A Leaf Inoculation Method for Detection of *Xanthomonas oryzae* pv. *oryzicola* from Rice Seed

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ABSTRACT

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A leaf-inoculation method for detecting *Xanthomonas oryzae* pv. *oryzicola* in rice seed was developed and tested. The method is based on inoculating leaf segments on agar with seed washings in a moist chamber. Freshness of inoculated leaf segments is maintained by addition of 75 to 100 ppm benzimidazole to the 1% water agar after sterilization. Using cells from pure cultures, the minimum inoculum concentration to initiate leaf streak lesions on the segments was found to be 10^3 CFU/ml. Inoculum prepared from seeds harvested from severely infected mother plants induced lesions typical of leaf streak, followed by bacterial ooze. With 4 g of seed, the incubation period was 3.5 days and bacterial ooze appeared within 5 days. The minimum amount of seed needed to detect the bacterium based on seed washings varied according to disease severity of the mother plants. The bacteria isolated from lesions on the leaf segments were confirmed as *X. oryzae* pv. *oryzicola* after bacteriological, serological, and Biolog tests were conducted. The results confirm that the detached-leaf method is as simple and reliable as the immuno-radiometric assay and inoculation on intact pot-grown rice plants in the greenhouse.

Additional keywords: bacterial leaf streak, disease diagnosis, quarantine

Bacterial leaf streak (BLS) of rice, caused by *Xanthomonas oryzae* pv. *oryzicola* (3,4,15), has been considered a quarantine disease in China since the 1960s and has gained greater importance in many Asian countries. The bacterial pathogen is both seedborne and seed-transmitted (4,5,9,13). The risk of BLS spread has increased with the movement of rice germ plasm in rice improvement activities at the international and national levels. In the late 1950s, the disease was reported in only two provinces of south China (4,7,17). The disease has since spread from the southern to the western, eastern, and central parts of the country, covering more than 10 provinces (7,12,17). Recently, it has become one of the four major diseases of rice in China (the other three being blast, sheath blight, and bacterial blight); the damage caused by the disease is, in many instances, as severe as that caused by bacterial blight (7,12,13,17).

Seed movement must be regulated on the basis of sound biology, and seed detection methods must be well established to effectively restrict dissemination of most pathogens (5,8,17). Methods for detection of seedborne bacteria, such as the growing-on and plant injection or inoculation tests (5,14,17,18), produce variable results. An immuno-radiometric assay (IRMA; 19), enzyme-linked immunosorbent assay (ELISA), and monoclonal antibodies (1,17,19) have been developed to detect *X. oryzae* pv. *oryzicola*. However, there are limitations to the use of these methods in routine seed-health testing of a large volume of seed samples. More importantly, these methods do not differentiate living from dead cells. Cross-reactions with *X. oryzae* pv. *oryzae* and other bacteria are observed in some cases (17–19). These techniques also require specific laboratory equipment and processing cost is high. Therefore, none of these methods is regularly used in routine rice seed health testing (5,10,11).

This paper describes a rice leaf-inoculation method for detecting *X. oryzae* pv. *oryzicola* in rice seed. This method is practical and useful for the detection of bacteria and is used routinely in some plant quarantine stations in China.

MATERIALS AND METHODS

Samples for assay. Rice seed samples were collected from farmer's fields with

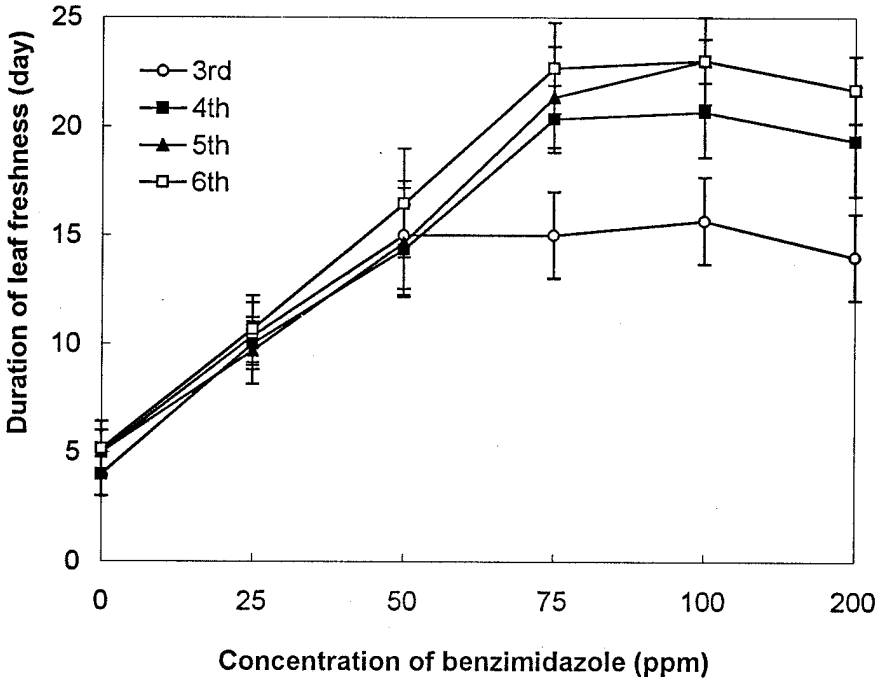

Fig. 1. Effect of benzimidazole incorporated in 1% water agar on the duration of freshness (greenness) of rice leaf segments incubated in petri dishes. Bars are standard deviations of three replications. (The figures of third, fourth, fifth, and sixth denoted the leaf position of the rice plant where leaf segments were taken in the experiments.)

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slight (1 to 10% of leaf area), intermediate (20 to 30%), and severe (more than 40%) BLS severity. To ensure efficiency, individual rice panicles with flag leaves displaying various degrees of disease symptoms of bacterial blight (BB) and BLS were collected from fields at crop maturity. Two sets of experiments were conducted in China and one was done independently at the International Rice Research Institute (IRRI; Los Banos, Philippines). Seed was collected from China (64 samples) and from the Philippines (52 samples), at 200 to 400 g per sample.

Rice leaves with BLS and BB lesions and pure cultures of *X. oryzae* pv. *oryzicola* strain BLS335 from the Philippines and S-872 from China were used for checks or antigen. Strain PXO 61, race 1 of *X. oryzae* pv. *oryzae*, and a strain of *Acidovorax avenae* subsp. *avenae (Pseudomonas avenae*) collected from IRRI, also served as checks.

Healthy seed of variety TN1 (Philippines) and Guang Ru-ai 4 (China) was grown in pots containing steamed soil and maintained in the greenhouse. Excessive N fertilizer was applied. Leaves from rice plants at the 3- to 6-leaf stage were used for the assay.

Leaf inoculation. To prepare the inoculum, 1 to 20 g of seed was used. Seeds were washed by shaking them for 2 h in sterile 1% peptone at a ratio of 1:5 (wt/vol). The suspension was filtered through filter paper (Whatman # 1) to remove the seeds. The suspension was centrifuged at 13,000 rpm for 5 min. The resuspended sediments were used as inoculum for the detection of bacteria. In a similar manner, inoculum was prepared from diseased leaves. Diseased leaves (2 to 4 g) from each sample were cut into small pieces and washed in 1% peptone at a ratio of 1:5 (wt/vol). The leaf pieces also were soaked in peptone water for 2 h, then removed by filtration. The suspension was centrifuged and the resuspended sediments were used for bacterial detection.

Rice leaves from the greenhouse-grown TN1 (in the Philippines) or Guang Ru-ai 4 (in China) were sampled at different growth stages from the third to the sixth leaf position and cut into 5- to 6-cm segments. The segments were washed twice with sterile distilled water, then placed adaxial-side-up on the surface of water agar plates. The water agar (1%, wt/vol) was prepared by adding, after sterilization, a suspension of benzimidazole to obtain concentrations of 25, 50, 75, 100, and 200 ppm in 25 ml medium per plate.

The leaf segments were inoculated by pricking them with small needles dipped into the leaf or seed wash suspensions. Some leaf segments were inoculated with pure cultures of *X. oryzae* pv. *oryzicola* or the other bacteria at 10^8 CFU/ml as controls.

Incubation and observation. The agar plates with inoculated leaf segments were

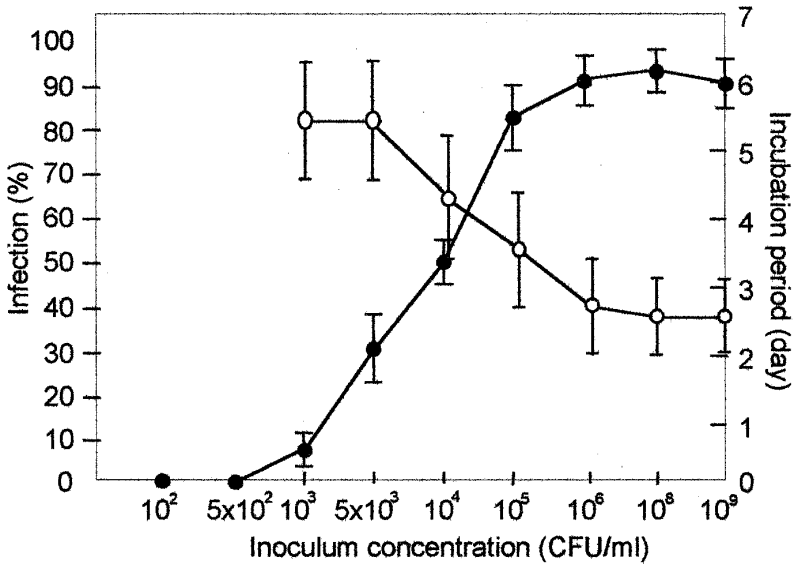

Fig. 2. Relationship of *Xanthomonas oryzae* pv. *oryzicola* inoculum concentration with ● = bacterial leaf streak severity and ○ = length of incubation period (time required for visible lesions to appear) of bacterial leaf streak on rice leaf segments in the leaf inoculation. Bars are standard deviations of three replications.

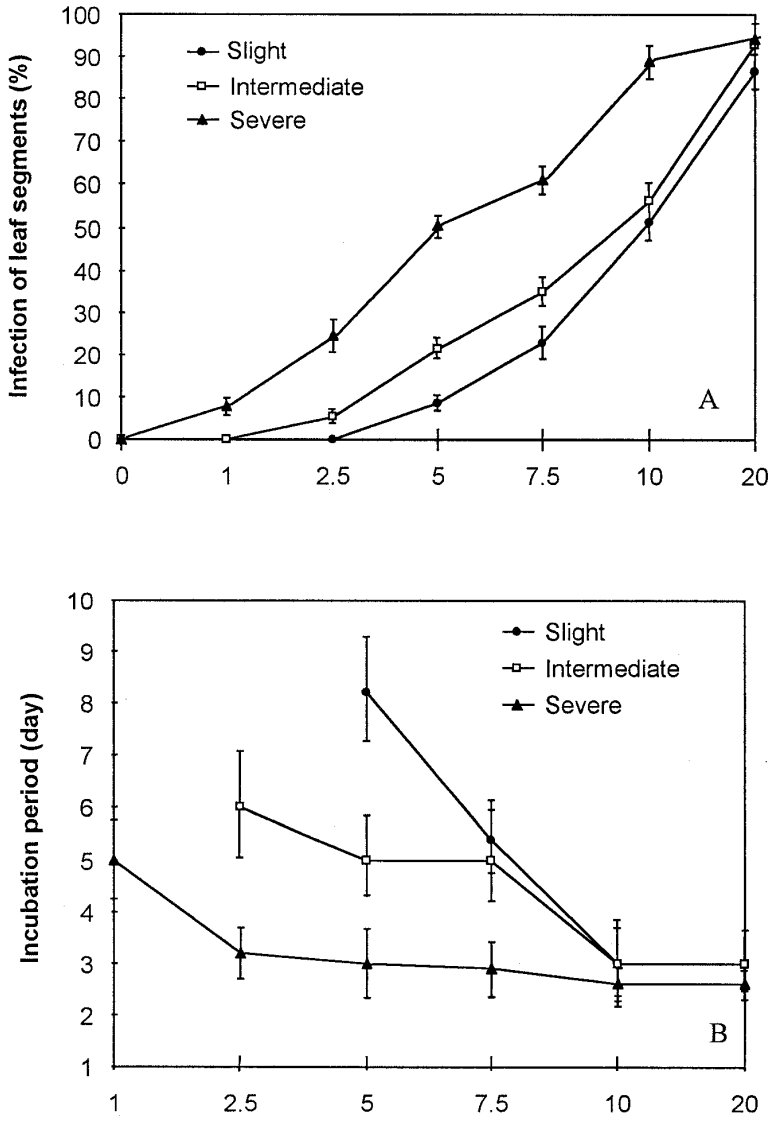

Fig. 3. Relationship of seed sample sizes (g) from mother plants with slight, intermediate, and severe bacterial leaf streak on **(A)** disease severity and **(B)** length of incubation period (time required for visible lesions to appear) in the rice leaf inoculation. Bars are standard deviations of three replications.

incubated in a growth chamber (Percival, Boone, Iowa) at 32/25°C (day/night) with 12 h/day of fluorescent light (GE Fluorescent Lamp, General Electric, Manila, Philippines). Moisture was maintained by gently spraying a fine mist of sterile distilled water on the segments each day. Symptom development, as well as the appearance of ooze, was monitored for 4 weeks after inoculation.

Confirmation tests. To confirm the reliability of the leaf assay method, bacteria were re-isolated from the leaf segments 2 to 3 days after inoculation. Bacteriological tests using the methods of Vera Cruz et al. (16) were followed.

The Biolog system (Microlog 1 Release 3.50, Biolog Inc., Hayward, CA), based on patterns of carbon source utilization, also was used to confirm identity of the bacteria. The modified procedures of Xie were followed (18).

The bacteria re-isolated from the lesions on the leaf segments also were confirmed by the tube precipitin test (micro-agglutination) following the procedures of Ball (2). A twofold serial dilution series (1:2 to 1:512) of antiserum against *X. oryzae* pv. *oryzicola* was produced and used for confirmation. Another serological method—IRMA (19)—was used to compare results obtained from the leaf inoculation method. The IRMA combined the antibody in the antiserum with the protein A labeled with radioactive I^{125}. When this radioactive I^{125}-labeled antiserum was used in the test, the higher the inoculum concentration of *X. oryzae* pv. *oryzicola*, the higher the radioactive reading that was registered.

RESULTS

The duration of the green appearance of the leaf segments on water agar with benzimidazole added was prolonged when compared with the check. Duration of the green appearance was increased as concentration of benzimidazole increased, up to 75 ppm (Fig. 1). However, at a concentration higher than 75 ppm, duration was not increased for leaves taken from any position.

Inoculum concentration for inducing lesions. To determine the minimum amount of *X. oryzae* pv. *oryzicola* required to initiate BLS lesions on the leaf segments, bacteria were re-isolated from the diseased leaf segments and re-inoculated at different concentrations to the leaf segments placed on water agar amended with 75 ppm benzimidazole. With an increase in inoculum density, the number of BLS lesions also increased (Fig. 2). At inoculum concentrations lower than 10^3 CFU/ml, no visible lesions were observed on the leaf segments at the end of the experiments, 10 days after inoculation. An inoculum concentration of 10^3 CFU/ml with an incubation period (the time required for visible lesions to appear from inoculation) of 6

Fig. 4. Bacterial ooze (O) on streak lesions of leaf segments 14 days after inoculation with pure culture of *Xanthomonas oryzae* pv. *oryzicola* isolated from seed wash of rice seeds estimated with 10^6 CFU/ml (leaf segments 1 and 2) and 10^3 CFU/ml (leaf segments 3 and 4).

Table 1. Bacteriological tests of *Xanthomonas oryzae* pv. *oryzicola* (Xoc), *X. oryzae* pv. *oryzae* (Xoo), and *Acidovorax avenae* subsp. *avenae (Aaa)* re-isolated from lesions of leaf segments

Tests	Xoc[u]	Xoc[v]	Xoo[w]	Xoo[x]	Aaa[y]
Gram staining	–[z]	–	–	–	–
Fluorescence	–	–	–	–	–
Nitrate reduction	–	–	–	–	+
Oxidation-fermentation	o	o	o	o	o
Oxidase	–	–	–	–	–
Starch hydrolysis	+	+	+	+	v
Acetoin production	+	+	–	–	
Growth on L-alanine as carbon source	+	+	–	–	
Growth on 0.2% vitamin-free casamino acids	+	+	–	–	
Resistance to 0.001% cupric nitrate	–	–	+	+	

[u] Isolate re-isolated from the leaf segments showing bacterial leaf streak symptoms by inoculation with bacteria from diseased seeds.
[v] Pure culture of *X. oryzae* pv. *oryzicola.*
[w] Isolate re-isolated from the leaf segments showing bacterial blight symptoms by inoculation with bacteria from diseased seeds.
[x] Pure culture of *X. oryzae* pv. *oryzae.*
[y] Isolate re-isolated from the leaf segments showing bacterial leaf streak symptoms by inoculation with bacteria from diseased seeds.
[z] + = positive, – = negative, o = oxidative, v = variable.

Table 2. Biolog confirmation of *Xanthomonas oryzae* pv. *oryzicola*, *X. oryzae* pv. *oryzae*, and *Acidovorax avenae* subsp. *avenae* re-isolated from the leaf segments showing different symptoms

		Biolog[x]	
IRRI acc. no.[y]	Leaf assay[z]	Identity	Similarity
11336	BLS	*X. oryzae* pv. *oryzicola*	0.82
11337	BLS	*X. oryzae* pv. *oryzicola*	0.78
11338	BLS	*X. oryzae* pv. *oryzicola*	0.72
11339	BLS	*X. oryzae* pv. *oryzicola*	0.70
11340	BLS	*X. oryzae* pv. *oryzicola*	0.81
11341	BLS	*X. oryzae* pv. *oryzicola*	0.75
11335	BLB	*X. oryzae* pv. *oryzae*	0.77
08899	BLP	*A. avenae* subsp. *avenae*	0.80

[x] Bacteria were re-isolated from the lesions and identified by Biolog.
[y] International Rice Research Institute accession number.
[z] Shows the lesions of bacterial leaf streak (BLS), bacterial leaf blight (BLB), and bacterial leaf stripe (BLP) on the leaf segments 2 to 4 days after inoculation by leaf assay.

days appears to be the critical concentration for lesion induction based on the leaf inoculation method. At inoculum concentrations higher than 10^5 CFU/ml, the number of lesions did not increase significantly with the increase in inoculum concentration, and the incubation period remained unchanged.

Sample size of rice seeds for leaf inoculation. It appears that inoculum thresholds (minimum amount of infected seeds needed to induce the disease) varied according to levels of disease severity on mother plants from which seeds were obtained. For positive detection based on the leaf inoculation method, the minimum amount of seed obtained from mother plants with slight, intermediate, and severe BLS infection was 5.0, 2.5, and 1.0 g, respectively, (Fig. 3A). The shortest incubation period on the leaf segments also varied with sample size and severity of infection of the mother plants (Fig. 3B). It took 10 g of seed from slightly and moderately infected mother plants to produce the shortest incubation period, while only 2.5 g of seed from severely infected mother plants was needed (Fig. 3). Ten grams of seed harvested from severely diseased mother plants yielded maximum BLS lesions on the leaf segments and the shortest incubation period, 3.5 days. To produce a similar number of BLS lesions on the leaf segments required 20 g of seed from mother plants with slight and intermediate BLS infection.

Confirmation test. The lesions produced on the leaf segments were typical of BLS caused by *X. oryzae* pv. *oryzicola.* The BLS lesions could also be distinguished using a stereomicroscope. At 7 to 14 days after inoculation with *X. oryzae* pv. *oryzicola*, numerous tiny drops of bacterial ooze formed on the lesions (Fig. 4), which are distinctly smaller and more numerous than those caused by *X. oryzae* pv. *oryzae*, while *A. avenae* subsp. *avenae* did not produce any bacterial ooze. Bacteria were re-isolated from streak lesions and their identity was confirmed by comparing them with *X. oryzae* pv. *oryzicola*, *X. oryzae* pv. *oryzae*, and *A. avenae* subsp. *avenae* in several bacteriological tests (Table 1).

The test tube precipitin test (micro-agglutination) revealed that a dilution of 10^6, 10^7, and 10^8 CFU/ml of the re-isolated bacteria showed strong positive precipitin reaction with all dilutions of the antisera against *X. oryzae* pv. *oryzicola* tested. No positive reaction was observed with cell suspensions from the cultures of *A. avenae* subsp. *avenae* or the three isolates of *X. oryzae* pv. *oryzae.* One of the isolates of *X. oryzae* pv. *oryzae* showed a positive reaction, indicating that a cross-reaction between the antisera of *X. oryzae* pv. *oryzicola* and that of *X. oryzae* pv. *oryzae* is common. All six of the selected isolates from leaf segments showing BLS lesions also were confirmed by Biolog to be *X. oryzae* pv. *oryzicola* (Table 2).

Comparison of in vitro leaf segment and intact leaf inoculation. The incubation period and time of ooze appearance did not differ between inoculations of leaf segments in vitro and leaves on intact plants in the greenhouse. Lesions and bacterial ooze formed on leaf segments in vitro with all the inocula tested, but formed on intact plants only with the highest inoculum concentration (Table 3).

Comparison with IRMA. Inoculum prepared from the same rice seeds was used to detect the presence of *X. oryzae* pv. *oryzicola* by the leaf assay method and by IRMA. Both methods were sufficiently sensitive to detect bacteria from the seeds at all levels of disease severity tested. However, a positive reaction for *X. oryzae* pv. *oryzicola* also was noted with the seeds from disease-free plants of Xiang-hu 24 using IRMA and from seeds of variety Zhi 10 from mother plants with lesions of bacterial bight (*X. oryzae* pv. *oryzae* positive), whereas *X. oryzae* pv. *oryzicola* was not detected by the leaf assay method. In another seed sample from a disease-free source, no *X. oryzae* pv. *oryzicola* was observed by leaf assay, while variable results were obtained by IRMA. Negative results were obtained using both methods in only one seed sample of Zhi 10 from

Table 3. Comparison of in vitro rice leaf inoculation with intact leaf inoculation in the greenhouse for detection of *Xanthomonas oryzae* pv. *oryzicola* from seeds and leaves by incubation period and ooze appearance[x]

		Leaf assay		Greenhouse inoculation	
Inoculation material	**Amount material**	**Incubation (days)**	**Ooze production (days)**	**Incubation (days)**	**Ooze production (days)**
Diseased seeds	1.0 g	5.0 a[y]	10.1 a	no lesion	–[z]
Diseased seeds	2.0 g	3.0 b	8.2 b	no lesion	–
Diseased seeds	4.0 g	3.0 b	5.0 c	8.0 a	–
Diseased seeds	8.0 g	2.5 b	5.0 c	6.0 b	10.0 a
Diseased leaves	2.0 g	3.0 b	5.0 c	5.0 b	10.0 a
Diseased leaves	4.0 g	2.5 b	5.0 c	5.0 b	7.0 b
Pure culture	10^8 CFU/ml	3.0 b	6.0 c	5.0 b	8.0 b

[x] All the diseased seeds and leaves were collected from severely infected individual plants with bacterial leaf streak.
[y] Means in columns followed by the same letter are not significantly different at $P = 0.05$ by the least significant difference test.
[z] – = not observed at end of experiment.

Table 4. Comparison of the rice leaf assay and an immuno-radiometric assay (IRMA) for detecting *Xanthomonas oryzae* pv. *oryzicola* in rice seeds

	Results of IRMA			
Rice cultivars	**BLS scale in field[y]**	**Xs/Xb[z]**	**Reaction**	**Results of leaf assay**
Qing-lian 16	7.2	4.23	+	+
Shan-you 6	7.0	2.26	+	+
Jun-xie	7.5	3.32	+	+
IR36	4.5	1.85	+	+
Xiu-shui 48	3.4	3.70	+	+
Shan-you 63	5.0	1.45	v	+
Xiu-shui 11	1.0	1.70	+	+
Xiang-hu 24 (disease-free seeds)	0	2.12	+	–
IR26 (disease-free seeds)	0	1.42	v	–
Zhi 10 (disease-free seeds)	0	1.21	–	–
Zhi10 (bacterial blight-infected)	0	2.54	+	–
Zhi 10 (bacterial blight-infected)	0	1.16	–	–

[y] BLS = bacterial leaf streak.
[z] When the Xs/Xb value was higher than 1.50, the samples were considered "+" (infected by *X. oryzae* pv. *oryzicola)*; 1.30 to 1.50 as "v" (suspected infection), and <1.30 as "–" (not infected by the bacterium).

disease-free mother plants (Table 4). In another case, when the seed sample coming from the mother plants with severe BLS infection was treated with hot air at 80°C for 10 min to kill the bacteria, a positive reaction was observed with IRMA.

DISCUSSION

An in vitro assay for the presence of *X. oryzae* pv. *oryzicola* successfully detected the bacteria in washings from seed. A critical aspect of this assay is to prevent senescence of the leaf segments before the time required (3 to 8 days) for lesion and ooze formation from low concentrations of inoculum. This was achieved by the addition of 75 ppm benzimidazole to the agar on which inoculated leaves were incubated.

Incubation period is governed by various factors inherent in the bacterial pathogens (species, mode of entry, inoculum density, and type of disease); the host plants (species, age, and organ of infection); and environmental conditions of infection (temperature and humidity; 6). In the leaf assay for *X. oryzae* pv. *oryzicola*, inoculum density was the key factor because other factors were fixed under most favorable conditions. The minimum concentration needed to induce lesions was 10^3 CFU/ml, with an incubation period of 5 to 6 days, but the percentage of leaf segments infected at this density was low. At inoculum density above 10^5 CFU/ml, the percentage of leaf segments developing lesions reached the maximum and the incubation period was the shortest.

In the leaf inoculation method, sample size in relation to seed contamination or infection is another key factor in detecting *X. oryzae* pv. *oryzicola* in rice seed. The lower the disease severity on mother plants, the greater the sample size of rice seed required for detection. Our results showed that the minimum sample size required for the detection of *X. oryzae* pv. *oryzicola* is from 1 to 5 g and that it varies with the disease severity of the mother plants. In reality, however, we often lack information on BLS severity in routine seed health testing for rice seed certification. Based on our data, 10 g of rice seed is recommended as the minimum sample size to detect *X. oryzae* pv. *oryzicola* using the leaf inoculation method.

Symptoms of bacterial leaf spots generally appear when the bacterial population reaches 10^6 CFU/ml at the infection court, if the leaf tissue is congested with water (5). Incubating the leaf segments on agar provides conditions suitable for bacterial multiplication and increases the likelihood that any bacterium present will multiply to the population level required for lesion development. Therefore, even at relatively low inoculum concentration, leaf streak lesions may be produced after a sufficient time of incubation of the leaf segments. In comparison, intact leaves of rice plants grown in the greenhouse may not provide as favorable an environment. The leaf inoculation method is as fast and as efficient in testing a large numbers of seed samples as inoculation of intact leaves.

With respect to the other methods, the leaf inoculation method is more reliable and economical for detecting *X. oryzae* pv. *oryzicola*. IRMA is a more sensitive and faster method than leaf inoculation in detecting the bacteria, but false positive reactions were sometimes noted among seed samples from *X. oryzae* pv. *oryzae*-infected mother plants. This may be due to the cross-reaction of the antisera to *X. oryzae* pv. *oryzicola*, which shares a common antigen with strains of *X. oryzae* pv. *oryzae* (19). In general, IRMA could not differentiate dead bacterial cells from living ones. The leaf inoculation method is currently being used in plant quarantine stations in China and for routine seed health testing at IRRI.

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