J. Gen. Plant Pathol. 68 : 356–362 (2002)

Alternative Route of Infection for Bacterial Seedling Blight of Rice Caused by *Burkholderia plantarii*

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ABSTRACT

***Burkholderia plantarii*, the pathogen of bacterial seedling blight of rice, was detected in paddy water. Its concentration rose in July and August. The bacterial concentration in the paddy water was always higher along levees than at distances more than 5 m from levees. Confirmed to be released into water when graminaceous weeds were immersed, *B. plantarii* survived for at least 4 days at 30°C. *B. plantarii* was splashed at least 30 cm upward by rain splash in the field. Harvested seeds, which had been sprayed with *B. plantarii* released from graminaceous weeds at the flowering stage, retained the bacteria. Bacterial seedling blight occurred when the seeds were then sown in nursery boxes. These results indicated that graminaceous weeds growing on levees of paddy fields are a source of infection of the disease and that rice seeds are infected through the paddy water.**

(Received May 23, 2002 ; Accepted September 1, 2002)

Key words : rice, *Burkholderia plantarii*, paddy water, infection source, levee weed.

INTRODUCTION

Seedling blight of rice, caused by the bacterium *Burkholderia plantarii*[18], was first detected in Chiba Prefecture of Japan in 1982[1]. Since then, it has been found in various locations except some parts of the south and west[2,6]. The disease occurs only in nursery boxes used for mechanical transplanting. Outbreaks sometimes occur at commercial rice seedling centers and can cause severe damage.

B. plantarii is seedborne and considered to persist in the basal parts of the rice plant in the field[2,5,16]. The bacterium is speculated to then move to upper parts of plants and infect rice seeds[3].

B. plantarii has been isolated from several graminaceous weeds[12], one pteridophytous weed and three dicotyledonous weeds[17] growing on the levees of paddy fields. However, the mode of transmission from the weeds to rice seeds has not yet been clarified. Miyagawa reported that *B. plantarii* was released into reservoir water from graminaceous weeds growing on the embankment[7]. Because miscellaneous host weeds generally flourish on the levees of paddy fields, *B. plantarii* might be released into the paddy water in the same way as in the reservoir. In this report, we examined the population fluctuations and distribution of *B. plantarii* in paddy water and the release of the bacterium from carrier weeds. We demonstrated that levee weeds and paddy water are sources of infection for the disease.

MATERIALS AND METHODS

Detection of *B. plantarii* in paddy water Paddy water was skimmed using a sterilized glass screw bottle. One hundred ml of water was screened for *B. plantarii* using the method described previously[7]. Briefly, the paddy water was filtered through a 5-μm membrane filter and then a 0.45-μm membrane filter. The latter filter was subsequently placed on a plate of AFG medium[2] with antibiotics and incubated for 3 to 4 days at 30°C. Colonies producing characteristic red-purple crystals[2] were enumerated using a binocular microscope.

Identification of isolates To confirm that the colonies were *B. plantarii*, pathogenicity tests and PCR detection were implemented as follows. Each colonies was scratched from the membrane filter with a needle, and suspended in a small amount of sterilized distilled water. Single colonies were isolated by two additional streakings onto AFG plates. The isolated colonies were transplanted onto PPGA plates[9]. The bacteria were suspended in skim milk broth (skim milk 10 g, sodium glutamate 1 g, distilled water 100 ml, autoclaved for 15 min at 115°C) and stored at −20°C until used.

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The isolates were multiplied on PPGA plates at 30°C for 2 days. The bacterial cells were suspended in sterilized distilled water and adjusted to 10^4 cfu/ml. The rice seeds were inoculated as described previously[7]. If no clear symptoms of disease appeared, the bacterial concentration was re-adjusted to 10^6 cfu/ml and the pathogenicity test was repeated.

A PCR-based technique was also used to identify *B. plantarii*. A set of primers (supplied by Dr. H. Sawada, unpublished) that were specific to *B. plantarii* were used to amplify bacterial 16S-23S rDNA spacer fragments. The bacterial colonies grown on PPGA plates were suspended in a small amount of sterilized distilled water. One microliter of bacterial suspension (10^8 cfu/ml) was mixed with 20 μl of reaction mixture. PCR amplification and gel electrophoresis for analyzing PCR products were performed following the method of Takeuchi *et al.*[15] *B. plantarii* (MAFF 301723) was used as the type strain.

Sampling of paddy water In 1999, paddy water was collected from six paddy fields in Kozan Town, Sera County, Hiroshima Prefecture. The area of the fields was approximately 2000 to 4000 m^2. The adjacent fields were located downstream of a reservoir infested with *B. plantarii*[7]. Paddy water was skimmed at five sampling sites along the levee of each field every 10 days from April to August 1999. When the paddy field was dry, sampling was cancelled. Paddy water more than 5 m from the edge of the levee was skimmed six times from July 26 to August 23, 1999 at five random sites in each field to compare with the water along the levee.

In 2000, 50 paddy fields located within an approximately 15-km range along a country road in Sera Country were sampled five times at 15-day intervals from May to August for the presence of *B. plantarii*. When there was no water, samples were collected from a nearby field. Therefore, sampling was not always conducted in the same fields, and the total number of fields examined was over 50.

The release of *B. plantarii* into water Five kinds of graminaceous weeds shown in Table 2 were collected from the levees in paddy fields in Fig. 1 in September 1999 and placed in the bottom of Wagner pots (1/2000 a) for 2 weeks until the start of the experiment. These weeds did not have any visible symptoms on stems and leaves. Ten liters of deionized water was gently poured into the pots so as not to muddy the water, then the plants were immersed in the water at a depth of about 20 cm. The supernatant was skimmed 0, 1 and 6 hr later and used for detecting *B. plantarii* as described earlier except for the amount of water taken. Because preliminary tests showed that too many colonies of *B. plantarii* appeared on a membrane filter when 100 ml of water was filtered, 1 ml of the supernatant was added to approximately 50 ml of sterilized water to facilitate the filtration. These plants did not show any visible symptoms on stems and leaves at the experiment started.

The longevity of *B. plantarii* released in water To examine the duration of the infectivity of *B. plantarii* released from host plants, the longevity of *B. plantarii* in water was investigated as follows. The perennial graminaceous weed *Molinia japonica* (Hack.) Hayata, previously confirmed as a carrier of *B. plantarii*, was collected in Kozan Town. The plants were transplanted into plastic containers (L, W, H=40, 30, 10 cm) that were then placed outdoors. Eight liters of deionized water was slowly poured into the container until it was brimming full. Two hours later, 1 liter of supernatant was skimmed off using a suction pump. After filtration through gauze to remove contaminants such as fallen leaves, the water was kept in an incubator at 30°C for 10 days. One or 0.2 ml of the water was removed daily, and the concentration of viable cells of *B. plantarii* was examined as described earlier. The experiment was carried out in July and November 2001 using three containers.

Bacterial dispersion under field conditions Experiment 1: A small shallow pool approximately 2 m^2 wide and 3 cm deep was made using a polyethylene sheet in an open field. The water sampler as a trap for the bacteria consisted of a 7-cm-diameter funnel inserted in a 100-ml glass bottle. Five props were installed in the pool. Two samplers were fixed using a clamp to each prop at a height of 30, 60, 90,120 or 150 cm from the water surface. The bacterial suspension of *B. plantarii*, approximately 10^7 cfu/ml, was poured into the pool right before rainfall. After rainfall, the water trapped in the glass bottles was examined for *B. plantarii*. The experiments were repeated twice in June 1998. The precipitation was measured by the meteorological facility in our institute.

Experiment 2: The experiment was carried out in farmers' paddy fields twice during July and August 1998. The props with water samplers were installed between rice hills grown in the closest row to a levee. Similar to experiment 1, water samplers were installed before and recovered after rainfall. In the paddy fields in Fig. 1, 14 props were installed. All water samplers were fixed at a height of 30 cm. In another paddy field in the suburbs of Fukuyama City, six props were installed and water samplers were fixed at 30 and 60 cm.

Testing seed transmission of the pathogen The water containing *B. plantarii* from *M. japonica* (Hack.) Hayata was obtained as previously mentioned. After checking for the presence of *B. plantarii*, the water was preserved in a refrigerator for 3 days until the results came out. Thirty ml of water was sprayed onto the spikes of rice plants (cv. Kinmaze) at the time of ear emergence and flowering. The remaining water was re-examined for

the concentration of *B. plantarii*. Deionized water was used as a control. Ten hills in a Wagner pot (1/5000 a) were used per treatment. The rice plants were covered with a polyethylene bag and kept at 25°C for 2 days, and then they were transferred to a glass house until harvest. To check for infection, the harvested seeds (3 g) were soaked in 30 ml of sterilized water in a vacuum for 1 hr. The concentration of bacteria released in the water was measured by the filter method mentioned earlier. The remaining seeds (10 g) were soaked at 25°C for 4 days and then at 30°C overnight to promote germination. One hundred budding seeds were sown in commercial soil packed in a small container (L, W, H=10, 6, 3 cm) and kept at 30°C and 80% humidity for about 10 days. The number of blighted seedlings and those with typical symptoms were scored. The experiments were carried out twice with three repetitions.

RESULTS AND DISCUSSION

Confirmation of isolates as *B. plantarii*

Because bacteria were isolated from paddy water or wild weeds without symptoms, 69 randomly selected isolates were examined for their identity as *B. plantarii* by both the pathogenicity test and PCR technique. Typical symptoms of rice seedling blight appeared after inoculation with all the isolates. All PCR products were identical in size to that of the type strain (approximately 200 bp). Considering the results and a previous report[7], we concluded that the characteristic colonies (producing red-purple crystals) isolated throughout this study were *B. plantarii*.

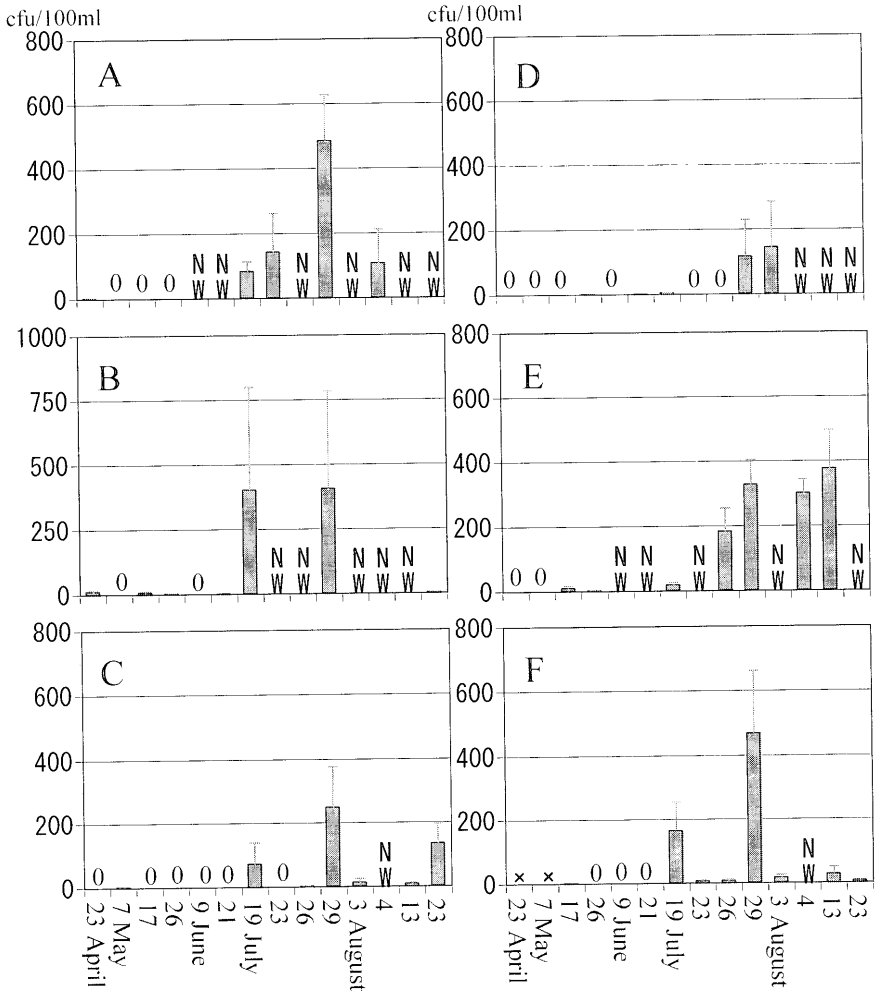

Fig. 1. Fluctuation in the concentration of *B. plantarii* in paddy water of six fields during the rice-growing season of 1999. Error bars indicate standard errors. 0, *B. plantarii* was not detected; NW, not investigated because there was no paddy water; ×, not investigated because of farm work.

Detection of *B. plantarii* found in paddy water along levees

In 1999, the presence of *B. plantarii* in paddy water was examined in six fields (Fig.1). *B. plantarii* was detected frequently in paddy water from July to August, but was absent or hardly detected from April to June (Fig. 1). The concentration of *B. plantarii* in the same paddy field greatly fluctuated with the sampling time and between sites in the same field.

We confirmed that *B. plantarii* was widely distributed in the region surveyed in 2000. The frequency of *B. plantarii* in the fields was 9/50 (detected fields/investigated fields) in May and 11/50 in June. The frequency increased to 25/50 in July and 34/50 in August. The mean and maximum values of the concentration increased similarly (Table 1).

***B. plantarii* released from graminaceous weeds**

B. plantarii was detected in water immediately after water was poured into the pots with Southern crab grass, Blady grass or Japanese lawn grass. One and 6 hr later, it was also detected in water of another pot with Blady grass. On the other hand, *B. plantarii* was not detected in water of pots with Eulalia or *Paspalum thunbergii*. The maximum concentration of *B. plantarii* in water was approximately 1200 cfu/ml (Table 2), about 100 times higher than that in paddy water. In paddy fields, *B. plantarii* released from weeds was probably diluted by a huge amount of irrigation water.

Detection of *B. plantarii* in paddy water inside paddy fields

The concentration of *B. plantarii* in paddy water was compared between the edge and the middle of the field. The results are shown in Fig. 2. The concentration was always lower in water that was more than 5 m from the levees than from that near levees. Especially, in the case of A2, B2, C2, C6 and F2 in Fig. 2, *B. plantarii* was not detected deep within the field, while it was abundant at the edge. In the fields indicated by symbols E1 to E5 in Fig. 2, *B. plantarii* was detected toward the middle of the field. Irrigation water frequently runs into this field.

Table 1. Detection frequency and concentration of *Burkholderia plantarii* in paddy water in 2000

Sampling date	Frequency of field detected[a)]	Concentration of *B. plantarii*	
		Mean±S.E.[b)]	Maximum[b)]
2000 May 30	9/50	2.3±10.6	63
June 16	11/50	1.4±4.6	26
July 3	32/50	40.0±105.1	662
July 17	25/50	47.0±149.4	676
August 7	34/50	107.4±265.6[c)]	1530[c)]

a) No. of field with *B. plantarii*/No. of field investigated. Fields were randomly chosen each time.
b) Numerical unit is cfu/100 ml.
c) Too many colonies of *B. plantarii* collected from one paddy field on August 7 appeared on the membrane filter. Therefore, these numerical values were based on the other 49 fields.

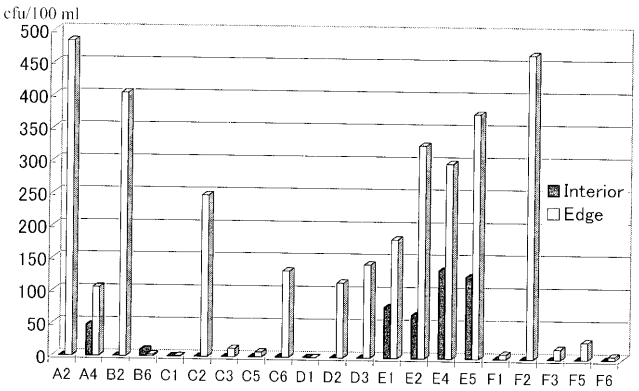

Fig. 2. Comparison of population of *B. plantarii* between the edge and interior of paddy field. Letters on the X-axis correspond to the paddy field of Fig. 1. The accompanying numerals indicate the investigation date; 1, 26 July; 2, 29 July; 3, 3 August; 4, 4 August; 5, 13 August and 6, 23 August in 1999.

Table 2. Release of *Burkholderia plantarii* from graminaceous weeds into water

Weeds		Time after immersing in water		
Scientific name	(Common name)	0 hr	1 hr	6 hr
Digitaria ciliaris[a)]	(Southern crab grass)	873[b)]	1040	1271
Digitaria ciliaris[a)]	(Southern crab grass)	10	28	58
Imperata cylindrica[a)]	(Blady grass)	0	164	167
Imperata cylindrica[a)]	(Blady grass)	298	240	151
Zoysia japonica	(Japanese lawn grass)	28	234	332
Miscanthus sinensis	(Eulalia)	0	0	0
Paspalum thunbergii		0	0	0

a) Each weed was removed from separate paddy fields.
b) Numerical unit is cfu/ml.

However, the bacterial concentration was always lower than along the levee.

The temperature is high in July and August and suitable for the growth of bacteria. This is likely the reason why the concentration and detection frequency of *B. plantarii* in paddy water increased during this season. Previously, *B. plantarii* was detected in water of a local reservoir[7]. *B. plantarii* might be carried from the reservoir to the paddy fields by irrigation water. If it is assumed that most of the *B. plantarii* detected in paddy water is derived from irrigation water, they must be uniformly distributed in the paddy field. However, *B. plantarii* was hardly detected at all over 5 m from the levee. In addition, it was confirmed that *B. plantarii* was released from the graminaceous weeds that grew on the levee. Considering these results, most of the *B. plantarii* detected in paddy water seems to originate in levee weeds. In paddy field E in Fig. 2, *B. plantarii* was detected more than 5 m from a levee. In this case, the paddy water circulated well in the field during irrigation.

The concentration of *B. plantarii* greatly fluctuated between sampling dates. It might be high when the water level of the paddy field is high enough to immerse the weeds on a levee; for example, immediately after irrigation or rainfall. *B. plantarii* was absent or hardly detected when the water level was very low. In addition, the concentration of *B. plantarii* greatly depended upon the site on the levee. As shown in Table 2, all the graminaceous weeds on a levee did not always carry *B. plantarii*. Therefore, the concentration seemed to be high around bacteria-carrying plants.

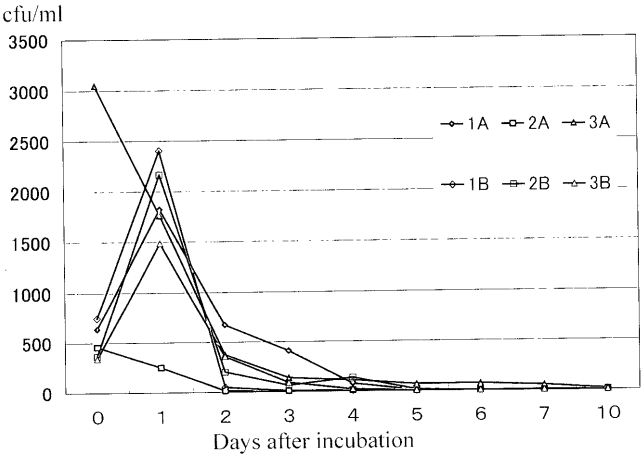

Fig. 3. The longevity of *B. plantarii* released into water from *Molinia japonica* and kept at 30°C. Numerals in explanatory remark indicate different plant containers. A and B indicate different experiments conducted in July and November 2001, respectively.

The longevity of *B. plantarii* in water

The initial concentration of *B. plantarii* released from a graminaceous weed, *M. japonica*, was between about 300 and 3000 cfu/ml. One day after incubation at 30°C, the concentration fluctuated greatly between the repetitions. However, it decreased continuously 2 days after incubation in all cases. *B. plantarii* was hardly detected after 4 or 5 days (Fig. 3).

It is not clear why the concentration of *B. plantarii* fluctuated 1 day after incubation. Though the water temperature of the paddy fields was not measured, it seemed to be around 30°C in July and August in the western region of Japan. The earlier results suggested that *B. plantarii* released in the paddy water would start to die out within few days. Tagami *et al.*[14] investigated the viability of *Xanthomonas campestris* pv. *oryzae* suspended in paddy or river water. They reported that the viable count of bacteria decreased markedly after 18 hr, and was 0.74 to 0.05% of the initial concentration after 36 hr. Thus, the decrease of viable cells in paddy water seemed to be a common phenomenon among certain bacteria, probably because of unfavorable environments. However, *B. plantarii* is expected to survive with certainty for several days in paddy water.

Bacterial dispersion in the field

Experiment 1: The results are shown in Table 3. The amount of rainfall was 29 mm in 10 hr in test A and 9 mm in 8 hr in test B. The concentration of *B. plantarii* in the pool water after rainfall was 2×10^4 and 6×10^4 cfu/ml in tests A and B, respectively. In both experiments, *B. plantarii* was detected from the water samplers fixed at a height of 30 cm. *B. plantarii* was rarely detected at a height over 60 cm (Table 3).

Experiment 2: In this experiment, the rainfall was not measured, but the amount of water caught in the sampler through a 7-cm-diameter funnel was approximately 20 to 40 ml in two tests in the field in Fig. 1, and approximately 10 ml in two tests in Fukuyama City. *B. plantarii* was

Table 3. The upward dispersion of *B. plantarii* by rain splash under field conditions

	Height from source (cm)				
	30	60	90	120	150
Test A[a]	430[b]	0	0	0	0
	286	0	0	0	0
Test B[a]	381	0	0	0	0
	671	2	0	1	0

a) For weather conditions during tests and the bacterial concentration of the infection source in a pool, see the text.

b) Total bacterial number of *B. plantarii* trapped in water samplers. The numerical unit is colony forming unit.

detected in four of 14 samplers in one of the two field tests in Fig. 1. The number of viable cells was 984, 62, 41 and 7 cfu. *B. plantarii* was detected in one sampler of six at a height of 30 cm in one of two tests in Fukuyama City. The viable cell number was 551 cfu. However, it was not detected at a height of 60 cm in two tests.

It was confirmed from these results that *B. plantarii* was rain-splashed to a height of at least 30 cm in open fields. The effect of wind was not considered in these experiments. With stronger wind and rain, minute raindrops containing *B. plantarii* were likely to scatter to higher parts of plants. In addition, wind might bend rice panicles so that rain splash can reach them. Investigations using a simulated rain tower and wind tunnel[4] would provide evidence for this.

Seed transmission of the pathogen

The concentration of *B. plantarii* in water samples obtained from *M. japonica* was approximately 700 and 1700 cfu/ml (Table 4), about 100 times higher than the concentration in paddy water, but nearly equal to that released from other weeds shown in Table 2. Bacterial numbers isolated from harvested seeds differed from one to the other. *B. plantarii* was detected at more than 10^5 cfu per g of seeds from the seeds inoculated with water samples No. 1 and No. 3. Disease occurrence was confirmed only in No. 1 and No. 3 seeds. The diseased seedling rate was about 90% in test A and about 30% in test B (Table 4).

The results indicated that this disease would arise when harvested seeds, sprayed with water containing *B. plantarii* released from host weeds, were sown in nursery boxes. The disease was not confirmed in the case of seeds No. 2. Bacterial numbers isolated from seeds of No. 2 were much lower than those of No. 1 and No. 3 (Table 4). Therefore, disease could not arise because of low numbers of the pathogen. It is well known that antagonistic microorganisms influence the multiplication of *B. plantarii*[8,11,13]. These microorganisms might repress the multiplication of *B. plantarii* after inoculation in the case of seeds No. 2 and bacterial numbers could not increase in seeds at harvest.

After rice seedlings were transplanted into fields, *B. plantarii* decreased in the above ground parts of rice plants, but persisted in the basal parts[2,5,16]. Azegami[3] speculated that *B. plantarii* moves from the basal to upper parts in association with plant growth or *via* a capillary phenomenon.

However, our results suggested an alternative route of infection as follows. *B. plantarii* latently inhabits graminaceous weeds on levees, probably around the basal parts of the weeds. When the level of paddy water is high enough to cover these basal parts or probably during rainfall, *B. plantarii* is released into paddy water. *B. plantarii* disperses by rain splash and wind, latently infecting rice panicles. When the rice seeds carrying the bacterium are sown in nursery boxes, the disease occurs under high temperature and high humidity. In support of this hypothesis, Satou and Matuda[12] isolated *B. plantarii* from the basal parts of several graminaceous weeds. Recently, Ohara *et al.*[10] reported that *B. glumae*, the pathogen of bacterial grain rot of rice, was detected from paddy water and irrigation water. Paddy water may also play an important role as the infection source of other diseases, such as bacterial grain rot and palea browning caused by *Erwinia ananas*. Further research on paddy water and nearby weeds would clarify these disease cycles.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. H. Sawada of the Natl. Inst. for Agro-Environmental Sciences for allowing us to use his newly designed primers for *B. plantarii*. We also would like to thank Dr. H. Koganezawa for critical reading of the manuscript.

Table 4. Incidence of bacterial seedling blight using seeds inoculated at flowering stage with *B. plantarii* released from weeds

Inoculum	Bacterial concentration of inoculum[a]	Bacterial number isolated from harvested seeds[b]	Disease seedling rate (%)	
			Test A[c]	Test B[c]
No. 1	1742	$1.5\pm1.1\times10^6$	92.0	39.4
No. 2	690	$6.0\pm2.3\times10^3$	0	0
No. 3	747	$7.8\pm5.3\times10^5$	87.5	33.9
Control	0	ND[d]	0	0

a) Numerical unit : cfu/ml.
b) Numerical unit : cfu/g seeds, mean±standard error.
c) Test A and test B were repetitions.
d) ND : not detected.

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