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Isolation, Purification and Identification of *Xanthomonas oryzae* pv. *oryzae*

Bhukya Srinivas[1*], V. A. Patil[2], Emmadi Venu[3], Hariprasath M.[1], Pooja Purushotham[1], Emani Rajeswari[6], Yerram Vasanth[4], Katravath Srinivas[6], K. Aravind[6], Eadulla Praveen Kumar[5] and K. Rajesh[6]

[1]Dept. of Plant Pathology, NMCA, NAU, Navsari, Gujarat (396 450), India
[2]Dept. of Plant Pathology, MRRC, SWMRU, NAU, Navsari, Gujarat (396 450), India
[3]Dept. of Plant Pathology, BCKV, Mohanpur, West Bengal (741 252), India
[4]Dept. of Genetics and Plant Breeding, BHU, Varanasi (221 005), India
[5]Dept. of Plant Pathology, Dr. RPCAU, Pusa, Samastipur, Bihar (848 125), India
[6]Dept. of Plant Pathology, PJTSAU, Rajendranagar,Hyderabad,Telangana (500 030), India

Corresponding Author

Bhukya Srinivas
e-mail: bhukyasrinivas954@gmail.com

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Abstract

The present study was conducted during *kharif* (July-November, 2020) at the Department of plant pathology, N. M. College of Agriculture, Navsari Agricultural University, Navsari, Gujarat, India to isolate, purify and identify the pathogen responsible for rice bacterial blight. The pathogen was isolated from infected rice leaves and purified through streaking techniques, with its cultural and morphological characteristics utilized for identification. The analysis revealed that colonies grown on nutrient agar media exhibited traits consistent with *Xanthomonas oryzae* pv. *oryzae*, appearing raised, creamy yellow, smooth, entire and buttery as cultural characteristics and the bacterium as possessing a rod-shaped structure, gram-negative staining, encapsulation and non-spore formation as morphological characteristics. Furthermore, a pathogenicity test was conducted on the susceptible rice cultivar GR-11, cultivated in pots under controlled conditions within a net house was successfully satisfying Koch's postulates. Rice (*Oryza sativa* L.) is one of the important cereal crop and a crucial dietary staple grown extensively in Gujarat, where it plays a significant role in ensuring food security and sustainability. However, rice bacterial blight remains a persistent threat, leading to substantial losses in both quantity and quality. To address this issue the investigation provided valuable insights into understanding the pathogen, thereby contributing to the development of cost-effective solutions for managing rice bacterial blight, which helps in increasing crop yield to the rice growing farmers of Gujarat.

Keywords: Isolation, purification, identification, staining, *Oryza sativa*, *Xoo*

1. Introduction

Rice holds paramount importance globally, serving as a vital staple crop crucial to human sustenance and economic value. Nutrient-wise, rice is a notable source of various essential components, with 100 g containing approximately 7.5 mg of protein, 1.9 mg of fat, 0.9 mg of crude fiber and providing 360 calories. Additionally, it contains essential minerals such as calcium (32 mg), phosphorus (221 mg), iron (1.6 mg) and potassium (9 mg) (Verma and Shukla, 2011). *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is a significant pathogen responsible for bacterial blight (BB) in rice (Feng et al., 2009). Among the various diseases affecting rice, *Xoo* is notably one of the most detrimental bacterial pathogens (Lee et al., 2005; Mansfield et al., 2012). Bacterial blight caused by *Xoo* is prevalent across regions including Australia, Africa, Latin America, the Caribbean and the United States (Mew et al., 1993; Mizukami and Wakimoto, 1969). Rice is a staple food crop that feeds over 70% of the world's population and is the most cost-effective source of protein and energy. India has the highest rice acreage with an annual production of 135.76 mt in 2022–23 (Anonymous, 2023). In Gujarat it is cultivated under area of 0.84 mha with production of 1.93 mt (Anonymous, 2019). The process of isolating *Xoo* involves collecting samples from rice plants exhibiting typical symptoms of bacterial blight during the panicle initiation stage Najeeya et al. (2007). 24 isolates of *Xoo* were obtained from various rice cultivation regions within the Andaman Islands Sakthivel et al. (2015). *Xoo* was isolated from collected samples using the direct plating method. Fourteen cultures were subsequently purified

using streaking techniques. These cultures were characterized based on various factors, including colony morphology, cell morphology and gram staining. The colonies exhibited diverse characteristics such as size, shape and surface appearance, with colors ranging from pale yellow, yellow, off-white, reddish, to creamy Nawaz et al. (2020). The symptoms further progress to include the occurrence of pale-yellow leaf symptoms, which are correlated with the virulence of *Xoo* isolates and the susceptibility of rice genotypes Hsieh et al. (2007). Additionally, a clipping technique for *Xoo* inoculation, involving the clipping of rice leaf tips with bacterial suspension-dipped scissors Kauffman et al. (1973). The pathogenicity of 13 isolates of *Xoo* was confirmed through inoculation experiments Gupta et al. (1986). The pathogenicity of *Xoo* was further characterized by employing standard leaf clipping techniques, which resulted in bleached, hypersensitive like reactions preceding a general yellowing or browning of the tissue Schaad et al. (1996). Additionally, in pathogenicity tests conducted with different isolates on six varieties, both clip and pin-prick methods of leaf inoculation were deemed non-significant Najeeya et al. (2007). The emergence of bacterial blight in rice was first documented by farmers in the Fukuoka region of Japan in 1884. Srinivasan et al. (1959) reported BB caused by *Xoo* in the Maharashtra state of India. Typical vascular disease systemic in nature, with infections occurring in the nursery during seedling stage transplanting and later during the booting or heading stage. The 'Kresek' phase, observed during the seedling stage, is particularly destructive (Chahal, 2005). The bacterial blight of rice manifests annually in endemic to epidemic forms in the southern regions of Gujarat (Anonymous, 2018). Though resistant varieties are available for the bacterial blight disease the occurrence of races and resistance to these races is not understood completely. The research work had been aimed for the isolation purification and identification of *Xanthomonas oryzae* pv. *oryzae* which may help in understanding disease development mechanisms.

2. Materials and Methods

The research was undertaken during the *kharif* (July-November, 2020), at the Department of Plant Pathology, Navsari Agricultural University, Navsari, Gujarat, India. The study focused on the isolation, purification and identification of *Xoo*.

2.1. Collection of samples

Diseased leaves exhibiting typical symptoms of bacterial blight were gathered from rice cultivars GR-11 and Taichung Native-1 (TN-1). These samples were collected in brown paper bags from Main Rice Research Centre, Navsari Agricultural University, Navsari and from farmers' fields in Gujarat.

2.2. Symptomatology

The various samples of bacterial blight disease were carefully examined for symptoms and documented following confirmation of pathogen presence within the collected field samples. Typically, the disease initiates as leaf tip blight approximately 4–6 weeks after transplanting, characterized by straw-colored blight along the edges of the leaves. The blight typically begins at the tip and progresses downward on both sides of the leaf blade, leading to tissue necrosis and desiccation. In severe cases, the blight may extend to the leaf sheath and stems. The onset of the "Kresek" phase occurs if infection arises during germination or early plant growth stages, often resulting in complete plant death as the infection progresses. Infected plants may either rapidly perish or exhibit partial recovery, producing new tillers.

2.3. Isolation and purification of pathogen

The isolation of the pathogen was conducted using fresh infected leaves of rice plants obtained from Main Rice Research Centre, Navsari Agricultural University, Navsari. These samples exhibited characteristic leaf blightening, with bacterial oozing observed from the cut sections during microscopy. Diseased portions containing healthy tissues were excised into 1.5–2 cm pieces. These segments underwent surface sterilization for 30 seconds using a 0.25% sodium hypochlorite solution, followed by three rinses with sterilized distilled water under aseptic conditions to remove residual traces. The sterilized pieces were placed on microscopic slides and further sectioned with a sterilized blade. Drops of sterilized distilled water were added to the cut sections on the slide to create a bacterial suspension. This suspension was then streaked onto Wakimoto's potato semi-synthetic medium using a sterilized wire loop. Inoculated plates were subsequently incubated at room temperature (27±2°C) for 48 hours. Following bacterial colony growth, typical *Xoo* colonies were selected and transferred to nutrient agar slants. The obtained pure cultures were preserved and maintained on Wakimoto's potato semi-synthetic medium throughout the investigation.

2.4. Identification of pathogen

2.4.1. Cultural characters

To characterize the pathogen or bacterium isolated from diseased rice leaves, various morphological and cultural traits of the bacterium were examined. Standard bacteriological methods were utilized for all experiments. Characteristics such as colony color, brightness, growth consistency and type were carefully observed and recorded.

2.4.2. Morphological characters

The following tests were conducted Gram's staining, acid-fast staining, capsule staining and spore staining. The staining reactions and shapes of the bacteria were documented. Morphological characteristics, including bacterial color and spore formation were observed in 7 days old cultures and compared with descriptions provided in relevant literature.

2.4.3. Pathogenicity test

To confirm the pathogenicity of the isolated pathogen (following Koch's postulates) from infected rice leaves, a

pathogenicity test was conducted in a net-house environment. Large earthen pots with a diameter of 30 cm were thoroughly cleaned with tap water, disinfected using a 4% formaldehyde solution and then sun-dried for seven days on the laboratory terrace. These pots were filled with double-sterilized soil at a rate of 4 kg pot^{-1}. Seedlings were then raised in pots filled with sterilized soil and covered with plastic within the net house. Healthy GR-11 rice seeds were surface-sterilized with a 0.25% sodium hypochlorite solution for 30 seconds and rinsed three times with sterilized distilled water. These sterilized seeds were then sown into the prepared pots, with one seedling per pot and covered with plastic to prevent air borne infections. Each pot was labelled, watered gently and arranged within the net house, with each pot covered with polyethylene bags to prevent airborne infections from germination until the end of the experiment. All necessary agronomic practices were implemented to ensure the proper growth of the rice plants in the pots.

2.4.4. Inoculation

The bacterial blight of rice disease was artificially induced using the clip inoculation method. A standard inoculum consisting of an aqueous bacterial suspension of 10^9 cell ml^{-1} was employed for the pathogenicity test.

2.4.5. Preparation of standard inoculam

To achieve uniform bacterial growth, 0.1 ml of the isolated pathogen's bacterial suspension was spread onto previously solidified 20 ml nutrient agar plates using a sterilized spreader, placed in 90 mm Petri plates. Following a 72 hours incubation period, 10 ml of sterilized distilled water was added to each plate, scraped uniformly and the growth from one plate was transferred into 100 ml of sterilized distilled water, yielding a bacterial suspension with a concentration of approximately 10^9 cell ml^{-1}. This aqueous bacterial suspension (10^9 cell ml^{-1}) served as the standard inoculum for the clip inoculation method during the pathogenicity test. The pots were thoroughly watered in the morning until saturation and bacterial inoculation (10^9 cell ml^{-1}) was conducted in the evening using the standard clip inoculation technique Kauffman et al. (1973), following 50 days of sowing. In this technique, the upper leaf tip portion (1–3 cm) was cut with scissors dipped in bacterial suspension. Twenty leaves per pot (representing one hill per pot) were clip-inoculated, with each treatment replicated four times (i.e., four pots). Leaves clipped using scissors dipped in distilled, sterilized water served as the control. Each pot was covered with a plastic bag to prevent airborne infections and they were sprayed with distilled water to maintain high humidity levels. Observations on the development of bacterial blight symptoms were recorded from 10 days after inoculation until harvest.

3. Results and Discussion

3.1. Isolation and purification of pathogen

The presence of the pathogen was confirmed through a meticulous examination of hand sections of diseased tissue under a microscope. Samples exhibiting bacterial oozing during microscopy were identified as infected with bacterial blight from the leaves (Figure 1). These identified samples were subsequently air-dried at room temperature and preserved for further analysis. The methodology employed in this study aligns with previous research. Soosairaj et al. (2015) isolated *Xoo* from infected rice leaf samples displaying symptoms of bacterial blight. The isolated leaf samples, when plated, produced light yellow, mucoid, round and smooth bacterial colonies on nutrient agar medium after 48–72 hours of incubation at 28±2°C. Similarly, Shankar et al. (2016) isolated *Xoo* from infected leaves, exhibiting typical symptoms of bacterial blight. Isolation was achieved using the streak plate method on modified Wakimoto's medium, resulting in well-separated, yellow mucoid colonies of the bacterium on medium after 48 hours of incubation at 27±1°C. Furthermore, Bhutto et al. (2018) isolated *Xoo* from diseased samples using the direct plating method. The isolated cultures were purified and characterized with colonies appearing small, medium and large and exhibiting irregular, circular and filamentous shapes on nutrient agar media. After 24 hours of incubation at room temperature (27±2°C), yellow colonies were observed. The typical bright yellow colonies were then transferred onto nutrient agar slants. Following incubation of the slants at room temperature, isolation from diseased plants yielded pure cultures, which were maintained on Wakimoto's potato semi-synthetic medium throughout the investigation. The observations made in this study correspond with findings from prior research. Naqvi (2019) delineated the symptoms of bacterial blight, noting the emergence of small water-soaked lesions that transition to a yellowish-white color.

Figure 1: Close up view of bacterial blight of rice under field condition

3.2. Identification of pathogen

The cultural and morphological examinations were conducted in accordance with the specified procedures and the obtained results were compared to the standard description of *Xoo*.

3.2.1. Cultural characterization

Cultural characteristics of the isolated bacteria were

documented after incubation for 48 hours on nutrient agar at room temperature (27±2°C). The analysis revealed that colonies grown on the nutrient agar media exhibited traits consistent with *Xoo*, appearing as raised, creamy yellow, smooth, entire and buttery (Table 1 and Figure 2).

Table 1: Cultural characters of the bacterium on nutrient agar medium

Sl. No.	Characters	On nutrient agar medium
1.	Colony colour	Waxy creamy yellow raised (figure 2)
2.	Consistency	Butterceous
3.	Growth of colony	Circular
4.	Luster	Bright
5.	Surface of growth	Smooth
6.	Margin of colony	Entire

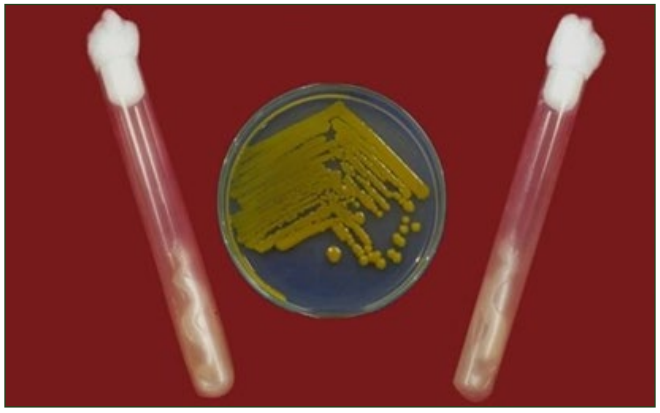

Figure 2: Pure culture of *Xanthomonas oryzae* pv. *oryzae*

3.2.2. *Morphological Characterization*

Additionally, the morphological assessment identified the bacterium as possessing a rod-shaped structure, gram-negative staining, encapsulation and non-spore formation (Table 2 and Figure 3).

Table 2: Morphological characters of the bacterium

S l . No.	Characters	Results
1.	Microscopic examination of infected tissue	Bacterial ooze seen from cut end
2.	Shape of bacteria	Rod shape
3. The staining		
	Gram's staining	-ve
	Acid fast staining	Non-acid fast
	Capsule staining	Capsulated
	Spore staining	Non-spore former

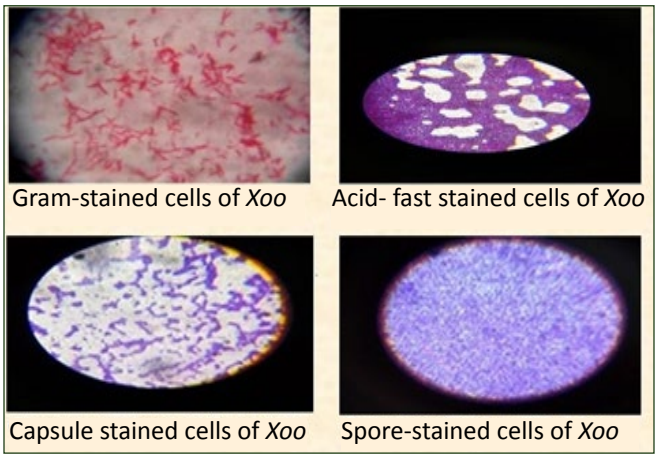

Figure 3: Different staining techniques of *Xanthomonas oryzae* pv. *oryzae*

3.3. *Pathogenicity test and symptom development*

The pathogenicity test of the isolated pathogen,was conducted on the susceptible cv. GR-11. Rice plants were cultivated in pots under controlled conditions within a net house. The Navsari isolate of *Xoo* was cultured on nutrient agar medium for 72 hours, after which a bacterial suspension with a concentration of 10^9 cell ml^{-1} was prepared in distilled sterilized water. Following 50 days of sowing, 10 leaves pot^{-1} were inoculated using the clip inoculation method. Artificial inoculation for the bacterial blight of rice disease was achieved using the prepared aqueous bacterial suspension (10^9 cell ml^{-1}) as the standard inoculum for the pathogenicity test. The results of the clip inoculation, as presented in Table 3 and Figure 4, demonstrated that the inoculated *Xoo* pathogen effectively infected the leaves, inducing blight symptoms within six days post-inoculation. The symptoms of tip blight progressed along both sides of the leaf blade, characterized by wavy margins between the blighted yellow area and the healthy tissue. Within 20 days, the yellowing and blighting of the leaf blade extended to cover approximately two-thirds of the leaf area. In contrast, control leaves inoculated with distilled sterilized water (without *Xoo* bacteria) remained healthy throughout the duration of the experiment. These findings are consistent with previous research. Jabeen et al. (2012) conducted pathogenicity tests to ascertain the pathogenic nature of *Xoo* isolates, observing hypersensitivity reactions in tobacco (*Nicotiana rustica* L.) plants using injection infiltration techniques.

Table 3: Pathogenicity test of *Xoo* in net house

Sl. No.	Pathogen	No. of leaves clip	
		Inoculated	Infected
1.	*Xanthomonas oryzae* pv. *oryzae*	10	10
2.	Control (without bacterial suspension)	10	0

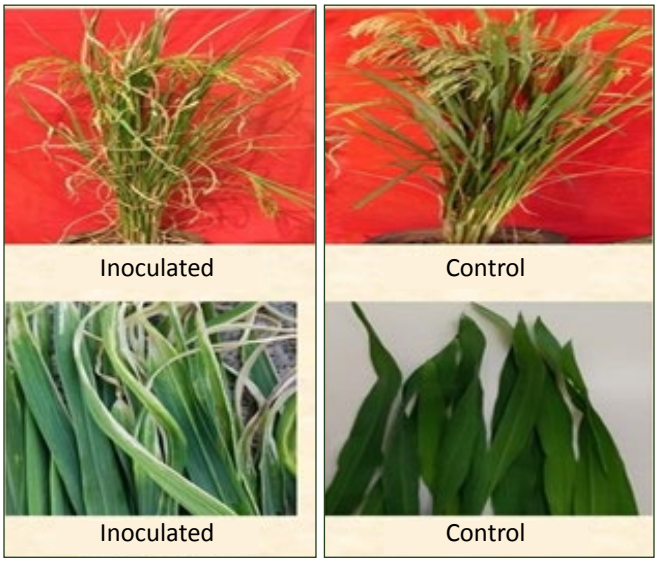

Figure 4: Pathogenicity test of *Xanthomonas oryzae* pv. *oryzae*

4. Conclusion

The investigation assessed Isolation, purification and identification of *Xanthomonas oryzae* pv. *oryzae* revealed that colonies grown on nutrient agar media exhibited traits consistent with *Xoo*, appearing raised, creamy yellow, smooth, entire and buttery as cultural characteristics and the bacterium as possessing a rod-shaped structure, gram-negative staining, encapsulation and non-spore formation as morphological characteristics. Furthermore, a pathogenicity test was conducted on the susceptible rice cultivar GR-11, cultivated in pots under controlled conditions within a net house was successfully satisfying Koch's postulates.

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