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Survival and transmission of *Xanthomonas oryzae* pv. *oryzae* in rice seeds

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ABSTRACT

Xanthomonas oryzae pv. *oryzae* is a major pathogen of rice that causes bacterial leaf blight (BLB) disease and is a great threat to rice production worldwide. Transmission of *X. oryzae* pv. *oryzae* from seed to seed is remain in enigma. In this study, seed transmission was investigated to detect and identified X. oryzae pv. *oryzae* in rice seeds of BR11 collected from the naturally BLB infected field and the transmission of *X. oryzae* pv. *oryzae* from seed to plant to seed during November, 2014 to November, 2015 at Seed Pathology Centre, Bangladesh Agricultural University, Mymensingh. The results indicated the survival ability of *X. oryzae* pv. *oryzae* for one season to another for at least 10 months but the survivability of the bacterium decreased with the increasing of storage time. The transmission of *X. oryzae* pv. *oryzae* was carried out through transplanting of the seedlings raised from the infected seeds. *X. oryzae* pv. *oryzae* was then detected in the seeds harvested from the infected plants raised from the seedlings produced from the previously harvested BLB infected seeds. These results clearly indicated the transmission of *X. oryzae* pv. *oryzae* from seed to plant to seed carryover at least from one season to another.

Keywords: Seed, transmission, rice, bacterial leaf blight

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1 Introduction

Xanthomonas oryzae pv. *oryzae* is a major pathogen of rice and is a threat to rice production in both temperate and tropical rice-growing regions, due to its high epidemic potential (Mew, 1987). Yield losses of 10–20% are common and losses of 50–70% have been recorded in severely infected fields (Mew, 1987; Mew et al., 1993). The spread of bacterial leaf blight (BLB) occurs through plant debris (Guo et al., 1980), wild rice (Aldrick et al., 1973), weeds (Valluvaparidasan and Mariappan, 1989) and water (Singh, 1971; Srivastava, 1972). During the last few years, a serious BLB epidemic occurred in the many areas of Bangladesh with significant yield losses around 50–70% especially in irrigated hybrid varieties possibly due to the emergence of new races (Alam et al., 2016; Islam et al., 2016).

The presence of *X. oryzae*. pv. *oryzae* in infected seeds (Mew et al., 1993) and disease transmission from seeds have been demonstrated (Chattopadhyay and Mukherjee, 1971). However, other scientists report controversy about its transmission (Goto et al., 1988; Unnamalai et al., 1988), probably due to the limited usefulness and accuracy of techniques used in detecting low numbers of viable cells of the pathogen

(Singh and Rao, 1977). The BLB bacterium is considered as a quarantine organism and is subject to phytosanitary regulations in many countries (FAO, 2014). Seed transmission of the closely related organism, *X. oryzae*. pv. *oryzicola*, the bacterial leaf streak pathogen, has already been established. However, survival period and possibility of seed transmission of *X. campestris* pv. *oryzae* (presently known as *X. oryzae* pv. *oryzae*) were studied by Thri Murty and Devadath (1984). They observed that the bacterium survived for longer (170–180 days) in kharif than rabi (120–130 days) harvested seed. Seed to seed transmission of *X. oryzae* pv. *oryzae* were studied by Sakthivel et al. (2001). They detected and identified *X. oryzae* pv. *oryzae* from the naturally infected seeds of cvs Jaya and TN1. They recovered the bacterium up to 4 months and 9 months from naturally infected seeds of cvs Jaya and TN1, respectively. They also identified the BLB bacterium was also detected in seedlings, mature plants and seeds collected from plants raised from naturally infected seeds. Detection of *X. oryzae*. pv. *oryzae* in symptomless plants using primers TXT and TXT4R were reported. However, detection of pathogen in non-symptomatic seedlings is of importance in certification programs, for both domestic and international quarantine, because a latent population can lead to serious epidemics under favorable conditions. Early detection of diseases, using rapid methods, is important for assessing the health status of a rice nursery before the transplantation of seedlings to fields.

Isolation of *X. oryzae*. pv. *oryzae* from rice plant and seed by conventional techniques is often difficult, usually due to the masking effect of fast-growing, yellow-pigmented bacteria. Agar media used in the isolation of the BLB pathogen are not selective enough to eliminate fast-growing contaminants (Ming et al., 1991). Biochemical tests (Cruz et al., 1984), serological assays (Benedict et al., 1989), fatty acids and metabolic profiling (Chase et al., 1992; Merieux and Balme-les Grottes, 1993) have been used in the identification of the pathogen. These assays however, have shortcomings including lack of sensitivity and specificity. Therefore, detection techniques of *X. oryzae* pv. *oryzae* in the infected rice seeds will certainly build up strong phytosanitary regulations to restrict the entrance of such dangerous pathogen into the country through importing high yielding rice varieties from abroad and the investigation into the mode of transmission of this bacterium through seeds will be useful to develop an effective strategy to control the disease in the field. The present study has been designed to detect and identify *X. oryzae* pv. *oryzae* in rice seeds (BR11) collected from the naturally BLB infected field and to study the transmission of *X. oryzae* pv. *oryzae* from seed to plant to seed.

2 Materials and Methods

Bacterial leaf blight infected rice seed samples were collected from a selected BLB infected rice (cv. BR11) field at Sutiakhali, Mymensingh and the harvested seeds were stored until May, 2015. BLB infected rice fields were selected based on the symptoms of the disease as described by Reddy (1984). Before collection of seed samples, diseased and healthy area of two plots were identified. Then 1 m^2 area of each plot was measured and seeds were collected, dried and stored at room temperature for conducting experiments.

2.1 Detection of in rice seeds

2.1.1 Detection from whole grains

On yeast extract calcium carbonate (YDC) medium The seed samples were surface sterilized with 1% sodium hypochlorite solution for 3 min, followed by repeated washing with distilled water (3 times) and blot-dried, then plated directly (25 seeds/plate) on to YDC; yeast extract (10 g), calcium carbonate (20 g), agar (20 g) in 950 mL distilled water and dextrose (20 g) in 50 mL distilled water, the two solutions were autoclaved separately and mixed well when temperature of the medium was 50 °C (ISTA, 2016). Plated seeds incubated at 28 ±2 °C for 24–72 h and observed for the presence of bacterial colonies based on the morphological characters such as shiny, raised, mucoid, pale yellow to straw yellow (Nino-Liu et al., 2006). The suspected colonies were subjected to pathogenicity and biochemical tests for confirmation of *X. oryzae* pv. *oryzae*. The experiment was carried out in four replicates of 100 seeds each and repeated twice.

Using liquid assay method One hundred seeds of each sample was ground to coarse powder and suspended in 200 mL of sterile saline (0.85% sodium chloride) and kept for 2 hr on a rotary shaker at 150 rpm. The samples were serially diluted in 10^{-6} concentration and spread 50μL of diluted suspension on YDC agar plates. The plates were incubated at 26 ± 2 °C for 3 to 4 days and observed for presence of small, shiny and yellow colonies. Number of colony forming unit (CFU) were counted and the total CFU were counted by multiplying with dilution factor (Mortensen, 2000; Razak et al., 2009).

2.1.2 Detection from kernels

Three layers of filter paper was placed in each petridish and it was soaked in 0.15 g of carbendazim solution and plates were sterilized at 121 °C for 20 min. The kernels from 100 seeds were removed and 25 kernels were placed on filter paper, the plates were incubated at 28 ± 2 °C for 24 h. Then plates were

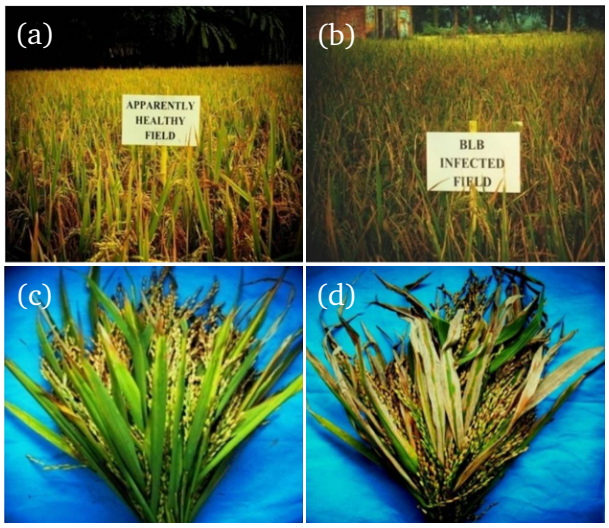

Figure 1. BLB infected rice fields from where seed samples were collected. (a) apparently healthy rice field, (b) a BLB infected rice field, (c) apparently healthy rice panicles, and (d) BLB infected rice panicles

transferred into deep freeze for 12–18 h, later incubated at 28 ± 2 °C for 72 h and observed for the development of yellow mucoid bacterial colonies on the kernels (Sakthivel et al., 2001).

2.2 Detection in seedlings

Seeds were soaked in water for a few minutes; four replicates of 50 seeds were put on the paper towel in equal distance and incubated at 28 ± 2 °C for 9 days. After 9 days seedlings were examined for typical symptoms of BLB disease (ISTA, 2016). Then leaf sections were surface sterilized with 1% (w/v) sodium hypochlorite for 3 min followed by repeated washing with distilled water; blot dried and ground in sterile distilled water. After serial dilution up to 10^{-6}, 50 μl of the final dilution was plated on YDC agar plates. The plates were incubated at 28 ± 2 °C for 24–72 h, and after 48 hr plates were observed for the presence of bacterial colonies, and pure culture was done on YDC slants. All bacterial isolates were maintained in YDC slants at 4 °C for short term storage.

2.3 Transmission of *X. oryzae* pv. *oryzae*

The transmission of *X. oryzae* pv. *oryzae* from seed to plant to seed was studied by raising seedlings from the previous year (2015) stored BLB infected rice (cv. BR11) seeds. Seedlings raised from the apparently healthy seeds were used as control. After transplanting of the seedlings raised from both healthy and infected seeds in the earthen pot containing sterilized soils, the plants were examined periodically for the appearance of BLB symptoms. Then the plants were allowed to grow until maturity and the seeds were harvested after ripening of the paddy. The seeds harvested from both infected and healthy rice plants were subjected to detection and identification of *X. oryzae* pv. *oryzae* based on morphology, pathogenicity and through a series of biochemical tests.

2.4 Determination of seedling vigor

To assess the effect of *X. oryzae* pv. *oryzae* infection on the seedlings vigor, the vigor index (VI) were calculated from the seedlings of healthy and infected seedlings raised from healthy and BLB infected seeds using formula: Vigor index = Mean root length + Mean shoot length × Germination (%).

2.5 Biochemical characterization

Biochemical tests (potassium, starch, Tween 80 hydrolysis, egg yolk reaction, anaerobic growth and oxidase) were done following the method described by Suslow (1982) ,Cowan (1974), Sierra (1957), McClung and Toabe (1947), Hugh and Leifson (1953) and Kovacs (1956), respectively.

3 Results

3.1 Occurrence of *X. oryzae* pv. *oryzae*

The BLB symptoms appeared as lesions coalesce and become yellowish-white with wavy edges which initially appeared as pale-green to grey-green water-soaked streaks near the leaf tip and margins. Eventually, the whole leaf was affected, became whitish or greyish and then died. Then the seeds were collected after harvesting the crop at maturity and the seeds were then preserved at room temperature until the next Aman growing season. *X. oryzae* pv. *oryzae* was identified as mucoid and straw-color to yellow color colonies at the third day. Usually after 3-–4 days, colonies of *X. oryzae* pv. *oryzae* on YDC were seen around the seeds as circular, entire, smooth, convex, opaque, and straw yellow color (Fig. 2a,b,c,d).

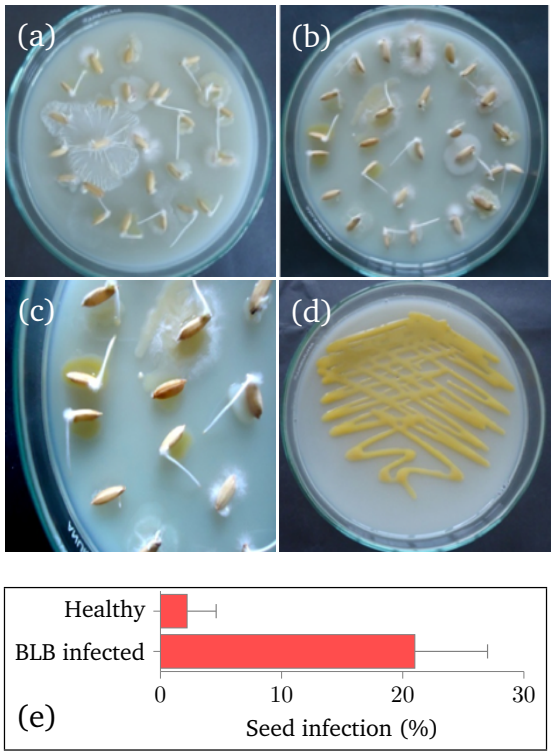

Figure 2. Growth of X. oryzae pv. oryzae (a) on apparently healthy and, (b) on infected seeds, (c) enlarged view of colony growth of *X. oryzae* pv. *oryzae* on the infected seeds plated on YDC medium, (d) a pure culture of *X. oryzae* pv. *oryzae* on YDC medium, and (c) percent seed infection by *X. oryzae* pv. *oryzae* on YDC plates from apparently healthy and infected rice seeds. Data are the averages of four replications consisting 25 seeds in each replication.

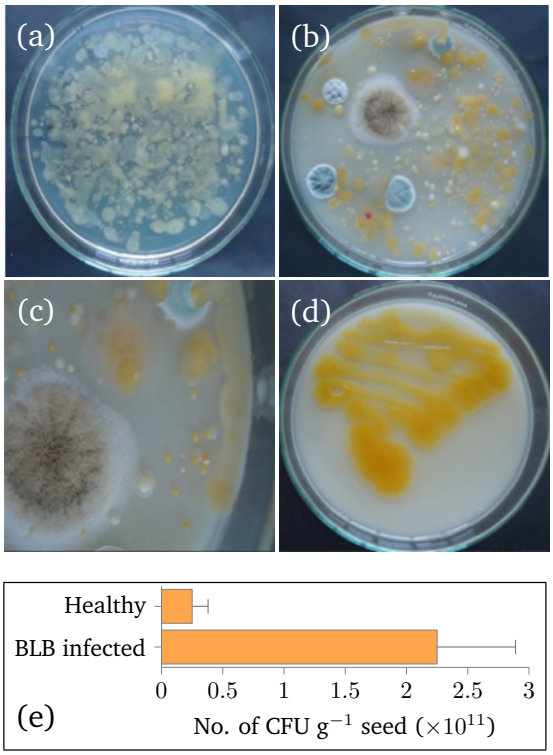

Figure 3. Yellow mucoidal colony growth of *X. oryzae* pv. *oryzae* on YDC agar medium at 10^{-6} dilution of the ground seed extracts. (a) Apparently healthy seeds, (b) infected seeds with 10^{-6} dilution, (c) enlarged view of colony growth of *X. oryzae* pv. *oryzae* on YDC agar plate, (d) colony growth of *X. oryzae* pv. *oryzae* on YDC agar plate, and (e) purified *X. oryzae* pv. *oryzae* on YDC agar plate. Colony forming unit (CFU) of *X. oryzae* pv. *oryzae* g^{-1} seeds as determined by dilution plate technique.

Colonies reached 1-2 mm after 5–7 days and their survival on solid media is short.

The percent infection of *X. oryzae* pv. *oryzae* in the infected rice seeds from the whole grains on YDC medium was 21% (Fig. 2e). However, detection of *X. oryzae* pv. *oryzae* infection was notices on the apparently healthy seeds which was 6% (Fig. 2e). *X. oryzae* pv. *oryzae* was detected and identified as mucoid and straw-colored to yellow in color on the third or fourth day as smooth, convex, opaque, and pale yellow at first, straw yellow color later colonies (Fig. 2a,b,c,d). The colony forming unit (CFU) on YDC medium counted from BLB infected seeds was 23.37×10^{10} (Fig. 2e). However, 2.17×10^{10} CFU g^{-1} seed was obtained from the healthy seeds. The colonies of *X. oryzae* pv. *oryzae* were detected and identified on the kernels plated on three layers of filter papers placed in each petridish as yellow mucoid *X. oryzae* pv. *oryzae* colonies on kernel (Fig. 2a,b). The *X. oryzae* pv. *oryzae* were observed 18% and 3% in the infected and healthy kernels, respectively (Fig. 2c). The colonies of *X. oryzae* pv. *oryzae* were detected and identified on the seedlings raised on the blotter contained in the plastic trays by liquid assay method as yellow mucoid colonies (Fig. 5a∼e).

3.2 Transmission from seed to seed

X. oryzae pv. *oryzae* was identified in the seedlings raised from infected seeds by selected with yellow and mucoid colonies as described above. After transplanting the seedlings in the earthen pot separately for both apparently healthy and infected seeds, the symptoms of BLB were observed periodically. The first symptoms of BLB were noticed in the plants raised from infected seeds at maximum tillering stage as straw color blighted area with inner wavy margin. The dried seeds harvested from plants raised from

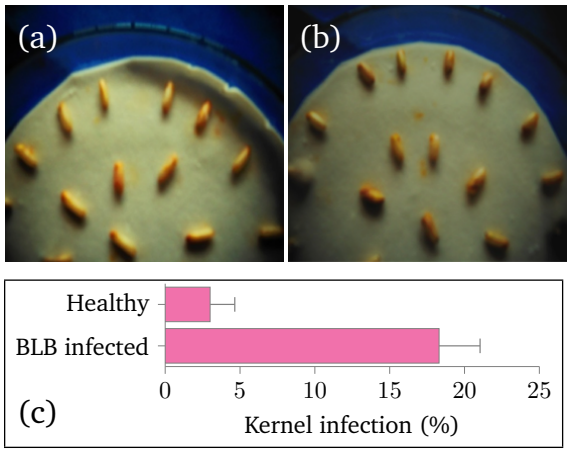

Figure 4. Plates with kernels on blotter papers soaked in carbendazim solution. A). Enlarged view of healthy kernels with X. oryzae pv. oryzae colonies and B) infected kernels with the colony growth of X. oryzae pv. oryzae. C) Percent kernel infection by X. oryzae pv. oryzae on YDC plates from apparently healthy and infected rice seeds. Data are the averages of four replications consisting 25 seeds in each replication.

both healthy and infected seeds were then tested in the presence of *X. oryzae* pv. *oryzae*. The *X. oryzae* pv. *oryzae* were detected and identified by using the seeds plated on YDC plates and kernels plated on blotter papers (data not shown) and liquid assay method using seedlings raised from infected and healthy seedlings as described earlier (Fig. 3a,b,c). The percent seed infection were obtained 14 and 26 for previously stored and freshly harvested seeds, respectively. But the results revealed that comparatively lower percent of seed infection was recorded from the stored seeds as compared to the freshly harvested seeds. These results indicates the decreasing trend of *X. oryzae* pv. *oryzae* survivability. However, *X. oryzae* pv. *oryzae* was also detection and identified from both categories of healthy seeds (Fig. 7a,b,c).

3.3 Occurrence in the seed kernels and harvested seeds

The results revealed that comparatively lower percent of seed infection was recorded from the stored seeds as compared to the freshly harvested seeds and indicates the decreasing trend of *X. oryzae* pv. *oryzae* survivability.

The results comparatively lower percent of seed infection was recorded from the freshly harvested seeds as compared to the stored seeds. These results is somewhat confusing with the decreasing trend of *X. oryzae* pv. *oryzae* survivability over time. However, it might be due to lower number of viable cells of *X. oryzae* pv. *oryzae* in the freshly harvested seeds.

3.4 Vigor of infected and healthy seeds

Comparatively higher vigor index was calculated in the seedlings raised from the stored (865.73) and freshly harvested (866.4) seeds from healthy plants as compared to the seedlings produced from stored and freshly harvested seeds from BLB infected plants with values 766.65 and 637.5, respectively (Fig. 2c). However, the vigor index was observed lowest in the stored BLB infected seeds as compared to that of freshly harvested BLB infected seeds. These results collectively indicated that *X. oryzae* pv. *oryzae* infection has a tremendous impact on the seedling vigor which determines the potentiality of seedlings for higher yield.

3.5 Results of pathogenicity and biochemical tests

On the basis of colony morphology, all the colonies of *X. oryzae* pv. *oryzae* isolates were tested for pathogenicity using the susceptible rice cv. IR-24. of the all colonies tested develop symptoms after 14 days of inoculation. On the basis of colony morphology, all the colonies of *X. oryzae* pv. *oryzae* isolates were tested for pathogenicity using the susceptible rice cv. IR-24. of the all colonies tested develop symptoms after 14 days of inoculation. Treatment of bacterium with 3% KOH demonstrates the conformation of the gram reaction. All the colonies tested showed thread like slime after vigorous mixing the bacterial growth with 3% KOH solution on a glass slide and pulling the toothpick up to observed the viscosity of the bacterium (Fig. 5a).

Oxidase test determines whether a bacterium has cytochrome oxidase, a compound that is present in most plant saprophytic bacteria. All the colonies isolates tested, were found delayed positive since they failed to produce the desired color (purple) with oxidase solution and impregnated strips production after one minute application (Fig. 6b). *X. oryzae* pv. *oryzae* isolates when incubated for 7 days to determine starch hydrolysis test demonstrated clear zones when plates were stained with Lugols iodine. Of all colonies tested gave positive results (Fig. 6c).

Egg yolk test is based on the observation that the enzyme lecihinase can break down the phospholipid emulsion of egg yolk, liberating a turbid zone of free fats around the colonies. This zone stains greenish-blue with copper sulfate. Such zones or color was visible for all the colonies of *X. oryzae* pv. *oryzae* tested

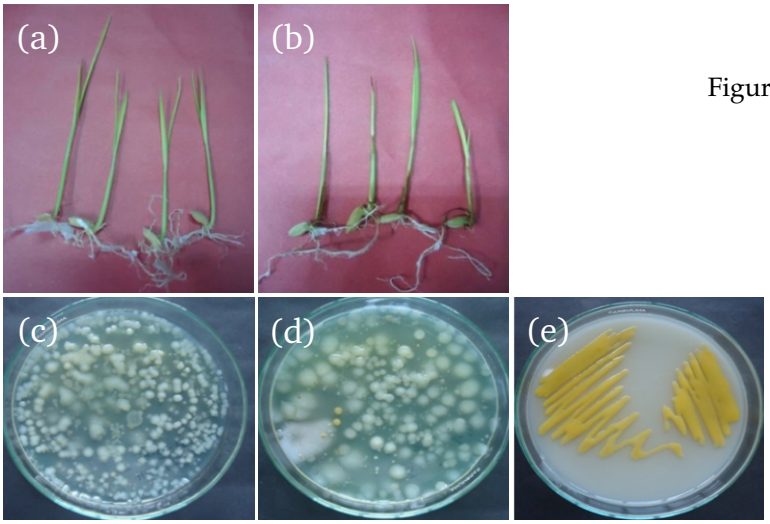

Figure 5. Identiifcation of *X. oryzae* pv. *oryzae* from rice seedlings raised from (a) healthy and (b) infected grains with kresek symptoms indicated by arrow. Colonies of *X. oryzae* pv. *oryzae* on YDC plate as detected by liquid assay method from (c) healthy and (d) infected seeds, and and (e) a pure culture of *X. oryzae* pv. *oryzae*.

(Fig. 6d). Some selected colonies obtained from the infected rice seeds were subjected for Tween-80 hydrolysis and all showed positive reaction by production of opaque zones around the colonies. These opaque zones were much clear on the third and fourth day of inoculation in Tween-80 plates (Fig. 6e). Anaerobic growth test is differential test for aerobic and non-aerobic bacteria. In our experiment none of the isolates tested gave positive anaerobic activity. This test indicated the true aerobic nature of the bacterium (Fig. 6f).

4 Discussion

The symptoms appeared as lesions coalesce and become straw color blighted area with wavy edges. Eventually, the whole leaf affected, becomes whitish or grayish and then dies. Transmission of *X. oryzae* pv. *oryzae* from seed to plant to seed were investigated in this study by preserving the seeds at room temperature (25 °C) until the next rainfed growing season. *X. oryzae* pv. *oryzae* was detected and identified as mucoid and pale yellow color colonies on the stored seeds. The percent *X. oryzae* pv. *oryzae* infected rice seeds were recorded on YDC medium was around 21% and the per cent colony forming unit (CFU g^{-1} seeds) on YDC medium was 23.37×10^{10}. However, *X. oryzae* pv. *oryzae* was also detected in the apparently healthy seeds which was 6% in case of intact rice seeds and 2.17×10^{10} CFU g^{-1} seeds.

The colonies of *X. oryzae* pv. *oryzae* were detected and identified on the kernels placed on three layers of filter papers and the per cent *X. oryzae* pv. *oryzae* were observed in the kernels as 18%. The colonies of *X. oryzae* pv. *oryzae* were detected and identified on the seedlings raised on the blotter contained in the plastic trays by liquid assay method. *X. oryzae* pv. *oryzae* was detected and identified in the infected rice seeds following the methods described above previously by Lu et al. (2014). However, the per cent incidence of *X. oryzae* pv. *oryzae* in the rice seeds were lower in the seeds stored previously than the freshly harvested seeds. Survival period and possibility of seed transmission of X. campestris pv. *oryzae* (Presently known as *X. oryzae* pv. *oryzae*) were studied by Thri Murty and Devadath (1984). They observed that the bacterium survived for longer (170–180 days) in kharif than rabi (120–130 days) harvested seed. They also noticed that the percentage of infected seeds was higher in kharif than rabi. The infected seeds when sown failed to produce the symptoms on respective seedlings due to the low number of bacterial population but they assumed that infected seedlings though not produce symptoms on the seedlings directly but serve as a source of inoculums from season to season.

The transmission of *X. oryzae* pv. *oryzae* studied by transplanting of seedlings raised from the previously harvested infected seeds. *X. oryzae* pv. *oryzae* was detected and identified in the seedlings raised from infected seeds. The plants were examined periodically for the symptoms of BLB after transplanting. The first symptoms of BLB were noticed in the plants raised from infected seeds at maximum tillering stage i.e. at around two months after transplanting as straw color blighted area with inner wavy margin. *X. oryzae* pv. *oryzae* were then detected using the methods as described earlier from the harvested infected dried seeds. The colonies of *X. oryzae* pv. *oryzae* were then purified and subjected to a series of biochemical tests. *X. oryzae* pv. *oryzae* were identified by a number of biochemical tests such as KOH solubility test, Starch hydrolysis test, Tween-80 hydrolysis test, oxidase test, anaerobic growth test, egg yolk reaction etc.

Previously seed to seed transmission of *X. oryzae* pv. *oryzae* were studied by Sakthivel et al. (2001). They detected and identified *X. oryzae* pv. *oryzae* from the naturally infected seeds of cvs Jaya and TN1. They recovered the bacterium up to 4 months and 9 months from naturally infected seeds of cvs Jaya and

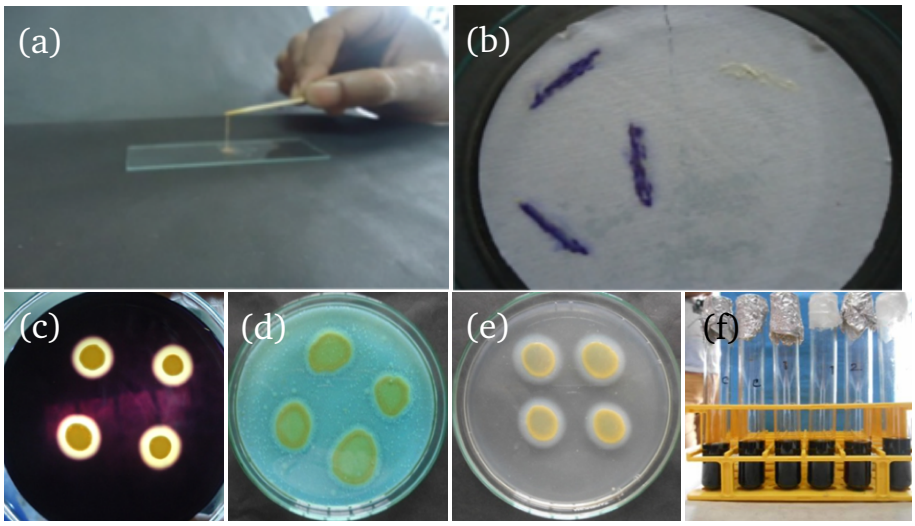

Figure 6. Identification of *X. oryzae* pv. *oryzae* by biochemical tests . (a) KOH test solubility test for Gram reaction (Suslow, 1982), (b) one day old bacterial colony, grown on nutrient agar as described previously, supplemented with 1% glucose was used in this assay, (c) starch hydrolysis test showing the cleared zones around the colonies of *X. oryzae* pv. *oryzae* which confirmed starch hydrolysis as indicated by arrows, (d) production of turbid zone of free fats around the colonies of *X. oryzae* pv. *oryzae* on nutrient agar plates containing 10% of egg yolk suspension,(e) *X. oryzae* pv. *oryzae* colonies on sterilized basal medium, and (f) anaerobic growth test of *X. oryzae* pv. *oryzae*, positive reaction indicates color change occurred from blue to yellow. The medium was spot cultured and incubated for three days at 27 °C.

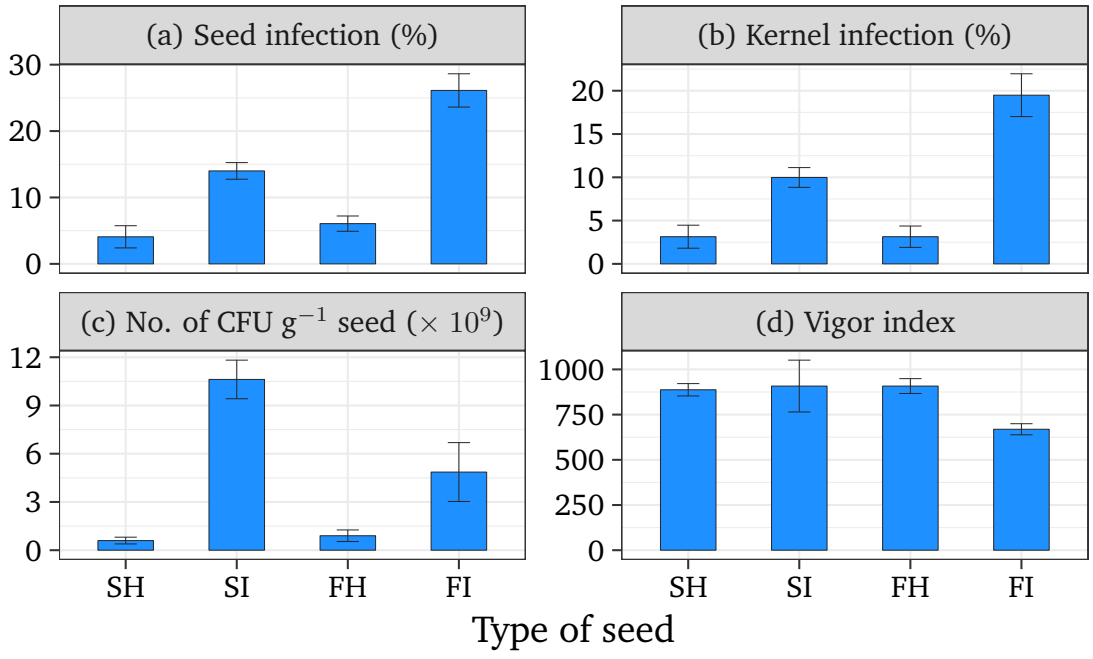

Figure 7. (a) Percent seed infection by *X. oryzae* pv. *oryzae* on YDC plates from apparently healthy and infected rice seeds, (b) percent kernel infection by *X. oryzae* pv. *oryzae* on YDC plates from apparently healthy and infected rice seeds, (c) colony forming unit (CFU) of *X. oryzae* pv. *oryzae* g^{-1} of seeds as determined by dilution plate technique, and(d) seedling vigor of seedlings raised from healthy and BLB infected seeds. Data are the averages of four replications consisting 25 seeds in each replication. SH = stored healthy seeds, SI = stored infected seeds, FH = freshly harvested healthy seeds, FI = freshly harvested infected seeds.

TN1, respectively. They also identified the BLB bacterium was also detected in seedlings, mature plants and seeds collected from plants raised from naturally infected seeds. Moreover, the presence of plant pathogenic bacteria in symptomless plants (latent infection) and the transmission of bacteria from seed to seed has been documented (Thomas and Graham, 1952; Tabei, 1967). Based on our results, PCR based detection technique can be used to detect the infection of *X. oryzae* pv. *oryzae* in symptomless plants. Detection of pathogen in non-symptomatic seedlings is of importance in certification programs, for both domestic and international quarantine, because a latent population can lead to serious epidemics under favourable conditions. Early detection of diseases, using rapid methods, is important for assessing the health status of a rice nursery before the transplantation of seedlings to fields. The findings of this study also revealed the distribution of different races of *X. oryzae* pv. *oryzae* all over the country because of the transmission of the pathogen through seeds as observed previously by Alam et al. (2016) and Islam et al. (2016).

5 Conclusions

It can be concluded that *X. oryzae* pv. *oryzae* causes BLB disease of rice is transmitted from seed to plant to seed for at least as a source of inoculum from one season to another. However, the study needs to be repeated in other rice cultivars grown in both rainfed and irrigated season with different storage conditions to check the possibility of different survival rate.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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