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Diversity of Gram negative bacteria antagonistic against major pathogens of rice from rice seed in the tropic environment

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Abstract: With the use of a seed washing technique, more than 4000 Gram negative bacteria were isolated by two improved isolation methods from 446 batches of 1 kg rice seed samples obtained from 22 provinces in the Philippines. They were initially characterized on the basis of colony morphology and results of biochemical and pathogenicity tests. Six hundred and fifty-two strains were further identified by Biolog, from which 133 were selected for fatty acid methyl ester (FAME) analysis together with 80 standard reference! strains. Sixteen species or types of *Pseudomonas* and 17 genera of non-pseudomonads were identified, more than one third of which have not been recorded in rice. The most predominant species observed were *P. putida* and *P. fulva*. About 17% of the strains of *Pseudomonas* and 2% of the non-pseudomonads were antagonistic to one or more fungal or bacterial pathogens of rice. Rice seed is an important source of biological control agents.

Key words: Rice seed, Non-pathogenic bacteria, Antagonistic bacteria, *Pseudomonas*
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INTRODUCTION

Rice seed harbors a dozen of plant pathogenic bacteria (Goto *et al.*, 1988; Xie *et al.*, 1998). The bacterial pathogens cause a range of rice diseases, some very destructive while others have no effect on rice crop growth. In this study, we found a large number of bacteria associated with rice seed that appeared to have antagonistic effect on some of the major fungal pathogens of rice. So far a large number of plant pathogenic bacteria were reported from rice seed, yet there is no information to suggest that among the microbial community of rice seed, those that possess the antagonistic ability are also potential source of biological control agents for disease management. Surveys of antagonistic bacteria from rice seed are rare, especially for Gram negative bacteria, but seed could be an important source of naturally occurring biological control agents that are valuable natural resources in management of rice pathogens. The present study was therefore targeted to determine the extent of the Gram negative antagonistic bacteria associated with rice seed in the tropics using seeds collected from the Philippines as a case study.

MATERIALS AND METHODS

Standard reference strains of phytobacteria and fungal pathogens and their maintenance

Fifty-two bacterial reference strains of phytobacteria including 19 type strains from the BCCMTM/LMG Culture Collection Laboratory for Microbiology, Ledeganchstraat, University of Gent, Belgium (RUG) and 28 bacterial reference strains, two fungal pathogens (*Rhizoctonia solani* and *Sarocladium oryzae*) and three bacterial pathogens (*Acidovorax avenae* subsp. *avenae*; *Burkhoderia glumae*; *P. fuscovaginae*) from the International Rice Research Institute, Philippines (IRRI) were used in the experim-

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ents. The bacteria were maintained in peptone sucrose medium for short term use and for long term storage, all the cultures were maintained as lyophilized culture.

Rice seed sampling and processing

Rice seeds were sampled during the period between harvesting of the wet season crop and sowing of the dry season crop. Four hundred and forty-six seed samples (1 kg each) were collected from 22 provinces in the Philippines from 1994 to 1996. All samples were maintained at 25 ℃ at 60% relative humidity before isolation. Isolation was done within a month after sampling.

Isolation of bacteria from rice seeds and pathogenicity test

Seed washing and seed crushing isolation was used. For seed washing isolation 50 g of rice seeds from each sample were soaked in 100 ml sterile distilled water with 0.025% Tween 20 and shaken for 48 h at 30 °C until the seeds began to germinate. One ml of the suspension was taken to make a serial dilution of King's medium B (KMB) and nutrient agar medium (NA) with four replications. Representative and unique colonies were picked up after 3 d of incubation at 28 °C. They were purified on peptone potassium medium (PPM) agar plates and maintained on agar slants for further test. Seed crush isolation and pathogenicity test followed those of Xie (1996).

Test for antagonistic against rice pathogens

Three hundred and three nonpathogenic bacterial isolates from rice seeds were tested for their ability to inhibit growth of 3 bacterial pathogens-*A. avenae* subsp. *avenae* (causing bacterial brown stripe), B. *glumae* (causing grain rot or glume blight), *P. fuscovaginae* (bacterial sheath brown rot), and 2 fungal pathogens *R. solani* (causing sheath blight) and *S. oryzae* (sheath rot). Procedures for the test followed those of Xie (1996).

Identification of bacteria from rice seeds

After purification of the strains, the identification process started with checking of colony morphology, Gram staining and checking of fluorescence under 365 nm long wave UV light. Bacterial isolates from rice seeds were separated into several representative groups for further identification. Standard bacteriological procedures (Mew *et al*., 1994) were used for all strains.

The Gram negative bacteria (GN) were further identified by a numerical taxonomic method, Biolog (Biolog Inc., 3447 Investment Blvd., Suite 3, Hayward, CA 94545, USA). The GN MicroPlates with 96 wells were inoculated with a bacterial suspension (Optical Density at 590 nm of 0.25). The plates were incubated at 30 °C for 48 h. Biolog GN database (version 3.5) was used to determine the identity of the isolates (Xie, 1996).

Gas chromatographic analysis of fatty acid methyl esters (FAMEs). Extraction and preparation of FAMEs were performed following the method of Stead (1989). FAMEs fingerprints were identified by using a microbial identification system software package (MIS version 4.15 obtained from Microbial ID, Inc., Newark, Delaware) and a calibration mixture of known standards.

RESULTS

Re-identification of the reference strains associated with rice seed

Among 52 reference strains of RUG (representing 22 species), 41 strains were correctly re-identified by Biolog except the strains which were not included in the Biolog GN database version 3.5 inasmuch as species *B. glumae* and *B. plantarii* which were misidentified as *B. gladioli* and *B. caryophylli*, respectively, with low similarity. Aside from species *B. glumae*, *B. plantarii*, and *P. fragi*, which did not occur in the FAMEs database version 4.15, *P. fluorescens*, *P. fulva*, *P. marginalis* pv. *marginalis*, *P. fuscovaginae*, *P. tolaasii*, and *P. viridilivida* could not be differentiated from *P. putida* in FAMEs. The Biolog identification of 28 bacteria reference strains (14 species) of rice from IRRI matched the original identity with higher Biolog similarity (0.52 – 0.98) excluding the 3 strains of *B. glumae* identified as *B. gladioli* with lower similarity (0.31 – 0.33).

Identification and isolation frequency of *Pseudomonas* spp. from rice seed

More than 4000 bacterial isolates were isolat-

ed from the samples, of which 2915 isolates were identified as belonging to genus *Pseudomonas*. After preliminary characterization, a total of 652 Gram negative bacterial isolates were selected for the Biolog test. The FAMEs data on 133 isolates (data not shown) from rice seeds showed results similar to those obtained with reference strains. Sixteen species or types of *Pseudomonas* were identified by Biolog with similarity above 0.50 (below 0.5, no identification was given; only an indication of probably related species). Aside from the nine species previously isolated from the rice plants, six species or types of *Pseudomonas* have never been recorded in rice seed in the Philippines (Table 1). *P. putida* A1, which was isolated from 9 out 10 seed samples, was the most predominant species. Three fluorescent species—*P. fulva*, *P. resinovorans*, and *P. putida* B1—were found in about 25% – 45% of the seed lots (Fig.1). *P. resinovorans* was the first time reported from rice seed. *P. fuscovaginae*, the causal organism of bacterial sheath brown rot, was isolated from 9.8% of the seed samples.

Table 1 Identification of some non-pathogenic *Pseudomonas* spp. isolated from 446 batches of rice seeds in the Philippines[1]

No.	Biolog identity	Biolog similarity	FAME similarity
1	*P. putida A*1	0.50 – 1.00	0.50 – 0.82
2	*P. fulva*	0.50 – 0.78	–[2]
3	*P. resinovorans* *	0.50 – 0.72	–
4	*P. aeruginosa*	0.50 – 0.92	0.50 – 0.82
5	*P. putida B*1	0.50 – 0.91	0.50 – 0.60
6	*P. viridilivida A*	0.50 – 0.78	–
7	*P. fragi* *	0.50 – 0.85	No match
8	*P. fluorescens C*	0.50 – 0.94	0.50 – 0.85
9	*P. corrugata*	0.50 – 0.52	–
10	*P. mendocina* *	0.50 – 0.85	0.50 – 0.70
11	*P. pseudoalcaligens* *	0.50 – 0.52	–
12	*P. fluorescens B*	0.50 – 0.77	–
13	*P. maculicola* *	0.50 – 0.82	–
14	*P. fluorescens A*	0.50 – 0.85	0.50 – 0.85
15	*P. marginalis*	0.50	–
16	*P. tolasii* *	0.70	–

[1] The total isolates of *Pseudomonas* for Biolog and FAMEs test were 519 and 111 respectively; [2] the similarity was below 0.40. * The species or types have not been recorded yet from rice seeds in the Philippines

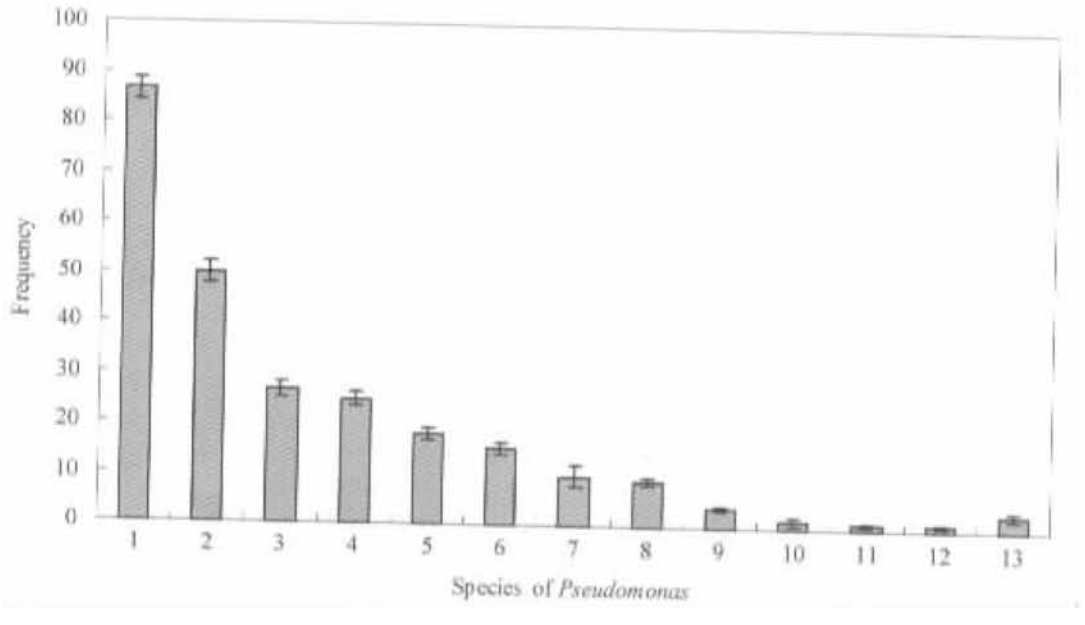

Fig.1 Isolation frequency of *Pseudomonas* spp. from 446 seed samples of rice in the Philippines (1 = *P. putida* A1; 2 = *P. fulva*; 3 = *P. putida* B1; 4 = *P. resinovorans*; 5 = *P. aeruginosa*; 6 = *P. viridilivida* A; 7 = *P. fuscovaginae*; 8 = *P. fragi*; 9 = *P. fluorescens* C; 10 = *P. corrugata*; 11 = *P. mendocina*; 12 = *P. pseudoalcaligens*; 13 = *P. tolasii* + *P. fluorescens* B + *P. maculicola* + *P. fluorescens* A + *P. marginalis*). Vertical bars denote standard deviation of the means

Identification and isolation frequency of nonpseudomonads from rice seed

Seventeen genera or groups of non-pseudomonads were identified by Biolog with similarity of 0.50 – 0.91, which belong to *Acidovarax*, *Acinetobacter*, *Agrobacterium*, *Brevundimonas*, *Burkholderia*, *Enterobacter*, *Escherichia*, *Flavimonas*, Gilardi pink gram negative rod, *Gluconobacter*, *Klebsiella*, *Kluyvera*, *Pantoea*, *Serratia*, *Sphingobacterium*, *Stenotrophomonas* and *Xanthomonas*. Species *Acin*. *baumannii* genospecies 2, *Acin*. *calcoaceticus* genospecies 13, *Acin*. *calcoaceticus* genospecies 1, and Gilardi pink Gram negative rod showed higher isolation frequency ranging from about 15% to 31%. *Acid*. *a*. subsp. *avenae* isolation frequency was 12.9%, that of *B*. *glumae* was 2.9% and those of others were still lower.

Antagonistic effect of non-pathogenic bacteria on selected rice pathogens

After the characterization of bacterial isolates from rice seeds, and pathogenicity test, 303 representative nonpathogenic bacteria were tested for antagonism to two fungal pathogens and three bacterial pathogens of rice. The highest number of antagonists (29%) was observed against *B*. *glumae*, followed by that against *P*. *fuscovaginae* (Fig.2). Percentage of antagonists against the two fungal pathogens, *R*. *solani* and *S*. *oryzae* was 13.2% – 19.8 % of the total isolates tested. The lowest number of antagonists to *A*. *a*. subsp. *avenae* was noted against these five major pathogens of rice.

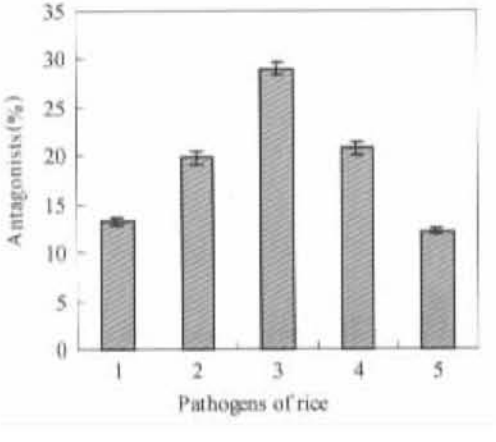

Fig.2 Percentage of 303 bacterial isolates from rice seeds in the Philippines showing antagonism to five major pathogens of rice (1 = *S*. *oryzae*; 2 = *R*. *solani*; 3 = *B*. *glumae*; 4 = *P*. *fuscovaginae*; 5 = *A*. *avenae* subsp. *avenae*)

Two hundred and eight out of 303 strains tested were *Pseudomonas* spp. and 95 were other species. About 12% – 19% of the total strains antagonistic against the two fungal pathogens belongs to *Pseudomonas* spp. and only 1% of the antagonists belong to other species (Table 2). Within each species, *P*. *aeruginosa* and *P*. *resinovorans* showed the highest number of antagonists and the largest inhibition zones against *R*. *solani* and *S*. *oryzae*. Among the species there were relatively higher number of antagonists and larger inhibition zones against one of the two fungal pathogens. Three antagonistic bacterial strains out of 95 strains of non-pseudomonads tested were observed in species *Ac*. *c*. *genospecies* 13, *Ac*. *c*. *genospecies* 2 and *En*. *gergoviae*. The largest inhibition zone (18 mm in diameter) was found in strain 9409 of *P*. *resinovorans*. Eleven strains with inhibition zones of 10 mm – 16 mm were antagonistic to both *R*. *solani* and *S*. *oryzae*.

Higher number of antagonistic bacterial strains against the three bacterial pathogens were observed from *P*. *aeruginosa*, *P*. *putida* A1, and *P*. *resinovorans*. Strains from other *Pseudomonas* spp. showed higher antagonistic effect against one of the three pathogens (Table 3). The inhibition zones against the bacterial pathogens were smaller compared with those against the two fungal pathogens. Fifteen strains were not only antagonistic against one of the fungal pathogens but also against more than one of the bacterial pathogens. They belong to four species, namely, *P*. *aeruginosa*, *P*. *putida* A1, *P*. *resinovorans* and *P*. *fluorescens* C. Non-pseudomonads were observed to have the lowest number of antagonists against the three bacterial pathogens. Strain 10707 of *P*. *aeruginosa* was antagonistic against the five pathogens. Strain 9409 of *P*. *resinovorans* was antagonistic against four of the pathogens except *A*. *a*. subsp. *avenae* against which the lowest number of antagonists was detected.

Table 2 Some non-pathogenic antagonistic bacterial strains from rice seeds showing inhibition zone against the 2 fungal pathogens, *R. solani* and *S. oryzae*, the causal organism of sheath blight and sheath rot of rice, respectively

Species	Number of strains tested	Against *R. solani*		Against *S. oryzae*	
		% of antag*	Inhibition zone (mm)	% of antag	Inhibition zone (mm)
[1] *Ac. c. genospecies* 13	18	5.6	11.7	5.6	11.1
[2] *Ac. c. genospecies* 2	15	6.7	15.0	6.7	12.0
[3] *En. gergoviae*	13	7.7	11.2	7.7	11.6
P. aeroginosa	15	60.0	12.3 – 16.7	46.7	11.3 – 16.0
P. fluorescens C	6	50.0	15.0 – 16.3	0	0
P. fulva	24	29.2	11.0 – 14.7	8.3	11.6 – 13.0
P. maculicola	3	33.3	16.0	33.3	11.3
P. putida A1	101	15.8	11.0 – 15.3	17.0	10.0 – 15.2
P. putida B1	20	10.0	12.3 – 13.0	5.0	11.6
P. resinovorans	28	57.1	11.0 – 18.0	28.6	10.0 – 14.1
P. stutzeri	3	33.3	11.0	0	0
P. viridilivida A	8	25.0	14.0 – 15.2	12.5	15.0

* Percentage of antagonists to total strains in a species tested; [1] = *Acinetobacter calcoaceticus* genospecies 13; [2] = *Acinetobacter baumannii genospecies* 2; [3] = *Enterobacter gergoviae*

Table 3 Some non-pathogenic antagonistic bacterial strains from rice seeds showing inhibition zone against 3 bacterial pathogens, *P. fuscovaginae*, *A. a.* subsp. *avenae* and *B. glumae*, the causal organism of bacterial sheath brown rot, bacterial brown stripe and bacterial grain rot of rice, respectively

Species	No. of strains tested	Against *P. fuscovaginae*		Against *A. a.* subsp. *avenae*		Against *B. glumae*	
		% of anta.	Inhibition zone (mm)	% of anta.	Inhibition zone (mm)	% of anta.	Inhibition zone
Ac. c. genospecies 13	18	11.2	10.2 – 11.0	5.6	9.1	11.2	7.8 – 9.7
Ac. b. genospecies 2	15	6.7	9.0	6.6	8.4	6.7	8.2
En. gergoviae	13	7.7	11.2	7.7	8.6	15.4	9.1 – 10.7
P. aeroginosa	15	33.3	10.0 – 11.6	33.3	7.6 – 10.2	53.3	7.0 – 11.3
P. fluorescens C	6	16.7	10.0	0	0	0	0
P. fulva	24	20.8	7.4 – 11.7	16.7	7.1 – 11.0	37.5	7.6 – 10.0
P. maculicola	3	33.3	11.0	0	0	33.3	11.3
P. putida A1	101	31.7	7.6 – 11.6	18.0	7.0 – 10.0	40.6	9.3 – 10.5
P. putida B1	20	15.0	7.3 – 10.0	10.0	8.1 – 9.2	35.0	7.6 – 11.0
P. resinovorans	28	35.7	9.0 – 11.8	14.3	7.3 – 11.1	50.0	9.6 – 11.0
P. stutzeri	3	33.3	11.0	0	0	0	0
P. viridilivida A	8	12.5	8.2	12.5	9.1	25.0	8.3 – 9.7

DISCUSSION

In a survey of Gram negative bacteria associated with rice seeds, we collected more than 446 kg of rice seeds representing 446 seed lots from 22 rice-growing provinces in the Philippines. Two main methods, the seed washing technique developed by the authors and seed crushing isolation, were used to isolate the bacteria from all seed samples. Each method had its own advantages and disadvantages. We found that with the seed washing technique, the more fluorescent and fast-growing bacteria were easily isolated even if they occurred at low density. The number

of species and number of colonies of fluorescent and fast-growing bacteria obtained by this method were significantly higher than those obtained by using other methods . However, more colonies of the slow-growing bacteria, such as *B. glumae*, *A. avenae* ss. *avenae*, *X. oryzae* pv. *oryzae*, and *X. oryzae* pv. *oryzicola*, could be recovered with the crushing isolation method. For isolation of pathogenic bacteria, less than 5 g of rice seeds (sometimes, even individual seeds are reported) were usually used with the crushing method (Cottyn *et al*., 1996). In our case, we used more than 50 g of rice seeds for each isolation. It provided greater chances of getting different bacterial species. Since one of our objectives was to survey all possible Gram negative bacteria from the discolored and non-discolored rice seeds, it was necessary to use several isolation methods on available media with relatively large amount of rice seeds.

We isolated and characterized more than 4000 Gram negative bacteria from the 446 seed samples. Forty-two species or types belonging to 18 genera were identified in the present study, around one third of which have not been recorded in rice (Mew *et al*., 1994; Cottyn *et al*., 1996; 2001). Some isolates still had to be differentiated using other methods, in spite of the 80 standard reference strains used. Species of *Pseudomonas* were predominant among the 42 species or types, with 16 classified under the genus *Pseudomonas*. Our data clearly showed the great diversity of Gram negative bacteria associated with rice seeds. About 91% of total bacterial isolates were nonpathogenic, of which about 80% from rice seeds neither affected the growth of rice plants nor inhibited the spread of pathogenic organisms and about 20% of the nonpathogenic bacteria were antagonistic to one or more pathogenic fungi or bacteria. Nine species or types of *Pseudomonas* and three species of nonpathogenic bacteria were involved in the antagonistic relationships. *P. aeruginosa*, *P. fluorescens* C, and *P. putida* A1 reportedly served as biological control agents against fungal pathogens (Mew *et al*., 1986.). In the present study, some strains of these three species from rice seeds were observed to be antagonistic to several fungal and bacterial pathogens. *P. resinovonans*, *P. putida* B1, and *P. viridilivida* A., with a relatively high isolation frequency, also inhibited the growth of some pathogenic fungi and bacteria. The data showed that most of these antagonistic bacteria belonged to *Pseudomonas*. *P. resinovorans*, reported to be isolated from a special soil (Balows *et al*., 1992) was found to be widely distributed as a rice seed contaminant in the Philippines. It holds promise as a good biological control agent because some isolates were antagonistic not only to fungal but also to bacterial pathogens.

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