The Pharma Innovation Journal 2023; 12(9): 2285-2290

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(9): 2285-2290

www.thepharmajournal.com
Received: 16-06-2023
Accepted: 19-07-2023

Kishan Debnath
Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Gopalakrishnan C
Department of Rice, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Kannan R
Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Kannan M
Department of Entomology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Manonmani S
Department of Rice, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Corresponding Author:
Kishan Debnath
Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Isolation, characterization and assessment of virulent pattern of *Xanthomonas oryzae* pv. *oryzae* the causal agent of bacterial leaf blight of rice from different parts of Tamil Nadu

Kishan Debnath, Gopalakrishnan C, Kannan R, Kannan M and Manonmani S

Abstract
Rice is the most important and extensively grown food crop grown in India. Bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) is the most destructive bacterial disease in different rice growing regions of India resulting in crop losses up to 50%. From the major rice growing areas of Tamil Nadu, 8 isolates of Xoo have been isolated during 2022-2023 to assess their virulence pattern. Sequencing and blast analysis confirmed that all the isolates were *Xanthomonas oryzae* pv. *oryzae*. The molecular characterisation of the isolates was carried out by PCR amplification of the hpaA gene using XOF and XOR primers, and all the isolates were amplified at 534 bp. The susceptible rice cultivar TN1 was inoculated with all the isolates, and all the isolates produced typical symptoms of BLB, including white to yellow stripes with wavy margins on the leaves. However, the severity of the disease varied significantly among them, from the most severe isolate, TNXOO1, to the least severe isolate, TNXOO8. Incorporating novel resistance genes into commercial rice cultivars is urgently needed to combat BLB due to the rising prevalence of highly virulent isolates of Xoo in Tamil Nadu.

Keywords: *Xanthomonas oryzae* pv. *oryzae*, bacterial leaf blight, rice, virulent isolates, TN1

Introduction
Rice (*Oryza sativa*) serves as the primary staple for over half of the global population. One of the most significant threats to rice cultivation is *Xanthomonas oryzae* pv. *oryzae* (Xoo), highlighting its critical importance (Liu *et al.*, 2014) [14]. If, bacterial infection develops during the tillering stage of the crop, losses may vary from 30–50% to 100% (Khan *et al*., 2012) [11]. After being initially documented in Japan in 1884 (Nino-Liu *et al*., 2006) [19], the BLB of rice has now been reported in all other nations that grow rice (Naqvi, 2019) [18]. In India, Bacterial leaf blight is widespread in nearly all the rice ecosystem in India. The initial documentation of this disease in India was made by Srinivasan *et al*, in 1959 [27]. According to Ou (1985) [20] and Akhtar *et al*. (2003) [1], there are two different types of symptoms associated with rice bacterial blight: leaf blight phase and kresek phase. Systematic infection is a characteristic of the Kresek phase. One to two weeks after transplanting, the symptoms typically become apparent; the leaves turn yellowish green, quickly wither, and roll up. At the tillering stage, the initial symptom of bacterial leaf blight appears on the leaf margins that start at the leaf tip and move down through the base of the leaves, causing the leaf to dry out, rolling inward and twisting of the diseased portion of leaf (Rangaswami *et al.*, 2004) [23]. Bacterial leaf blight mostly occurs first in lower leaves and then moving up to the upper ones (Goto, 1992; Cha, 1982) [8, 5]. The development of diseases is mostly facilitated by higher doses of nitrogen, one of the three most important fertilizer ingredients (Cha, 1982) [5]. Higher temperatures have been reported to promote disease incidence, although extreme heat and drought have been found to decrease disease development (Webb *et al.*, 2010) [26]. At 25 to 30 °C, disease symptoms begin to manifest in rice plants that have been infected with the pathogen, but not at 17 °C (Muko *et al*., 1957) [17]. When exposed to several strains of Xoo, this was observed in both susceptible and resistant Indica and Japonica rice varieties (Horino *et al.*, 1982) [9]. In addition, BLB has been found to be a serious issue in the production of hybrid rice in nations like China and India (Quibod *et al*., 2020) [22]. The initial crucial phase in addressing this disease involves the characterization of the pathogen.

The outbreak and severity of this disease in Tamil Nadu are not consistent throughout the rice growing areas (Chandraprakash *et al.*, 2018) [5]. The current study aims to isolate different isolates of Xoo from major rice growing areas of Tamil Nadu and identification of such isolates by biochemical tests, PCR techniques and assess their virulence pattern.

Materials and Method

Studied area

This study was carried out at Department of Plant Pathology, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India. Samples of BLB infected leaves were collected from eight districts of Tamil Nadu namely-Coimbatore (10.99N, 76.917E), Thanjavur (10.45N, 75.387E), Tiruvarur (10.80N, 79.68E), Tiruvallur (13.15N, 79.92E), Villupuram (12.17N, 79.50E), Cuddalore (11.80N, 79.78E), Tirunelveli (9.28N, 77.56E) and Madurai (9.94N, 78.15E)

Isolation of of *Xanthomonas oryzae* pv. *oryzae*

Rice leaves infected with bacterial leaf blight were collected from above locations and 14 bacterial pathogens were isolated according to method mentioned by Bradbury, 1970 with minor modifications. The blighted leaf samples were surface sterilized with 70% ethanol for 60 seconds and washed 2-3 times with sterile distilled water. Infected leaf samples were cut into small bits of approximately 5-10 mm in size by using a sterile scalpel and incubated for 20-30 min in vials containing sterile distilled water. After the incubation period, a loopful of water containing the bacteria were streaked on Nutrient Agar (NA) plates and incubated at 28 ℃ for 48-72 hours. Pale yellow mucoid round colonies appeared on NA plates which are then picked individually and purified 2-3 times to obtain the pure culture.

Biochemical characterization of bacterial isolates

All the bacterial isolates were characterized based on the following biochemical tests-1) Gram's staining, 2) Citrate utilization test, 3) lysine utilization test, 4) ornithine utilization test, 5) urease test, 6) phenylalanine deamination test, 7) nitrate reduction test, 8) H2S production test, 9) glucose utilization test, 10) adonitol test, 11) lactose utilization test, 12) arabinose utilization test, 13) sorbitol utilization test. Biochemical tests number 2-13 were carried out by using rapid biochemical test kit (KB002 HIAssortedTM Biochemical test kit for gram negative rods).

Molecular confirmation of Xoo through PCR

The genomic DNA from the isolated bacteria was obtained using the technique given by Gabriel and De Feyter (1992). The DNA obtained from the isolated samples was utilized as a template for a polymerase chain reaction (PCR), which was carried out using specific forward and reverse primers targeting *Xanthomonas oryzae* pv. *oryzae*. The molecular identification of the bacteria involved amplifying the 16S rRNA gene from the isolated bacterial samples. This was achieved using the forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3'), resulting in an amplicon size of 1537 bp (Mondal *et al*., 2013). The PCR was conducted for 40 cycles with a reaction volume of 10 µl, comprising 5 µl of master mix, 1 µl each of forward and reverse primers, 1 µl template DNA (200 ng/µl) and 2 µl of double sterile distilled water. Amplification was carried out through following temperature profile: initial denaturation at 94 ℃ for 5 min, denaturation at 94 ℃ for 1 min, annealing at 55 ℃ for 45 sec, extension at 72 ℃ for 1 min and final extension at 72℃ for 10 Min.

For species specific primers, XOF forward primer (5'-ATGCCGATCACCATGCCGAT-3') and XOR reverse primers (5'-TGGCCTTGTCGTACGAGCTC-3') were used for bacterial isolates. PCR reactions were performed. The PCR was conducted within a reaction mixture of 10 µl consisting of 5 µl of master mix, 1 µl template DNA, 1 µl of each forward and reverse primers and 2 µl of double sterile distilled water.

Multiplex PCR for positive Xoo isolates were carried out by using 2 sets of primer 1) Xo3756F (5'-CATCGTTAGGACTGCCAGAAG-3') and Xo3756R (5'-GTGAGAACCACC GCCATCT-3') primers 2) Xoo80F (5'-GCCGCTAGGAATGAGCAAT-3') and Xoo80R (5'-GCGTCCTCGTCTAAGCGATA-3') primers that was suggested by IRRI, Philippines. Multiplex PCR was employed to concurrently identify Xoo while distinguishing it from its close counterpart, *Xanthomonas oryzae* pv. *oryzicola* (Xoc). Among the isolates tested, seven were verified as Xoo. Additionally, all these isolates exhibited blight symptoms on rice, with variations in the intensity of symptoms.

Agarose gel electrophoresis of amplified DNA fragments

The amplified DNA segments were subjected to horizontal electrophoresis using 1.2% agarose gel submerged in TAE buffer, following the procedure outlined by Martins *et al*. (2005) [15]. PCR products were loaded onto the gel in 5 µl portions. The gel was then treated with ethidium bromide stain and captured through UV illumination at a wavelength of 320 nm.

Assessment of virulence pattern of Xoo isolates

For pathogenicity testing bacterial isolates namely TNXOO1, TNXOO2, TNXOO3, TNXOO3, TNXOO4, TNXOO4, TNXOO5, TNXOO6, TNXOO7 and TNXOO8 were artificially inoculated on 30 days old transplanted seedlings of susceptible rice cultivar TN1 by leaf clipping method (Kauffman *et al*., 1973) [10]. Seeds of TN1 cultivar were transplanted to 15cm diameter earthen pots and were grown under glasshouse conditions for 1 month. For inoculations bacterial suspensions were prepared in sterile distilled water at 10^9 CFU/mL and inoculations were done by using a sterile scissor. Leaves of control plants were treated with sterile distilled water and the leaves were observed daily for 3 weeks after inoculation. Pathogens were re-isolated from leaves showing typical BLB symptoms and cultured on nutrient agar (NA) plates for further studies. Lesion length was calculated on 3, 14 and 21 days after inoculation by using the following scale.

Disease ratings	Lesion size (% of leaf length)
0	0
1	>1-10
3	>11-30
5	>31-50
7	>51-75
9	>76-100

Percent disease index (PDI) on leaves were calculated by applying the formula:

$$\text{Percent disease index} = \frac{\text{Sum of individual ratings}}{\text{Total numbers of leaves examined}} X \frac{100}{\text{Maximum Grade}}$$

Results

Isolation and and morphological characterisation of Xoo

From the above-mentioned locations of Tamil Nadu 14 different isolates of Xoo have been isolated from the BLB infected fields (Fig. 1) and produced different colony characters. On NA media, the colonies showed up as spherical, convex, glistering yellow and slimy colony with uniform margins. Extracellular polysaccharide (EPS) was produced by all the isolates, as evidenced by the colonies' slimy appearance (Fig. 2).

Fig 1: A &B) BLB infected fields, C) Sample collection for isolation of bacterial pathogen

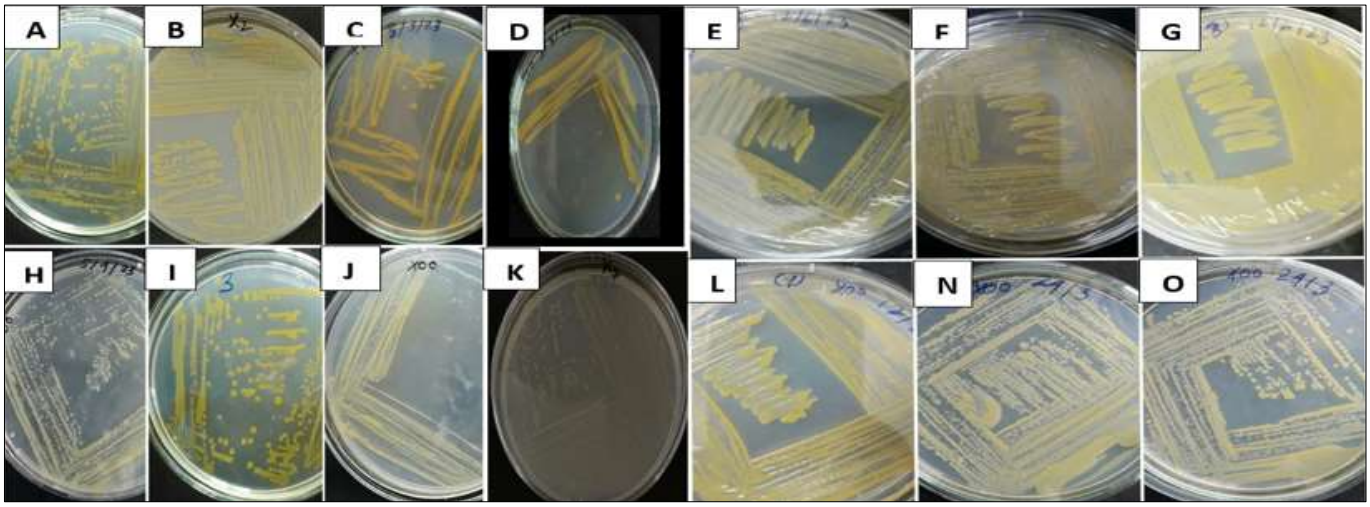

Fig 2: Colony morphology of 14 bacterial isolates from A-O

Biochemical characterisation of Xoo

All 14 bacterial isolates were tested for Grams staining (Fig.3) and 12 different biochemical tests (Fig. 4) as mentioned above and the results from biochemical tests are presented in table 1.

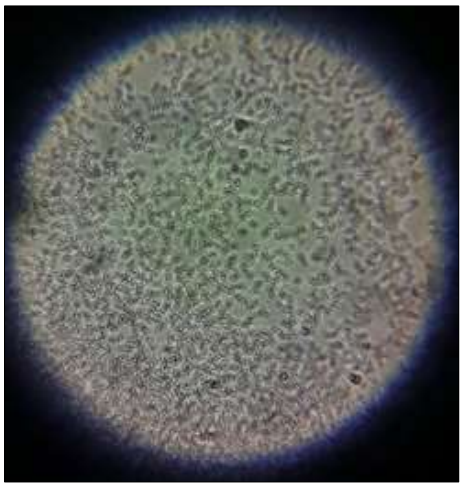

Fig 3: Grams staining

Fig 4: Biochemical test kit

Table 1: Biochemical characterisation of Xoo isolates

S. No	Biochemical tests	Results
1	Gram's staining	–
2	Citrate utilization test	+
3	Lysine utilization test	–
4	Ornithine utilization test	+
5	Urease test,	±
6	Phenylalanine deamination test,	–
7	Nitrate reduction test,	–
8	H2S production test	–
9	Glucose utilization test,	+
10	Adonitol test	–
11	Lactose utilization test	+
12	Arabinose utilization test	–
13	Sorbitol utilization test	±

-Negative reaction; + Positive reaction; ± some isolates showing positive reaction

Confirmation of *Xanthomonas oryzae* pv. *oryzae* through PCR

14 bacterial isolates were confirmed by 16S rRNA gene primers (27F and 1492R) and on 1.2% agarose gel all 14 isolates produce a band approximately 1537 bp (Fig.5A). Eight isolates of Xoo were identified by specific primers XOF and XOR which produced a PCR product of 534 bp (Fig.5B). Multiplex PCR with two sets of primers Xo3756F&R and Xoo80F&R also produced PCR products of 331bp and 162bp respectively (Fig. 5C). Sequencing was done on each of the PCR-positive results to further confirm the Xoo. The BLAST tool was used to evaluate the sequence (www.ncbi.nlm.nih).

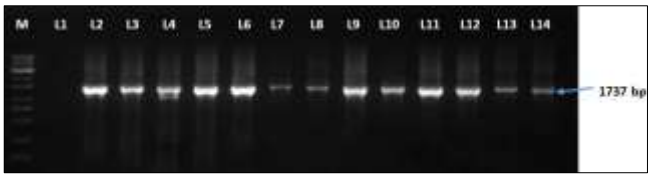

Fig 5A: Lane M-1kb ladder; Lane 1-14 bacterial isolates from BLB infected leaves

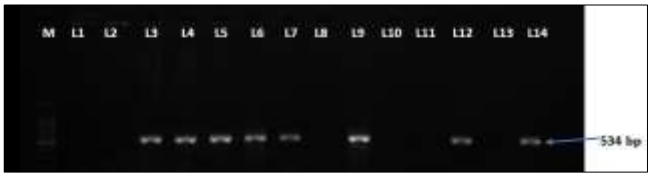

Fig 5B: Lane M -100 bp ladder; Lane 3,4,5,6,9,12,14 Xoo isolates from BLB infected

Fig 5C: Lane M-100bp ladder, Lane1-8 & 9 are positive Xoo isolates for multiplex PCR

Assessment of virulence pattern of Xoo isolates

To assess their virulence, all the Xoo isolates were inoculated on the susceptible rice cultivar TN1 (Fig. 4). After 15 days of inoculation, all the isolates exhibited the characteristic symptoms, including white to yellow stripes with wavy edges on the leaves (Fig. 5). Disease severity of 8 Xoo isolates were calculated and presented in Fig.6.

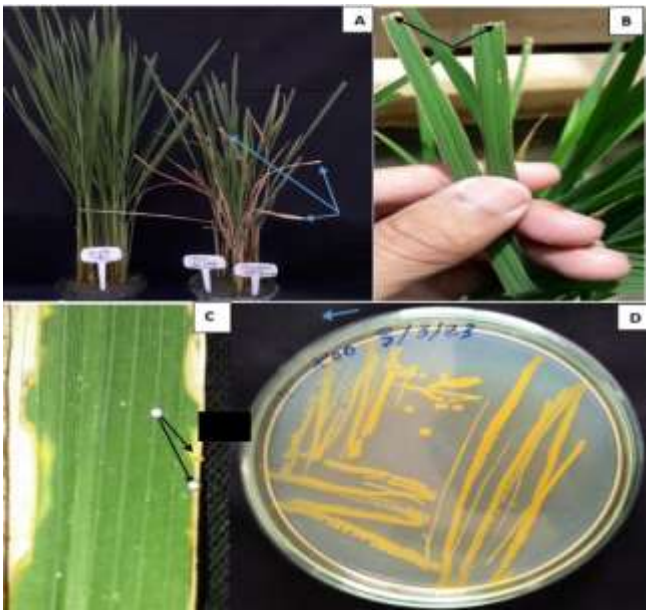

Fig 4: A) Healthy and inoculated control for pathogenicity test, B) Initiation of symptom expression 3 days after inoculation, C) Bacterial ooze in the inoculated leaves, D) Re-isolation of the bacterial pathogen from the artificially inoculated leaf samples

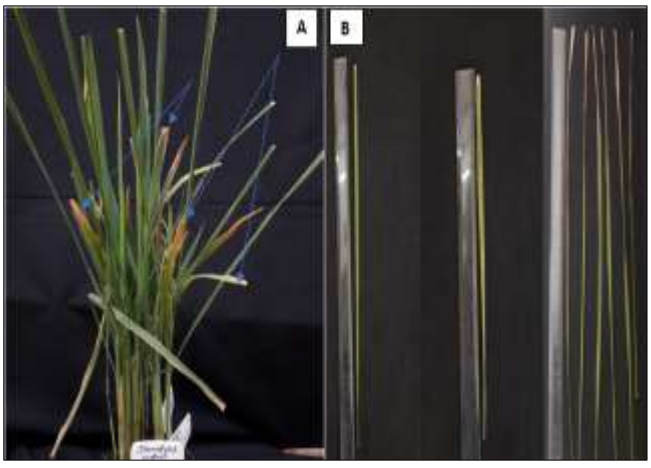

Fig 5: A) Typical symptom of BLB on TN1 cultivar of rice, B) Symptoms expression on artificially inoculated plants by leaf clipping method and measuring lesion length on 3rd, 7th and 15th days after inoculation

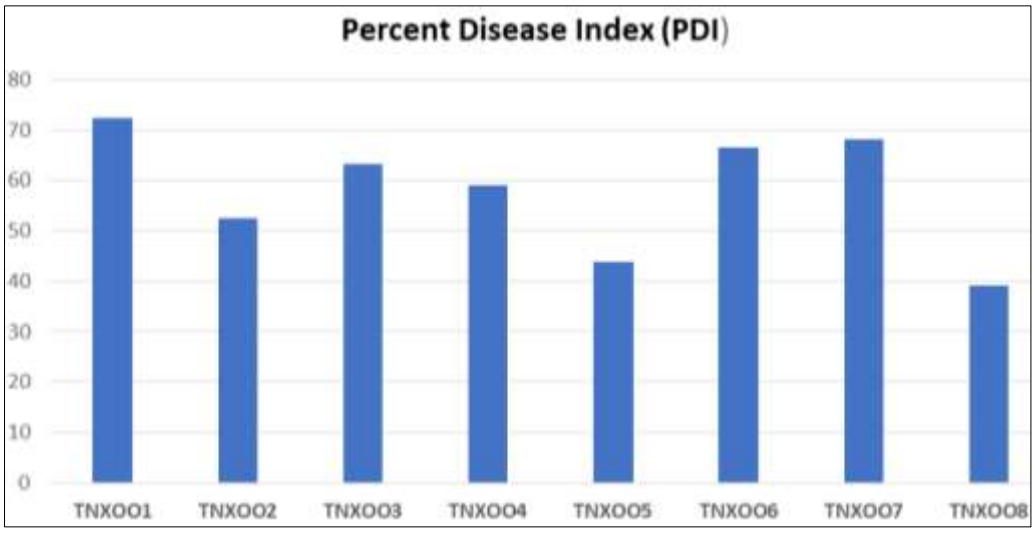

Fig 6: Virulence pattern of Xoo isolates on TN1

Discussion

According to Arshad *et al*. (2015) [2], one of the major diseases affecting rice plants and causing yield losses of up to 80% was BLB. In all the sampling locations of Tamil Nadu, BLB was prevalent and causing severe damage to the crop from seedling to maturity. We have identified *X. oryzae* pv. *oryzae* isolates based on morphological, biochemical, pathogenicity tests, and PCR techniques using specific primers.

Eight isolates of Xoo produced typical blighted symptoms and considerable differences were observed in virulence pattern on inoculated plants. These results showed that isolates derived from various locations differ significantly in their degree of virulence. The highest severity was recorded in TNXOO1 isolate, followed by TNXOO7 and the lowest severity in TNXOO8 isolate. Both the pathogen and the pathovars of *X. oryzae* can be detected using the XOF and XOR primers which produces a 534 bp PCR product. Because of crop age and climatic factors, the severity of disease differed amongst different rice growing zones of Tamil Nadu. According to research by Philip and Devadt (1980) [21], certain isolates exhibit severe symptoms during the seedling stage, while others become aggressive as the crop approaches maturity. Similar findings were also reported in Korea by Cha et al. in 1982 [5], who noted that young age plants had the highest incidence. A cell density of 10^4–10^6 cells/mL was shown to be necessary for the initiation of the disease symptom, although the number of bacterial cells needed for symptoms development in young seedlings varied significantly (Mew, 1987) [16]. The bacterium found in the seed of susceptible varieties is a possible source for additional transmission of BLB to other rice growing areas, which makes the seed-borne nature of Xoo in rice a major problem in the seed supply chain, trade and plant quarantine. In general, antibacterial antibiotics treatments and resistant rice varieties were used, to control BLB disease but emergence of new virulent races of Xoo possess a challenge particularly during epidemic form of the disease (Kumar *et al*., 2020) [12]. The present study indicates significant differences among isolates of Xoo isolated from various locations of Tamil Nadu during 2023 in terms of colony morphology, biochemical reaction and virulence pattern. Virulence of all the eight isolates differed significantly from highly virulent isolate TNXOO1 to less virulent isolate TNXOO8. However, all the eight isolates showing pathogenic reaction on artificially inoculated TN1 plants. Incorporating novel resistance genes into commercial rice cultivars is needed to combat BLB due to the rising prevalence of virulent isolates in Tamil Nadu.

References

1. Akhtar MA, Zakeri M, Abassi FM. Inoculum build-up of bacterial blight of rice in rice-wheat cropping area of Punjab in relation to zero tillage Asian J Plant Sci. 2003;2:548-550.
2. Arshad HMI, Saleem K, Khan JA, Rashid M, Atiq M, Alam SS, et al. Pathogenic diversity of *Xanthomonas oryzae* pv. *oryzae* isolates collected from Punjab Province of Pakistan. Eur. J Plant Pathol. 2017;147(3):639-651.
3. Blight disease of rice in India. The International Rice Commission, Newsletter. 17, 27-33.
4. Bradbury JF. Isolation and preliminary study of bacteria from plants. Int. J Pest Manag. 1970;16:632-637.
5. Cha KH, Kim YS, Kim HI, Lee DK, Kim MS. Effects of application levels of Chandraprakash S, Manonmani K, Revathy N, Devi EK. Management of Bacterial Leaf Blight Disease of Paddy (*Oryza sativa*) Caused by *Xanthomonas oryzae* pv. *oryzae* Using Bacteriophages. (2018) International Journal of Current Microbiology and Applied Sciences; c1982. p. 3066-3080.
6. Cha KH, Kim YS, Kim HJ, Lee DK, Kim MS. Effects of application levels of fertilizer on the susceptibility to bacterial leaf blight, yield and quality of grains in nineteen rice cultivars in Jeonnam Region. Korean journal of applied entomology. 1982;21(4):216-221.
7. Gabriel DW, De Feyter R. RFLP analyses and gene tagging for bacterial identification and taxonomy. In: Molecular Plant Pathology, A Practical Approach, SJ Gurr, MJ McPherson and DJ Bowles (eds). Oxford, UK: IRL Press; c1992. p. 51-66.
8. Goto M. Fundamentals of bacterial plant pathology. San Diego. Calif. USA: Acad. Press; c1992. p. 342.
9. Horino O, Mew TW, Yamada T. The effect of temperature on the development of bacterial leaf blight on rice. Japanese Journal of Phytopathology. 1982;48(1):72-75.
10. Kauffman HE, Reddy APK, Heisk SPV, Maraca SD. An improved technique for evaluating resistance of rice

varieties to *Xanthomonas oryzae*. Plant Dis. Rep. 1973;57:537-541.
11. Khan JA, Siddiq R, Arshad HMI, Anwar HS, Saleem K, Jamil FF. Chemical control of bacterial leaf blight of rice caused by *Xanthomonas oryzae* pv. *oryzae*. Pakistan. Phytopathol. 2012;24:97-100.
12. Kumar A, Kumar R, Sengupta D, das SN, Pandey MK, Bohra A, et al. Deployment of genetic and genomic tools toward gaining a better understanding of rice-*Xanthomonas oryzae* pv. *oryzae* interactions for development of durable bacterial blight resistant rice. Front. Plant Sci. 2020;11:1152.
13. Lee CN, Hu RM, Chow TY, Lin JW, Chen HY, Tseng YH, et al. Comparison of genomes of three *Xanthomonas oryzae* bacteriophages. BMC Genomics. 2007;8:442.
14. Liu W, Pan LQ, Zhao HJ, Jia YL, Wang YL, Yu XP, et al. Molecular detection of *Xanthomonas oryzae* pv. *Oryzae*, *Xanthomonas oryzae* pv. *oryzicola*, and *Burkholderia glumae* in infected rice seeds and leaves. Crop J. 2014;2:398-406.
15. Martins ML, Araújo EF, Mantovani HC, Moraes CA, Vanetti MCD. Detection of the apr gene in proteolytic psychrotrophic bacteria isolated from refrigerated raw milk. International J Food Microbiol. 2005;102:203-211.
16. Mew TW. Current status and future prospects of research on bacterial blight of rice. Annu. Rev. Phytopathol. 1987;25:359-382.
17. Muko H, Kusaba T, Watanabe M, Tabei H, Tsuchiya Y. Effect of major environmental factors on the development of bacterial leaf blight of rice. Japanese Journal of Phytopathology. 1957;29:13-19.
18. Naqvi SAH. Bacterial leaf blight of rice: An overview of epidemiology and management with special reference to Indian sub-continent. Pak. J Agric. Res. 2019;32(2):359.
19. Niño-Liu DO, Ronald PC, Bogdanove AJ. *Xanthomonas oryzae* pathovars: Model pathogens of a model crop. Mol. Plant Pathol. 2006;7(5):303-324.
20. Ou SH. Rice diseases in Common wealth mycological Instt. 2nd ed (England: Kew. Surrery); c1985. p. 61-96.
21. Philip R, Devadath S. Effect of inoculum concentration of wild and mutant colony types of *Xanthomonas oryzae* on the disease development in rice. Oryza. 1980;17:38-41
22. Quibod IL, Atieza-Grande G, Oreiro EG, Palmos D, Nguyen MH, Coronejo ST, *et al.* The green revolution shaped the population structure of the rice pathogen *Xanthomonas oryzae* pv. *oryzae*. The ISME Journal. 2020;14(2):492-505.
23. Rangaswami G. *In vitro* and *in vivo* activities of streptocycline on bacterial blight of rice caused by *Xanthomonas oryzae*. Indian phytopathology; c1971. p. 145-152.
24. Srivastava D, Rao T. Seed Transmission and epidemiology of bacterial blight disease of rice in North India. Indian Phytopathol. 1964;17:77-78.
25. Cruz VCMV, Gossele F, Kersters K, Segers P, Mooter VDM, Swings J, Ley JD. Differentiation between *Xanthomonas campestris* pv. *oryzae*, *Xanthomonas campestris* pv. *oryzicola* and the bacterial "brown blotch" pathogen on rice by numerical analysis of phenotypic features and protein gel electrophoregrams. J General Microbiol. 1984;130:2983-2999.
26. Webb KM, Ona I, Bai J, Garrett KA, Mew T, Vera Cruz CM, Leach JE. A benefit of high temperature: increased effectiveness of a rice bacterial blight disease resistance gene. New Phytologist. 2010;185(2):568-576.
27. Srinivas MN. The dominant caste in Rampura. American anthropologist. 1959 Feb 1;61(1):1-6.