DETECTION OF *XANTHOMONAS ORYZAE* PV. *ORYZAE* FROM RICE SEEDS THROUGH BIO-PCR TECHNIQUE IN PADDY FIELDS OF GUILAN PROVINCE IN NORTHERN IRAN

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Abstract

Xanthomonas oryzae pv. *oryzae,* the causal agent of bacterial leaf blight (BLB) in the rice has been occurring as an increasing and important problem in Iran in recent years. Despite the fact that it is one of the main pathogens of rice in northern Iran, the biology of this bacterium in the Caspian Sea agro-ecosystem region is poorly understood. In this study, samples of infected plants were collected from different areas of Guilan province during the spring and summer of 2005–2006. In order to isolate bacteria, infected tissues of leaves, stems and roots were crushed in peptone water and 100 µl of this juice was cultured on NA and YDC media containing cycloheximide (50 µg/ml). After 48 to 72 hours bacterial colonies were selected and purified. Their morphological, physiological and biochemical characteristics were tested. A polymerase chain reaction (PCR) technique was developed to detect the presence of *X. o.* pv. *oryzae* in rice seeds as well as to study the transmission of this bacterium from seed to plant. Primers TXT and TXT4R from an insertion sequence (IS1113) of the pathogen were used to amplify a 964-bp DNA fragment. *X. o.* pv. *oryzae* was detected from the seed washes and DNA extracted from the seed washes of naturally infected seeds of cv. Hashemie. When the naturally infected seeds were stored at ambient temperature, the pathogen was recovered from them up to 10 months later. The BLB bacterium was also detected in seedlings, mature plants and harvested seeds of plants raised from naturally infected seeds. To the best of our knowledge, this is the first record of seed-borne feasibility of bacterial leaf blight agent in Iran.

Key words: *Xanthomonas oryzae* pv. *oryzae*, rice, seed-borne, PCR

INTRODUCTION

Bacterial leaf blight in rice caused by *Xanthomonas oryzae* pv. *oryzae* (Swings et al., 1990) has become an increasingly important problem affecting rice production worldwide (Zhao et al., 2007). Bacterial blight disease is a major rice disease in tropical Asian countries where high-yielding rice cultivars are often highly susceptible to the disease (Yashitola et al., 1997). Bacterial blight is one of the most economic serious diseases of lowland irrigated rice and can cause yield losses up to 50% (Gnanamanickam et al., 1999). Bacterial blight is a vascular disease in which bacteria enter through the hydathode, divide in the epithem and move to the xylem (Nino-Liu et al., 2001). The bacterial blight disease pathogen is seed-borne, although the extent to which it is transmitted through the seed has been questioned (Sakthivel et al., 2001). A PCR assay for amplification of *X. o.* pv. *oryzae* DNA using primers derived from a repetitive mobile element IS1113 could not detect the pathogen DNA from seeds collected from infected plants (Yashitola et al., 1997). The presence of *X. o.* pv. *oryzae* in infected seeds (Zhao et al., 2007) and disease transmission from seeds have been demonstrated (Reddy, 1983). However, probably due to the usefulness and limited accuracy of techniques used in detecting low numbers of viable cells of the pathogen (Singh and Rao, 1997) other scientists report controversially about its transmission (Goto et al., 1988). Biochemical tests (Vera Cruz et al., 1984), serological assays (Benedict et al., 1989), fatty acids and metabolic profiling (Chase et al., 1992) were used in the identification of the pathogen. Nevertheless, these assays have some vices including lack of sensitivity and specificity (Sakthivel et al., 2001). Due to the problems encountered in conventional methods, polymerase chain reaction (PCR) technology has found wide application in detecting plant pathogenic bacteria (Schaad et al., 1995). In India, general recommendations given to farmers include the use of seed treatment and prevention measures, including cultural practices and the production of disease-free nursery plants (Reddy, 1995). Seed movement must be regulated on the basis of sound biology, and seed detection methods must

be well established to restrict dissemination of most of the pathogens effectively (Ogawa and Khush, 1989). Different methods for detection of seed borne bacteria, such as the growing-on and plant injection or inoculation tests (Leach et al., 1990), produce various results. An immune-radiometric assay (Reddy and Reddy, 1989), Enzyme-linked Immune Sorbent Assay (ELISA), and monoclonal antibodies (Adhikari et al., 1995) have been developed to detect *X. o.* pv. *oryzicola*. However, there are some limitations in 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 cells from dead cells. Cross-reactions with *X. o.* pv. *oryzae* and other bacteria are observed in some cases (Reddy and Reddy, 1989). These techniques also require specific laboratory equipment and as a result, processing cost is high. Therefore, none of these methods is regularly used in routine rice seed health testing (Kauffman et al., 1972). It is evident that induction of disease resistance in rice was accelerated following treatment with acibenzolar-S-methyl (Babu et al., 2003). The present study focuses on rapid detection of *X. o.* pv. *oryzae* in rice leaves and seeds by Bio- PCR and also investigation of its transmission from seeds to rice plants.

MATERIALS AND METHODS

Bacterial isolation from rice seeds

Rice seeds were sampled randomly from paddy fields in different regions of Guilan province (Lahijan, Astane, Kiashahr, Soume Sara, Rasht, Fouman, Roudbar and Anzali) in the South of Caspian Sea during the spring and summer of 2005–2006 and 30 samples of each field were tested. To isolate bacterial pathogens from seeds, 50 g of each sample was soaked overnight in 150 ml sterile saline (0.85% NaCl, 0.01% Tween 20) at 4 °C. Then 100 µl aliquot of this liquid was streaked on Luria Peptone Glucose Agar (LPGA) and Yeast Dextrose Carbonate (YDC) media containing cycloheximide antibiotic (50 µg/ml). From each seed sample, three single colonies were isolated and one isolate/field was selected as a representative for this study. For long-term storage, the purified strains were grown in peptone sucrose and frozen at –80 °C in 20% glycerol. The strains were revived on Luria Peptone (LP) (Difco) medium for biochemical tests, DNA isolation and pathogenicity tests.

Pathogenicity test on rice

Rice seeds cv. Hashemie was sown in plastic boxes, and 3 weeks later, seedlings were transplanted to 30 cm diameter plastic pots. Rice plants were grown under greenhouse conditions for 3 months. For inoculation, bacterial suspensions were prepared in 10 ml of sterile distilled water at 1×10^9 CFU/ml. To test the pathogenicity of the isolates, plants with fully expanded leaves were inoculated by the leaf-clipping method (Kauffman et al., 1973). The instrument used to inoculate the rice plants to the bacterium was scissors. Before using the scissors, it was sterilized using 70% ethanol. The scissors were dipped in the bacterial suspension and used to cut inoculate the rice plant. The inoculated plants were covered by a polythene bag for 24 h, and incubated at 27 °C with 12 h light cycle. Lesions were observed on the leaves 14 days after inoculation (Backer, 2002). Individual leaves were ground in 3 ml of sterile distilled water. Then the suspensions were appropriately diluted and 50-µl aliquot was spotted on duplicate LPGA plates. Controls were treated with sterile distilled water.

Biochemical and physiological tests

Isolates were characterized on the basis of the following tests: Gram (Sulsow et al., 1982), oxidative/fermentative test (Hugh and Leifson, 1953), production of fluorescent pigment on King's B medium, hypersensitive reaction (HR) in tobacco and geranium leaves, oxidase test, levan formation, catalase, urease, gelatin liquefaction, salt tolerance (5% and 7%) and gas formation from glucose (Lelliot and Stead, 1987). In addition, tests were performed for arginine dihydrolase, hydrogen sulfide production from peptone, reducing substance from sucrose, tyrosinase casein hydrolysis, indole production, lecithinase, starch hydrolysis, aesculin and Tween 80 hydrolysis and optimal growth temperature was conducted (Schaad et al., 2001). The presence of DNase was tested on DNA agar (Diagnostic Pasteur, France). Carbohydrate utilization using Ayer basal medium was carried out and the results were recorded daily over a period of 8 days (Hildebrand 1998). For each defined test in this study, a representative isolate was deposited in the Collection Française de Bactéries Phytopathogèns (CFBP) culture collection. This reference isolate was considered as a typical isolate of *X. o.* pv. *oryzae.*

Detection of pathogen by BIO-PCR in naturally infected rice seeds

To detect *X. o.* pv. *oryzae* in naturally infected seeds of cv. Hashemie, 500 g of seeds were soaked overnight in 750 ml of 0.01% Tween 20 at 4 °C (Sakthivel et al., 2001; Schaad et al., 1995). Samples of 100 µl of seed extract were plated onto PSA in duplicates and incubated for 2 days at 29 °C. Then plates were washed three times

by 1 ml of sterile distilled water and a 35-µl sample of the washes was used in PCR assays. To study the survival of the pathogen in naturally infected seeds, the seeds were stored at ambient temperature and subjected to a PCR test every 2 weeks.

Transmission studies

To test the transmission of *X. o.* pv. *oryzae* from naturally infected seeds to seedlings, 500 infected seeds of cv. Hashemie were germinated and 50 seeds were planted into 10 pots, each containing sterile soil mixture. The pots were maintained at 30–35 °C with 12 h dark/light cycles. Seedlings were observed for BLB symptoms. Leaf samples were collected after 45 days. At maturity, seeds were collected and subjected to a BIO-PCR test (Sakthivel et al., 2001).

DNA Extraction

For bacterial DNA extraction, the isolates were grown overnight in nutrient broth (Merck, Darmstadt, Germany), at 26 °C and the DNA was extracted as described by Martins et al. (2005). One tube of 1.5 ml was used to centrifuge the cells at 13 000 × g for 5 min and the pellet was suspended in 200 µl Tris 0.1 mol/L and added with 200 µl of lysis solution (NaOH 0.2 N and 1% SDS), mixed and added 700 µl of phenol/ chloroform/ isoamyl alcohol (25 : 24 : 1 v/v/v), homogenized and centrifuged for 10 min at 13 000 × g .To precipitate DNA, 700 µl of cold isopropanol was added and spun, washed in 70% ethanol and centrifuged. Precipitated DNA was dried at room temperature and suspended in 100 µl of water. The method described by Ausubel et al. (1996) was performed comparing 30 isolates. The samples from the both methods were electrophoresed on 1.5% agarose gels, stained with ethidium bromide and photographed under UV.

Primers and PCR conditions

Primers TXT (5′-GTCAAGCCAACTGTGTA-3′) and TXT4R (5′-CGTTCGCGCCACAGTTG-3′) were made from an insertion sequence element, IS1113 of *X. o.* pv. *oryzae* (Sakthivel et al., 2001), with a predicted PCR product of 964 bp. PCR assays were performed with a PTC-225TM thermo cycler (Master cycler gradient, Germany). Mg++ concentration and other conditions were optimized to test the DNA template and the bacterial cells in PCR. All amplifications were carried out in a final volume of 50 µl containing 1.5 mM MgCl2, 0.2 mM of each dNTP, 1 M of each primer, 1 unit of *Taq* polymerase enzyme (Promega Corp., Madison,WI), 5 µl of thermo-DNA buffer (Promega Corp., Madison,WI) and 200 ng of DNA or 35 µl of suspension of bacteria (seed washes). PCR-water (Sigma) was used in the mixtures and the preparation of PCR reactions was carried out in a laminar-flow hood. Each PCR experiment included a control without DNA. Reactions were run for 30 cycles, each consisting of 1 min at 95 °C, 1 min at 56 °C, 1 min at 72 °C, with ini-

Table 1: Phenotypic characteristics of *Xanthomonas oryzae* pv. *oryzae* strains tested

Characteristics	Iranian isolates	Reference isolate CFBP 2532
Gram reaction	–	–
Oxidative/Fermentative	–	–
Fluorescent pigment	–	–
HR on tobacco and geranium	+	+
Leaf blight on rice	+	+
Pectinase	–	–
Arginine dihydrolase	–	–
Levan formation	+	–
Catalase	–	+
Tween® 80 hydrolysis	–	+
Oxidase	–	–
Starch hydrolysis	–	–
Gelatin hydrolysis	+	+
Aesculin hydrolysis	±	+
DNase activity	±	+
Indole formation	–	–
H_2S from cysteine	–	–
Casein hydrolysis	±	+
Urease	±	–
MR/VP	–	–
Utilization of:		
L-lysine	–	–
Citrate	+	+
lecithinase	–	±
Growth in 5% NaCl	+	
Acid from:		
L-Arabinose	–	–
Inositol	–	–
Mannitol	+	±
Xylose	+	±
Trehalose	–	–
Maltose	+	
L-tartrate	–	–
Galactose	+	±
D-Sorbitol	±	±
Sucrose	–	–

– negative reaction or no growth; + positive reaction or growth; ± some isolates positive

tial denaturation of 5 min at 95 °C and final extension of 10 min at 72 °C. Amplified PCR products were separated on a 1.5% agarose gel using TBE buffer and then stained by ethidium bromide and visualized and photographed under UV light (312 nm) using a Gel Documentation System, GDS 8000 (BioRad., California, USA).

RESULTS

Biochemical, biological and physiological test

By and large, among all the obtained isolates from collected samples 19 isolates produced HR on tobacco and geranium and leaf blight on rice. All isolates were gram, oxidase, catalase, pectinase, arginine dihydrolase negative and levan positive. All isolates produced acid from xylose, maltose, galactose and mannitol and were capable to hydrolyze gelatin. However, all isolates reacted differently in urease activity and hydrolysis of aesculin and casein. Moreover, the isolates of *X. o*. pv. *oryzae* for the presence of DNase were tested on DNA agar (Diagnostic Pasteur, France). Results from the phenotypic tests are presented in Table 1.

Pathogenicity test

All isolates of *X. o*.pv. *oryzae* caused leaf blight on the surface of rice leaves two weeks after inoculation. Bacterial leaf blight appeared on the leaves of young plants, as pale-green to grey-green water soaked streaks near the leaf tip and margins. These lesions coalesced and became yellowish-white with wavy edges. Leaf sheaths and culms were attacked. Typical spike symptoms caused by bacterial leaf blight are also known as brown necrosis on seeds. The seeds showed a dull, brownish black area on the unripe ear heads. These symptoms did not occur in the control plants.

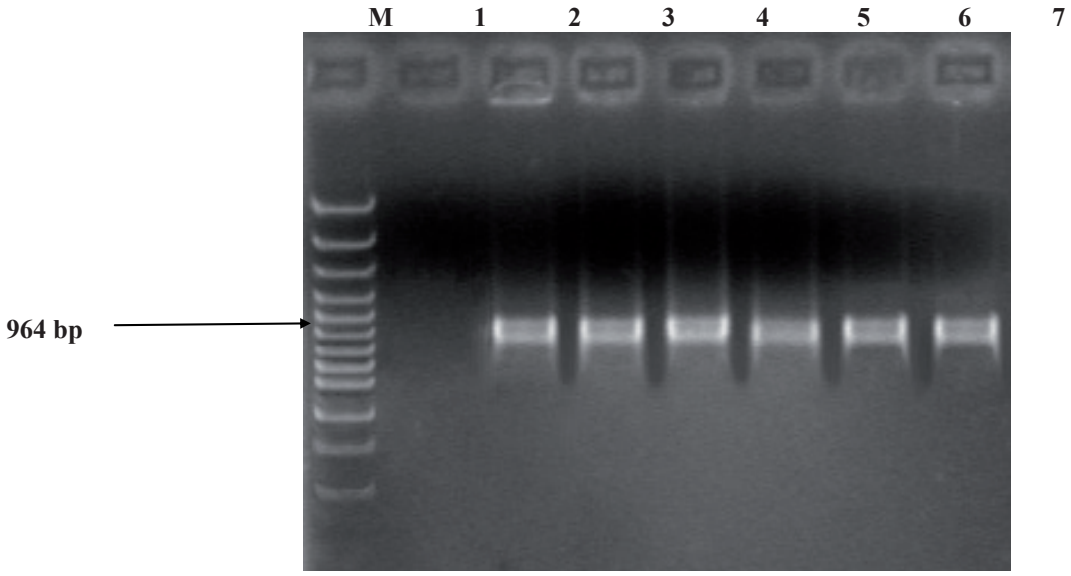
Detection of pathogen in leaf symptoms of inoculated rice plants

The bacterial leaf blight lesions of 5–10 cm were observed 2 weeks after clip inoculation with isolates of *X. o.* pv. *oryzae*. When genomic DNA from inoculated leaves, upper symptomless leaves, or seedlings developed from artificially infected seeds were used as template, a 964-bp fragment of pathogen was detected in PCR assays. No amplification was observed from the DNA of un-inoculated control plants, or seedlings raised from un-inoculated seeds (Figure 1).

Detection of pathogen by BIO-PCR in naturally infected rice seeds

The bacterial leaf blight pathogen was detected by PCR in naturally infected seeds of cv. Hashemie. When these naturally infected seeds were stored at ambient temperature, *X. o.* pv. *oryzae* was detected from them up to 40 weeks (Figure 1).

Figure 1: Detection of *X. o.* pv. *oryzae* by PCR in naturally infected seeds and plants with specific primers of TXT and TXT4R, *Arrow,* indicates the 1-kb size fragment of the 1-kb ladder. *Lane1,* control negative (distilled water); *lane 2, X. o.* pv. *oryzae* (CFBP 2532) showing the amplification the approximately 752 bp,, *lane 3 to 5,* agar plate–washings from seeds collected from infected plants of cv. Hashemie, *lane 6 and 7,* DNA of symptomatic leaves of cv. Hashemie



Transmission studies

The bacterial leaf blight symptoms were observed in the plants developed from naturally infected seeds of cv. Hashemie. Also *X. o.* pv. *oryzae* was detected by PCR in infected leaves and seeds at maturity (Figure 1).

DISCUSSION

Based on morphological, phenotypical, nutritional characteristic, pathogenicity tests and PCR using specific primers, we identified causal agent of bacterial blight of rice as *X. o*.pv. *oryzae*. All isolates of *X. o*.pv. *oryzae* produced blight on rice. No significant differences were observed in the degree of blight on inoculated plants. These results can be indicative of that isolates obtained from different fields do not differ in their degree of virulence. Our results showed that PCR and BIO-PCR techniques could be used to detect *X. o.* pv. *oryzae* in rice seeds and plants. Primers (TXT and TXT4R) used in this study did not amplify DNA from other pathogenic or saprophytic bacteria of rice, with the exception of *X. o.* pv. *oryzicola*, since IS1113 is also present in *X. o.* pv. *oryzicola* (George et al., 1994). PCR techniques with these primers can be applicable to detect both pathogens; and pathovars of *X. oryzae* can be differentiated by ligation-mediated PCR (George et al., 1994). Therefore, PCR techniques based on TXT and TXT4R primers can be used to detect strains of *X. o.* pv. *oryzae* with different geographical origins (Sakthivel et al., 2001). A PCR technique was successfully used to detect viable cells of *X. o.* pv. *oryzae* in naturally infected seeds (cv. Hashemie) up to 10 months after harvest. Transmission of *X. o.* pv. *oryzae* from naturally infected seeds (cv. Hashemie) to seedlings was demonstrated. Although PCR is a highly sensitive technique, it cannot differentiate dead cells from live cells (Ghasemie et al., 2008; Sakthivel et al., 2001). This limitation is a major concern and affects PCR application in quarantine laboratories. This problem was solved in an earlier investigation (Schaad et al., 1995) and in this study, by employing a BIO-PCR technique of plating seed or seed extract onto agar prior to the application of PCR and using plate-washing (bacteria) as the template. This method could eliminate the problem of false-negative results due to PCR-inhibitors in the plant or seed extracts and also avoid false-positive results due to dead cells. BIO-PCR could be employed without the need for time-consuming DNA extraction and isolation of *X. o.* pv. *oryzae* from seeds (Sakthivel et al., 2001). Generated data in this study demonstrates the seed-borne nature and transmission of *X. o.* pv. *oryzae*, the causal agent of the bacterial leaf blight disease of rice. Since high humidity and temperature are important factors for *Xanthomonas oryzae* pv. *oryzae* to cause bacterial leaf blight in rice (Nino-Liu et al., 2006), in this research, collection of infected samples and then isolation were fulfilled in the north of Iran. It should be noted that most of isolates (13 isolates) were obtained from areas close to Caspian Sea. However, the occurrence and incidence of this disease on rice have not been studied in different geographic regions of Iran. To best of our knowledge, this is the first report of transmission of bacterial leaf blight of rice by seeds in Iran. Study on the biological control of bacterial blight on rice by antagonistic isolates in different parts of Iran and using resistant cultivars could be a study case for future research.

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Received for publication on May 12, 2009
Accepted for publication on April 12, 2011

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