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A *Pseudomonas viridiflava*-Related Bacterium Causes a Dark-Reddish Spot Disease in *Glycine max*

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A virulent *Pseudomonas viridiflava*-related bacterium has been identified as a new pathogen of soybean, one of the most important crops worldwide. The bacterium was recovered from forage soybean leaves with dark-reddish spots, and damage on petioles and pods was also observed. In contrast, common bean was not affected.

Soybean (*Glycine max* [L.] Mer. In), a subtropical leguminous plant native to southeastern Asia, is one of the most important crops for both human food and animal feed worldwide. Three bacterial species are recognized as soybean pathogens: *Pseudomonas syringae* pv. glycinea, *Xanthomonas axonopodis* pv. glycines, and *Curtobacterium flaccumfaciens* pv. flaccumfaciens, which cause soybean bacterial blight, soybean bacterial pustule, and bacterial tan spot, respectively (3, 7). In Spain, a nonproducer country for soybean, only the last species has been sporadically detected in common bean (*Phaseolus vulgaris* L.) (5).

Asturias is a farming region in the north of Spain where cattle breeding for meat and milk production is an important economic activity. The Regional Service of Agrofood Research and Development (SERIDA) has an ongoing project to assess the suitability of different varieties of forage plants as cattle feed. In one of the experimental orchards, dark-reddish spots were noticed in the leaves of soybean, which was one of the plants under study. With time the lesions extended to the whole plant, and the intensity of the damage was such that the entire culture had to be removed.

In order to identify the pathogen responsible for the disease, four lots of symptomatic leaves were processed for microbiological analysis, through tissue dilacerations followed by soaking of the material in sterile water during 2 h. After plating on King B agar, fluorescent and nonfluorescent colonies were obtained and tested for Gram staining and biochemical traits. Isolates showing identical features were considered a single strain. In total, three strains, LPPA 221, LPPA 222, and LPPA 223, were differentiated. As common features, they were Gram-negative rods, oxidative in Hugh-Leifson medium, positive for the tobacco hypersensitivity, esculin, casein, gelatin, betaine, trigonelline, L-lactate, quinate, mannitol, erythritol, and *m*-inositol tests, and negative for the levan, oxidase, arginine dihydrolase, sucrose, adonitol, homoserine, D-tartrate, and sorbitol tests (Table 1). The three strains differed only with regard to fluorescence in King B agar and pectinolytic activity. On the basis of their overall characteristics, they could be tentatively assigned to *Pseudomonas*, but their biochemical profiles did not precisely match those expected for any other *Pseudomonas* species, including previously reported soybean and/or common bean pathogens, such as *P. syringae* pv. glycinea, *P. syringae* pv. phaseolicola, *P. syringae* pv. syringae, and *P. viridiflava* (Table 1).

The sequences of nearly complete 16S rRNA genes determined for the three soybean strains (4) proved to be identical (GenBank accession number FM865870 for LPPA 221) and more closely related (99% identity) to 16S rRNA gene sequences of *P. viridiflava* (accession no. AM909660), *Pseudomonas corrugata* (AF348508), and *Pseudomonas tolaasii* (AF348507). However, the soybean strains shared a higher number of biochemical traits with *P. viridiflava* (Table 1) than with *P. corrugata* (a nonfluorescent bacterium, positive in the oxidase test and weakly positive for arginine dihydrolase activity) or with *P. tolaasii* (fluorescent, positive for the oxidase and the arginine dyhidrolase reactions, and negative for hypersensitivity in tobacco leaves).

For a more precise identification of the new soybean pathogens, multilocus sequence analysis (MLSA) based on their 16S rRNA, *rpoD*, *gyrB*, and *gltA* genes was also performed (8, 15). Sequences were edited and aligned using the software program MEGA version 5 (18), and phylogenetic trees were constructed for the concatenated sequences of the four genes (Fig. 1) and for each individual gene (not shown), using the neighbor-joining method with genetic distances computed by Kimura's two-parameter model (9). Homologous sequences obtained from databases or generated in the present work were included as controls. The selected sequences corresponded to *P. viridiflava*, strains of *P. syringae* belonging to the five clades described for this species, *P. tolaasii*, and *P. fluorescens* (13, 19). In all trees, the sequences of the soybean strains clustered together and appeared to be more closely related to *P. viridiflava* than to any other *Pseudomonas* species, including *P. syringae* pv. glycinea, *P. syringae* pv. phaseolicola, and *P. syringae* pv. syringae.

The three soybean strains were also analyzed by pulsed-field gel electrophoresis (PFGE) performed with the restriction enzymes PmeI (11 U, 6 h, 37°C; TaKaRa Biomedicals) and SwaI (11 U, 6 h, 30°C; TaKaRa Biomedicals) under the conditions described by San José et al. (14). As shown in Fig. 2A, the PFGE profiles generated with each of the two enzymes were indistinguishable and were clearly different from those obtained for *P. viridiflava* CECT 458 and *P. syringae* pv. syringae CECT 4429, included as controls. Similarly, the three strains could not be differentiated by randomly amplified polymorphic DNA (RAPD) typing performed

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TABLE 1 Biochemical features of *Pseudomonas* strains from forager soybean

Strain[a]	Species	Original host	Result of biochemical test[b]																				
			F	L	O	P	A	T	ES	C	G	S	AD	B	H	TR	D-Ta	L-La	Q	M	ER	SO	I
LPPA 221		*Glycine max*	−	−	−	−	−	+	+	+	+	−	−	+	−	+	−	+	+	+	+	−	+
LPPA 222		*Glycine max*	+	−	−	−	−	+	+	+	+	−	−	+	−	+	−	+	+	+	+	−	+
LPPA 223		*Glycine max*	+	−	−	+	−	+	+	+	+	−	−	+	−	+	−	+	+	+	+	−	+
Controls																							
*	*P. syringae* pv. glycinea	*Glycine max*	+	+	−	−	−	+	−	unk	−	+	unk	−	−	+	−	−	+	v	−	−	+
CECT 321	*P. syringae* pv. phaseolicola	*Phaseolus vulgaris*	+	+	−	−	−	+	−	−	−	+	−	+	−	+	−	−	+	−	−	−	−
CECT 4429	*P. syringae* pv. syringae	*Syringa vulgaris*	+	+	−	−	−	+	+	+	+	+	−	+	−	+	+	+	−	+	+	+	+
CECT 458	*P. viridiflava*	*Phaseolus* sp.	+	−	−	+	−	+	+	+	+	−	−	+	−	+	+	+	+	+	+	+	+
LPPA 79	*P. viridiflava*	*Phaseolus vulgaris*	+	+	−	v	−	+	+	v	v	−	−	+	−	+	+	+	+	+	+	+	+
LPPA 522	*P. tolaasii*	*Agaricus bisporus*	+	−	+	−	+	−	−	+	+	−	+	+	+	−	+	+	+	+	+	+	+
*	*P. corrugata*	*Lycopersicon esculentum*	−	−	+	−	+	v	−	unk	+	+	−	+	unk	+	v	+	unk	+	−	−	+

[a] LPPA, Laboratory of Phytopathology of the Principality of Asturias; CECT, Spanish Type Culture Collection; *, results according to Schaad (16) for *P. syringae* pv. glycinea and according to Sutra et al. (17) and Catara et al. (2) for *P. corrugata*.

[b] F, fluorescence; L, levan production; O, oxidase reaction; P, pectinolytic activity, A, arginine dihydrolase; T, tobacco hypersensitivity; ES, esculin; C, casein; G, gelatin; S, sucrose; AD, adonitol; B, betaine; H, homoserine; TR, trigonelline; D-Ta, D-tartrate; L-La, L-lactate; Q, quinate; M, mannitol; ER, erythritol; SO, sorbitol; I, *m*-inositol; −, negative; +, positive; unk, unknown; v, variable.

with primers A (AGCAGCGCCTCA), OPB-6 (TGCTCTGCCC), OPB-10 (CTGCTGGGAC), OPS-19 (GAGTCAGCAG), primer-21 (GTGAGCGTC) and primer-6 (GAAACAGCGG) (12, 11, 6, 10, 1) (see Fig. 2B for primer-21, primer-6, and OPB-10).

To demonstrate that the new disease was caused by the *P. viridiflava*-related bacteria, LPPA 221, LPPA 222, LPPA 223, and two control *P. viridiflava* strains (CECT 458 and LPPA 79) (4) were artificially inoculated on healthy seedlings of both forage

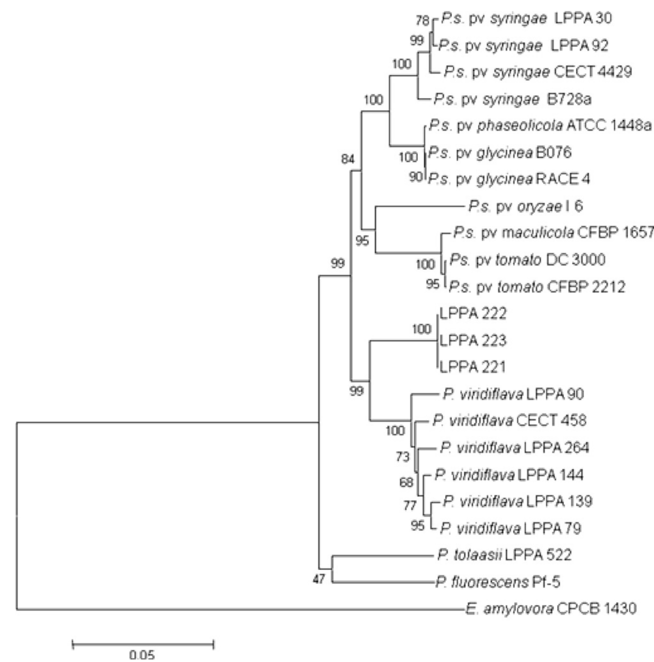

FIG 1 Phylogenetic consensus tree of the soybean *Pseudomonas* strains based on the nucleotide sequences of the 16S rRNA, *rpoD*, *gyrB*, and *gltA* genes. The scale bar represents the number of substitutions per site. The number shown next to each node indicates the percent bootstrap values of 1,000 replicates. Sequences from *Erwinia amylovora* were treated as the outgroup. The topological characteristics of the phylogenetic trees produced for individual genes were almost identical (data not shown). *P.s.*, *P. syringae*.

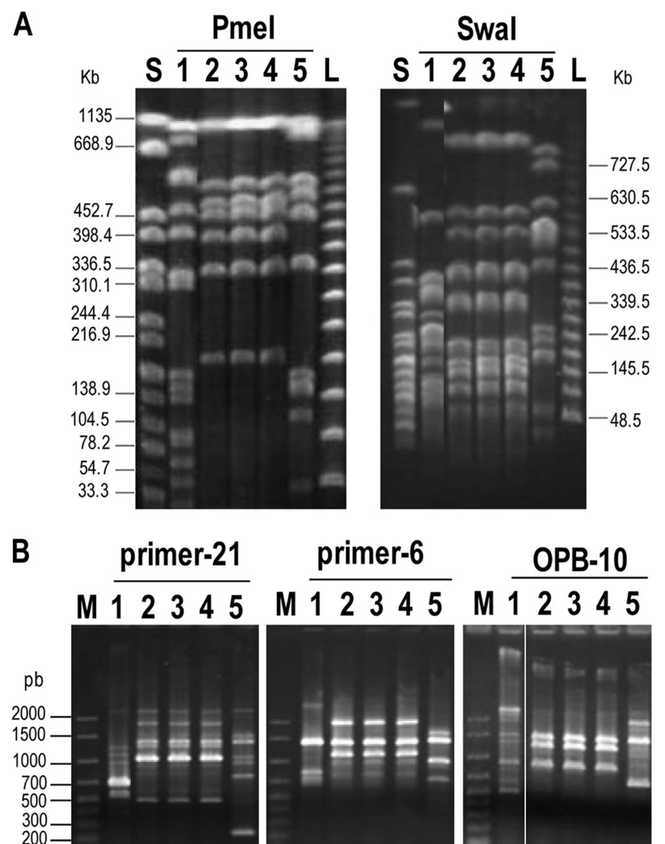

FIG 2 PFGE and RAPD analysis of soybean strains. (A) PFGE using PmeI and SwaI. Lane 1, *Pseudomonas syringae* pv. syringae CECT 4429; lane 2, LPPA 221; lane 3, LPPA 222; lane 4, LPPA 223; lane 5, *Pseudomonas viridiflava* CECT 458; lane S, *Salmonella enterica* serovar Braenderup H9812 digested with XbaI; lane L, lambda ladder PFG marker (New England BioLabs); the last two are used as size standards. (B) RAPD analysis performed with the indicated primers. Lane M, 50- to 2,000-bp BioMarker EXT size marker (Bioventures Inc., Murfreesboro, TN); lane 1, *Pseudomonas syringae* pv. syringae CECT 4429; lane 2, LPPA 221; lane 3, LPPA 222; lane 4, LPPA 223; lane 5, *Pseudomonas viridiflava* CECT 458.

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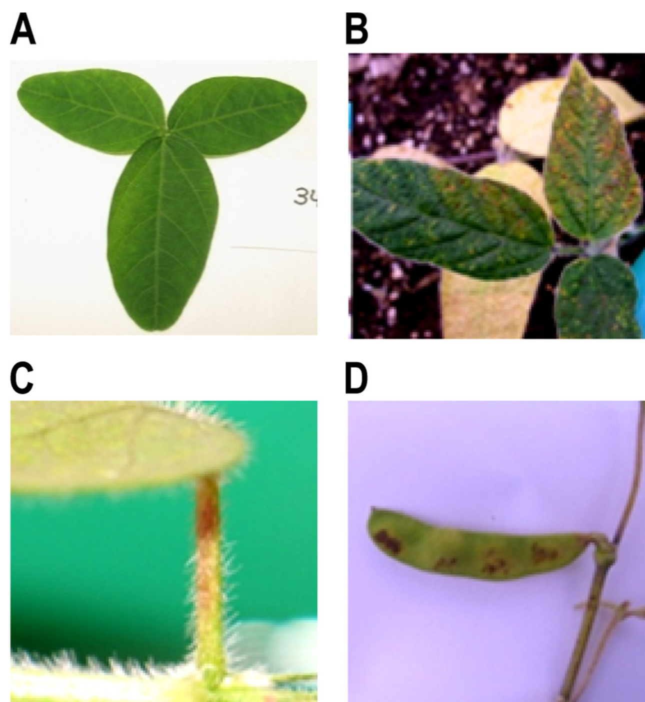

FIG 3 Bacterial dark-reddish spot disease in soybean. Healthy leaves inoculated with sterile water (A) and damage in leaves (B), petioles (C), or pods (D) caused by strain LPPA 223 are shown.

soybean and common bean. For each strain, 10 seedlings were sprayed with a bacterial suspension (10^8 CFU/ml) in yeast extract-peptone-glucose broth. The seedlings were maintained at 22°C during 1 month, with a 16/8 photoperiod and 80% relative humidity. They were initially kept under transparent plastic bags for 48 h, to saturating humidity. Seedlings treated with sterile distilled water were included as controls, and the experiments were carried out twice. The strains from forage soybean experimentally reproduced the dark-reddish spots characteristic of the natural infection in leaves, and damage in petioles and pods was also noticed (Fig. 3). The bacterium was reisolated from inoculated plants, hence fulfilling Koch's postulates. In contrast, symptoms were not observed either in leaves or in petioles or pods of common bean (not shown).

To the best of our knowledge, the *P. viridiflava*-related bacterium described in this study has not been previously reported as a soybean pathogen. Considering the importance of soybean as human food and in animal feed and the high virulence of the new pathogen, further surveillance should be performed.

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