Pseudomonas viridiflava Associated with Root and Crown Rot of Alfalfa and Wilt of Birdsfoot Trefoil

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ABSTRACT

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Strains of *Pseudomonas viridiflava* were associated with crown and root rot of alfalfa (*Medicago sativa*) and wilt of birdsfoot trefoil (*Lotus corniculatus*). Inoculation studies demonstrated that a test strain was capable of inducing necrosis and stunting of the roots of alfalfa, red clover (*Trifolium pratense*) and birdsfoot trefoil when the roots were wounded before inoculation. These studies also showed that the alfalfa strain induces severe root and crown rot of alfalfa.

In 1977, a survey was completed in Pennsylvania of microorganisms associated with crown and root rots of alfalfa (*Medicago sativa* L.). Specific fungal and bacterial populations were isolated on common microbiological media including Nash and Snyder's medium (16), acidified potato-dextrose agar, yeast extract carbonate agar (YDCA) (12), King's medium B (8), and nutrient agar (18). Green fluorescent pseudomonads producing blue extracellular pigment on YDCA were isolated from two of these samples. In preliminary investigations, we demonstrated that these organisms induced a hypersensitive response in tobacco leaves and rotting of potato slices; these responses indicated a potential role for these organisms in the root rot complex and provided the rationale to identify the causal organism. Based on the preliminary results of both physiological and nutritional experiments, we hypothesized that the bacterium was *Pseudomonas viridiflava* (3,4).

In 1978, bacteria with similar characteristics were isolated from alfalfa obtained near the original site in Wyoming County, PA. In 1980, isolations from mature birdsfoot trefoil (*Lotus corniculatus*) plants with wilt symptoms from Centre County, PA, yielded similar bacteria.

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This report summarizes the biochemical, physiological, and pathological tests that show that the bacteria isolated from the diseased alfalfa and birdsfoot trefoil are pathogenic and identical to *P. viridiflava*.

MATERIALS AND METHODS

The pathogen. The original strains were from alfalfa cultivar WL-210 seeded in 1971. Plants selected randomly in a production field were kept under ice until examinations and isolations were completed. Isolations were made from the section of upper taproot extending from 2.5 to 3.5 cm below the crown. Each root section was surface-sterilized, samples of internal tissue were comminuted in sterile 0.1 M phosphate buffer (pH 7.0), and loopfuls of the suspension were streaked onto plates of King's medium B and YDCA.

P. viridiflava strains 1208, Pv5 (ATCC 13222), 1209, and Pv6 (ATCC 13273), obtained from R. S. Dickey, Department of Plant Pathology, Cornell University, and *P. syringae* pv. *tomato* strains Pt22 and Pt66, obtained from S. W. Kim, Pennsylvania Department of Agriculture, Bureau of Plant Industry, Harrisburg, were used to confirm the interpretation of the biochemical and physiological reactions of the unknown organism.

Biochemical and physiological tests. Levan production and the presence of arginine dihydrolase activity, nitrate reduction, gelatin liquefaction, 2-ketogluconate production, and oxidase reaction were tested as described by Misaghi and Grogan (15). Tests for the presence of lipase and the utilization of citrate were conducted as described by Skerman (20). Pectin liquefaction at pH 5 and 8.5 were determined using the method described by Hildebrand (6). Nutritional tests were conducted with the basal medium and technique described by Lukezic (12).

The guanine plus cytosine mole ratio (%GC) in the DNA was determined using the purification procedure of Marmur (14) and the ultraviolet absorbance-temperature profile method of Mandel and Marmur (13). Thermal melting values were calculated as described by Knittil et al (9). Determinations were completed on three strains.

Pathogenicity tests. For these tests, bacteria stored in sterile tap water were transferred to YDCA and increased for 24 hr before use. The roots of 1-yr-old greenhouse alfalfa plants, cultivar Saranac, were freed of potting soil by washing. A 1.5-mm-diameter hole was drilled 6.5 mm deep directly into the crown from the top toward the root. About 0.1 ml of a test suspension (10×10^6 cells per milliliter) of the bacteria in sterile tap water was placed into the hole. After inoculation, the plants were repotted and replaced in the greenhouse. Six uninoculated control plants were similarly drilled and the holes filled with surface water from sterile medium. Each strain was inoculated into six plants; 30 days later, the roots were washed and evaluated for disease development.

Virulence of an alfalfa strain of the test bacteria on alfalfa cultivar Arc, birdsfoot trefoil cultivar Dawn, and red clover (*Trifolium pratense* L.) cultivar Kenland was tested using the slant-board technique of Leath and Kendall (10). A birdsfoot trefoil strain was tested on birdsfoot trefoil. The test plants were grown in perlite for about 14 days, then transferred to slant-board units and kept saturated with a nutrient solution. Two weeks later, the roots were spread and a single root was inoculated with the test strain by stabbing with a 26-gauge hypodermic needle. Four plants were inoculated with each strain. One week later, the roots were evaluated for necrosis, water-soaking, and elongation. Preliminary tests conducted with bacterial suspensions similar to those used in the mature alfalfa studies were inconclusive because the bacteria were apparently flushed from the inoculation sites by the nutrient solution. This problem was eliminated by stabbing roots with a needle dipped directly into bacteria colonies from young cultures.

Stems of greenhouse-grown lettuce (*Lactuca sativa*), radishes (*Raphanus sativus*), tobacco (*Nicotiana tabacum*), and tomato (*Lycopersicon esculentum* Mill.) and immature fruit of bean (*Phaseolus vulgaris*), green pepper

(*Capsicum frutescens*), and tomato were inoculated by injection with a suspension of the test bacteria. The inocula in sterile tap water were adjusted to 20% transmission at 420 nm (10^9 cells per milliliter) before use. Plastic bags were placed over the plants on a greenhouse bench for 24 hr. The fruit were placed for 24 hr in an incubation chamber with the humidity maintained near saturation. Disease development was evaluated 5 days later by measuring the length of the necrotic lesions that formed at the inoculation site.

RESULTS

Symptoms. The original alfalfa plants that yielded the bacteria had a light brown dry rot of the crown and upper taproot. Streaks of light brown extended beyond the rot into the vascular system for about one-third of the root. In birdsfoot trefoil, the tops were wilted and the crowns yellowish gray. Isolations on Nash and Snyder's medium and acidified potato-dextrose agar did not yield fungi. Mixtures of bacteria, however, including some green fluorescent colonies on King's medium B, were detected when subsamples of the plant material were plated on media suitable for their growth.

The pathogen. Six strains of a *Pseudomonas* sp. that produced a gray-blue pigment were isolated from the rotted crowns of alfalfa plants and crowns of wilted trefoil plants. These strains produced convex rugose colonies on YDCA. This character, however, was not stable because the colonies became small, convex, and cream-colored after repeated transfers. The gray-blue pigment faded as the colonies aged. On King's medium B, the colonies were opaque, convex, shiny, and semifluid after 48 hr and produced a bright green, diffusible, fluorescent pigment. The %GC mole ratio in the DNA of three alfalfa strains was 59. Biochemical and nutritional tests (Table 1) identified these strains as *P. viridiflava* according to the scheme used by Billing (1) and Sands et al (17). We have noted strain PSU 531 and PSU 670 as typical of the strains tested.

Pathogenicity tests. In inoculated alfalfa crowns, the periderm adjacent to the inoculation site collapsed and turned dark brown (Fig. 1). Below the inoculation site, the cambial area was dark brown but the periderm retained the original shape and color. The discolored cambial area formed a cylinder around the xylem tissue. Inside the cylinder next to the inoculation site, the xylem rotted and turned light brown. The test strain was reisolated from the discolored material. The crown tissue adjacent to the control sites was discolored; however, this did not extend more than 2 mm beyond the inoculation site. Typical saprophytic bacteria were isolated from this tissue.

Injection of the forage bacterial strains into the stems of tomato plants caused a confined brown wet rot of the cortical

Table 1. Tests used to compare strains of bacteria isolated from alfalfa and birdsfoot trefoil with *Pseudomonas viridiflava*

Tests	Alfalfa isolate PSU531	Trefoil isolate PSU670	*P. viridiflava* Cornell PV5 + PV6	*P. syringae* pv. *tomato* PT22 + PT66
Oxidase[a]	–[b]	–	–	–
Potato rot[c]	+	+	+	–
Levan[a]	–	–	–	+
Arginine dihydrolase[a]	–	–	–	–
Nitrate reduction[a]	–	–	–	–
Gelatin liquefaction[a]	+	+	+	+
2-Ketogluconate[a]	+	+	+	+
Lipases[d]	+	+	+	–
Pectinases[e]	+	+	+	–
Utilization as carbon and nitrogen source[c]				
Alanine	–	–	–	–
DL-Asparagine	+	+	+	+
Ethanolamine	–	–	–	–
DL-Threonine	–	–	–	–
Utilization as carbon source[c]				
Adonitol	–	–	–	–
D-Arabinose	–	–	–	–
L-Arabinose	+	+	+	+
iso-Butyrate	–	–	–	–
Citrate	+	+	+	+
Dextrose	+	+	+	+
Ethanol	–	–	–	–
Homoserine	–	–	–	–
D-(–)-Lactate	+	+	+	+
L-(–)-Lactate	+	+	+	+
L-(–)-Lysine	–	–	–	–
Malonate	+	+	+	+
DL-Propionate	–	–	–	–
Sodium propionate	–	–	–	–
Saccharin	–	–	–	–
D-Sorbitol	+	+	+	+
Sucrose	–	–	–	+
D-(–)-Tartrate	+	+	+	+
L-(+)-Tartrate	–	–	–	–
Trehalose	–	–	–	–

[a] As described by Misaghi and Grogan (15).
[b] – = Negative reaction or the same as controls; + = positive reaction.
[c] As described by Lukezic (12).
[d] As described by Skerman (20).
[e] As described by Hildebrand (6).

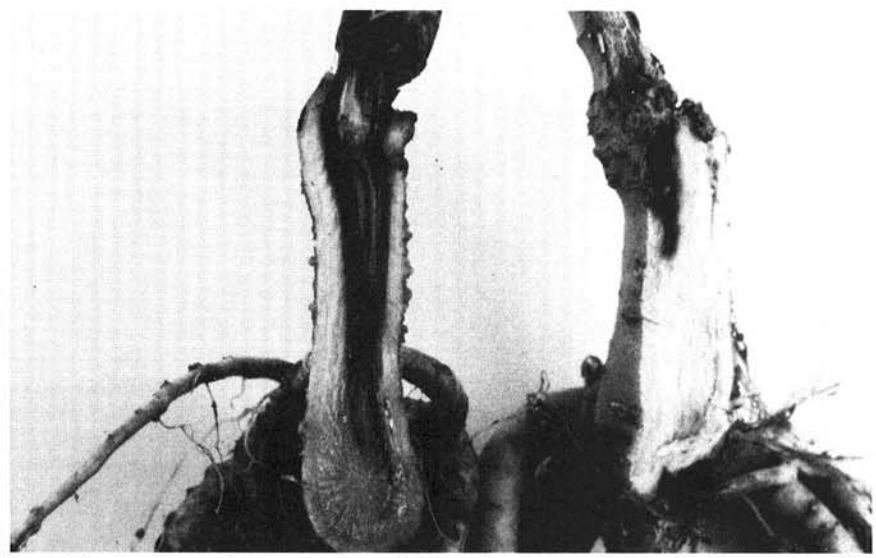

Fig. 1. Discoloration and rot present in an alfalfa root and crown 30 days after inoculation with a strain of *Pseudomonas viridiflava* from alfalfa. The plant on the right was treated with sterile water.

Table 2. Necrosis of plant tissues wound-inoculated with *Pseudomonas* strains[a]

Plant tissue	Bacterial strains: Alfalfa strain PSU531	Trefoil strain PSU670	*P. viridiflava* Cornell PV6	*P. syringae* pv. *tomato* PT22	Control
Alfalfa crown	58	14	ND[b]	ND	6
Tomato stem	4	4	0	45	1
Tobacco stem	0	40	0	40	0
Lettuce stem	20	10	0	20	0
Tomato fruit	5	0	0	15	0
Bean fruit pod	20	20	10	50	0
Pepper fruit	25	50	0	ND	0
Tobacco Leaves	+[c]	+	−	+	−

[a] Length of necrosis in millimeters, including the inoculation site. Means of six sites.
[b] ND = Not determined.
[c] + = Hypersensitive response; − = did not produce a hypersensitive response.

Table 3. Extent of necrosis and root elongation of three forage legumes 1 wk after inoculation with strains of *Pseudomonas viridiflava*[a]

	Alfalfa cv. Arc		Birdsfoot trefoil cv. Dawn		Red Clover cv. Kenland	
	Necrosis (cm)	Root elongation (cm)	Necrosis (cm)	Root elongation (cm)	Necrosis (cm)	Root elongation (cm)
Alfalfa strain (PSU 531)	0.5[b,c]	7.1	0.8	4.0	2.0	3.0
Birdsfoot strain (PSU 6570)	ND[d]	ND	0.3	10.9	ND	ND
Control	0.1	15.3	0	14.3	0.1	15.5

[a] Plants were grown using the slant-board method of Leath and Kendall (10).
[b] Length of necrosis was the amount of tissue showing discoloration and water-soaking after inoculation by stabbing with a teasing needle covered with bacteria cells from a 24 hr culture. Each inoculation site was 2 cm from the root tip. Root elongation was determined by measuring from the inoculation site to the root tip at the time of evaluation.
[c] Means of four sites.
[d] ND = Not determined.

tissue. The necrosis was minor, however, compared with the necrosis induced by *P. syringae* pv. *tomato* (Table 2). In tobacco, the trefoil strain caused necrosis equal to the *P. syringae* pv. *tomato* strain, but the alfalfa strain was avirulent. The main veins of lettuce were damaged equally by the alfalfa strain and *P. syringae* pv. *tomato*. In tomato fruit, only the alfalfa strain and *P. syringae* pv. *tomato* caused necrosis. The alfalfa strain induced necrosis was similar to that induced by *P. syringae* pv. *tomato* but not nearly as extensive. Water-soaked soft brown lesions were induced in bean fruit pods and pepper fruit by the test strains used. The type strain (Cornell Pv6) was avirulent on all of the test plants except the bean fruit pods, in which it induced water-soaked soft brown lesions. Of the strains tested, only the type strain did not induce a hypersensitive response in tobacco leaves (Table 2).

The alfalfa strain induced small necrotic lesions surrounded by water-soaked areas in wounded tissue in the young roots of alfalfa, red clover, and trefoil grown on slant boards. In addition to the necrotic lesions, the alfalfa strain reduced root elongation to about one-third of that which occurred in the controls (Table 3). The trefoil strain affected root elongation only mildly but produced necrotic lesions in the trefoil roots.

DISCUSSION

The bacterial strains from alfalfa and trefoil were morphologically, biochemically, and physiologically identical to authentic strains of *P. viridiflava*. *P. viridiflava* is apparently an opportunistic pathogen of a wide variety of plants (3,7,21,22) in which it causes leaf and fruit spots. With the exception of the report by Hunter and Cigna (7), however, this is the first report of *P. viridiflava* associated with root disorders of growing plants. Hunter and Cigna (7) induced necrosis and rot of parsnip roots but were unable to recover the test strain consistently. We were able to recover the test strain from inoculated material, except when the bacterial suspension was used to inoculate alfalfa roots using the slant-board method.

In contrast with Wilkie et al (22) but in agreement with Burkholder (3) and Lelliott et al (11), we had a great deal of difficulty in maintaining virulence. Passing the strains through compatible hosts and reisolating them just before screening helped, but we were unable to revive the virulence of the Cornell strains or increase the virulence of the trefoil strains to the level of the alfalfa strains. These strains were virulent when used by Burkholder (3).

Just how widespread *P. viridiflava* is in the alfalfa-growing areas is not known. Nevertheless, the observation that the alfalfa strains could be isolated from the same site at two different growing seasons indicates persistance and involvement of the bacteria with the plant. Unfortunately, *P. viridiflava* is easily confused with *P. marginalis* when observed cursorily on King's medium B. Because *P. marginalis* is commonly present in diseased alfalfa crowns (2), it is easy to overlook the occurrence of *P. viridiflava* in field surveys. Separation can be made by the oxidase test or by culturing the strains on YDCA. Although rarely encountered on alfalfa roots, the green fluorescent bacteria of the *P. syringae* nomenspecies can be separated from those of the *P. viridiflava* group by the absence of pectinase in the former.

Bookbinder et al (2) proposed that *P. viridiflava* may have a synergistic relationship with certain endoparasitic nematodes that would contribute to the root rot complex of alfalfa.

The observation that *P. viridiflava* has a role in crown decay of forage legumes confirms recent articles reporting that bacteria can have a primary role in these disorders (5,19).

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LITERATURE CITED

1. Billing, E. 1970. *Pseudomonas viridiflava* (Burkholder, 1930; Clara, 1934). J. Appl. Bacteriol. 33:492-500.
2. Bookbinder, M. G., Bloom, J. R., and Lukezic, F. L. 1982. Interactions among selected endoparasitic nematodes and three pseudomonads on alfalfa. J. Nematol. 14:105-109.
3. Burkholder, W. H. 1930. The bacterial diseases of the bean: A comparative study. Cornell Univ. Agric. Exp. Stn. Mem. 127:1-88.
4. Dowson, W. J. 1939. On the systemic position and generic names of the Gram-negative bacterial plant pathogens. Zentralbl. Bakteriol. Parasitenkd. und Infektionskr. Infektionskr. II Abt. C; 177-193.
5. Gaudet, D. A., Sands, D. C., Mathre, D. E., and Ditterline, R. L. 1980. The role of bacteria in the root and crown rot complex of irrigated sainfoin in Montana. Phytopathology 70:161-167.
6. Hildebrand, D. C. 1971. Pectate and pectin gels for differentiation of *Pseudomonas* sp. and other bacterial plant pathogens. Phytopathology 61:1430-1436.
7. Hunter, J. E., and Cigna, J. A. 1981. Bacterial blight incited in parsnip by *Pseudomonas marginalis* and *Pseudomonas viridiflava*. Phytopathology 71:1238-1241.
8. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.
9. Knittil, M. D., Black, C. H., Sandine, W. E., and Fraser, D. K. 1968. Use of normal probability paper in determining thermal melting values of deoxyribonucleic acid. Can. J. Microbiol. 14:239-245.
10. Leath, K. T., and Kendall, W. A. 1978. Fusarium root rot of forage species: Pathogenicity and host range. Phytopathology 68:826-831.
11. Lelliott, R. A., Billing, E., and Hayward, A. C. 1966. A determinative scheme for the fluorescent

plant pathogenic pseudomonads. J. Appl. Bacteriol. 29:470-489.
12. Lukezic, F. L. 1979. *Pseudomonas corrugata*, a pathgoen of tomato, isolated from symptomless alfalfa roots. Phytopathology 69:27-31.
13. Mandel, M., and Marmur, J. 1968. Use of ultraviolet absorbance-temperature profile for determining the guanine plus cytosine content of DNA. Methods Enzymol. 12:195-206.
14. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3:208-218.
15. Misaghi, I., and Grogan, R. G. 1969. Nutritional and biochemical comparisons of plant-pathogenic and saprophytic fluorescent pseudomonads. Phytopathology 59:1436-1450.
16. Nash, S. N., and Snyder, W. C. 1962. Quantitative estimations by plate counts of propagules of the bean root rot *Fusarium* in field soils. Phytopathology 52:567-572.
17. Sands, D. C., Schroth, M. N., and Hildebrand, D. C. 1980. Pseudomonas. Pages 36-44. in: Laboratory Guide for Identification of Plant Pathogenic Bacteria. N. W. Schaad, ed. American Phytopathological Society, St. Paul, MN. 72 pp.
18. Schaad, N. W. 1980. Identification schemes. Pages 1-11 in: Laboratory Guide for Identification of Plant Pathogenic Bacteria. N. W. Schaad, ed. American Phytopathological Society, St. Paul, MN. 72 pp.
19. Shinde, P. A., and Lukezic, F. L. 1974. Isolation pathogenicity and characterization of fluorescent pseudomonads associated with discolored álfalfa roots. Phytopathology 64:865-871.
20. Skerman, V. B. D. 1967. A Guide to the Identification of the Genera of Bacteria. 2nd ed. Williams and Wilkins Co., Baltimore, MD. 303 pp.
21. Suslow, T. V., and McCain, A. H. 1981. Greasy canker of poinsettia caused by *Pseudomonas viridiflava*. Plant Dis. 65:513-514.
22. Wilkie, J. P., Dye, D. W., and Watson, D. R. W. 1973. Further hosts of *Pseudomonas viridiflava*. N.Z. J. Agric. Res. 16:315-323.