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Virulence Spectrum and Molecular Characterization of *Xanthomonas oryzae* pv. *oryzae* Infecting Rice

Yuvaraj Nandhikeswaran[1], Akshaya Subbaiah Balamurali[1], Rageshwari Selvaraj[1], Melvin Joe Manoharan[2]

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ABSTRACT

Background: Bacterial leaf blight (BLB) is a major bacterial disease of rice, causing serious yield losses in endemic regions. Continuous surveillance and characterization of pathogen variability are essential for effective disease management.

Methods: A roving survey was conducted during 2025 in major rice-growing areas of the Chengalpattu district, Tamil Nadu. Diseased samples were collected from different rice varieties and subjected to isolation using the maceration technique. The per cent disease index (PDI) was calculated and documented. Morphological, cultural, pathogenicity analysis and molecular characterization (16S rRNA sequencing) were performed to confirm the pathogen. Virulence variability among ten isolates was assessed using clip inoculation on rice variety CO51.

Result: The disease severity ranged from 40 to 85%, with CO51 recording the highest susceptibility (85%) among the reported varieties. All the study isolates exhibited circular, smooth, yellow, mucoid colonies on Nutrient Agar and were Gram-negative rods. Pathogenicity tests reproduced typical BLB symptoms and fulfilled Koch's postulates. Molecular analysis confirmed 100% similarity with *Xanthomonas oryzae* pv. *oryzae* in the database. Significant variation in virulence was observed, with isolate 1(YN*X001*) recording the highest PDI. The study confirmed the prevalence of virulent BLB strains in Chengalpattu district and highlights the need for integrated disease management strategies.

Key words: Bacterial leaf blight, Molecular characterization, Rice, Variability, Virulence, *Xanthomonas oryzae* pv. *oryzae*.

INTRODUCTION

Rice is a staple food for millions of people worldwide. In Asia, its importance is even greater, as over 90% of the population relies on rice as a daily dietary essential. In India, rice is of utmost importance as the main cereal crop. It occupies 42% of the nation's cultivated land. Rice is grown over the largest area in India, covering 43.78 million hectares (FAO, 2023) and globally ranks second in production, with a yield of 118.43 million tonnes, behind China.

One of the biggest challenges in rice production is the disease threat caused by harmful fungi, bacteria and viruses. Among these, bacterial leaf blight (BLB) stands out as the most severe disease of rice, causing annual yield losses ranging from 20% to 50% and up to 70% under severe epidemic conditions (Nino-Liu *et al*., 2006; Mew *et al*., 1993). This devastating disease is caused by the pathogen *Xanthomonas oryzae* pv. *oryzae* and it can drastically reduce yield if not properly managed (Nagaraju *et al*., 2007; Das *et al*., 2014).

The first recorded outbreak of rice BLB was reported from Japan during 1884, when local farmers spotted its devastating effects (Onasanya *et al*., 2010). In the present scenario, the disease has become a serious epidemic in many parts of the world, with severe cases leading to crop losses of up to 50%.

The *Xanthomonas* pathogen infects rice plants by entering through hydathodes, stomata, or wounds on the roots and leaves (Nino-Liu *et al*., 2006). Once inside, it causes leaf wilting and disrupts photosynthesis, leading to yield losses of 20-50% (Adhikari *et al*., 1995, White and Yang, 2009). Infected plants often produce immature grains, which break more easily during milling, resulting in a higher proportion of broken rice (Khan *et al*., 2014).

[1]Department of Plant Pathology, SRM College of Agricultural Sciences, SRM Institute of Science and Technology, Baburayanpettai, Chengalpattu-603 201, Tamil Nadu, India.
[2]Department of Soil Sciences and Agricultural Chemistry, SRM College of Agricultural Sciences, SRM Institute of Science and Technology, Baburayanpettai, Chengalpattu-603 201, Tamil Nadu, India.

Corresponding Author: Rageshwari Selvaraj, Akshaya Subbaiah Balamurali, Department of Plant Pathology, SRM College of Agricultural Sciences, SRM Institute of Science and Technology, Baburayanpettai, Chengalpattu-603 201, Tamil Nadu, India.
Email: rageshws@srmist.edu.in; akshayas2@srmist.edu.in

ORCIDs: 0000-0002-7735-630X, 0000-0002-5158-1461

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MATERIALS AND METHODS

Survey for the assessment of BLB disease

A roving survey has been conducted across different zones of Chengalpattu district to collect infected rice leaf samples

with bacterial leaf blight. The samples were collected from different rice varieties, including CO 51, Ponni and Gundu. Each sample was labelled with an isolate number, place of collection, variety, geographical coordinates (latitude and longitude) and the PDI was calculated. The collected samples were transported to the laboratory for the successful isolation of the pathogen (Mordue, 1982).

Assessment of disease severity and calculation of per cent disease index (PDI)

Disease severity of bacterial leaf blight (BLB) was evaluated using the standard evaluation system (SES) for Rice developed by the International Rice Research Institute (2013). The severity of infection was assessed by visually scoring the affected leaves based on the proportion of leaf area showing BLB symptoms (Table 1).

The PDI was calculated using the following formula:

$$\text{PDI (\%)} = \frac{\Sigma \text{ Disease ratings}}{\text{Number of leaves observed} \times \text{Maximum disease grade}} \times 100$$

Isolation of *Xanthomonas oryzae* pv. *oryzae*

The bacterial leaf blight pathogen was isolated following the maceration technique given by Klement *et al.* (1990) and Schaad *et al.* (2001). Freshly infected leaf bits (0.5-1 cm) were excised and surface-sterilized with 1% sodium hypochlorite for 30 seconds. The leaf bits were rinsed three times with sterile distilled water and blotted dry using sterile tissue paper. The leaf bits were transferred into a sterile mortar containing 5-10 mL of sterile distilled water and gently macerated using a sterile pestle. From the prepared dilutions, one mL of the sample was carefully transferred into a sterile petri dish. Each plate was overlaid with about 15-20 mL of molten cooled (45-50°C) nutrient agar (NA), with a gentle swirl. The inoculated plates were incubated at 28±2°C for 48-72 hours.

Following incubation, the representative colonies were selected and purified using the streak plate method (Benson, 1990). Quadrant streaking was performed on fresh Nutrient Agar plates to obtain the pure culture and incubated again at 28±2°C for 48-72 hours.

Morphological identification of the pathogen

The incubated plates were observed for bacterial colonies. The colonies depicting *Xanthomonas* were sub-cultured. The morphological characters of the study isolates were recorded.

Pathogenicity test (Koch's postulates)

To confirm the pathogenic nature of the isolates, Koch's postulates were carried out under controlled conditions. The bacterial suspension of a single colony was prepared on NA broth and adjusted to approximately 10x-10y CFU ml^{-1}. A 48-hour-old virulent culture of all ten isolates was prepared separately and inoculation was accomplished using clip inoculation technique as described by Kauffman *et al*. (1973). The virulence of each isolate was studied based on symptom expression and severity of the symptom.

Molecular characterization of *Xanthomonas oryzae* pv. *oryzae*

The virulent isolate was identified and subjected to molecular characterization. Genomic DNA was extracted using the modified protocol described by Sambrook *et al*. (1989). Actively growing bacterial cultures were lysed in extraction buffer containing Tris-HCl, EDTA and SDS, followed by protein precipitation and purification. The quality of the DNA was verified through spectrophotometric analysis and gel electrophoresis.

To study the molecular identity, the 16S rRNA gene was amplified using universal bacterial primers27F and 1492R, targeting a region of approximately 1300-1500 bp. PCR amplification was performed in a 25 µL reaction mixture containing genomic DNA, PCR buffer, MgCl, dNTPs, primers, Taq polymerase and nuclease-free water. Thermal cycling was performed for 35 cycles with an annealing temperature of 55°C for 30 seconds. The products were gel electrophoresed and resolved in 1% agarose gel.

The purified PCR product was subjected to Sanger sequencing at Barcode Biosciences Pvt. Ltd., Bengaluru. The obtained sequence was submitted to GenBank database for retrieving the accession number. BLASTn analysis and phylogeny were performed by retrieving the sequences from the NCBI database using MEGA 12.0 0 (Kumar *et al.,* 2024).

RESULTS AND DISCUSSION

Survey for the assessment of blb disease

Disease severity varied considerably across locations and varieties, ranging from 40% to as high as 85% from January 2025 to May 2026 (Table 1). Among the varieties surveyed, the variety CO 51 was found to be severely affected, recording the highest incidence of 85%, suggesting its high susceptibility. In contrast, Ponni and Gundu exhibited moderate levels of infection (40 to 65%). The differences in disease severity observed among the surveyed fields may be due to the varieties cultivated, pathogen density, weather parameters and different agronomical practices.

Typical symptoms observed in infected fields included yellowing that began at the leaf tips, water-soaked lesions

Table 1: Disease grade table (International Rice Research Institute, 2013).

Grade	Diseased leaf area (%)
0	No symptom
1	1-5
3	6-12
5	13-25
7	26-50
9	51-100

along the leaf margins and gradual drying of affected tissues. As the disease advances, leaf spots shift from yellow to a pale straw colour. The prominent symptom, *viz*. wavy, irregular edges on the lesions was observed in all the field (Fig 1). Over time, individual spots merge, creating large blighted patches with those characteristic undulating borders.

Similar symptoms, including tip yellowing, water-soaked streaks along leaf margins, irregular lesions with wavy margins and progressive drying, were earlier reported by Ou (1985); Nino-Liu *et al.* (2006); Kauffman *et al.* (1973) and Ezuka and Kaku (2000). These observations reinforce the infection process, where the pathogen typically enters through hydathodes or wounds as reported by Swings *et al.* (1990) and subsequently colonizes the xylem vessels, enabling systemic spread within the plant. The survey revealed that BLB incidence in Chengalpattu district ranged from 40% to 85% in the rice varieties CO 51, Gundu and Ponni, showing the highest incidence of 85% in CO 51, followed by Gundu (45%-65%) and Ponni (40%) (Table 2). These results are consistent with earlier reports by Mew *et al.* (1993) and Nagaraju *et al.* (2007), who documented severe BLB outbreaks in susceptible cultivars under favourable conditions. The roving survey conducted in different rice-growing locations of Chengalpattu district revealed considerable variation in bacterial leaf blight severity. The disease was prevalent in all surveyed areas, although the level of infection differed among locations and cultivars. Similar observations were reported by Kanipriya *et al*. (2024), who identified diverse pathotypes and virulence patterns among *Xanthomonas oryzae* pv. *oryzae* isolates collected from different rice-growing regions of Tamil Nadu. The similarity is likely due to comparable agro-climatic factors such as high humidity, warm temperatures and intensive cropping systems that favour rapid pathogen spread.



Fig 1: Leaf symptom with wavy margins.

Isolation of *Xanthomonas oryzae* pv. *oryzae*

The pathogen was successfully isolated on NA media using the maceration technique. All isolates produced uniform colonies that were circular, smooth, slightly raised, bright yellow in colour due to the production of Xanthomonadin pigment, shiny in appearance and distinctly mucoid as a result of extracellular polysaccharide secretion (EPS) (Table 3). These characteristic features confirmed the pathogen's identity as *Xanthomonas oryzae* pv. *oryzae.* The colony characters documented were on par with the reports of Schaad *et al*. (2001) and Mew *et al.* (1993), where they observed bright yellow smooth colonies.

Morphological identification of the pathogen

After an incubation for 48-72 hours the colonies appeared circular with smooth, entire margins and showed a slightly convex to raised profile measuring 1-3 mm in diameter. They displayed a bright, uniform yellow pigmented, shiny colonies with mucoid consistency. The yellow coloration was stable across all isolates, a hallmark of *Xanthomonas oryzae* pv. *oryzae*. Growth on NA was moderate to profuse, producing clearly distinguishable colonies with or without spreading or swarming behaviour (Fig 2). The colony traits observed here align closely with the work of Kanipriya *et al*. (2024), who documented the distinctive yellow, mucoid colonies of *Xanthomonas oryzae* pv. *oryzae* from rice fields across Tamil Nadu. Furthermore, Karan *et al*. (2026) emphasized the importance of colony morphology and cultural characteristics as practical and reliable preliminary criteria for the identification and characterization of plant-associated bacterial pathogens.

The colonies were distinctly mucoid in texture, a feature that aligns with earlier descriptions by Schaad *et al.* (2001)

Table 2: Isolates of *Xoo* from different locations of the Chengalpattu district, Tamil Nadu.

Isolate name	Place of collection	District	Variety	Geo-location	PDI (%)
Yn*Xoo*1	Sirupairpandi	Chengalpattu	CO51	Lat 12.409604°; Long 79.737719°	84.44
Yn*Xoo*2	Porpanankaranai		CO51	Lat 12.402266°; Long 79.738255°	71.11
Yn*Xoo*3	Sirupairpandi		CO51	Lat 12.409864°; Long 79.73776°	78.89
Yn*Xoo*4	Pakkam		Ponni	Lat 12.476596°; Long 79.858269°	40.56
Yn*Xoo*5	Pakkam		Gundu	Lat 12.402266°; Long 79.738255°	44.44
Yn*Xoo*6	Sothupakkam		Gundu	Lat 12.44258°; Long 79.839386°	64.44
Yn*Xoo*7	Seethapuram		CO51	Lat 12.403699°; Long 79.83766°	48.89
Yn*Xoo*8	Sothupakkam		CO51	Lat 12.442482°; Long 79.839389°	46.67
Yn*Xoo*9	Kilathivakkam		CO51	Lat 12.375211°; Long 79.726141°	61.11
Yn*Xoo*10	Chengalpattu		CO51	Lat 12.37637°; Long 79.719836°	57.78

and Mew *et al*. (1993). The yellow pigmentation is attributed to Xanthomonadin, a pigment known to protect bacterial cells against oxidative stress and ultraviolet radiation, thereby enhancing survival under field conditions. The mucoid consistency reflects EPS secretion, which plays a critical role in virulence by facilitating adhesion, biofilm formation and xylem blockage Denny (1995). The production of EPS is considered an important virulence determinant that enables successful colonization and systemic movement of the pathogen within host tissues. Similar observations on colony morphology and pathogen variability were reported by Kanipriya *et al.* (2024).

Microscopic examination using Gram staining further confirmed the cellular characteristics. Under oil immersion (100X), the bacterial cells-stained pink, indicating their Gram-negative nature.

They appeared as short, slender rods (bacilli), measuring approximately 0.5-0.8 µm in width and 1.0-2.0 µm in length. Cells were mostly observed singly, occasionally in pairs and rarely in short chains. The consistent rod-shaped morphology and Gram-negative reaction strongly supported the identification of the isolates as *Xanthomonas oryzae* pv. *oryzae* (Fig 3). The morphological characteristics observed in this study correspond well with the taxonomic descriptions of *Xanthomonas oryzae* pv. *oryzae* outlined by Bradbury (1986); Schaad *et al*. (2001). Midha *et al*. (2017) also reported that diverse isolates of this pathogen consistently exhibit the traits of Gram negative, rod shaped bacteria. Adding to this, Jerish *et al*. (2022) highlighted the importance of pathogen diversity in bacterial leaf blight and stressed the value of characterizing pathogen populations to improve disease management strategies. Although the isolates examined here shared similar colony morphology, earlier studies have shown that such outward resemblance does not guarantee uniformity in pathogenic potential. In reality, morphologically alike isolates may vary considerably in their virulence and disease causing behaviour (Midha *et al*., 2017; Jerish *et al*., 2022; Kanipriya *et al*., 2024).

Pathogenicity test (Koch's postulates)

Pathogenicity assay using clip inoculation method on rice variety CO 51 successfully reproduced the typical BLB symptoms. Inoculation was performed 40 DAS (Days after sowing). Symptom expression was initially observed 10-15 DAI (Days after inoculation). Water-soaked streaks developed near the clipped leaf tips, followed by lesion elongation, yellowing and eventual drying of the infected portions. In contrast, control plants inoculated with sterile

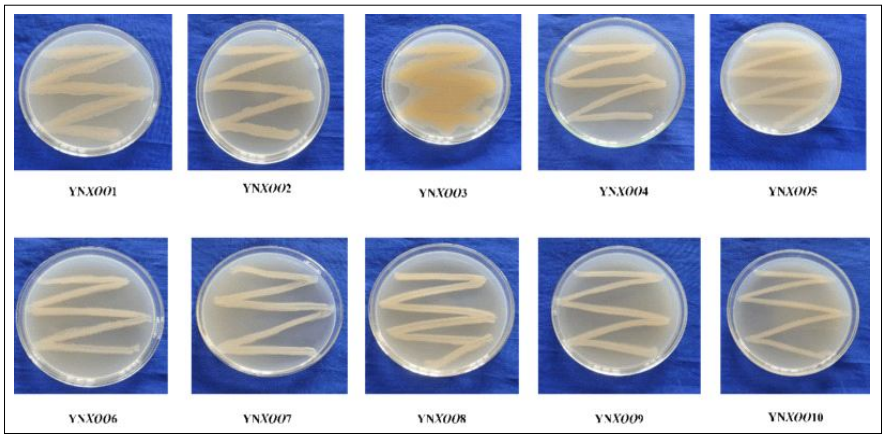


Fig 2: Isolates of *Xanthomonas oryzae* pv. *oryzae*.

Table 3: Morphological characteristics of isolates.

Isolate	Colony structure	Colony colour	Colony texture	Xanthomonadin pigment
Yn*Xoo*1	Circular, convex	Bright yellow	Smooth, mucoid	Present
Yn*Xoo*2	Circular	Yellow	Smooth, glistening	Present
Yn*Xoo*3	Slightly irregular	Yellow	Mucoid	Present
Yn*Xoo*4	Circular	Pale yellow	Smooth	Present
Yn*Xoo*5	Circular	Bright yellow	Mucoid	Present
Yn*Xoo*6	Circular	Light yellow	Smooth	Present
Yn*Xoo*7	Circular	Creamy yellow	Slightly mucoid	Present
Yn*Xoo*8	Circular	Pale yellow	Smooth	Present
Yn*Xoo*9	Circular	Light yellow	Smooth	Present
Yn*Xoo*10	Circular	Pale yellow	Smooth, slightly mucoid	Present

distilled water remained completely healthy throughout the observation period.

Re-isolation of the bacterium from symptomatic leaves yielded colonies identical to the original isolates, thereby satisfying Koch's postulates and confirming the causal role of the pathogen (Fig 4).

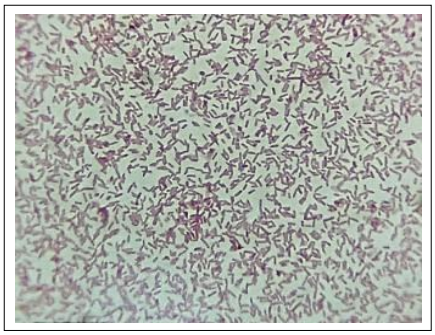


Fig 3: Gram-negative rods depicting the nature of *Xanthomonas oryzae* pv. *oryzae*.

The virulence of ten isolates of *Xanthomonas oryzae* pv. *oryzae* was tested under controlled conditions using the rice variety CO 51. The plants produced classic signs of bacterial leaf blight, including water-soaked streaks near the clipped leaf tips, yellowing and gradual drying along the leaf margins. The severity of disease varied noticeably among the isolates. Based on visual observations and the calculated PDI, the isolates were classified into distinct virulence categories (Table 4).

Pathogenicity test outcome aligns with Kauffman *et al.* (1973), who standardized the method for reliable pathogenicity confirmation. The reproduction of identical symptoms and successful re-isolation of the pathogen fulfil Koch's postulates, validating the causal link. The clip method's effectiveness lies in its ability to mimic natural infection through hydathodes, ensuring uniform inoculum

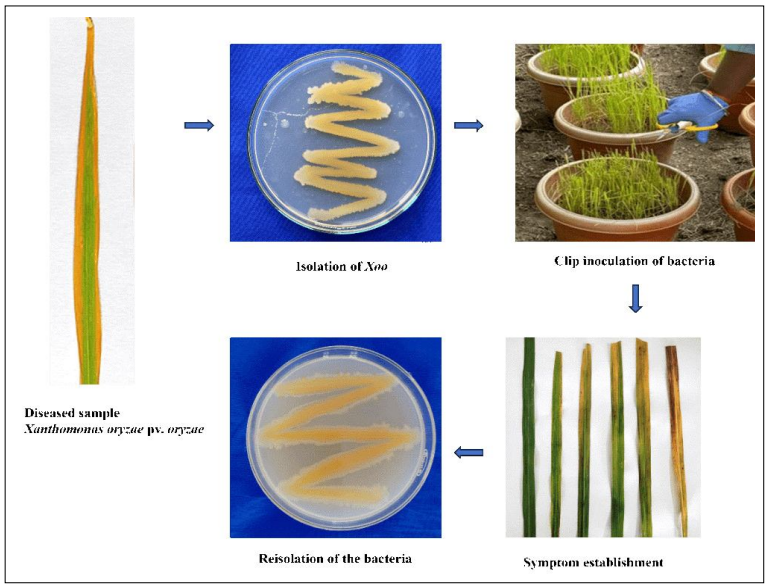


Fig 4: Proving koch's postulate.

Table 4: Study on the virulence of isolates of *Xanthomonas oryzae* pv. *oryzae*.

Isolate category	Isolate(s)	Virulence level	Symptom expression	Days	Disease progression	PDI (%)	Mean disease score (0-9 SES scale) (Koch's postulate)
Highly virulent	YnXoo1	Very high	Rapid symptom appearance with intense yellowing and extensive leaf drying	10-12 days	Very fast	84.44	7.60%
Highly virulent	YnXoo2, YnXoo3	High	Well-developed lesions spreading along the leaf blade	12-14 days	Fast	71.11	6.40-7.10
Moderately virulent	YnXoo4-YnXoo10	Moderate	Mild to moderate symptoms with limited lesion spread	14-18 days	Moderate to slow	40.56 -64.44	3.65-5.80
Control	-	None	No symptoms observed	No symptoms up to 20 days	No disease development	0.00	0%

delivery. A key finding was the variation in virulence among the ten isolates, with Isolate 1 showing the highest PDI. Similar variability has been reported by Shanti *et al.* (2001) and Wang *et al.* (1996), who attributed differences to genetic diversity and environmental selection pressures.

Molecular characterization of *Xanthomonas oryzae* pv. *oryzae*

The virulent isolate was subjected to DNA extraction using the lysis buffer method as described by Sambrook *et al.* (1989). The integrity of the DNA was confirmed through agarose gel electrophoresis before proceeding to amplification. The 16S rRNA gene was targeted using universal bacterial primers 27F and 1492R, which successfully amplified a fragment of approximately 1330 base pairs, producing a single clear band on the gel.

The purified PCR product was sequenced and the resulting nucleotide sequence was analysed using BLASTn against the NCBI GenBank database. The sequence exhibited 100% similarity with reported strains of *Xanthomonas oryzae* pv. *oryzae*, in NCBI database thereby confirming the molecular identity of the isolate, which is consistent with genomic studies of Xoo strains (Bogdanove *et al.,* 2011). The validated sequence was subsequently deposited in the GenBank under accession number PX945785, providing a permanent molecular reference for future studies.

Phylogenetic analysis was performed with *Xoo* isolates reported worldwide and retrieved from database for analysis. The study isolate was found to be closely clustered with other isolates of *Xoo* at a bootstrap value of 70% and 1000 replications indicating high nucleotide similarity. *Pseudomonas syringae* pv. *phaseolicola* (FJ972539), was used as outgroup. All the isolates were segregated into two major groups. Group I included all the isolates of *Xoo* whereas Group II had outgroup. Under Group I all the isolates of *Xoo* diverged into different subgroups. Our study isolate PX945785 was found to be more closely associated with the isolates MZ714131 (Egypt), KX088299 (Andaman and Nicobar), OP071236 (Telangana), MW069710 (Kerala), PX658324 (Korea), PV759764 (Tirunelveli) and MH158537 (China) with 100% nucleotide identity. Interestingly, other isolates of Tamil Nadu including PP165072 (Chidambaram) and OR587908 (Coimbatore) was found to be clustered separately with an identity of 99% (Fig 5).

Phylogenetic analysis based on 16S rRNA gene sequences confirmed that the study isolate (PX945785, CGN) from rice, clustered within the *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) clade, forming a well-defined monophyletic group with other reference strains isolated from rice across diverse geographical regions. The tight clustering, supported by short branch lengths (0.02 substitutions per nucleotide position), indicates high sequence similarity and minimal evolutionary divergence among *Xoo* isolates, suggesting a conserved genetic makeup, as reported by Nino-Liu *et al.* (2006) and Triplett *et al.* (2016).

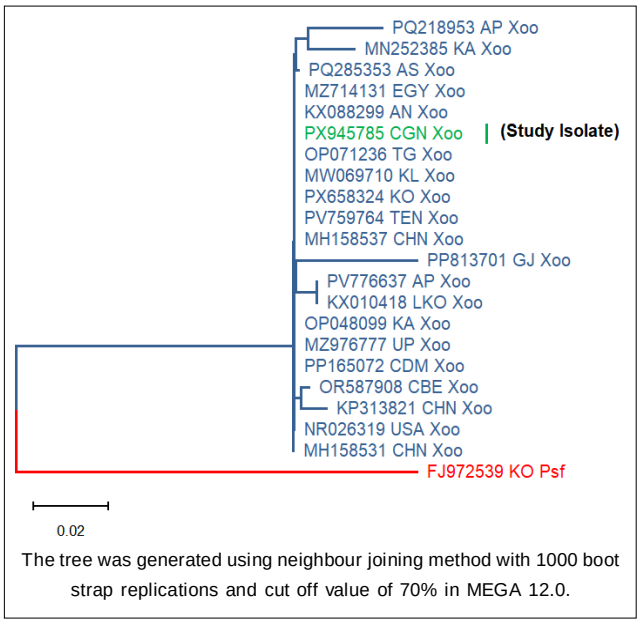
Similarly, Midha *et al.* (2017) have performed phylogenetic analysis for 106 *Xoo* strains from African, USA and Asia. Their study revealed that Indian *Xoo* strains, along with a few Asian strains, form a lineage distinct from USA and African strains.

Similarly, Koebnik *et al.* (2021) analysed multiple *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains from diverse geographical regions and reported clear lineage differentiation among strains. Their study showed that Asian *Xoo* strains, including those from India, tend to cluster separately from African strains, indicating geographic structuring and independent evolutionary trajectories within global *Xoo* populations.

The phylogenetic tree clearly distinguished the outgroup, forming a separate branch with greater evolutionary distance, thereby validating the accuracy of tree rooting and highlighting divergence from unrelated taxa. Within the *Xoo* cluster, the study isolate exhibited close genetic affinity with strains reported from Assam, Egypt, Telangana, Kerala and China, indicating a high degree of genetic conservation across geographically distant populations. Similar patterns of low genetic variability and widespread distribution have been documented by Lee *et al.* (2005) and Adhikari *et al.* (1995).

Further molecular validation was achieved by amplifying a ~1300-1500 bp 16S rRNA gene fragment, followed by BLAST analysis, which revealed 100% sequence similarity to authenticated *Xoo* reference strains.

Overall, the results provide strong molecular evidence that the isolate PX945785 is correctly identified as *Xanthomonas oryzae* pv. *oryzae*. Its close genetic relationship with previously reported strains from different geographical regions highlights the conserved nature of



The tree was generated using neighbour joining method with 1000 boot strap replications and cut off value of 70% in MEGA 12.0.

Fig 5: Phylogenetic analysis of the study isolates of *Xoo* using universal bacterial primers 27F and 1492R.

this pathogen. This genetic consistency further supports its role as the causal agent of bacterial leaf blight in rice.

CONCLUSION

Bacterial leaf blight (BLB) remains a major threat to rice cultivation in Chengalpattu district, with the susceptible variety CO 51 showing disease severity as high as 84.44%. Variations in disease severity across locations indicate that environmental conditions, cultivation practices and pathogen diversity strongly influence disease development. Cultural, morphological, pathogenicity and 16S rRNA analyses confirmed that BLB pathogens were closely related to established reference strains, though they differed markedly in virulence. Repeated cultivation of susceptible varieties may be contributing to the emergence of more aggressive pathogen populations. The prevalence of highly virulent isolates underscores the importance of continuous pathogen monitoring, cultivating resistant varieties and adopting integrated disease management practices.

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Disclaimers

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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