Phenotypic Diversity in Strains of *Pseudomonas solanacearum* Isolated from a Single Potato Field in Northeastern Florida

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ABSTRACT

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Eighty-five strains of *Pseudomonas solanacearum* isolated from diseased tubers and soil samples from a 0.3-ha field near Hastings, Florida, were heterogeneous for antibiotic production and sensitivity, pathogenicity, and carbohydrate utilization. Major variation was noted in antibiotic production and strain aggressiveness. All strains belonged to race 1, biovar 1. Eight strains belonged to one of three groups: 1) weakly aggressive to potato cultivars Atlantic (susceptible) and Ontario (moderately resistant), 2) highly aggressive to both potato cultivars, and 3) highly aggressive to Atlantic but weakly pathogenic toward Ontario. All strains were aggressive pathogens to the tomato cultivar Rutgers and the eggplant cultivar Black Beauty. Although the Florida strains belonged to biovar 1, they differed in ability to oxidize galactose, arabinose, and lactose. All strains elicited the hypersensitive response in leaves of the tobacco cultivar Bottom Special. When the 85 strains were tested for antibiotic production against 26 indicator strains, there were 23 phenotypes for antibiotic production and 12 for antibiotic sensitivity. Forty-six of the strains belonged to four groups that differed in ability to produce antibiotics against three of the indicator strains. Differences in antibiotic production and sensitivity were also evident among 13 strains tested against 10 exotic strains of *P. solanacearum*.

Additional keywords: bacteriocins, potato brown rot

Pseudomonas solanacearum (E. F. Smith) is a highly heterogeneous bacterial pathogen that causes severe wilting of many important crop plants, including tomato, tobacco, and eggplant (race 1), potato (races 1 and 3), banana (race 2), ginger (race 4), and mulberry (race 5) (4,18). Considerable variation has been observed among strains from different hosts and geographic regions in pathogenicity (3,4,26), biochemical properties (14), restriction fragment length polymorphisms (9), sensitivity to bacteriophages and bacteriocins (10,20,25), and lysogenicity and bacteriocinogenicity (10,20,25). Variation in pathogenicity to different plant species has been used as the basis for race designations. However, strains within each race may differ in their pathogenicity or aggressiveness toward cultivars of the same species (12,13,27). In addition, attempts to classify strains by biochemical and other in vitro tests have not resulted in consistent groupings that relate to apparent host specificity or preference. Most strains of race 3 (mainly pathogens to *Solanum tuberosum* L.), however, have a unique carbohydrate oxidation phenotype (15).

The taxonomic treatment of *P. solanacearum* has been based on strains isolated from widely diverse geographic locations and from different host species. This approach has resulted in a broad understanding of diversity among strains of the bacterium. In contrast, phenotypic variation among local populations of *P. solanacearum* has not been documented, although there has been one report that strains from localized areas in a single field can vary in pathogenicity toward different crop species (26). Additional research is needed in order to determine whether this type of variation is widespread and whether it involves variation in other phenotypic attributes.

We found considerable variability in aggressiveness and antibiotic production within strains of *P. solanacearum* isolated from infected potatoes or soil from a single potato field in northeastern Florida. These findings have important implications in terms of biological control of bacterial wilt. An abstract of this research was published previously (21).

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MATERIALS AND METHODS

Isolation of *P. solanacearum*. Strains of *P. solanacearum* were isolated from a 0.3-ha portion of a naturally infested field located at Yelvington Farm on the University of Florida Experiment Station, Hastings. Potatoes had been grown at this site for 20 successive seasons (D. P. Weingartner, *personal communication*). In 1984, three strains were isolated from soil samples and seven from infected tubers collected at random from a four-row plot 150 m long. In 1985, 64 strains were isolated from soil samples taken from 30 sites (1 × 4 m) selected at random in an adjacent plot in the same field. At each site, six independent, 40-cm-deep core samples were taken immediately before planting. From each core sample, 10 g of soil was serially diluted in 10 mM phosphate buffer (pH 7.2) and plated on SM-3 selective medium (22). A single colony of *P. solanacearum* was selected from the plates of each sample. Eleven additional strains were isolated in randomly selected samples of infected tubers from the same field.

Pathogenicity tests. Each strain of *P. solanacearum* was tested for pathogenicity on eggplant (*S. melongena* L. 'Black Beauty'). Inoculum was prepared from a suspension in 10 mM phosphate buffer (pH 7.2) of the colonies on a 48-hr CPG agar (11) plate culture. Bacterial suspensions were diluted with buffer to 10^9 cfu/ml (OD_{600nm} = 0.5). Two 6-wk-old plants were inoculated by the stem puncture technique with each strain (17). Plants were scored for absence or presence of wilt symptoms after 2 wk.

Eight randomly selected strains—five isolated from brown-rotted tubers in 1984 and 1985 (prefixed Br) and three isolated from soil in 1985 (prefixed S)—were also tested for pathogenicity on tomato (*Lycopersicon esculentum* Mill.) cultivar Rutgers (susceptible) and potato cultivars Ontario (moderately resistant) and Atlantic (susceptible) (16). Six 6-wk-old tomato plants (started from seed) and six 3-wk-old potato plants (started from sprouted tubers) were stem-inoculated with each strain (17). At weekly intervals, plants were rated for symptom development: 0 = no disease, 1 = 1–25% of the leaves wilted, 2 = 26–50% of the leaves wilted, 3 = 51–75% of the leaves wilted, and 4 = 76–100% of the leaves wilted (19).

Disease progress data from the strain aggressiveness tests were analyzed with the general linear models procedure of SAS (1). A univariate analysis of variance was performed for repeated measures over time with replicate effects

nested within strain (23). For tests where significant differences ($P \leq 0.05$) in isolate aggressiveness were observed, mean separations were estimated by the least significant difference (LSD) method (29).

Antibiosis tests. Each of 85 strains was tested for production of bacteriocinlike compounds against other Florida strains as well as against 10 strains of race 1 and race 3 obtained from other geographic areas (26 strains). Sterile toothpicks were used to transfer bacteria from individual colonies to CPG agar plates, which were then incubated at 28 C. After 40 hr, the plates were inverted and the cultures were exposed to chloroform vapor for 1 hr (7). Indicator strains were suspended in 4 ml of melted 0.7% CPG agar at 2×10^6 cfu/ml and poured over the original agar layer. Plates were incubated for 24 hr at 28 C and then checked for the absence of bacterial growth around each of the original colonies. Antibiotic sensitivity frequencies were calculated to determine the percentage of strains (85 total) that each indicator strain was sensitive to, and antibiotic production frequencies were calculated as the percentage of indicator strains that were sensitive to antibiotics produced by each strain. The 26 strains tested for both antibiotic sensitivity and production were compared, using Student's t test, to determine if strains isolated from tubers with brown rot or from soil differed in frequency of production or sensitivity to antibiotics.

Biovar tests. Strains were tested for ability to oxidize 13 different carbohydrates, as described previously (15).

Hypersensitive response test. All strains were tested for induction of a hypersensitive response in leaves of tobacco (*Nicotiana tabacum* L. 'Bottom Special'). Intercostal leaf areas of fully expanded leaves were infiltrated with 10^9 cfu/ml suspensions of each strain by means of a syringe fitted with a 27-gauge needle. Plants were kept at 28–30 C in a growth chamber set for a 14-hr photoperiod with an average light intensity of 350 $\mu E \cdot s^{-1} \cdot m^{-2}$. Leaves were examined for appearance of a typical hypersensitive response (tissue collapse) after 24 hr.

RESULTS

Pathogenicity tests. All strains caused wilting of eggplant. Each of the eight strains caused severe wilting of tomato cultivar Rutgers at 19 days after inoculation (Fig. 1), but significant differences ($P \leq 0.05$) in strain aggressiveness were not apparent (Table 1). Disease progress appeared slower in tomato plants inoculated with strain Br5, however.

Significant differences in aggressiveness of the strains were apparent in each of the tests on the potato cultivars (Table 2). By 3–5 wk after inoculation, most of the strains caused less disease in cultivar Ontario (Fig. 2A) than in cultivar Atlantic (Fig. 2B). Some strains (e.g., S4, Br1, Br2, and Br4) were highly aggressive on both cultivars, whereas strains S1 and S5 were highly aggressive on Atlantic but not on Ontario plants. Strains Br3 and Br5 were less aggressive than strains S1, Br1, Br2, Br4, and S4 when inoculated on both cultivars.

Antibiosis typing. Strains of *P. solanacearum* differed substantially in antibiotic production and sensitivity. When all 85 strains were tested for antibiotic production against 26 indicator strains, 24

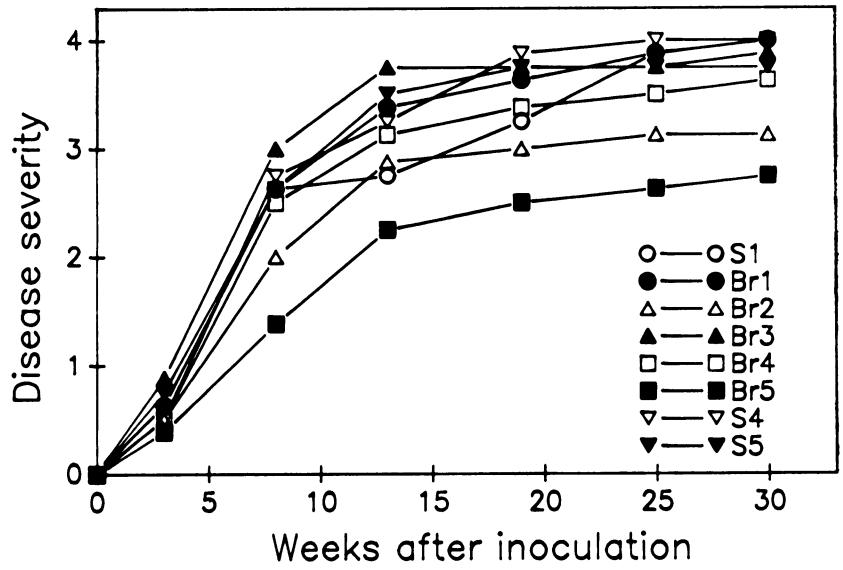

Fig. 1. Disease progress in plants of tomato cultivar Rutgers after stem inoculation with Florida strains of *Pseudomonas solanacearum*. Disease severity scale: 0 = no disease, 1 = 1–25% of the leaves wilted, 2 = 26–50% of the leaves wilted, 3 = 51–75% of the leaves wilted, and 4 = 76–100% of the leaves wilted (17).

Table 1. Analysis of variance[a] for the effect of strain of *Pseudomonas solanacearum* on disease severity in tomato cultivar Rutgers[b]

Source	df	Mean squares	*F* value	*P* value
Strain[c]	7	7.929	2.00	0.0710
Replicate (strain)	56	3.961	8.70	0.0001
Time	5	87.235	191.64	0.0001
Strain × time	35	0.315	0.69	0.9054
Error	280	0.455	···	···

[a] Univariate analysis of variance for repeated measures over time using type III sums of squares of the SAS general linear models procedure (1,22).
[b] Six-week-old plants were inoculated by the stem puncture technique (19) with a 10^9 cfu/ml suspension of each strain, placed in a 24 to 32 C greenhouse, and rated for disease severity at 3, 7, 13, 19, 25, and 30 days after inoculation. Disease severity scale: 0 = no disease, 1 = 1–25% of the leaves wilted, 2 = 26–50% of the leaves wilted, 3 = 51–75% of the leaves wilted, and 4 = 76–100% of the leaves wilted (17).
[c] Test of hypothesis that strains were similar in aggressiveness by type III sums of squares (1) using the means squares value for replicate (strain) effects as the error term.

Table 2. Analysis of variance[a] for the effect of strain of *Pseudomonas solanacearum* on disease severity in potato cultivars Ontario and Atlantic[b]

Cultivar	Source	df	Mean squares	*F* value	*P* value
Ontario	Strain[c]	7	16.53	7.14	0.0001
	Replicate (strain)	23	2.31	6.51	0.0001
	Time	4	20.99	57.81	0.0001
	Strain × time	28	1.81	5.06	0.0001
	Error	92	0.36	···	···
Atlantic	Strain[c]	7	10.34	21.73	0.0001
	Replicate (strain)	23	0.93	1.95	0.0134
	Time	4	33.02	69.34	0.0001
	Strain × time	28	0.95	1.99	0.0076
	Error	92	0.48	···	···

[a] Univariate analysis of variance for repeated measures over time using type III sums of squares of the SAS general linear models procedure (1,22).
[b] Three-week-old plants (from sprouted tubers) were inoculated by the stem puncture technique (19) with a 10^9 cfu/ml suspension of each strain, placed in a 24 to 32 C greenhouse, and rated for disease severity at 1, 2, 3, 4, and 5 wk after inoculation. Disease severity scale: 0 = no disease, 1 = 1–25% of the leaves wilted, 2 = 26–50% of the leaves wilted, 3 = 51–75% of the leaves wilted, and 4 = 76–100% of the leaves wilted (17).
[c] Test of hypothesis that strains were similar in aggressiveness by type III sums of squares (1) using the means squares value for replicate (strain) effects as the error term.

groups of strains differing in antibiotic production were obtained (Table 3). Forty-six of the strains belonged to four groups (groups 11, 16, 18, and 22) that were distinctly different in antibiotic production against three indicator strains: Br11, Br12, and S4. Although most of the strains (groups 8–24) produced antibiotics against only a few strains (≤27%), 14 (groups 1–7) produced antibiotics against more than half (≥54%) of the strains tested. In paired comparison tests, Br strains showed significantly greater ($P \leq 0.05$) antibiotic production and sensitivity frequencies than did S strains. Strains from diseased tubers had sensitivity and production frequencies of 0.204 and 0.306, respectively; strains from soil samples had sensitivity and production frequencies of 0.133 and 0.215, respectively.

Thirteen strains from Florida, selected at random, were tested for antibiotic production and sensitivity against 10 exotic strains of *P. solanacearum*. The Florida strains typed similarly against seven of the 10 strains (*data not shown*). The strains could be grouped into four separate phenotypes based on their reaction toward the remaining three exotic strains. On the basis of antibiotic production, nine of the strains belonged in group 1, two in group 2, and one each in groups 3 and 4. On the basis of antibiotic sensitivity, seven of the strains belonged in group 1, three in group 2, two in group 3, and one in group 4.

Hypersensitive response. All Florida strains induced a typical hypersensitive response in leaves of tobacco cultivar Bottom Special within 24 hr after infiltration.

Biovar characterization. Twenty-three of the strains were tested for ability to oxidize 13 carbohydrates (Table 4). The strains were typical of biovar I (15). The strains did not oxidize disaccharides or hexose sugar alcohols, except for mannitol, where weak oxidation was observed. Some strains weakly oxidized galactose and arabinose; only two of the strains oxidized lactose.

DISCUSSION

All strains of *P. solanacearum* that were isolated from northeastern Florida were pathogenic in eggplant and tomato. They belong to race 1 (5,12), the only race of *P. solanacearum* that has been reported to occur in Florida (32). Phenotypic variability among the strains was evident in three of the four tests, however. The most striking differences were in the pathogenicity tests on potato and the antibiosis tests. Although there were minor differences in ability of the strains to oxidize certain carbohydrates, all strains had the typical oxidation pattern of biovar I (15). Biovar I is a very heterogeneous group in which at least nine biochemical subbiovars have been proposed (14).

Based on the pathogenicity tests on potato, there appeared to be three different groups of strains: 1) those weakly aggressive in both potato cultivars (strains Br3 and Br5); 2) those strongly aggressive in both potato cultivars (strains S1, S4, Br1, Br2, and Br4); and 3) those strongly aggressive on potato cultivar Atlantic but weakly aggressive on potato cultivar Ontario (strain S5). In general, strains were more aggressive in tomato and eggplant than in potato.

The variability of aggressiveness of *P. solanacearum* strains from this field may have resulted from its use for potato cultivar trials during the past 20 consecutive years. Strains that carry genes for pathogenicity or aggressiveness toward different potato cultivars may have been selected and maintained in the population because of the presence of certain resistant or tolerant cultivars from year to year. Strains isolated from different areas of Florida have been reported as similar in pathogenicity toward potato, tomato, and eggplant but variable in patho-

Table 3. Antibiotic production groups among 85 strains of *Pseudomonas solanacearum* from Florida when tested against a random subset of 26 strains from the same location[a]

Antibiotic production group	Number of producing strains[b]	Sensitivity of indicator strains[c]												Antibiotic production frequency[d]
		1	2	3	4	5	6	7	8	9	10	11	12	
1	4	+	+	−	+	+	−	+	+	+	+	−	+	0.88
2	1	+	+	+	+	−	−	+	+	+	+	−	+	0.88
3	2	−	−	+	−	−	+	+	+	+	+	−	+	0.81
4	1	+	+	−	+	−	−	−	+	+	+	−	−	0.77
5	1	+	+	−	−	−	−	+	+	+	+	−	−	0.77
6	4	−	−	+	−	−	−	+	+	+	+	−	−	0.77
7	1	+	+	+	−	−	−	−	+	+	−	−	−	0.54
8	1	+	+	+	+	+	+	−	−	−	−	+	−	0.27
9	3	+	+	+	+	+	+	+	−	−	−	−	−	0.27
10	3	+	+	+	+	−	+	−	−	−	−	+	−	0.23
11	10	+	+	+	+	+	+	−	−	−	−	−	−	0.23
12	5	+	+	+	+	+	−	−	−	−	−	−	−	0.19
13	1	+	+	+	+	−	+	−	−	−	−	−	−	0.19
14	1	−	−	+	+	+	−	−	+	−	−	−	−	0.19
15	1	+	+	−	+	+	+	−	−	−	−	−	−	0.19
16	6	+	+	+	+	−	−	−	−	−	+	−	−	0.38
17	4	+	+	+	−	−	−	+	−	−	−	−	−	0.15
18	9	+	+	+	+	−	−	−	−	−	−	−	−	0.15
19	1	+	−	+	−	+	+	−	−	−	−	−	−	0.15
20	1	+	+	−	−	−	−	+	−	−	−	−	−	0.12
21	1	+	−	+	−	+	−	−	−	−	−	−	−	0.12
22	21	+	+	+	−	−	−	−	−	−	−	−	−	0.12
23	2	+	−	−	+	−	−	−	−	−	−	−	−	0.08
24	1	+	−	−	−	−	−	−	−	−	−	−	−	0.04
Antibiotic sensitivity frequency[e]		0.92	0.87	0.87	0.56	0.32	0.26	0.24	0.18	0.16	0.22	0.05	0.08	
Number of strains per antibiotic sensitivity group		1	1	1	1	1	1	1	2	9	6	1	1	

[a]Bacterial strains tested for antibiosis on CPG agar (11) using a modification of the soft agar overlay technique of Chen and Echandi (7).
[b]Number of *P. solanacearum* strains showing a unique phenotype for antibiotic production when tested against the indicator strains.
[c]In each column, *P. solanacearum* strains are grouped that have a unique phenotype for antibiotic sensitivity. + = Sensitive, − = insensitive.
[d]Percentage of 26 indicator strains sensitive to antibiotics produced by each strain.
[e]Percentage of 85 strains to which each indicator strain was sensitive.

genicity toward pepper, ginger, and sunflower (32). In Japan, Okabe and Goto (26) reported on variation in pathogenicity of *P. solanacearum* strains isolated from field soils artificially infested with different strains (lysotypes) of *P. solanacearum*. When eggplant, tobacco, and tomato were planted at single sites, strains that differed in pathogenicity (pathotypes) toward each plant species were recovered. The authors postulated that this type of variation occurs naturally in *P. solanacearum*. The appearance of strains that are highly pathogenic to banana in certain areas of Central America, where the pathogen is endemic on native *Heliconia* spp., is also indicative of natural variation within the indigenous population of the bacterium (4).

Based on our observations, the commonly observed "breakdown" of resistance (30) in the field may be due to selection of strains with different levels of aggressiveness from a highly diverse population of the bacterium in the soil. Indigenous heterogeneity of *P. solanacearum* populations may also explain why it has been so difficult to obtain stable sources of resistance to bacterial wilt in potato (30).

Significant strain diversity was also evident in the production of antibiotics, particularly among the Florida strains (Table 3). The antibiotics produced by these strains may be similar to bacteriocins reported in *P. solanacearum* (10). Smidt and Vidaver (28) have also shown that strains of *Clavibacter michiganense* subsp. *nebraskense* (Schuster et al) Davis et al, isolated from a single 1-ha field, vary in the production of bacteriocins. In both studies, a large proportion of the strains produced antibiotics, indicating it is plausible that this phenotype may have a functional role in the ecology of bacteria. The ability to produce antibiotics may be important for survival and may give a competitive advantage to certain strains for infection and colonization of host tissue. In studies with other phytopathogenic bacteria, antibiotic (in some cases bacteriocin) production affected the relative population of certain strains in mixed-inoculation tests (2,24). Thus, biological control may be dependent on the ability of a particular strain to produce antibiotics.

Recent studies on biological control of bacterial wilt have involved the use of antibiotic-producing, avirulent strains of *P. solanacearum* (6,8,19,20,31). In view of the striking amount of heterogeneity among natural populations for antibiotic production and sensitivity, it would be appropriate to assess potential biological control agents for ability to inhibit the growth of all strains present in the area(s) where the agent is to be tested and to be certain that the agent is not sensitive to the antibiotics produced by indigenous strains.

Table 4. Carbohydrate oxidation patterns of 23 Florida strains of *Pseudomonas solanacearum*[a]

Carbohydrate	Oxidation pattern (no. of strains)[b] I (15)	II (4)	III (2)	IV (2)
Glucose	+	+	+	+
Sucrose	+	+	+	+
Fructose	+	+	+	+
Galactose	+	+	±	+
Arabinose	+	±	+	+
Glycerol	+	+	+	+
Inositol	+	+	+	+
Maltose	−	−	−	−
Lactose	−	−	−	+
Cellobiose	−	−	−	−
Mannitol	±	±	±	±
Sorbitol	−	−	−	−
Dulcitol	−	−	−	−

[a]Bacterial strains grown for 2 wk at 24 C on Hayward's basal medium (15) containing sugar concentrations of 1% (w/v).

[b]Cultures kept at room temperature and scored for color change 2 wk after inoculation. + = Positive, − = negative, ± = weak positive.

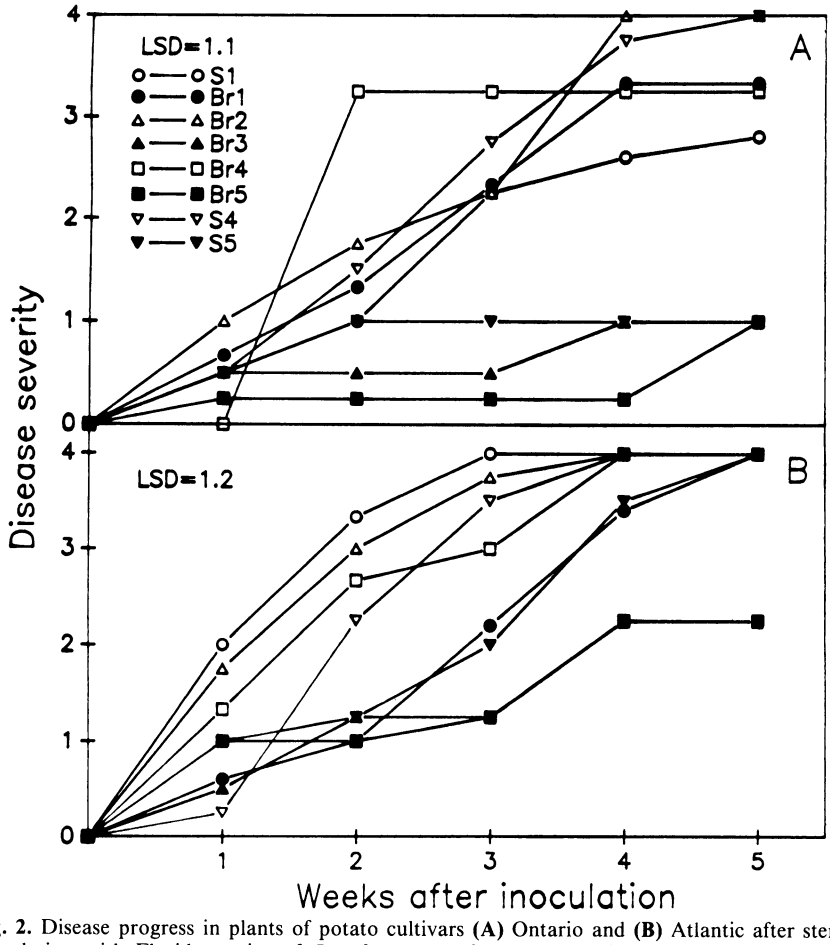

Fig. 2. Disease progress in plants of potato cultivars **(A)** Ontario and **(B)** Atlantic after stem inoculation with Florida strains of *Pseudomonas solanacearum*. Disease severity scale: 0 = no disease, 1 = 1–25% of the leaves wilted, 2 = 26–50% of the leaves wilted, 3 = 51–75% of the leaves wilted, and 4 = 76–100% of the leaves wilted (17). LSD = least significant difference ($P = 0.05$) calculated by repeated measures analysis of variance with the general linear models procedure of SAS.

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