, the pathogen population had been checked by monitoring the total number of colonies on Pseudomonas selective medium from sprout and water samples. However, our results indicated that *Pseudomonas* is not an epidemic pathogen and that culture on this selective medium was not appropriate because other bacterial genera were also detected. In addition, we found that culture using Erwinia selective medium was not able to differentiate between *Erwinia* and other genera.

The two bacterial identification methods, FAME-MIDI and 16s rRNA sequence analysis using BLAST, yielded mutually consistent results. Although only 13 isolates were subjected to 16s rRNA sequencing, there were no inconsistencies in the results obtained between these two methods. FAME-MIDI measures bacterial cell wall composition, while 16s rRNA resides inside the bacterial cell. Park *et al.* used the Biolog for identification of bacteria on sprout samples from different soybean cultivars (Park *et al.*, 1998), and found only *Pseudomonas* spp., *Bacillus* spp., and *Erwinia* spp. We also used the Biolog method on the same isolates

but the results were neither reliable nor repeatable. Although these methods are both time-consuming and expensive compared to culture on selective medium, they should be conducted for identification purposes, as this is the first and most important step to control the disease.

Various genera were previously reported to be involved in the decay of various fresh vegetables, including sprouts (Liao and Fett, 2001; Robbs *et al.*, 1996; Takao and Katao, 1986). These organisms were shown to be common regardless of the host plant. Most of the bacteria that cause rotting of sprouts can attack young seedlings, regardless of the species of the host seed. In conclusion, attempts to control soybean sprout decay should focus on bacteria other than the soybean pathogens found in the present study.

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