

Distribution of Fluorescent *Pseudomonas* spp. Causing Grain and Sheath Discoloration of Rice in Latin America

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ABSTRACT

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Pathogenic fluorescent *Pseudomonas* spp. were isolated from discolored rice seed and flag-leaf sheath samples received from Argentina, Bolivia, Brazil, Burundi, Chile, China, Colombia, Costa Rica, Cuba, Dominican Republic, Ecuador, El Salvador, Guatemala, Jamaica, Nicaragua, Panama, Peru, Philippines, Surinam, and Uruguay. No such pathogens were recovered from samples received from Thailand. *Pseudomonas syringae* pv. *syringae* (= *P. oryzae*) was found only in Chilean samples, whereas all the others yielded bacteria consistent with *P. fuscovaginae*. Antisera developed from *P. fuscovaginae* were not completely specific for this species, reacting as well with strains from Chile tentatively identified as *P. syringae* pv. *syringae*. However, the relationship between pathogenicity of strains and positive serological reaction was very high. The antisera were successfully used to increase the efficiency of pathogen isolation from diseased plant tissue. The presence of these pathogens and the dirty panicle disease of rice are correlated; however, not all cases of discolored grain should be ascribed to these pathogens.

Certain fluorescent *Pseudomonas* spp. have been shown to cause grain and sheath rot and discoloration of rice (*Oryza sativa* L.) in various parts of the world (1,5-7,13,15). Earliest reports were from Asia and Europe. In Europe, a bacterial sheath rot of rice caused by *P. oryzae* was first described in 1955 (5), but the name was changed later to *P. syringae* pv. *syringae* (*P. s.* pv. *syringae*)

(14). The disease was characterized by brown discoloration and rot of the flag-leaf sheath and discoloration of the grain. A similar disease was reported in 1960 in Japan and China. The pathogen was similar to *P. marginalis* (4). In Japan, bacterial sheath brown rot of rice was described with symptoms identical to those caused by *P. s.* pv. *syringae* (13). The causal agent was identified as the new species, *P. fuscovaginae* (7). Subsequently, this pathogen was isolated from discolored rice grains and sheaths in Burundi (1) and Colombia (15). Preliminary studies of Colombian strains indicate that the designation of *P. fuscovaginae* as distinct from *P. marginalis*

may be premature. Japanese strains (6) are distinct from *P. marginalis* in that they are negative for denitrification, levan formation from sucrose, β -glucosidase, pit formation on polypectate gel, and utilization of 2-ketogluconate, inositol, sorbitol, adonitol, and polygalacturonic acid, whereas *P. marginalis* is positive. Latin American strains are described as between the two (15) and are positive for levan formation from sucrose and utilization of inositol and sorbitol, variable for nitrate reduction, and negative for the other characteristics. Japanese and Latin American strains have been reported to have a broad potential host range, particularly among the Gramineae (6,16).

P. fuscovaginae is seed-transmitted (15) and may be an important component of the dirty panicle disease, or *manchado de grano*. This disease is of unknown etiology and is variably ascribed to soil nutritional problems and a host of fungal pathogens (10). *P. glumae* and *P. avenae* also cause rice grain discoloration (8). *P. s.* pv. *panici* causes a seedling leaf lesion. All of these species may be seedborne as well (8).

The fluorescent pseudomonad, similar to *P. fuscovaginae*, isolated only recently from diseased rice in the western hemisphere (15), is the only fluorescent pseudomonad yet to be reported as

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pathogenic on rice in this hemisphere. This study was undertaken to determine to what extent fluorescent pseudomonads pathogenic on rice are present in countries of the Caribbean and Central and South America. An attempt was made as well to assess their involvement in the dirty panicle disease as perceived by local agronomists.

MATERIALS AND METHODS

Antisera to three strains obtained from Colombia (CIAT accessions 163, 409, and 532) and the type culture of *P. fuscovaginae* (PDDCC 5940) were prepared as follows: Bacterial suspensions of 24-hr-old colonies grown on nutrient agar were washed by centrifugation three times in 0.85% saline solution; 0.2 ml of the suspension (10^9 cfu/ml) was injected into the ear vein of 6-mo-old New Zealand white rabbits at 4-day intervals until a total of 1 ml was injected. After an 8-day rest, the rabbits were bled at 7-day intervals and crude serum was obtained by centrifugation. Serum from each rabbit was obtained before the first injection of antigen for use as a check.

The crude antiserum was tested against strains of *Pseudomonas* spp. isolated from rice and commonly encountered contaminants by a precipitation test. For the test, 0.1 ml of a bacterial suspension (10^9 cfu/ml) was mixed with a 0.85% saline solution containing 0.1 ml of antiserum (1:40 dilution). Preliminary evaluation of different concentrations of the antiserum using the corresponding antigen (strain) showed the 1:40 dilution to give clear reactions. Reaction was evaluated after at least 3 hr of incubation. Specificity of the crude antisera was determined by testing the following bacteria: *P. avenae* (NCPPB 1011, 1392, 3354, 3355, 3356, 3357, 3358, and 2399, plus nine strains received from F. Winther, Danish Government Institute for Seed Pathology, Copenhagen), *P. fluorescens* biotype II (isolated from *Centrosema* spp. provided by J. Lene, CIAT), *P. fluorescens* (nonpathogenic strain from cassava provided by J. C. Lozano, CIAT), *P. fuscovaginae* (PDDCC 5940 [type culture], 5941, and NCPPB 3085 plus one strain from F. Winther), *P. glumae* (NCPPB 2981), *P. marginalis* (NCPPB 667 and 2644), *P. marginalis* pv. *alfalfae* (PDDCC 5708), *P. marginalis* pv. *marginalis* (PDDCC 3553), *P. marginalis* pv. *pastinaceae* (PDDCC 7709 and NCPPB 806), *P. putida* (from J. C. Lozano), *P. s. pv. japonica* (NCPPB 3093 and PDDCC 6305), *P. s. pv. pacini* (NCPPB 1498 and PDDCC 3955), *P. s. pv. phaseolicola* (provided by M. C. Pastor-Corrales, CIAT), *P. s. pv. striafaciens* (NCPPB 1898 and 2394; PDDCC 3961 and 4483), *P. s. pv. syringae* (*P. oryzaicola* originally from Klement) (PDDCC 3876 and 3877), *Erwinia herbicola* (from rice grain), *Xanthomonas oryzae* (CIAT 1163),

“brown blotch” of rice (CIAT 1173), and 41 nonpathogenic fluorescent cultures from rice grain.

With the permission of the Instituto Colombiano Agropecuario (ICA), samples of discolored rice grain and flag-leaf sheaths were obtained from rice scientists in tropical and subtropical areas, primarily in the Americas. Requests for material stated only that the grain be discolored and that the scientists considered them typical of the dirty panicle (*manchado de grano*) syndrome. The samples were to be sent with flag-leaf sheath if possible, and associated sheath rot was noted, if present. Samples of certified and breeder seed not showing severe discoloration were received as well.

Samples were rated for discoloration (GD) based on the following scale: 0 = healthy seed, 1 = seeds with only small spots restricted to hull, 2 = up to one-half of seeds in sample with hull discolored no more than 25%, 3 = 25–50% of seeds in the sample with hulls discolored 25% or more and some seeds completely discolored, 4 = 50–75% sample showing at least 25% hull discoloration and 25% of the seeds in the sample may be completely discolored, and 5 = 75% seeds discolored at least to 25% of hull and >25% seeds completely discolored.

Seventy-five to 100 seeds and sheaths from each sample were washed under running, chlorinated tap water for 1 hr, then air-dried. Fluorescent bacteria in the tissue were detected by plating on King's medium B (KB) and incubating for 48 hr at 24 C (11). Percent contamination in the seed samples was estimated as the percentage of seeds yielding fluorescent bacteria. Sheath samples were evaluated only as containing fluorescent bacteria or not.

Efficiency of recovering pathogenic fluorescent pseudomonads was enhanced by an enrichment technique. Colonies of fluorescent bacteria from seed and sheath tissue plated on KB were transferred directly to nutrient both and incubated for 24 hr. The resulting impure suspension was then assayed for precipitation reaction to the prepared antisera. Suspensions that reacted positively were then used as inoculum on healthy test plants, as described later. The pathogen was recovered by reisolation from symptomatic test plants and/or by purifying the original suspension.

Isolates of fluorescent bacteria from seed and sheaths were purified by dilution plating and serial transfer. Pathogenicity was tested on 15-day-old rice seedlings of cultivars CICA 8, Metica 1, Oryzica 1, and/or Fanny. The plants were grown in steam-sterilized soil from seed treated at 65 C for 6 days. This heat treatment is known to eradicate the pathogen from seed (15). Inoculation was via stem puncture using inoculum of 10^9 cfu/ml. Plants were incubated in a

screenhouse and symptoms evaluated after 7 days. Strains were considered pathogenic if necrosis extended at least 10 mm beyond the point of inoculation.

Pathogenic strains were subjected to the following physiological tests: fluorescence on KB, Gram stain, oxidase, arginine dihydrolase, and starch hydrolysis (11). The oxidase and arginine dihydrolase tests differentiate *P. syringae* pathovars from the *P. fluorescens* group (9).

Plants of Oryzica 1 in the boot stage grown from pathogen-free seed grown in sterile soil were inoculated with a suspension of 10^9 cfu/ml by spraying on the boot or by injection of 0.1 ml into the base of the boot. At least four tillers of each plant were inoculated by spraying, and one tiller was inoculated by injection. The plants were incubated in a moist chamber at 100% relative humidity at 23–25 C for 72 hr before being moved to a screenhouse. Discolored grain and sheath tissues were sampled after about 5 days for reisolation of bacterial pathogens and fungi. After the grain matured in the screenhouse, discolored tissue was again sampled for isolation of fungi and bacteria. Fungi were isolated directly from tissue incubated in a moist chamber or tissue plated on KB and V-8 medium.

RESULTS

The antisera are not specific to *P. fuscovaginae* compared with the known strains included in this study. Although the reaction is strongest for that species, weak reactions were occasionally obtained with unknown nonpathogenic strains (Table 1). *P. s. pv. syringae* (*P. oryzaicola*) (PDDCC 3876 and 3877) did not react with the antisera in this study. The relationship between pathogenicity and positive serological reaction was very high. Of 124 pathogenic fluorescent bacterial strains, 94 reacted strongly with all the antisera, 18 reacted weakly, and 12 failed to react. Of 41 nonpathogenic fluorescent bacteria, none reacted strongly, only two reacted weakly, and the remainder did not react. Non-fluorescent pathogenic strains did not react serologically. All fluorescent pathogens were gram-negative and negative for starch hydrolysis (Table 2). They varied in the results of oxidase and arginine dihydrolase tests.

Isolation of pathogenic fluorescent pseudomonads was generally easiest from sheath samples. Enrichment of fluorescent bacteria by transferring tissue from KB to nutrient broth enhanced the success of recovering pathogenic fluorescent bacteria and was superior to random selection of fluorescent colonies from material plated on KB. In general, isolation of the pathogens was difficult, particularly on older or badly deteriorated samples. *E. herbicola* was a ubiquitous contaminant and very difficult to separate from the pathogen. Many of the

fluorescent strains were nonpathogens, as others have reported (5,6). In some instances, fewer than 10% of the fluorescent strains from a given sample were pathogenic.

Samples of discolored rice seed and/or leaf sheaths were obtained from 21

countries, primarily in Latin America. Pathogenic fluorescent *Pseudomonas* spp. were obtained from samples from 20 of the countries (Table 2). No pathogen was recovered from material from Thailand. Occasionally, nonfluorescent pathogens were isolated and tentatively

identified as *P. avenae*, based on colony morphology and symptomatology (12). The fluorescent pathogens could be clearly separated into two groups: those positive for arginine dihydrolase and oxidase, consistent with *P. fuscovaginae*, and those negative for arginine dihydrolase and oxidase, consistent with *P. s. pv. syringae*. The latter was only isolated from samples from Chile. The antisera to *P. fuscovaginae* did react strongly with some Chilean strains but did not react with pathovar *syringae* strains from rice originally obtained from Klement (PDDCC 3876 and 3877).

In all instances, inoculation of the sheath containing the emerging panicle (boot) by aspiration yielded sheath rot and grain discoloration symptoms identical to those observed in the field and in the samples received. In instances where the panicle was partly emerged at inoculation, only the emerged portion showed grain discoloration. When sheath inoculation occurred 5 days or more before panicle emergence, emergence was poor, with the flag-leaf sheath severely discolored and necrotic and nearly all the grains severely discolored.

Fluorescent pathogenic bacteria were recovered from discolored inoculated sheath and grain tissue after 5 days, and fungi were not. As the plants matured, however, fungi were observed to colonize the tissue. The following fungi were isolated from the discolored mature rice grain and sheaths previously inoculated by spraying pathogenic pseudomonad strains: *Alternaria* spp., *Cladosporium* spp., *Cochliobolus miyabeanus* (= *Helminthosporium oryzae*), *Curvularia* spp., *Epicoccum purpurea*, *Fusarium moniliforme*, *Gibberella* sp., *Nigrospora* spp., and *Septoria* spp. From rice plants inoculated with pathogenic pseudomonads by injection into the boot, the same fungi (except *Septoria* spp.) were isolated, as well as *Sarocladium oryzae* (= *Acrocyllindrium oryzae*) and *Sphaerulina* spp.

In Table 3, data are presented showing that high frequencies of fluorescent bacteria are significantly less likely in samples with slight glume discoloration and significantly more likely when there is substantial glume discoloration. Similarly, samples with relatively clean grain (GD 0-2) are much less likely to yield pathogenic fluorescent bacteria than severely discolored samples (GD 3-5) (Table 3).

DISCUSSION

Pseudomonas spp. are the principal bacteria known to infect rice grain and sheaths. Although they have been known for many years, their importance, relationships, and distributions have not been studied extensively (8). From the results of this study, it is clear that these pathogens are more widely distributed and probably more important than previously thought.

Table 1. Reactions of crude antisera from four strains of *Pseudomonas fuscovaginae* to strains of *Pseudomonas* spp. using precipitation test

Species ^a	Strains tested (no.)	Antiserum ^b			
		163 ^c	409	PDDCC 5939	532
<i>Erwinia herbicola</i>	14	—	—	—	—
<i>P. avenae</i>	18	—	—	—	—
<i>P. fluorescens</i> (nonpathogenic)	1	—	—	—	—
<i>P. fluorescens</i> (<i>Centrochema</i>)	1	—	—	—	—
<i>P. glumae</i>	1	—	—	—	—
<i>P. fuscovaginae</i>	7	+++	+++	+++	+++
<i>P. marginalis</i>	2	+ ^d	+ ^d	+ ^d	+ ^d
<i>pv. alfalfae</i>	1	—	—	—	—
<i>pv. marginalis</i>	1	—	—	—	—
<i>pv. pastinaceae</i>	2	—	—	—	—
<i>P. putida</i>	1	—	—	—	—
<i>P. syringae</i>					
<i>pv. japonica</i>	2	—	—	—	—
<i>pv. panici</i>	2	—	—	—	—
<i>pv. striafaciens</i>	4	—	—	—	—
<i>pv. syringae</i>	2	—	—	—	—
<i>Pseudomonas</i> spp.	41	± ^e	± ^e	± ^e	± ^e
<i>Xanthomonas oryzae</i>	2	—	—	—	—
Brown blotch pathogen	1	—	—	—	—

^a See text for strain origins. *Pseudomonas* spp. = fluorescent nonpathogenic strains.

^b Antigens were *P. fuscovaginae*; 163, 409, and 532 are CIAT strain numbers.

^c +++ = Strong reaction, forming massive coagulated precipitate; + = fine precipitate; ± = weak reaction with some precipitate, but some bacteria surviving 24 hr and obviously not reacted; and — = no precipitate.

^d Only NC PPB 2644 was positive.

^e Forty-one fluorescent nonpathogenic strains from rice grain of which two reacted weakly.

Table 2. Origin of samples of discolored rice grain from which pathogenic strains of fluorescent *Pseudomonas* sp. were obtained by CIAT (all strains reacted positively with *P. fuscovaginae* antiserum)

Country	No. of samples ^a	Cultivar ^b	Symptom ^c			
			ShR	GD	Arg ^d	Ox ^d
Argentina	3	Bl, IRGA 409, Bluebonnet 50	+	+	+	+
Bolivia	1	Bl	+	+	+	+
Brazil	33	Bl, IRGA 409, 410, CICA 8, CICA 9, IAC 164, Bluebelle	+	+	+	+
Burundi	1	+	+	+
Chile	11	Bl, Ovacion, Oro Quella	+	+	—	—
China (P.R.)	5	Bl, Evanguai	+	+	+	+
Colombia	116	Bl, CICA 4, CICA 8, Metica 1, Oryzica 1	+	+	+	+
Costa Rica	4	CR 201	...	+	+	+
Cuba	2	Bl	...	+	+	+
Dominican Republic	1	Bl	+	+	+	+
Ecuador	9	Bl, INIAP 7, INIAP 10	...	+	+	+
El Salvador	1	CENTA A-2	+	+	+	+
Guatemala	2	Bl, IRGA 409	+	+	+	+
Jamaica	2	Inglés, CICA 8	+	+	+	+
Nicaragua	3	Bl, CICA 8	+	+	+	+
Panama	2	Bl, CR 1113	...	+	+	+
Peru	1	...	+	+	+	+
Philippines	3	Bl, IR 58, JKAU	+	+	+	+
Surinam	2	Camponi, Eloni	+	+	+	+
Uruguay	7	...	+	+	+	+

^a Number of samples from which pathogenic isolates were recovered.

^b Bl = breeding lines; ... = unknown.

^c ShR = sheath rot and/or discoloration, ... = sheath samples not received, and GD = grain discoloration.

^d Arg = arginine dihydrolase test; Ox = oxidase test.

The arginine dihydrolase-positive, oxidase-positive *Pseudomonas* spp. pathogenic on rice (hereafter referred to as *P. fuscovaginae*) are very widely distributed, particularly in Latin America, whereas *P. s. pv. syringae* is restricted to Chile. This country is historically and geographically isolated from the rest of Latin America in terms of rice production. The rice varieties grown in Chile were introduced from southern Europe, whereas those from the rest of the continent were introduced primarily from North America and Asia, where *P. fuscovaginae* was first described. There has been almost no exchange of rice germ plasm between Chile and the rest of the continent until very recently. Sheath rot of rice caused by *P. s. pv. syringae* (= *P. oryricola* Klement) was described first from southern Europe and may have been introduced via seed from there. However, because both *P. fuscovaginae* (16) and *P. s. pv. syringae* (5) have a broad range of potential hosts, this distribution may reflect environmental limitations rather than geographical origins. Detailed studies are under way to characterize these strains and establish the relationships among pathogenic fluorescent *Pseudomonas* spp. in Latin America.

The combination of partially and totally discolored, poorly emerged panicles that was observed after controlled spray inoculations is also observed in the field. Under field conditions, however, a lack of relationship is sometimes observed between sheath and grain discoloration. This has been most commonly observed when environmental conditions have not favored early infection of the boot and the panicle emerges before sheath rotting. Apparently, the pathogen can cause only slight discoloration of the grain when infection is late.

Failure to recover the pathogen cannot lead to a firm conclusion excluding

bacterial involvement in sheath rot and grain discoloration. Klement (5) reported that only 5–15% of the strains from rice were pathogenic, and Miyajima (6) reported as low as 1%. Much of the difficulty in recovering the pathogen is from overgrowth by contaminants, such as *E. herbicola*. Because it is difficult to recover the pathogen, some samples in this study from which we did not recover pathogenic bacteria may well have contained them. Thus, there is a bias against establishing a relationship between fluorescent *Pseudomonas* spp. and grain discoloration. The skewed distribution of percent fluorescent bacteria per sample toward severe grain discoloration is suggestive of a causal relationship (Table 3). The samples from which the pathogens were isolated were disproportionately skewed toward severe discoloration (Table 3) and were sent by the cooperators as typifying the dirty panicle disease. The evidence is therefore strong for fluorescent bacterial involvement in the dirty panicle syndrome.

The dirty panicle syndrome is poorly defined and it would be simplistic in the extreme to attribute all cases of grain discoloration to these bacteria. Rice grain may be discolored because of virus infection before flowering, such as by rice *hoja blanca* virus (3,8), or by severe soil stress (10). Fields in Latin America on highly acid upland soils or flooded acid soils with high iron content frequently have been observed by the authors to show severe grain discoloration if the rice lines are not adapted to these conditions. These symptoms are sometimes found in the absence of fluorescent bacterial pathogens. *P. avenae*, which has been detected in seed lots from many countries (12), may be involved in grain discoloration to a high degree in some cases. A range of pathogenic fungi may cause grain spotting but rarely cause the severe symptoms observed to be caused by these

bacteria. However, of the fungi described as causing grain discoloration (10), some may actually be secondary, because we recovered many of those commonly named from lesions known to be caused by bacteria.

The economic importance of the pathogens in Latin America cannot be determined from this study. In some countries (e.g., Argentina), the individuals providing the samples indicated that the affected plants were rare in production fields. Respondants from Brazil and Chile stated that severity varies from year to year, as reported by others in the literature (1,2,5,6). Because *P. fuscovaginae* and *P. s. pv. syringae* favor relatively cool, wet weather (5), the large rice-producing areas of temperate South America may be subject to severe outbreaks when conditions favorable to disease development coincide with panicle emergence and adequate inoculum. That some of the samples in this study from which the pathogen was recovered were collected from commercial seed production fields suggests that inoculum frequently may be introduced to farmers' fields via infected seed.

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Table 3. Association of slight and severe grain discoloration (GD) with percent contamination of seed samples with fluorescent bacteria and with recovery of pathogenic fluorescent bacteria from the samples

Ratios ^a	No. of samples ^b		
	GD 0-2 ^c	GD 3-5 ^c	Total ^d
Seeds with fluorescent bacteria (%)			
<50:≥50	171:62	128:190	
(χ^2)	(51.0)	(12.1)	551
Fluorescent pathogen			
Not present:present	218:91	39:104	
(χ^2)	(44.7)	(29.5)	460

^aChi-square test based on expected ratio of 1:1 in all cases; probability ($P = 0.001$) is that the observed deviation for this ratio is from chance.

^bSample sizes: 75–150 seeds.

^cGD: 0 = healthy seed; 1 = seeds with only small spots restricted to hull; 2 = one-half of sample with seeds discolored up to 25%; 3 = 35–50% sample seed discolored 25% or more, some seeds completely discolored; 4 = 50–75% sample showing at least 25% hull discoloration, 25% of sample may be completely discolored; and 5 = 75% seeds discolored at least to 25% of hull, >25% seeds completely discolored.

^dNumbers of samples differ because not all samples assayed for fluorescent bacteria were subjected to isolation procedure.

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