Isolation, Pathogenicity and Characterization of Fluorescent Pseudomonads Associated with Discolored Alfalfa Roots

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ABSTRACT

Isolations made from discolored roots of stunted alfalfa plants of cultivars resistant to bacterial wilt caused by *Corynebacterium insidiosum* consistently yielded fluorescent pseudomonads and Erwinias. In greenhouse tests, alfalfa seedlings inoculated with the pseudomonads sometimes developed yellow leaves which later wilted or the entire plant suddenly collapsed and died.

The pseudomonads were nonspore-forming, gram negative, capsulated, rods with three to six polar flagella. All six isolates of pseudomonads reacted similarly to most of the physiological and biochemical tests to which they were subjected. The oxidase-positive, alfalfa pseudomonads differed significantly from *Pseudomonas chichorii* in seven of twelve physiological tests. They differ from *P. marginalis* only in their ability to cause hypersensitivity in tobacco, and in their inability to produce arginine dihydrolase. We think that the fluorescent pseudomonads from alfalfa roots are a variety of *P. marginalis* and propose the name *P. marginalis* var. *alfalfae*.

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During the fall of 1970, in areas of eastern Pennsylvania, alfalfa (*Medicago sativa* L.) cultivars normally considered resistant to bacterial wilt caused by *Corynebacterium insidiosum* were showing typical bacterial wilt symptoms. Diseased plants were stunted with small pale green leaves and a comparatively greater number of shoots. When the cortical tissue of the tap root was removed, the vascular cylinder showed a moist yellowish or pale brown discoloration. These symptoms, in most part, are typical of bacterial wilt caused by *C. insidiosum* (6, 9, 14, 19). However, sections of discolored roots cultured on beef-lactose agar (BLA), according to Kreitlow's method (18), in most cases did not yield the typical blue pigment producing *C. insidiosum* but consistently yielded fluorescent pseudomonads and Erwinias along with species of *Fusarium*.

In 1931, Zhavoronkova (30) described a bacterial disease of sweet clover, lentils, alfalfa, and sainfoin which she characterized as root-rot and withering. The bacterium was studied and named *Bacterium radiciperda* Jav. Rodigin and Petrov (23) confirmed the identity of the pathogen from sweet clover. Savulescu (25) proposed the name *Pseudomonas radiciperda* (Javoronkova) Savulescu in 1947. Unfortunately not enough physiological data was included in these references to determine classification by current practice. The problem is further compounded by Gorlenko's statement "*Bacterium radiciperda* described by I. P. Zhavoronkova is probably identical with *Corynebacterium insidiosum*" (10).

The study was undertaken to identify the two groups of gram negative bacteria isolated from discolored roots of stunted alfalfa plants along with species of *Fusarium* and to see whether they have an independent role as pathogens of alfalfa. Observations on various characters of *Erwinia* isolates and the results of interactions of these two groups of isolates with certain root pathogens of alfalfa will be reported separately elsewhere.

MATERIALS AND METHODS.—During the spring and fall of 1970 through 1972, stunted alfalfa plants with small, pale-green leaves and discolored roots, but no crown rot, were collected from the central, southeastern, and south-central areas of Pennsylvania. Sixty-one diseased plants and 15 apparently healthy plants were collected during the spring and summer 1972 and used for intensive isolation work. The plants were placed on ice immediately after digging. In most cases, samples were cultured on the day following collection; however, when delays occurred the cleaned roots were stored at −10 C until cultures could be made.

Isolations were made during 1970-1971 on beef-lactose agar (BLA). In the majority of cases isolations did not yield the typical blue pigment-producing *C. insidiosum*, but consistently yielded a white, non-pigmented bacterium. To assist in distinguishing between the white isolates and *C. insidiosum*, King's A and B media (15) were included in 1972 isolations. Acid potato-dextrose agar (APDA) was included to detect the presence of associated fungi. The samples for testing were taken from tap roots that had a yellowish or pale-brown discoloration of the woody cylinders. The samples were from a 5-cm-long piece of tap root taken 5.0 - 7.5 cm below the crown. The pieces were surface-sterilized with 1:500 mercury bichloride in 50% ethyl alcohol for 3-5 min depending on root diam, followed by three changes of sterile water (18). Each piece was transferred to a dry petri

plate; the cortex and thin-sections from both ends were removed and discarded. The remaining vascular cylinder was cut into five transverse sections and each section, in turn, cut into five pieces. One piece from each section was aseptically placed in petri dishes containing BLA, APDA, and King's A and B media. The remaining pieces were pooled for each sample and crushed in 5 ml of sterile water. After 15 min, a loopful of the suspension was streaked on BLA, and King's A and B media. The plates were incubated in the dark at 21 C. Observations on the presence of different organisms and subcultures to various media were made after 7 days of incubation.

Pathogenicity tests.—Isolations made from the discolored roots consistently yielded fluorescent pseudomonads and Erwinias, the former being more prevalent. Purification of the isolates was completed by selecting colonies from streak plates, then streaking these on fresh agar plates. This process was repeated three times. Ten isolates of fluorescent pseudomonads, selected on the basis of oxidase reaction and pigmentation on King's B medium, were used in preliminary pathogenicity tests. These were representative of the pseudomonads occurring in the roots. Twelve- to 15-day-old seedlings of alfalfa [cultivar (cv.) DuPuits] grown under sterile conditions in sand, in test tubes (25 × 200 mm) at 19-23 C, were inoculated with the different isolates. Before inoculation, the seedling roots were injured with a fine, sterile needle (3). One ml of a bacterial suspension (approximately 4.4×10^8 cells/ml) from a 3-day-old culture on nutrient yeast-dextrose agar (NYDA) (26) was added to each tube. Five tubes, containing four seedlings each, were inoculated with each isolate. The controls consisted of the same number of seedlings, treated in the same manner except that sterile water was used. Observations of symptoms were recorded 4 wk after inoculation.

Two inoculation tests were completed in the greenhouse. In the first, six isolates (P-1, P-2, P-3, P-4, P-5, and P-6) were used on the basis of their virulence, as determined in a preliminary pathogenicity test. Only isolates P-1, P-3, and P-6 were used in the second inoculation test. In both tests, 3- and 8-wk-old seedlings of cultivars DuPuits and Buffalo were inoculated according to the bare-root soak method of Cormac et al. (5). A suspension of bacteria in water from a 3-day-old culture on King's B medium diluted to approximately 4.4×10^8 cells/ml was used as inoculum. Twenty and 30 seedlings of each of the two cultivars were inoculated with each isolate in test 1 and 2, respectively. The controls were treated in the same manner, except that sterile water was used. The inoculated seedlings were transferred to the greenhouse bench after inoculation. To determine if the bacteria can affect other plants, young red clover (*Trifolium pratense* L. 'Pennscott'), tomato (*Lycopersicon esculentum* Mill.), sweet pepper (*Capsicum frutescens* var. *grossum* Sendt.), and eggplant (*Solanum melongena* L.) were inoculated using the root dip technique.

Observations on symptom development were made 10, 20, and 30 days after inoculation. Reisolations were made on King's B medium from recently wilted plants, and from plants showing other symptoms of infection such as yellowing, stunting, or root discoloration.

Characterization of the bacteria.—Origin of cultures.—Six representative isolates of the pseudomonads (P-1, P-2, P-3, P-4, P-5, and P-6) from the discolored roots of stunted alfalfa plants were selected on the basis of the results of the pathogenicity tests. Cultures of *P. marginalis, P. cepacia, P. caryophylii, P. alliicola, P. tabaci, P. marginata,* and *P. phaseolicola* were included as positive and negative checks in certain physiological and nutritional tests.

Stock cultures of the isolates were maintained in sterile tap water at room temp. Unless indicated otherwise, 48-h-old cultures of the pseudomonads grown on King's B medium were used to inoculate the test media. The inoculum consisted of one loopful of a bacterial suspension. Cultures in characterization tests were incubated in the dark at 27 C for the period specified under each test.

Morphological characters.—Young cultures (18-24 h) were used for morphological characterization. Gram morphology was determined by the method outlined by Skerman (27). Motility was checked by making hanging drop preparations of young broth and agar cultures. The presence of capsules was determined by Tyler's modification of Anthony's method (4). The pattern of flagellation was determined by electron microscopic observation of bacteria from 18- to 24-h-old cultures in nutrient dextrose broth (Difco nutrient broth 8 g/liter; dextrose 10 g/liter) that were negatively stained with 1.0% PTA (phosphotungstic acid) pH 7.0.

Biochemical and physiological tests.—Except for carbohydrates, which were sterilized by Millipore filtration, the test media were sterilized at 121 C for 15 min.

—1) Levan production.—Plates of Oxoid nutrient agar and Difco nutrient agar both containing 5.0% sucrose were streaked with the isolates. Three days after inoculation, isolates that produced large white, domed mucoid colonies were considered to be levan producers (20, 21).

—2) Oxidase production.—Kovac's method (17) was used to test for the presence of oxidase. A loopful of bacteria from 48-h-old cultures grown on nutrient agar (20) and King's B medium (24) was smeared on filter paper previously soaked with 1.0% (w/v) aqueous solution of N,N′-dimethyl-p-phenylene-diamine with a platinum needle. Production of a dark-purple color in 10 sec indicated the presence of oxidase.

—3) Potato soft-rot test.—Transverse sections, of potato tuber cylinders 7-8 mm thick and 15 mm in diam were placed aseptically in petri dishes, and sterile water was added until the sections were half immersed. Five sections per isolate were inoculated with a heavy bacterial suspension placed in a nick made in the center of each section. After 48 h, sections were tested for soft-rot by teasing. A positive result was recorded if most of the section had rotted (20, 21). At least five uninoculated sections from each potato tuber were included as controls.

—4) Arginine dihydrolase production.—About 10 ml of Thornley's arginine medium 2A (29) in screw-capped test tubes were sterilized by autoclaving. The pH was adjusted to 7.2 with NaOH. Duplicate tubes were stab-inoculated with 48-h-old cultures. One tube was sealed

with sterile, melted, and cooled Vaseline to a depth of 5 mm. The screw cap of the other tube was left loose. The anaerobic formation of alkali from arginine was detected by a change in color of the indicator after 4 days. This change suggested the presence of all or part of the arginine dihydrolase system (20, 21).

—5) Hypersensitivity reaction on tobacco leaves.—Older, lower leaves of tobacco (*Nicotiana tabacum* 'White Burley') were injected with a bacterial suspension using a modification of Klement's method (16). Two bacterial concns, from 48-h-old cultures, in sterile tap water adjusted to optical density 0.2 (approximately 1.1×10^8 cells/ml) and 0.4 (approximately 2.2×10^8 cells/ml) at 650 nm were used as inoculum. After leaf infiltration, the plants were transferred to a growth chamber and maintained at 21 C. Observations were recorded after 1, 2, 3, and 4 days after infiltration.

—6) Protease production.—Gelatin liquefaction was tested by stabbing tubes of Difco nutrient gelatin and checking 1, 5, 10, and 14 days after inoculation. The cultures were placed at 4 C for 30 min before recording the results. Liquefaction was positive if the medium flowed readily when tubes were tilted (20, 21).

Gelatin hydrolysis was confirmed by streaking duplicate plates of nutrient agar (Difco) with 0.4% gelatin with each isolate and testing with an acidic $HgCl_2$ solution (27).

—7) Amylase production.—Plates of nutrient agar containing 1.0% soluble starch were streaked once and incubated at 27 C. After 2, 4, and 10 days, one plate of each isolate was flooded with iodine solution. The presence of a clear area around the bacterial growth indicated starch hydrolysis.

—8) Lipase production.—The method of Misaghi and Grogan (21), using Tween 80 (polyoxyethylene sorbitan monooleate), was used to determine lipase production. The plates were streaked and incubated at 27 C. The presence of an opaque halo (due to precipitation of calcium oleate) around the bacterial growth within 7 days, indicated hydrolysis of the detergent.

—9) Nitrate reduction.—Tubes of nitrate broth (containing Difco Bacto peptone, 1.0%; nitrite-free KNO_3, 0.1%) were inoculated and incubated at 27 C. After 1 and 5 days, the cultures were tested for nitrate reduction following the method outlined by Shaffer (26).

—10) Acid from carbohydrates.—Ayers, Rupp, and Johnson's medium C (1) was used for acid production from sucrose. A 10.0% (w/v) aqueous solution of sucrose sterilized by Millipore filtration was added aseptically to the hot, sterile, basal medium to give a final concn of 1.0% (w/v). Slope cultures were made and a change in the indicator color to yellow after 2, 3, and 7 days was scored as a positive reaction (1, 20).

Production of acid and gas from glucose was determined according to the method outlined by Shaffer (26) using the brom cresol purple indicator instead of phenol red. Cultures were observed for acid and gas production after 4, 6, and 10 days.

—11) Pigment production.—Pigment production by pseudomonads was determined by streak inoculation of slopes of King, Ward, and Raney's medium A and medium B (15). Cultures were examined for pigmentation after 1, 2, 3, and 10 days of incubation at 27 C. Ultraviolet light was used to check for fluorescence.

—12) Catalase.—One ml of H_2O_2 (3%) (v/v) was poured over the surface of a 24-h-old agar slope culture. The release of bubbles of oxygen from the surface indicated the presence of catalase (27).

—13) Oxygen relations.—Duplicate tubes of deep NYDA were inoculated while in fluid condition at 45 C with an inoculum dilute enough to permit discrete colonies and rotated to mix the inoculum with the medium. Bacteria were considered aerobic if they were growing upon the surface or the upper layer of the medium. They were considered facultative anaerobic if they grew throughout medium (7, 26).

—14) Anaerobic fermentation of glucose.—The mode of carbohydrate metabolism was determined by the method of Hugh and Leifson (13). Duplicate tubes of the solidified medium about 4 cm in depth were stab-inoculated. After inoculation one of the pair of tubes was covered with a layer of sterile, melted, and cooled Vaseline to a depth of 2.5-10.0 mm. Cultures were examined after 20, 48, and 96 h for the type of glucose metabolism.

—15) Indole production.—Tubes of 1.0% tryptone (Difco) were inoculated with one drop of bacterial suspension. Strips of dry filter paper previously soaked in a saturated solution of oxalic acid were inserted with the cotton plugs in such a position that they did not touch the surface of the medium. The presence of indole was indicated by the pink color of the filter paper strips (26).

—16) Hydrogen sulphide production.—The production of hydrogen sulphide was tested by the lead acetate test-strip technique. The cultures were grown on peptone (Bacto-tryptone) water. The blackening of the edges of the test strip within 3 to 7 days indicated the production of hydrogen sulphide (4, 26).

—17) Methyl red test.—The ability of different isolates to produce acid from glucose in amounts sufficient to reduce the pH to 4.2 or less and to maintain this low pH for at least 4 days was determined by using the glucose-phosphate-peptone-water method outlined by Skerman (27). Cultures were tested for acid production 2 and 4 days after inoculation by adding a few drops of methyl red solution. The presence of red color was taken as a positive indication and yellow color as a negative indication.

—18) Voges-Proskauer reaction.—The ability of different isolates to produce acid from glucose and subsequently to convert it to acetylmethylcarbinol or 2:3-butylene glycol, both neutral substances, was determined by the method outlined by Skerman (27). Cultures grown in glucose-phosphate-peptone water were tested for the presence of a neutral substance with 1 ml of a 10.0% KOH solution, 2 and 4 days after inoculation. The presence of pink color was scored positive and no coloration, negative.

—19) Test for pectolytic enzymes.—The production of pectolytic enzymes was detected by pit formation on polypectate gel according to the method of Hildebrand (11). Plates of sodium polypectate medium adjusted to three different pH's (medium A pH 4.9 - 5.1; medium B pH 6.9 - 7.1; medium C pH 8.3 - 8.5) were stored for 3 days until the surface of the gels were dry. Duplicate plates, each with two isolates per plate, were hand inoculated

with masses of bacterial and incubated at 27 C. The activity of each isolate was rated according to the degree of pitting 6 days after inoculation.

—20) Nutritional tests.—The organic compounds tested as substrates were: cellobiose, salicin, sorbitol, glycine, L-alanine, L-valine, L-proline. The standard mineral base of Misaghi and Grogan (21) was used. Substrates containing both carbon and nitrogen were added singly (0.1%, w/v) to the standard mineral base to test for its utilization as sources of both carbon and nitrogen. Each of the sugars, (0.2%, w/v) was tested in a standard mineral base containing (0.1%, w/v) ammonium sulfate as the nitrogen source. All test media were solidified with (1.0%, w/v) Colab Epi agar. Sugars were sterilized by Millipore filtration and added to the medium before pouring. Plates containing about 30 ml medium were kept for 5 days for drying and detection of any contamination. Plates were hand-inoculated with four isolates per plate and incubated at 27 C. Control plates lacking a nitrogen or carbon source or both were similarly inoculated. The plates were scored visually for growth 2, 4, 6, and 21 days after inoculation.

TABLE 1. Percentage of dead plants of two cultivars of *Medicago sativa* L. 20 days after inoculation with fluorescent pseudomonads in the greenhouse

Isolate	Cultivar 'DuPuits'		Cultivar 'Buffalo'	
	Test I[a]	Test II[b]	Test I	Test II
P-1	35	3	45	0
P-2	55		15	
P-3	60	13	50	37
P-4	40		40	
P-5	45		30	
P-6	50	16	50	7
Check	0	0	0	0

[a]Percentage based on 20 plants/isolate, age of seedlings when inoculated 3 wk after planting.

[b]Percentage based on 30 plants/isolate, age of seedlings when inoculated 8 wk after planting.

TABLE 2. Activity of six isolates of fluorescent pseudomonads from the discolored alfalfa roots on polypectate gels at different pH levels

Isolate	Medium A[a] (pH 4.9-5.1)	Medium B (pH 6.9-7.1)	Medium C (pH 8.3-8.5)
P-1	MW[b]	MW	MW
P-2	MW	MW	MW
P-3	NP-S	MW	MW
P-4	S-MW	MW	MW
P-5	MW	MW	MW
P-6	MW	MW	MW

[a]Medium made and designated according to Hildebrand (10).

[b]Activity rated according to degree of pitting produced. MW = moderate to wide, indicates a pit with radius of > 1 mm from colony margin; S = slight, indicates a pit of < 1 mm in diameter from colony margin and NP = no pit formed.

RESULTS.—*Isolations.*—Twenty-eight of the 61 diseased plants yielded predominantly fluorescent pseudomonads and different *Fusarium* spp. Fluorescent pseudomonads alone were recovered from nine samples, whereas they were present in 12 plants along with a white, non-pigmented *Erwinia* and *Fusarium* spp. Fluorescent pseudomonads were present along with *C. insidiosum* and with *Erwinia* in one and three plants, respectively. One plant yielded *Erwinia* and *Fusarium* and in another plant *C. insidiosum* was present along with species of *Fusarium*. Nonfluorescent yellow bacteria along with species of *Fusarium* were recovered from three plants, while three plants yielded only species of *Fusarium*. In summary, 53 diseased plants yielded fluorescent pseudomonads. In the group of 15 apparently healthy plants, six yielded fluorescent pseudomonads alone and another six plants yielded fluorescent pseudomonads along with *Fusarium* spp. In two healthy plants, fluorescent pseudomonads were present along with *Erwinia* and species of *Fusarium*, while one sample yielded species of *Fusarium* and *Erwinia*.

Pathogenicity tests.—The preliminary pathogenicity test with 12- to 15-day-old seedlings in tubes showed that all 10 isolates of fluorescent pseudomonads produced symptoms ranging from moderate root necrosis to death of plants, within 4 wk of inoculation whereas the controls were free of any symptoms.

In the greenhouse inoculation tests, the pseudomonads caused a higher percentage of deaths in 3-wk-old seedlings in comparison with 8-wk-old seedlings (Table 1). The bacteria often caused sudden death of the plants, and in a few cases, gradual yellowing and wilting. The roots of dead plants in most cases had soft-rot of tap and feeder roots. Surviving plants of both cultivars in both tests appeared stunted and chlorotic. Control plants of both the cultivars had dark green leaves and were growing vigorously.

Reisolation made from newly wilted or yellowed plants usually yielded the bacterium originally inoculated.

The bacteria were not pathogenic on the other plant species tested under the conditions of the experiment.

Characterization of the bacteria.—

—1) Morphological characters.—All isolates of fluorescent pseudomonads were nonspore-forming, gram-negative, medium-sized rods with round to slightly pointed ends. All produced capsules and were motile in hanging drop preparations. Electron micrographs revealed the presence of three-to-six polar flagella.

—2) Physiological characterization tests.—The *Pseudomonas* spp. isolates were aerobic, as they grew in the upper layer and on the medium surface. They were levan-positive on both media and also produced a light-pink diffusible pigment on these media. The isolates were oxidase-positive on nutrient agar and King's B medium. On King's B medium, they produced a yellow-fluorescent pigment. In the test for lipase, the pseudomonads produced a faint, opaque halo in 3 days and a thin, light-pink opaque halo 5-8 mm wide in 7 days. The bacteria were able to show some reduction of nitrate 24 h after inoculation, and had completely reduced nitrate by the fifth day. They were catalase-positive.

The pseudomonads were strong liquefiers of gelatin. Complete liquefaction of gelatin medium was

accomplished within 14 days after inoculation. The bacteria produced a clear zone of 10-12 and 20-25 mm around the colony in 3 and 7 days after inoculation, indicating rapid hydrolysis of gelatin.

The isolates did not produce arginine dihydrolase, indole, or H_2S. The methyl red reaction, and Voges-Proskauer test results were also negative, even after 11 days of incubation.

The organisms produced a positive hypersensitive reaction at both inoculum concns within 24 h after leaf infiltration. They were capable of completely softening potato slices in 72 h.

—3) Action on carbohydrates.—The bacteria were not capable of hydrolyzing starch within 10 days after inoculation. However, they did produce acid without gas from glucose and sucrose. Test completed to determine if glucose was metabolized oxidatively or fermentatively showed that acid was first produced at the surface, and then extended gradually downward into the medium of the open tube. The Vaseline-covered media were unchanged, which indicates that the glucose was oxidized.

—4) Production of pectolytic enzymes.—Moderate-to-wide pits were formed on Hildebrand's media B and C by the pseudomonads. On medium A, isolate P-3 produced slight pitting, whereas remaining isolates produced moderate to wide pitting (Table 2).

—5) Nutritional tests.—Sorbitol was utilized by all the six isolates of the fluorescent pseudomonads. Good growth occurred with P-1, P-3, P-4, and P-5 isolates on cellobiose, whereas P-2 and P-6 grew slowly on this substrate. All isolates produced good growth in salicin, except P-2 which was unable to utilize that compound. All isolates except P-3 and P-6 grew slightly on glycine and proline, respectively. Isolate P-3 grew well on glycine, whereas P-6 was unable to utilize L-proline. Except for P-1 and P-6, which produced scanty growth, all isolates grew well on L-alanine. Isolates P-2 and P-6 were unable to utilize L-valine whereas remaining isolates grew only slightly on valine (Table 3).

DISCUSSION.—*Taxonomic relationship of fluorescent pseudomonads from discolored alfalfa roots.*—All the isolates of the pseudomonads tested reacted similarly in almost all of the physiological and biochemical tests to which they were subjected. Slight variation in reactions to a few of the tests are not unusual, since these isolates were from samples collected from different localities, and at different times. Similar variation among the isolates of other phytopathogenic bacteria have been reported by others (7, 20, 21, 24).

Except for the report of *P. radiciperda* causing wilt or withering of alfalfa (23, 30), no other *Pseudomonas* spp. resembling the isolates under study has been reported. Descriptive literature (8, 23, 30) on *P. radiciperda* is

TABLE 3. Utilization of organic compounds by six isolates of fluorescent pseudomonads from discolored alfalfa roots

	As carbon source			As carbon and nitrogen source			
Isolate	Cellobiose	Salicin	D-sorbitol	Glycine	α-alanine	L-valine	L-proline
P-1	+[a]	+	+	±	±	±	±
P-2	±	0	+	±	+	0	±
P-3	+	+	+	+	+	±	±
P-4	+	+	+	±	+	±	±
P-5	+	+	+	±	+	±	±
P-6	±	+	+	±	±	0	0

[a]Visual scoring of growth; 0 = growth no greater than control plate without C or N source; + = good growth, ± = slight growth but more than on control plate (27).

TABLE 4. Comparison of certain physiological and nutritional characteristics of fluorescent pseudomonads from discolored alfalfa roots with *Pseudomonas marginalis* and *P. cichorii*

Organism	Levan	Oxidase	Potato-rot	Arginine dihydrolase	Tobacco hypersensitivity	Lipase	NO_3 reduction	Acid from sucrose	Pigments	α-alanine utilization	Sorbitol utilization	Sodium polypectate	
												pH 5	pH 8.5
1. Alfalfa isolates	+[a]	+	+	−	+	+	+	+	F[b]	±(2[c])+(4)	+	±(1)+(5)	+
2. *P. marginalis*[d]	+	+	+	+	−	+	+	+	F	+	+	−	+
3. *P. cichorii*[d]	−	+	−	−	+	−	− or +	−	F	−	−	−	−

[a]Positive reaction or utilizes for growth; − = negative reaction or cannot utilize for growth.
[b]Yellow fluorescent pigment.
[c]Number in parentheses refers to number of isolates giving that reaction.
[d]Data from Lelliott et al. (19) and Hildebrand and Schroth (11).

insufficient, as many of the determinative characters now employed, were not available when that pathogen was first described. Lack of information on some of the major characters like oxidase reaction, potato soft-rot, arginine dihydrolase, hypersensitivity, NO_3 reduction, and others, have made comparisons difficult. All attempts to obtain authentic culture of *P. radiciperda* from the original source or from the known collections of phytopathogenic bacteria failed. Due to these difficulties, our alfalfa isolates were compared with two known oxidase-positive members of the phytopathogenic fluorescent pseudomonads.

Lelliott et al. (20) proposed a determinative scheme the 'LOPAT' reactions, based on a differential use of the levan, oxidase, potato, and arginine tests, and the tobacco hypersensitivity test as the main characters with 2-keto-gluconate, lipase, NO_3 reduction, and acid from sucrose as subsidiary characteristics. Recently Hildebrand and Schroth (12), using other studies of pseudomonads (2, 20, 21, 22, 24, 28), have compiled additional tests that are also useful in the identification of phytopathogenic species of pseudomonads. Reactions of the alfalfa isolates to the physiological characters proposed by Lelliott et al. (20) and Hildebrand and Schroth (12) show that the alfalfa isolates differ from *P. cichorii* in seven of twelve characters (Table 4). A comparison with *P. marginalis* shows that the alfalfa isolates differ in their ability to cause hypersensitivity in tobacco, produce pitting in sodium polypectate medium at low pH, slight variation in utilization of L-alanine among isolates, and the inability to produce arginine dihydrolase. However, they react similarly to *P. marginalis* in the other tests. The positive hypersensitive reaction is quite important; it is regarded as an indication of pathogenic capability of the organism (16). In this respect, and in a few other characters mentioned above, they are distinct from the strains of *P. marginalis* reported in the literature, and as shown by tests in our laboratory. In view of these observations, it is proposed that the isolates of fluorescent pseudomonads from discolored alfalfa roots be considered as a variety of *P. marginalis*. The name *P. marginalis* var. *alfalfae* with isolate P-1 as the type culture is proposed.

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