Problems in Detection of *Xanthomonas oryzae* pv. *oryzae* in Rice Seed and Potential for Improvement Using Monoclonal Antibodies

S. S. GNANAMANICKAM, Centre for Advanced Studies in Botany, University of Madras, India 600025; T. SHIGAKI, Department of Plant Pathology, University of Hawaii, Honolulu 96822; E. S. MEDALLA and T. W. MEW, Department of Plant Pathology, International Rice Research Institute, Philippines; and A. M. ALVAREZ, Department of Plant Pathology, University of Hawaii, Honolulu 96822

ABSTRACT

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Four semiselective media that varied with respect to carbon source, amino acids, and antibiotics were evaluated for the growth of various strains of the rice bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae* in pure and mixed culture. Plating efficiencies in pure culture ranged from 40 to 90% on these media, and colonies developed in 3–7 days, depending on the strain of *X. o. oryzae*. In mixtures with *Erwinia herbicola* and *Pseudomonas putida* (1:1:1), *X. o. oryzae* was recovered only on XOS medium. Recovery of a faster growing *X. o. oryzae* strain (X1-5) was improved by adding 50–100 mg/L of FeEDTA to XOS medium, but recovery of other strains was reduced or unaffected. Despite the semiselectivity of XOS medium, sensitivity of the seed assay remains inadequate because even the faster growing *X. o. oryzae* strains were not recovered unless present in relatively high populations (2×10^5 to 1×10^6 cfu/ml) in the seed extract. When fluorescent pseudomonads were present at ratios greater than 60:1, *X. o. oryzae* was not detected. On plates crowded with rice seed contaminants, colonies of *X. o. oryzae* were identified by positive ELISA or immunofluorescence reactions with pathovar-specific monoclonal antibodies. Reactivity with monoclonal antibodies correlated well with pathogenicity tests and shortened the assay time required for presumptive identification of *X. o. oryzae*.

Bacterial blight of rice (*Oryza sativa* L.), caused by *Xanthomonas oryzae* pv. *oryzae* (Ishiyama) Swings et al (24), is a serious disease worldwide. Yield losses of 10–20% are common and have been as high as 74%, depending on location, season, weather, growth stage of the crop, and cultivar (13–15,18–20).

The bacterial blight pathogen is seedborne, although the extent to which it is transmitted through emerging rice seedlings has been questioned (16,25). Rice seed that is infested by *X. o. oryzae* may be discolored and poorly filled and thus have a poor market value, and seed that appears healthy may harbor low populations of the pathogen that later serve as the inoculum source (6,14). Introduction of *X. o. oryzae* in infested seed into an area that was previously blight-free presents a potential threat to commercial rice production (14).

The survival of *X. o. oryzae* in seed and its recovery from infested rice seed have recently been reviewed (13,21). Direct isolation from seed is difficult because *X. o. oryzae* grows slowly on media and does not compete with faster growing contaminant bacteria that may show colony characteristics and pigmentation similar to those of *X. o. oryzae*. Hsieh et al (9) used a streptomycin-resistant strain of *X. o. oryzae* to demonstrate isolation of the pathogen from rice seed. Wild-type strains of *X. o. oryzae* are not easily recovered from seed, however, and occurrence of nonpathogenic xanthomonads among other yellow contaminants of rice seed has increased the difficulty of assessing the epidemiological impact of seedborne *X. o. oryzae* (3,16).

In this study, we evaluated several media (4,5,28) for direct isolation of *X. o. oryzae* in the presence of common contaminants of rice seed and assessed the use of *X. o. oryzae*-specific monoclonal antibodies (MAbs) for confirming the identification of the pathogen.

MATERIALS AND METHODS

Bacterial strains. Eighty-seven strains of *X. o. oryzae* from India, the Philippines, other Asian locations, and the United States were evaluated for growth characteristics, virulence on rice cultivars, and reactivity with a panel of five MAbs. Three strains that represented the biological and serological diversity among these strains were selected for competition studies on media: a race 2 strain PXO86 (from the Philippines) that represented typical slow-growing Asian strains, an atypical fast-growing weakly virulent strain X1-5 (from C. F. Gonzalez, Texas), and an atypical fast-growing virulent race 1b strain G14 (from India). Several hundred bacterial contaminants were isolated from Philippine seed lots assayed from January to December 1990. Bacteriological tests were performed to identify approximately 40 strains (22,26). Fluorescent *Pseudomonas* sp. and *Erwinia herbicola* were commonly encountered. Presumptive identifications of pseudomonads and

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erwiniae were confirmed by the Biolog system (Biolog, Inc. Hayward, CA) and the API 20E system (Plainview, NY), respectively.

Evaluation of media for growth of *X. o. oryzae*. Components of media evaluated for the growth of *X. o. oryzae* are listed in Table 1. Kelman's tetrazolium chloride (TZC) agar (11) was modified to reduce the TZC level to 0.001%, and YCM medium (28) was supplemented with antibiotics and 0.001% TZC (modified YCM = YAT). Inoculum of strains PXO86 and X1-5 was prepared from 72-hr-old TZC agar cultures. The cells were washed from plates and suspensions were adjusted spectrophotometrically to A_{600} = 0.1, which was equivalent to approximately 2×10^8 cfu/ml. For evaluation of plating efficiency of pure cultures, 10 µl of the inoculum from the 10^{-4} to 10^{-7} dilutions was applied in spots (approximately 1 cm^2) onto replicate plates of the seven media using an EDP electronic pipette (Rainin Instruments Co., Woburn, MA). Plates were checked daily with dark-field illumination under 40× magnification, and colony-forming units were counted.

Recovery of *X. o. oryzae* in the presence of rice seed contaminants. Two representative rice seed contaminants, *Pseudomonas putida* strain A4378 and *E. herbicola* strain A4379, were used to evaluate the effect of contaminants on recovery of *X. o. oryzae* in mixed culture. Cell suspensions were adjusted spectrophotometrically to A_{600} = 0.1, serially diluted, and applied in 10-µl spots onto TZC agar plates to determine the number of colony-forming units per milliliter. Suspensions of *X. o. oryzae* were mixed with equal volumes of similarly diluted suspensions of *P. putida* or *E. herbicola* or both, and 100 µl was spread onto YAT, MXO, and XOS media to give an expected count of approximately 50, 33, 25, 20, and 10 cfu per plate for each bacterium. Plates were incubated for 7 days at 28 C, and the colonies of different bacteria were counted.

Effect of FeEDTA on recovery of *X. o. oryzae* in the presence of contaminant bacteria. The effect of FeEDTA on recovery of *X. o. oryzae* was tested to determine whether higher iron concentrations would improve its competitive advantage in the presence of contaminating pseudomonads. XOS medium was modified by adding FeEDTA at 5, 50, 100, 500, 750, or 1,000 mg/L and compared with XOS medium containing 1 mg/L of FeEDTA as originally described (4). Strains X1-5, PXO86, and G14 of *X. o. oryzae* were grown and harvested separately, then individually diluted in a 10-fold series to an expected 50 cfu/50 µl. The *X. o. oryzae* strains were separately mixed in 1:1 ratios with *P. putida* strain A4378 or *E. herbicola* strain A4379 (also grown, harvested, and diluted separately) using 50 µl of each strain from the appropriate dilution, and 0.1-ml aliquots were spread onto XOS medium containing FeEDTA. The experiment was repeated with four replications for each combination.

Detection of *X. o. oryzae* in extracts from artificially infested rice seed. Seed (1 or 5 g) of rice cultivars TN1, IR8, IR66, and IR74 (obtained from the International Rice Research Institute, Philippines) were soaked for 1 hr in 10 ml of sterile water; seeds were removed, and liquid was dilution-plated onto XOS medium (4) containing 1 mg/L of FeEDTA and TZC to determine numbers of contaminant bacteria and *X. o. oryzae*. No *X. o. oryzae* was detected in any of the seed lots, so separate lots were then artificially infested with strains G14 and X1-5. Ten seeds were removed from a 25-g seed sample. Inoculum ranging from 10^3 to 10^8 cfu/ml was applied to the seed coat in 1-µl droplets onto each of 10 seeds; controls received similar volumes of sterile phosphate-buffered saline (PBS). Infested seeds were incubated in a laminar flow hood for an hour at room temperature and for an additional 2 hr at 5 C to permit adsorption of bacteria or buffer onto the seeds. For each inoculum level, 10 infested seeds were then mixed into separate 25-g samples, and each sample was suspended in 50 ml of sterile PBS and gently ground for 20–30 sec in a mortar to break the seed coat. Subsamples of the extracts were plated immediately and again after shaking at 200 rpm on an orbital shaker for 2 hr at 5 C.

The seed extract was decanted and centrifuged at 12,350 *g* for 10 min. The supernatant was removed, the pellet was suspended in 1 ml of sterile PBS and serially diluted, and 100 µl was spread onto four replicate plates of XOS medium containing 1 mg/L of FeEDTA and 0.001% TZC. Identity of colonies was confirmed by ELISA (7) using *X. o. oryzae*-specific antibodies (3).

Limits of recovery by direct isolation onto agar media. Seeds of rice cultivar IR74 were infested as described above with suspensions ranging from 2,500 to 250,000 cfu/µl of *X. o. oryzae* strain G14. Treated samples were ground and assayed immediately or after incubation for 2 hr. Contaminant bacteria appearing on agar plates were counted for 7 days beginning 24 hr after plating. Colonies resembling those of strain G14 were counted after 7 days. Representative colonies were tested for reactivity with *X. o. oryzae*-specific MAbs using ELISA as outlined below. The experiment was repeated twice with four replications of each treatment.

Identification of *X. o. oryzae* by ELISA using monoclonal antibodies.

Table 1. Composition of culture media evaluated for growth and recovery of *Xanthomonas oryzae* pv. *oryzae*

Component	Medium[y]					
	TZC	WF-P	YCM	YAT	MXO	XOS
Bacto agar	17 g/L	17 g/L	17 g/L	17 g/L	15 g/L	17 g/L
$Ca(NO_3)_2 \cdot 4H_2O$		0.5 g/L				0.2 g/L
$FeSO_4 \cdot 7H_2O$		0.05 g/L				
Fe (EDTA)			1 mg/L	1 mg/L		1 mg/L
Glucose	5 g/L				1 g/L	
K_2HPO_4					0.8 g/L	2.0 g/L
KH_2PO_4			1 g/L	1 g/L	0.6 g/L	
L-glutamate (monosodium salt)			1 g/L	1 g/L		5 g/L
Methionine			0.1 g/L	0.1 g/L		
$MgCl_2 \cdot 6H_2O$			1 g/L	1 g/L		
$MgSO_4 \cdot 7H_2O$	0.1 g/L					
Na_2HPO_4		0.82 g/L				
NH_4Cl			1 g/L	1 g/L		
Peptone	10 g/L	5 g/L	2 g/L	2 g/L		2 g/L
Potassium bromide					10 g/L	
Rice starch					8 g/L	
Sucrose		20 g/L	10 g/L	10 g/L		20 g/L
Tryptone			5 g/L	5 g/L		
Yeast extract					0.7 g/L	
Cephalexin				20 mg/L	20 mg/L	20 mg/L
Cycloheximide				100 mg/L		100 mg/L
Gentamycin				2 mg/L	2 mg/L	
Kasugamycin				20 mg/L	20 mg/L	20 mg/L
Methyl green					60 µg/L	
Methyl violet 2B					30 µg/L	0.3 mg/L
Nystatin				300 U/L	300 U/L	
TZC[z]	10 mg/L			10 mg/L		

[y] TZC modified from Kelman (11), WF-P from Karganilla et al (10), YCM by Yuan (28), YAT = YCM + antibiotics and TZC, MXO from Gonzalez et al (5), and XOS from Di et al (4).

[z] Prepared as a 1% stock solution, filter-sterilized and added (1 ml/L) after other ingredients were autoclaved.

Colonies suspected of being *X. o. oryzae* were lifted with sterile toothpicks and resuspended in 100 μl of 0.05 M carbonate bicarbonate buffer (pH 9.6) in individual wells of polyvinyl chloride 96-well plates (Costar, Cambridge, MA, and Dynatech Labs, Chantilly, VA). These suspensions were dried in a circulating-air incubator at 37 C. ELISA (2) was conducted using genus-specific MAbs *X*1 and *X*11 (1), and *X. o. oryzae*-specific MAbs *Xco*-1, *Xco*-2, and *Xco*-5 (3). ELISA reactions were compared with those of reference strains (3) at 450 nm with a Titertek Multiskan plate reader. Readings above 0.15 were considered positive.

Immunofluorescence. Bacterial colonies that reacted positively to MAbs specific to *X. o. oryzae* in ELISA were examined by indirect immunofluorescence. A loopful of a 48-hr-old culture was added to 10 ml of PBS in a tube and vortexed, and 10 μl was spotted onto glass slides, air-dried, and fixed in absolute methanol for 15 min at −20 C. The slides were washed with PBS, MAb *Xco*-2 was added, and the slides were incubated for 20 min. Cells were rinsed with PBS and incubated for 20 min in rabbit-antimouse-FITC conjugate (Miles Scientific, Naperville, IL). Slides were washed and dried, and a drop of PBS-glycerol mounting buffer (3) was added. Cells were examined with an Olympus BH2 epifluorescent microscope.

Plant inoculations. Pathogenicity tests of rice seed contaminants and *Xanthomonas*-like colonies were conducted by pinprick inoculation of a susceptible rice cultivar (IR8) with a bacterial suspension containing 10^8 cfu/ml. Lesion lengths were measured 14 days after inoculation. Strains producing lesions 10–40 cm long were considered pathogenic, those producing lesions less than 10 and greater than 2 cm long were weakly pathogenic, and those producing lesions less than 2 cm long were nonpathogenic. To differentiate strains PXO86 and G14, suspensions were inoculated onto differential cultivars, and reaction patterns were recorded. Forty-day-old rice plants (cultivars IR8, IR20, IR1545, Cas209, and DV85) were clip-inoculated with 10^8 cfu/ml of *X. o. oryzae*. Control plants were clipped with scissors dipped in sterile PBS. Plants were evaluated 14 days after inoculation; those with lesions longer than 10 cm were recorded as susceptible. Characteristic reaction patterns of Philippine race 2 and Indian race 1b were used to confirm the identity of strains PXO86 and G14, respectively.

RESULTS

Evaluation of media for growth of *X. o. oryzae*. The maximum recovery of pure cultures of strain PXO86 occurred on the general plating medium, TZC agar. Plating efficiencies of the semiselective media YCM, YAT, XOS, MXO, and MXO plus TZC were 90, 88, 83, 44, and 40%, respectively (Table 2). Plating efficiencies for strain X1-5 were highest (75%) on YCM and XOS media. The addition of TZC to YCM, MXO, and XOS greatly aided in differentiating colonies and did not significantly reduce the plating efficiency. Colonies of the fast-growing atypical American strain, X1-5, developed 3–4 days after plating, whereas the Asian strain, PXO86, required 7 days. Most of the 87 additional Asian strains from the Philippines, India, and Nepal also required 6–7 days for colony formation on XOS medium. Strain G14 from India was atypical of Asian strains because colonies developed in 3–4 days on XOS medium.

Recovery of *X. o. oryzae* in the presence of rice seed contaminants. In mixed cultures of *X. o. oryzae* and *E. herbicola*, colonies of strains X1-5 and PXO86 were visible under 40× magnification on YAT and XOS at 3 and 7 days, respectively; few colonies were recovered on MXO (Table 3).

Plating efficiencies for pure cultures of *Pseudomonas* sp. on the three semiselective media ranged from 95 to 148% (data for TZC medium not shown). *Pseudomonas* sp. suppressed colony formation of the slower growing strain PXO86 on all media (Table 3). Recovery of the faster growing strain X1-5 on the XOS medium in the presence of *Pseudomonas* sp. was 84–144% of the expected number of colony-forming units. A few colonies of X1-5 were recovered on YAT medium, but MXO did not support any growth of X1-5 in the presence of the *Pseudomonas* strain.

In 1:1:1 mixtures of *Pseudomonas* sp., *E. herbicola*, and *X. o. oryzae*, strains PXO86 and X1-5 did not form colonies on YAT or MXO media (Table 3). Recovery of the faster growing *X. o. oryzae* strain X1-5 was 88–120% of the expected colony-forming units on XOS medium. No colonies of *E. herbicola* developed on XOS medium.

Effect of FeEDTA on recovery of *X. o. oryzae* in the presence of contaminant bacteria. Colonies of X1-5 were first visible under 10× magnification 3 and 4 days after plating onto XOS containing 50 and 100 mg/L, respectively, of FeEDTA (Fig. 1). Colonies were raised and glistening and retained this charac-

Table 2. Plating efficiency of *Xanthomonas oryzae* pv. *oryzae* on culture media[x]

Medium	Colony-forming units/ml × 10^4	
	Strain PXO86	Strain X1-5
TZC	220 a[y]	256 a
YCM	197 b	192 b
YCM/T[z]	166 bc	185 b
YAT	194 b	110 d
MXO	97 d	115 d
MXO/T[z]	87 d	102 d
WF-P	133 c	144 c
XOS	182 b	193 b
XOS/T[z]	186 b	176 bc

[x]Four 10-fold serial dilutions of a suspension containing approximately 2 × 10^8 cfu/ml were made and 10 μl was plated. Colony-forming units were counted after 7 days. Each figure is a mean of four replications.
[y]In a column, means for each medium followed by the same letter are not significantly different ($P = 0.05$).
[z]Tetrazolium chloride was added at a final concentration of 0.001%.

Table 3. Numbers of colonies of *Xanthomonas oryzae* pv. *oryzae* recovered on semiselective media when plated from suspensions containing equal numbers of contaminant bacteria[x]

Expected colony-forming units[y]	Observed colony-forming units/ml × 10^7					
	YAT		MXO		XOS/T[z]	
	PXO86	X1-5	PXO86	X1-5	PXO86	X1-5
X. o. oryzae + *Erwinia herbicola* (1:1)						
50	34	48	7	5	23	56
33	21	26	7	1	29	18
25	17	15	4	0	16	24
20	8	12	3	0	10	28
10	4	7	0	0	4	15
X. o. oryzae + *Pseudomonas* sp. (1:1)						
50	0	5	0	0	0	42
33	0	0	0	0	0	32
25	0	0	0	0	1	36
20	0	0	0	0	0	22
10	0	0	0	0	0	9
X. o. oryzae + *E. herbicola* + *Pseudomonas* sp. (1:1:1)						
33	0	0	0	0	0	29
25	0	0	0	0	1	30
20	0	0	0	0	0	20
10	0	0	0	0	0	0

[x]Colony-forming units were counted from four replicate plates after 7 days of incubation at 28 C.
[y]Calculated from the initial suspension (2 × 10^8 cfu/ml), which was then diluted to 50 cfu per plate. Mixtures at the stated ratios were based on colony-forming units in diluted suspensions of pure cultures of *Pseudomonas* and *Erwinia*.
[z]XOS with 1 mg/L of FeEDTA + 0.001% TZC.

teristic for 14 days. At FeEDTA concentrations higher than 100 mg/L, however, colonies were small, rough, and desiccated after 5–6 days. Strain PXO86 did not grow on XOS medium amended with more than 5 mg/L of FeEDTA (Table 4). Twelve additional typical Asian strains from the Philippines, India, and Nepal tested on these media all required 6–7 days for colony formation on XOS containing 1 mg/L but did not grow on XOS containing FeEDTA at concentrations of 50 mg/L or higher (F.-U. Rehman, *unpublished*). *E. herbicola* did not grow on XOS medium, whereas *Pseudomonas* colonies developed equally well at all concentrations (Table 4).

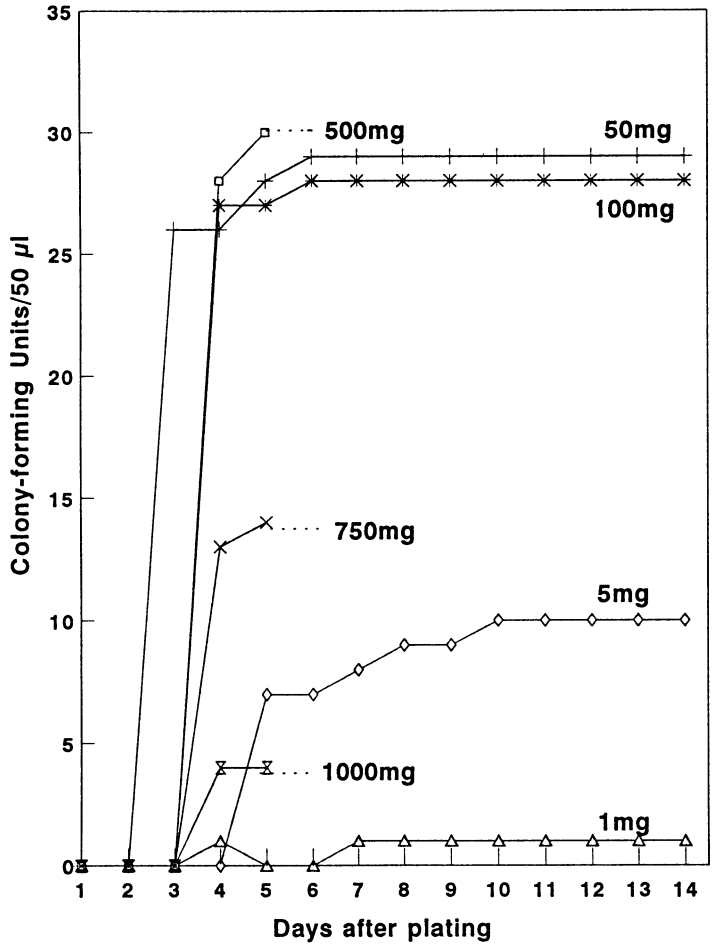

Fig. 1. Recovery of *Xanthomonas oryzae* pv. *oryzae* strain X1-5 on XOS medium containing various concentrations (milligrams per liter) of FeEDTA. A suspension of strain X1-5 was spread on the agar surface, and colonies were enumerated at daily intervals. With FeEDTA concentrations higher than 100 mg/L, colonies grew normally for 2–4 days, then became desiccated and difficult to recognize. With concentrations <100 mg/L, colonies were raised, mucoid, and typical of *Xanthomonas*.

In either pure or mixed culture, colony development of strain G14 was unaffected by FeEDTA concentration, and the colonies remained raised and glistening for the duration of the experiment. In contrast, the slow-growing Philippine strain PXO86 did not appear in mixed culture until 7 days after plating, and growth was poor at all levels of FeEDTA (Table 4).

Detection of *X. o. oryzae* in extracts from artificially infested rice seed. Plating seed extracts of TN-1, IR8, IR66, and IR74 onto XOS medium resulted in growth of large numbers of contaminant bacteria after 48 hr. *X. o. oryzae* strains X1-5, PXO86, and G14 added to seed lots to yield approximately 2,500 and 25,000 cfu/ml of seed extract were not recovered in several initial experiments. High numbers of contaminants (approximately 2.4×10^7 cfu/ml) were recovered from extracts of 25-g seed samples, and at these levels they suppressed growth of *X. o. oryzae*. The *Pseudomonas*-like contaminants had a colony diameter of 6–12 mm, which was reduced to 4–6 mm when the XOS medium was amended with 1.0 g/L of FeEDTA.

Limits of recovery by direct isolation onto agar media. In XOS medium containing 1 mg/L of FeEDTA and TZC, colonies of *X. o. oryzae* strains had characteristic rose-pink pigmentation and were mucoid. Colony counts were higher in extracts from seeds shaken for 2 hr than from extracts plated immediately after grinding the seed (*data not shown*). In the presence of contaminants, *X. o. oryzae* was recovered from high

Table 4. Effect of FeEDTA concentrations in XOS medium on the development of colonies of *Xanthomonas oryzae* pv. *oryzae* (X) strains X1-5, PXO86, and G14 in the presence of contaminant bacteria *Pseudomonas putida* (P) and *Erwinia herbicola*[w]

		Number of colonies recovered/ml × 10^6															
							Experiment 1						Experiment 2				
		Pure culture					X1-5		PXO86		G14		X1-5		PXO86		G14
Medium	FeEDTA[x] (mg/L)	X1-5	PXO86	G14	*Erwinia*	*Pseudomonas*	X	P	X	P	X	P	X	P	X	P	X	P
PSA	...	53	48**[y]	33	48**	38	34**	44	6**	26	17	21**	35**	63[z]	4	82[z]	18	59[z]
XOS	0	51	0	33	0	54	4	43	0	36	20	16	3	23	0	25	18	18
	1	40	29	21	0	49	2	59	0	34	28	41	0	55	0	40	18	40
	5	74	29	42	0	43	9	47	...	...	30	33	9	53	...	...	24	28
	50	54	0	28	0	45	36	45	0	30	24	43	34	40	1	15	18	30
	100	54	0	30	0	51	48	44	0	29	17	31	43	35	0	21	23	43
	500	58	0	25	0	44	39	48	0	32	26	40	4	34	0	37	23	32
	750	55	0	34	0	45	32	41	0	40	24	37	1	37	0	31	20	32
	1,000	52	0	34	0	46	11	44	0	43	32	27	2	41	0	28	22	27

[w] Initial inoculum (approximately 5×10^7 cfu/ml) was diluted to an expected 50 cfu per plate for each strain. Results were recorded for strain X1-5 after 6 days of growth on respective media. Suspensions were 1:1 mixtures of *X. o. oryzae* and *P. putida* in experiment 1 and 1:1:1 mixtures of *X. o oryzae*, *P. putida*, and *E. herbicola* in experiment 2. Counts for strains PXO86 and G14 were recorded on the seventh day.

[x] Colonies appeared rough and desiccated at concentrations greater than 100 mg/L.

[y] ** = *F* test significant at the 1% level; all others nonsignificant.

[z] *Pseudomonas* and *Erwinia* colonies combined because they could not be differentiated on PSA on the basis of morphology; *Erwinia* did not grow on XOS.

Table 5. Detection of *Xanthomonas oryzae* pv. *oryzae* strain G14 in rice (cv. IR74) seed extracts by direct isolation on XOS/T[x] medium in two experiments

Seed sample		Expected cfu/ml seed extract	Bacteria recovered (cfu/ml seed extract)[y]			
			Experiment 1		Experiment 2	
No.	Treatment		Contaminants	*X. o. oryzae*	Contaminants	*X. o. oryzae*
1	Control	0	5.8×10^7	0	5.3×10^7	0
2	Infested	2.5×10^4	1.4×10^7	0	1.6×10^7	0
3	Infested	2.5×10^5	2.1×10^7	0	1.7×10^7	0
4	Infested[z]	5.0×10^5	6.3×10^7	1×10^6	4.6×10^7	2×10^6
5	Infested[z]	2.5×10^6	2.9×10^7	6×10^6	3.9×10^7	5×10^6

[x] XOS with 1 mg/L of FeEDTA + 0.001% TZC.
[y] Mean colony-forming units of contaminants were counted after 3 days; *X. o. oryzae* was counted after 7 days. Data are averages of four replications.
[z] Sixteen representative colonies plated from these samples were identified as *X. o. oryzae* by ELISA and indirect immunofluorescence.

dilutions (10^{-4} to 10^{-5}) of seed extracts only when *X. o. oryzae* was present in concentrations of more than 1×10^6 cfu/ml (Table 5). In an identical study with strain X1-5, the lower limits of detection were 2×10^5 cfu/ml of seed extract (*data not shown*). Even when added at approximately 1×10^8 cfu/ml, the slower growing *X. o. oryzae* strain PXO86 was not recovered on XOS medium in the presence of contaminants.

Colonies of most common saprophytic bacteria were nonmucoid red (type 1, *Pseudomonas*-like) and red with yellow margins (type 2, *Erwinia*-like) on XOS medium amended with TZC. When the contaminant bacteria were grown separately or together in XOS broth, the pH of liquid culture, monitored over 6 days at 12-hr intervals, stayed within a range of 6.3–6.8. The liquid culture concentrated 50 times by vacuum drying and applied in 20-μl aliquots to standard antibiotic paper-disk assays did not inhibit strain PXO86 or G14.

Identification of *X. o. oryzae* by ELISA using monoclonal antibodies. The identifications of presumptive colonies of *X. o. oryzae* strains X1-5 and G14 recovered from rice seed extracts on XOS medium were confirmed as *X. o. oryzae* by species-specific MAbs *Xco*-1 and *Xco*-2. Antibody *Xco*-5 reacted positively to all 41 colonies tested from seed inoculated with strain X1-5 (Table 6). Sixteen colonies tested from seed inoculated with strain G14 gave positive reactions to antibodies *Xco*-1 and *Xco*-2 and negative reactions to *Xco*-5 (Table 6). Nine colonies that appeared to be *Xanthomonas* were positive for the genus-specific MAbs *X*1 and *X*11 but negative for all the *X. o. oryzae*-specific MAbs. They also were nonpathogenic on all rice cultivars.

Immunofluorescence. Bright immunofluorescence with MAb *Xco*-2 was observed for reference strains G14 and X1-5 and presumptive *X. o. oryzae* colonies recovered from rice seed. No fluorescence was observed for the nine nonpathogenic *Xanthomonas* sp. recovered from contaminated rice seed.

Plant inoculations. Sixteen colonies recovered from rice seed inoculated with strain G14 were pathogenic to rice cultivars IR8, IR20, IR1545, and Cas209 but not to DV85; this reaction pattern confirmed the identity of strain G14. Colonies recovered from seed inoculated with strain X1-5 were weakly pathogenic on these cultivars; lesions 2–3 cm long were visible 14 days after inoculation.

Table 6. Evaluation of monoclonal antibodies (MAbs) for distinguishing *Xanthomonas oryzae* pv. *oryzae* from rice seed contaminants when plated onto XOS medium containing 1 mg/L of FeEDTA + tetrazolium chloride (0.001%)

Colony color and tentative identification	Number of colonies tested	Reactivity with MAbs					Pathogenicity
		*X*1	*X*11	*Xco*-1	*Xco*-2	*Xco*-5	
Red and pink	161	−	−	−	−	−	−
Red with orange or yellow margins	128	−	−	−	−	−	−
Rose-pink *X. o. oryzae*	41	+	+	+	+	+	+[y]
Rose-pink *X. o. oryzae*	16	+	+	+	+	−	+[z]
Rose-pink *Xanthomonas*	9	+	+	−	−	−	−

[y] Weakly pathogenic (lesions 2–3 cm long).
[z] Characteristic bacterial blight, lesions 10–40 cm long.

DISCUSSION

Detection of seedborne *X. o. oryzae* on semiselective media has been a challenge because this pathogen grows very slowly and growth is suppressed by contaminating bacteria that abound on rice seed (16). Among the recently formulated semiselective media (4,5,28), XOS medium developed by Di et al (4) is the most effective for the direct isolation of *X. o. oryzae* from rice seed. A major advantage of XOS medium is suppression of *E. herbicola*, a common saprophyte on rice seed. This bacterium failed to grow on the XOS basal medium lacking FeEDTA or on XOS media amended with various concentrations of FeEDTA.

XOS medium failed to inhibit growth of fluorescent pseudomonads, however, and these commonly encountered rice seed contaminants suppressed recovery of *X. o. oryzae*. In mixed cultures with a representative fluorescent pseudomonad, the faster growing *X. o. oryzae* strains X1-5 and G14 were detected only when present in high levels in seed extracts, and when ratios of the pseudomonad to *X. o. oryzae* were greater than 60:1, the latter strains were not detected.

The prevalence of fluorescent pseudomonads on rice seed led us to examine the possibility that higher levels of FeEDTA in XOS medium may improve recovery of *X. o. oryzae* in mixed culture by reducing the competitive advantage of fluorescent pseudomonads (8,12,17, 23,27). At high FeEDTA concentrations, however, colonies of *X. o. oryzae* were flat and dry, possibly reflecting damage to the bacterial outer membrane (27). We conclude that while recovery of atypical fast-growing *X. o. oryzae* strains may have been improved by adding EDTA at 100 mg/L, recovery of typical Asian strains was reduced, and even XOS medium containing 1 mg/L was inadequate for detection of low populations of the slow-growing Asian strains.

Mew et al (16) recently showed that direct isolation of *X. o. oryzae* was possible from naturally infected rice panicles and highly infected immature rice grains on intact plants. However, in the numerous seed lots tested, we have not yet encountered natural infestation by *X. o. oryzae*. In contrast, nonpathogenic xanthomonads recovered previously from rice seed (3,4) were again detected in this study. The nonpathogenic xanthomonads can be distinguished from *X. o. oryzae* because the former reacted to *Xanthomonas*-specific MAbs *X*1 and

*X*11 but not to the pathovar-specific MAbs *Xco*-1 or *Xco*-2. Use of these antibodies following isolation on XOS would thus help to resolve the controversy as to the significance of seedborne *X. o. oryzae* and its transmission to emerging rice seedlings (16,25). Monoclonal antibodies also could be used to follow the progress of such wild-type strains through the seedbed to the subsequent crop.

Serological methods will facilitate identification of *X. o. oryzae* among the numerous rice seed contaminants prior to pathogenicity tests that require a minimum of 7–10 days for symptom development. Although pathogenicity tests are needed for final confirmation of seedborne pathogens, preliminary screening with MAbs would be a great advantage when large quantities of rice germ plasm must be checked for seedborne *X. o. oryzae*.

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