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Research Paper

Isolation and Identification of *Xanthomonas oryzae* pv. *oryzae* a Causative Organism for Bacterial Leaf Blight of Rice

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A B S T R A C T

The present work is focused on isolation, identification and pathogenicity test of *Xanthomonas oryzae* pv. *oryzae* (Xoo) a causative organism of Bacterial leaf blight (BLB) of rice. The diseased leaf samples were collected from different paddy fields of Karnataka A total of 72 bacterial isolates were isolated and identified as *Xanthomonas oryzae* pv. *oryzae* (Xoo) based on biochemical characterization. The growth of Xoo was checked on 6 different media such as Nutrient agar (NA), Yeast extract Dextrose Calcium Carbonate Agar media (YDCA), Modified D-5 agar media (M D-5), Yeast extract Nutrient Agar media (YENA), Peptone Sucrose Agar media (PSA) and Tryptone Soya Agar media (TSA). The pathogenic bacteria shown best growth on YDCA, NA, TSA, followed by PSA, YENA and poor growth was recorded on M D-5 media. The isolates were screened for pathogenicity tests by using clip inoculation method, cutting and dipping method for IR 64 rice variety. Among 72 isolates subjected for pathogenicity test, the isolates only one isolate was shown to be pathogenicity, the remaining isolates were moderately pathogenic.

***Key words*:** *Trichoderma, Xanthomonas oryzae*, Rice, Bacterial leaf blight, Germination

Bacterial Leaf Blight (BLB) of rice is one of the most destructive disease caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) which limits the annual rice production in both tropical and temperate regions of the world [1]. In the tropics, damage is more severe than in the temperate regions [2]. It was first observed in 1884 in Japan by the farmers and it is known to be one of the oldest disease in rice. The disease incidence of this disease was subsequently reported from other countries like Northern Australia, USA, different parts of the Asia, and Africa [3]. India stands next to China in the production of rice by producing 87.80 million tons where India has (43 million hectares) the largest area under rice cultivation. In India the BLB disease was first reported in 1951 [1]. Among top 10 bacterial plant pathogens Xoo in fourth position after *Pseudomonas syringae*, *Ralstonia solanacearum* and *Agrobacterium tumefacient* [4].

The severity of the disease is more in tillering stage resulting in the loss of yield up to 75%, but this disease can cause disease in the host even at seeddling, vegetative and reproductive which depends on factors such as location, rice cultivar and weather [5]. Loss of crop yield caused by the bacterial disease depends on virulence of the pathogen, the relationship between the host and the pathogen and also environmental factors. In India, BLB disease was observed in many important rice growing states like Andhra Pradesh, Bihar, Haryana, Kerala, Orissa, Punjab and Uttar Pradesh [6]. The bacterium exists in different races (or pathotypes) distinguished based on their behaviour on differential cultivars. New races are variable in bacterial virulence. The resistance offered by each variety of rice cultivar is different [7].

Considering the vast diversity of Xoo within India and other rice growing irrigated regions of Asia, typing of Xoo using molecular tools such as RFLP, PCR fingerprinting based on polymorphism in insertion sequences (IS) and Southern blotting for Transcription Activator Like Effector (TALE) gene content analysis become critical to determine the pathogen population structure [8]. Conventionally, identification of a plant pathogen involves the process of isolation of the pathogen, cultivation, and confirmation based on morphology of the colonies, bacteriological characteristics, microscopic observation, and by other means are time-consuming process. In addition, the detection process requires much equipment and chemicals, increasing the cost [9]. In recent years RAPD-PCR approach has been shown to be useful in classifying a number of microbial strains and species including various *Xanthomonas* spp. A polymerase chain reaction (PCR) technique was developed for detecting the presence of *Xanthomonas oryzae* pv. *oryzae*, the bacterial leaf blight pathogen in rice seed and for studying the transmission of this bacterium from seed to plant [10].

Xanthomonas oryzae pv. *oryzae* is a rod shaped, Gram negative bacteria. Individual cells vary in length from approximately 0.7µm to 2.0µm and width from 0.4µm to 0.7µm. Cells are motile by means of a single polar flagellum. Colonies on solid media are round, convex, mucoid and yellow in colour due to the production of the pigment called xanthomonadin. It is obligatory aerobic and does not form spores. Optimal temperature for growth is between 25 to 30°C

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[11]. In the present work the Xoo is isolated from different diseased paddy fields, identified, different media compositions are used to know the growth of the organisms and their pathogenicity was checked.

MATERIALS AND METHODS

Collection of diseased samples from rice fields

The infected leaf samples were collected from different paddy fields of Karnataka showing typical bacterial leaf blight symptom were collected from the rice growing regions. The infected leaf samples were collected in sterile polythene covers, labelled and stored at 4°C until further use. The infected plants were subjected for isolation and identification of bacterial pathogen.

Isolation of bacteria

The bacteria were isolated directly from infected rice plant. The infected parts of the leaves were cut in to 0.5 cm^2, surface sterilized with 1% Sodium hypochlorite for one minute followed by sterile distilled water wash for 3-4 times and blot dried. The sterilized leaf segments were inoculated on yeast extract dextrose calcium carbonate (YDC) agar medium, incubated at 28±2°C for 72 h. The plates were observed for convex, mucoid and yellow colored colonies [12].

Identification of isolated bacteria by biochemical and physiological tests

The identification of bacterial isolates were done based on morphological, microscopic, biochemical and physiological tests such as gram staining, catalase hydrolysis test, oxidase test, KOH test, gelatin liquefaction test, starch hydrolysis test, casein hydrolysis test and pectin hydrolysis test were carried out as described in the Bergey's Manual of Systematic Bacteriology (2001).

Gram staining

A thin bacterial smear was prepared on a glass slide, heat fixed and then stained with i.e., crystal violet stain for minute, washed with running tap water, flooded with iodine solution for 1 min, decolorized using (95%) ethanol, washed with running tap water, counter-stained with safranin stain for 30 seconds and washed with running tap water. The slides were dried and observed under microscope in oil immersion [13].

KOH (Potassium hydroxide) test

A drop of 3% KOH solution was added on a clean glass slide and a loopful of 24 h old bacterial culture grown on YDC was added, stirred well and observed for change in viscosity, and formation of thread like slime [13].

Catalase test

A drop of hydrogen peroxide was taken on a clean glass slide and 24 h old culture of bacteria was added and observed for the bubble formation. Formation of bubbles indicated catalase positive and no bubble formation indicated catalase negative [13].

Oxidase test

The bacteria was streaked on oxidase disc and observed for the development of purple colour within 10 seconds which indicated positive result and if no colour was developed after 60 seconds the test was indicated to be negative [13].

Starch hydrolysis test

The 24 h old bacterial culture was streaked on starch medium, incubated for 24 h at 28±2°C. After incubation, the plates were flooded with iodine solution and was observed for the zone of clearance [13].

Pectin hydrolysis test

The 24 h old bacterial culture was streaked on to Hankin's medium and incubated at 28±2°C for 24 h. After the incubation period the plates were flooded with hexadecyltrimethyl-ammonium bromide and was observed for the zone of clearance [13].

Casein hydrolysis test

The 24 h old bacterial culture was streaked on skim milk agar medium, incubated for 24 h at 28±2°C. After incubation, the plates were observed for the zone of clearance [13].

Gelatin liquefaction test

The gelatin hydrolysis test was performed to know ability of bacteria to hydrolyze gelatin by the production of gelatinase enzyme. Tubes containing the gelatin medium was stab inoculated with the test bacteria and was incubated at 28±2°C for 24 h followed by placing in the refrigerator for about 30 minutes. If there was production of the enzyme by the bacteria the tube remained liquid even after refrigeration indicating positive results [13].

Growth of Xanthomonas oryzae pv. oryzae on different media

To study the growth characteristics of the pathogen, various differential and selective/semi-selective media were used such as Nutrient agar(NA), yeast extract dextrose calcium carbonate agar media (YDCA), modified D.5 agar media (M D-5), yeast extract nutrient agar media (YENA), peptone sucrose agar media(PSA), and tryptone soya agar media (TSA) [14].

Pathogenicity test

Planting material and bacterial inoculum preparation

To prove the Koch's postulates, the pathogenicity test was carried out for all the 72 isolated bacterial strains in order to know whether the isolates from rice plants are pathogenic or not under artificial conditions. Systemic investigations was conducted in the present work to know the nature of disease development. Rice variety IR 64, susceptible to bacterial leaf blight, was obtained from National Seed Corporation, Bengaluru, India. A suspension of Xoo was prepared by adjusting the density of the organism suspension equal to that of the 0.5 McFarland standards by adding sterile distilled water [15].

Plant growth conditions and inoculation

The Seedlings of the rice genotype IR 64, were grown on sterilized moist filter papers in Petri plates following the standard blotter method as described by [16]. Seedlings of two weeks old were transplanted to small plastic pots. Once after the plants reached tillering stage the plants were transferred to bigger pots and these cultivars were subjected to clip inoculation method [2]. After inoculation, the plants were monitored at regular intervals of 24 h by noting down the appearance of disease symptoms and final data was documented after 14 days of post inoculation. Percent disease incidence was calculated according to formula as follows [17].

$$\% \text{ Disease incidence} = \frac{\text{Total lesion length}}{\text{Total leaf length}} \times 100$$

RESULTS AND DISCUSSION

Collection of diseased samples from rice fields

The infected leaf samples were collected from different districts of Karnataka like Ramanagara, Chikkaballapura, Kolar, Tumkur, Channapatna, Mandya, and Mysuru. A total of 247 diseased leaf samples from collected from infected rice plants.

Fig 1 Bacterial wilt infected paddy field in Kolar district (Gummakal) Karnataka, India

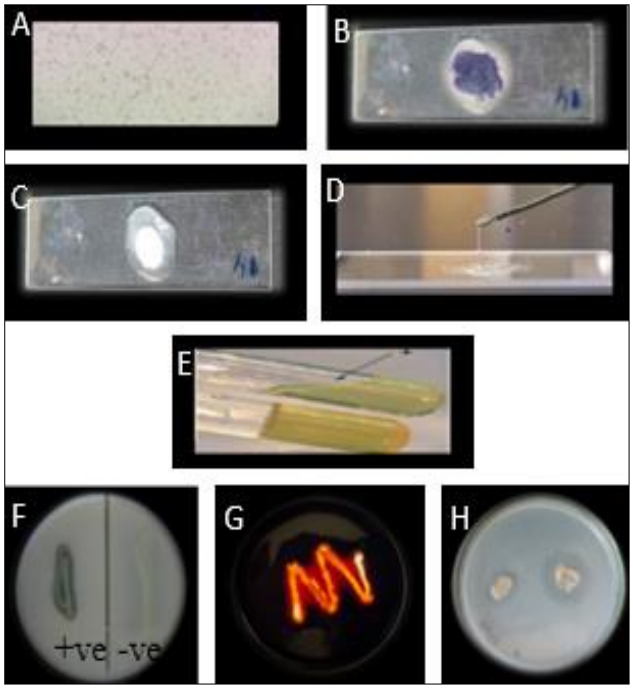


Fig 3 Biochemical identification of the isolated bacteria from infected leaf samples of rice

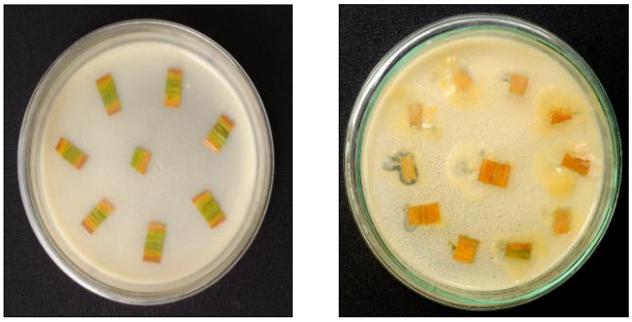
A-Gram's staining, B- Oxidase test, C- Catalase, D- KOH test, E- Gelatin liquefaction, F- Casein hydrolysis test, G- Starch hydrolysis test, and H- Pectin hydrolysis test.

Identification of isolated bacteria by Biochemical and physiological tests

Morphologically identified samples were pure cultured and further used for biochemical and physiological identification methods according to Bergey's Manual of Systematic Bacteriology (2001) for the tests such as Gram's reaction, oxidase test, catalase test, KOH test, starch hydrolysis test, casein hydrolysis test, gelatin liquefaction test and pectin hydrolysis test. The isolated bacteria were Gram-negative, short rods producing yellow-colored pigment. The

Isolation of bacteria from diseased samples

The infected leaf samples were collected from different parts of Karnataka, a total of seventy-two bacterial isolates were obtained from different infected fields of Karnataka. The infected leaf samples were plated on the media plate and incubated at 28±2°C for 72 h. The bacterial cultures recovered from the collected samples, showed typical Xoo bacterial colony characteristics such as yellow colour, mucoid, convex colonies on plating the samples as explained by [12] (Fig 1-2)



Before incubation After incubation

Fig 2 Plating of infected leaf samples for isolation of pathogenic bacteria

bacterial isolates tested were positive for catalase, oxidase, 3% KOH, gelatin liquefaction, starch hydrolysis and pectin hydrolysis [18]. The isolates were positive for all the tests except for the Gram's reaction, which showed it to be negative, indicating that it is a gram-negative organism (Fig 3).

Growth of Xanthomonas oryzae pv. oryzae on different media

The growth of the organism was checked on different media to check the growth rate of the organism. Nutrient agar (NA), yeast extract dextrose calcium carbonate agar media (YDCA), modified D.5 agar media (M D-5), yeast extract nutrient agar media (YENA), peptone sucrose agar media (PSA), and tryptone soya agar media (TSA) were used. The growth of organism was best in YDCA, NA, TSA, followed by PSA, YENA and poor growth or no growth was observed in M D-5 media. And according to [14] studies the yeast extract dextrose agar and potato sucrose agar (PSA) supported the excellent growth of *Xanthomonas oryzae* pv. *oryzae* (Fig 5).

Pathogenicity test of Xanthomonas oryzae pv. oryzae to prove Koch's postulates

The rice genotype IR 64 was used for pathogenicity test, where all the 35 isolates of bacterial strains isolated from the diseased samples were subjected to pathogenicity test in order to prove the Koch's postulate and clip inoculation method was carried out [2]. After few days of inoculation to the rice seedlings, the tested plant divulged typical bacterial leaf blight symptom like pale yellowish discoloration which was visible at the cut end of the leaves. The clip inoculation method was found to be effective in infecting the plant, though the lesion length varied depending on the bacterial strain. The diseased samples were collected and plated on to YDC media for re isolation and to prove the Koch's postulate. The bacterial colonies obtained depicted typical Xoo colony characteristics which were further confirmed by biochemical tests. Out of 35 isolates used for pathogenicity test, most of the isolates were weakly virulent and moderately virulent and only three virulent Xoo was found (Fig 4-5).

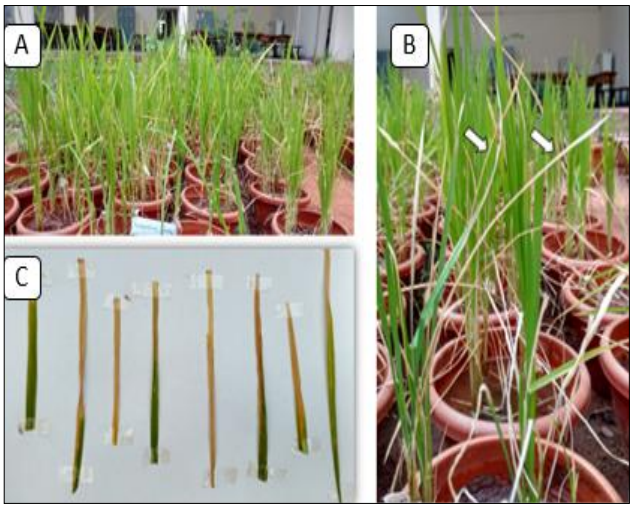


Fig 5 A- rice genotypes used for pathogenicity test, B-Inoculated rice plants showing symptoms, C- varied lesion length of infected leaves

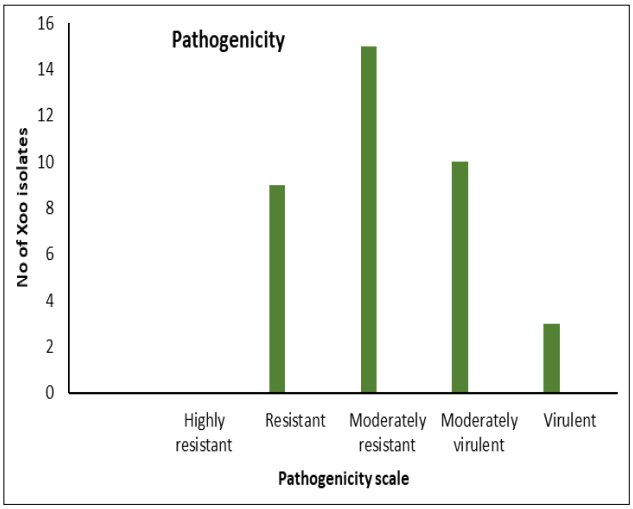


Fig 7 Pathogenicity variations in rice genotypes after inoculation with Xoo

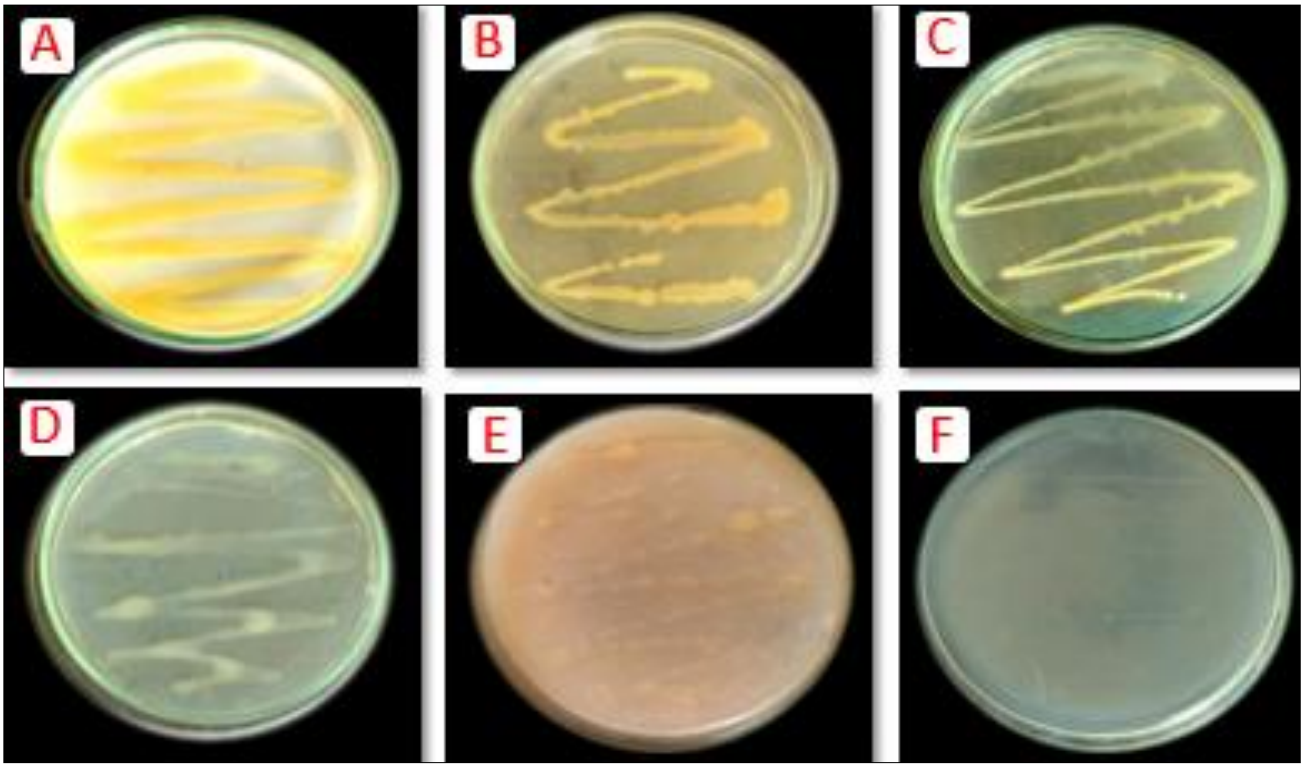


Fig 4 Growth of Xoo on different media: A-YDC, B-NA, C-TSA, D-PSA, E-YENA, F-M D-5 media

CONCLUSIONS

In the present work, seventy-two *Xanthomonas oryzae* pv. *oryzae* (Xoo) bacterial isolates were isolated and identified collected from different diseased paddy fields of Karnataka. Different media was used to know growth of the Xoo which supports the growth of the organisms. The Xoo isolates were subjected for the pathogenicity test in order to know the virulence of the pathogen.

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