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Prevalence and virulence of a noble strain of *Xanthomonas oryzae* pv. *oryzae* BLB 1 causing Bacterial Leaf Blight of Rice in North Bengal

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Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae*, remains a major constraint to rice production in tropical rice-growing regions. The present study was undertaken to investigate the incidence of BLB in major rice-growing areas of North Bengal and to characterize the associated pathogen based on morphological, biochemical, pathogenic and environmental response attributes. Systematic field surveys were conducted in four districts of North Bengal to check the incidence of BLB in these regions which indicated a varying level of infestation. Infected samples were also collected from all the location for isolation and characterization. All isolates produced yellow, circular colonies and exhibited Gram-negative reactions along with positive KOH solubility, confirming their bacterial nature. Biochemical profiling revealed considerable similarity among isolates, although distinct variations in certain metabolic reactions led to their segregation into discrete clusters. Pathogenicity evaluation on the susceptible rice cultivar BPT 5204 identified a single isolate as highly virulent, producing characteristic BLB symptoms and extensive lesion development. The influence of temperature and pH on the growth of the virulent isolate revealed optimal growth at 30 °C and pH 6–7, while extreme conditions significantly inhibited bacterial proliferation. This paper reports the prevalence of a noble strain of *Xanthomonas oryzae* pv. *oryzae* in North Bengal that has a potential of causing huge crop loss in this area. These findings contribute to a better understanding of the pathogen characterization and growth requirements which may aid in developing effective disease management strategies.

Keywords : Bacterial leaf blight, characterization, pH, rice, temperature

INTRODUCTION

Rice serves as a primary source of dietary energy for both rural and urban populations and constitutes the staple food for more than half of the global population (Khush, 2005). Despite its importance, rice productivity is constrained by several biotic stresses, among which bacterial leaf blight (BLB) is recognized as one of the most ancient and destructive diseases. The disease is especially prevalent in the tropical regions of Asia, where it can cause yield losses of up to 74% under favourable conditions.

BLB is caused by the gram-negative bacterium *Xanthomonas oryzae* pv. *oryzae* (Xoo), first reported by Ishiyama (1922).

The disease manifests differently across growth stages of the host plant, appearing as a vascular wilt during early growth and progressing to leaf blight at the flowering stage. The pathogen gains

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entry into the plant primarily through wounds or hydathodes and subsequently colonizes the xylem tissues, resulting in systemic infection (Wang *et al.* 2019). The bacterium produces a characteristic yellow, water-soluble pigment known as Xanthomonadin, along with extracellular polysaccharides (EPS), both of which play crucial roles in protecting the cells from desiccation and aiding their dispersal through rain splash (Mansfield *et al.* 2012).In the early phase of infection, lesions typically initiate at the leaf tips. As the disease advances, these lesions turn yellow and develop into elongated, irregular streaks with wavy margins. Eventually, the infection spreads across the entire leaf blade, leading to whitening followed by greyish saprophytic growth. Since West Bengal being the major producer of rice, majority of farmers in North Bengal cultivates rice during Kharif and Rabi season. However, the level of infestation of Bacterial leaf blight during Kharif season and the characterization of the pathogen in this zone is less documented. Therefore, the present study was carried out to investigate the incidence of BLB in major rice-growing areas of North Bengal, to characterize the pathogen and to evaluate the influence of pH and temperature on its growth behaviour.

MATERIALS AND METHODS

Survey and collection of diseased samples from rice fields

A field survey was conducted during 2024 across the major rice-growing regions districts of North Bengal viz., Cooch Behar, Uttar Dinajpur, Dakshin Dinajpur and Malda to evaluate the incidence of bacterial leaf blight (BLB) disease (Fig. 1). From each sampling location 20 random plants were selected for estimation of disease incidence. The infected leaf samples were also collected in sterile polyethylene bags with proper labelling and brought to the laboratory where they were stored at 4 °C until further use.

Isolation, purification and maintenance of bacteria isolates

Bacterial isolation was carried out directly from infected leaves showing typical bacterial leaf blight symptoms collected from each sampling site. Prior to isolation, a preliminary bacterial ooze test was performed by placing a small leaf segment containing both healthy and diseased tissue on a glass slide and observing it under a microscope. Following confirmation of bacterial exudation, the infected leaves were cut into uniform size (approximately 10 mm × 5 mm), surface-sterilized in 70% ethanol for one minute and subsequently rinsed three times with sterile distilled water (Srinivas *et al.* 2024; Arshad *et al.* 2015). The sterilized leaf tissues were then transferred to a clean slide with a drop of distilled water and kept it for one minute to facilitate the release of bacterial ooze. Using a sterile inoculation loop, the emerging bacterial oozes was streaked onto previously prepared autoclaved Wakimoto medium: Peeled Potato 300g, Sucrose 20g, Peptone 5g, Sodium dihydrogen phosphate 1.87g, Calcium Nitrate 0.5g, Agar 17g, Distilled Water 1 L, pH 6.8 (Wakimoto, 1955). The and incubated at 28 ± 1 °C for 48 hours. Isolates were purified through dilution streaking method and subsequently stored on nutrient agar slants at 4°C for short-term storage and in 20% glycerol at -80°C for long-term preservation.

Morphological and biochemical characterization

Morphological characterization of the bacterial isolates was carried out by examining colony morphology, including colour, shape, size, margin characteristics, and elevation. Biochemical profiling was performed using the Hi Media KB002 identification kit (Chowdappa *et al.* 2018). The assays included citrate utilization, starch hydrolysis, lysine utilization, urease activity, phenylalanine deamination, nitrate reduction, H_2S production, and carbohydrate utilization. For each test, 50 µL of a 24-hour broth culture of the respective isolate was inoculated into the designated wells of the kit and incubated at 28 ± 1 °C for 24 hours, after which colour changes in the reaction substrates were recorded. In addition, Gram staining and the KOH test were also performed as outlined by Jonit *et al.* (2016).

Pathogenicity test and virulence

A total of twentyone bacterial leaf blight (BLB) isolates were obtained and pathogenicity tests

were conducted on the susceptible rice cultivar Shampa Masuri (BPT 5204). Each bacterial isolate were adjusted to a concentration of 10^7CFU mL^{-1} and inoculated onto three leaves per seedling using the clip-inoculation technique as described by Kauffman *et al.* (1973) thirty-day-old rice plants grown in pots under controlled environmental conditions. Development of symptoms and disease development were recorded upto 20 days after inoculation. Lesion length and the percentage progression of the diseased leaf area were measured to assess the virulence of the isolates.

Molecular characterization of the most virulent BLB isolate

Genomic DNA from the most virulent isolate was extracted the DNeasy Ultra Clean Microbial Kit, following the protocol mentioned by Qiagen. The isolated DNA were then provided to Barcode Biosciences Pvt. Ltd for 16SrRNA sequencing and analysis. The 16Sr RNA gene sequences were deposited to NCBI GenBank database. The bioinformatics analysis was performed using NCBI BLAST (http://ncbi.nlm.nih.gov/blast) for identifying the isolate using NCBI reference sequence genomic database with 100% coverage of the query sequence. Reference sequences available in GenBank were retrieved, and a phylogenetic tree was generated using the neighbor-joining method implemented in MEGA 12 software to examine the evolutionary relationships among the strains.

Effect of pH and temperature on growth of BLB isolate

Effect of pH on growth of bacterial isolate was tested in nutrient agar medium adjusted to varying pH of 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 using 0.1 N sodium hydroxide or hydrochloric acid as required. To this 1 ml of bacterial suspension from a 10^5 dilution was inoculated onto the surface of the media and spread uniformly using a sterile spreader and incubated at 28°C ± 1 °C for 72 hours. Following incubation, colony development was assessed, and the colonies were counted and recorded for analysis. Similarly, the influence of temperature on the growth of the bacterial leaf blight (BLB) pathogen was assessed by incubating the inoculated nutrient agar plates at varying temperature viz., 20°C, 25, 30, 35, and 40°C, for a period of 72 hours. The number of colonies were recorded and analysed.

Statistical Analysis

All the data obtained was analysed using R studio (Version 4.4.2). The figures were generated using QGIS software (Version 3.40.12) and Phylogenetic analysis was conducted using MEGA X software (Version 12.0.11)

RESULTS AND DISCUSSION

Survey and collection of diseased samples from rice fields

A total of twenty-one locations across four districts of North Bengal were surveyed, revealing considerable variation in the incidence of bacterial leaf blight among the sampled sites (Table 1). During the survey it was found that infected rice leaves predominantly exhibited white to straw-coloured longitudinal stripes on one or both sides of the leaves. These lesions typically extended along the midrib and were marked by irregular, wavy margins (Fig. 1). Among the surveyed locations, the highest incidence of bacterial leaf blight was recorded at Nazirpur (51.11%) followed by Buniadpur (42.22%) in the Dakshin Dinajpur district and Rahutgaon (40.00%) in Malda district. The lowest disease incidence was recorded in Paikpara (11.11%) in the Dakshin Dinajpur district.

Isolation of bacterial isolates

A total of twenty-one different bacterial isolates was isolated on a Wakimoto medium and each isolate was coded as described in Table 1.

Morphological and biochemical characterization of isolated bacteria

Morphological characterization of all bacterial leaf blight (BLB) isolates was performed based on visual assessment of colony characteristics. The colonies exhibited different colour variations ranging from light yellow to deep yellow. These differences represent variation in the intensity of yellow pigmentation rather than a change in colony

Table 1: Geographical coordinates of sampling locations and sampling details

Sl No.	District	Location	Latitude/Longitude	Variety	Plant part	Disease Incidence (%)	Isolate Name
1	Dakshin Dinajpur	Nazirpur	25.31805/88.800568	Shampa Masuri	Leaf	51.11	BLB 1
2	Dakshin Dinajpur	Kumarganj	25.368962/88.753302	Shampa Masuri	Leaf	28.89	BLB 2
3	Dakshin Dinajpur	Paikpara	25.322297/88.811398	Swarna	Leaf	11.11	BLB 3
4	Dakshin Dinajpur	Majhian	25.311627/88.7672	Shampa Masuri	Leaf	20.00	BLB 4
5	Dakshin Dinajpur	Kasilabati	25.334298/88.709491	Shampa Masuri	Leaf	33.33	BLB 5
6	Dakshin Dinajpur	Chak Bhabani	25.38059/88.758912	Shampa Masuri	Leaf	24.44	BLB 6
7	Dakshin Dinajpur	Bolla	25.347615/88.706564	Shampa Masuri	Leaf	28.89	BLB 7
8	Dakshin Dinajpur	Buniadpur	25.384894/88.408556	Shampa Masuri	Leaf	42.22	BLB 8
9	Dakshin Dinajpur	Binsira	25.249495/88.898633	Swarna	Leaf	22.22	BLB 9
10	Malda	Deotala	25.28272/88.293663	Swarna	Leaf	33.33	BLB 10
11	Malda	Mahil	25.241593/88.234708	Swarna	Leaf	35.56	BLB 11
12	Malda	Mandilpur	25.067211/88.154409	Swarna	Leaf	31.11	BLB 12
13	Malda	Rahutgaon	25.072408/88.123964	Swarna	Leaf	40.00	BLB 13
14	Malda	Lakshmighat	25.056706/88.035816	Swarna	Leaf	24.44	BLB 14
15	Malda	Pichli	25.05636/88.053648	Swarna	Leaf	26.67	BLB 15
16	Uttar Dinajpur	Chhatrapur	25.631943/88.149797	Swarna	Leaf	28.89	BLB 16
17	Uttar Dinajpur	Basudebpur	25.610024/88.101924	Swarna	Leaf	26.67	BLB 17
18	Uttar Dinajpur	Sobhanpur	25.722623/88.182424	Swarna	Leaf	35.56	BLB 18
19	Coochbehar	Angrakata	26.39975/89.391307	Swarna	Leaf	26.67	BLB 19
20	Coochbehar	Kalarayerkuthi	26.434238/89.373221	Swarna	Leaf	20.00	BLB 20
21	Coochbehar	PurbaNilangibari	26.311319/89.312099	Swarna	Leaf	37.78	BLB 21

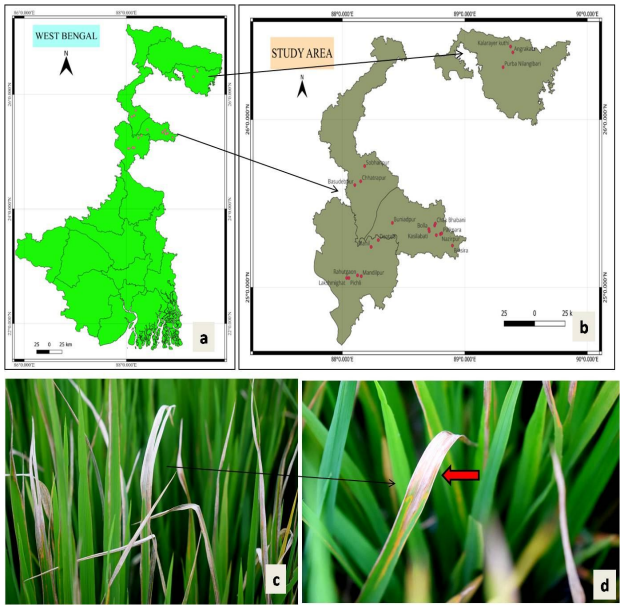

Fig. 1 (a-b) : Various locations of field survey and sampling areas for isolation of pathogen within West Bengal. **(c-d)-** Disease symptoms of Bacterial Leaf Blight (BLB) observed in rice cultivars in field condition

colour, and all isolates remained within the characteristic yellow phenotype of *Xanthomonas oryzae* pv. *oryzae*. All the isolates were small, circular with smooth margins and flat or slightly raised elevations (Fig. 2, Table 2), similar observations of bacterial isolates as yellow, smooth and mucoid colonies after 48 h of incubation were reported by Srinivas *et al.* (2024). They further reported raised, creamy-yellow colonies with smooth margins on Wakimoto medium. Comparable colony morphology was also documented by Soosairaj *et al.* (2015), who isolated Xoo from bacterial leaf blight–infected rice leaves and recorded light-yellow, round, and smooth colonies on nutrient agar after 48–72 h at 28 ± 2 °C. Shankar *et al.* (2016) obtained well-separated yellow mucoid colonies of *Xoo* on modified Wakimoto's medium within 48 h of incubation at 27 ± 1 °C. Likewise, Bhutto *et al.* (2018) reported the formation of yellow colonies of varying sizes and shapes on nutrient agar

Table 2 : Morphological Characterization of Bacterial Leaf Blight isolates of Rice

Isolate	Colony colour	Shape	Size	Margin	Elevation
BLB 1	Light yellow	Circular	Small	Smooth	Raised
BLB 2	Light yellow	Circular	Small	Smooth	Flattened
BLB 3	Light yellow	Circular	Small	Smooth	Flattened
BLB 4	Light yellow	Circular	Small	Smooth	Flattened
BLB 5	Light yellow	Circular	Small	Smooth	Flattened
BLB 6	Light yellow	Circular	Small	Smooth	Raised
BLB 7	Deep Yellow	Circular	Small	Smooth	Raised
BLB 8	Light yellow	Circular	Small	Smooth	Flattened
BLB 9	Light yellow	Circular	Small	Smooth	Flattened
BLB 10	Light yellow	Circular	Small	Smooth	Flattened
BLB 11	Light yellow	Circular	Small	Smooth	Flattened
BLB 12	Light yellow	Circular	Small	Smooth	Flattened
BLB 13	Light yellow	Circular	Small	Smooth	Flattened
BLB 14	Light yellow	Circular	Small	Smooth	Raised
BLB 15	Light yellow	Circular	Small	Smooth	Flattened
BLB 16	Deep Yellow	Circular	Small	Smooth	Raised
BLB 17	Deep Yellow	Circular	Small	Smooth	Raised
BLB 18	Light yellow	Circular	Small	Smooth	Raised
BLB 19	Light yellow	Circular	Small	Smooth	Raised
BLB 20	Light yellow	Circular	Small	Smooth	Raised
BLB 21	Light yellow	Circular	Small	Smooth	Raised

Growth recorded after 48h of growth in Wakimoto medium.

Table 3. Effect of pH and temperature on the growth of *Xanthomonas oryzae* pv. *oryzae*

pH	Mean Colony count	Temperature (° C)	Mean Colony Count
4	0.00 (1.00^{d})*	20	112.25 (10.64^{b})*
5	112.25 (10.60^{b})	25	307.25 (17.55^{a})
6	335.50 (18.34^{a})	30	332.75(18.27^{a})
7	299.75 (17.34^{a})	35	120.75 (11.01^{b})
8	31.50 (5.67^{c})	40	0.00 (1.00^{c})
9	0.00 (1.00^{d})	Sem ±	0.26
Sem ±	0.30	$CD_{0.01}$	1.08
$CD_{0.01}$	1.20	CV (%)	4.44
CV (%)	6.57		

*Figures in the parenthesis are √ (x+1) transformed values.
Values with common letters do not differ significantly as per Duncan's Multiple Range Test (DMRT) at 1% level.

following direct plating of infected samples, further supporting the cultural variability of *Xoo* under different growth conditions. Collectively, these similarities in colony morphology and growth behaviour across studies corroborate the identity of the pathogen isolated in the present investigation. All the isolates exhibited a Gram-negative reaction which was further confirmed by the formation of characteristic slime threads in the potassium hydroxide (KOH) solubility test. The colour change observed in the substrate wells of the biochemical test kit is presented in Fig. 2. The

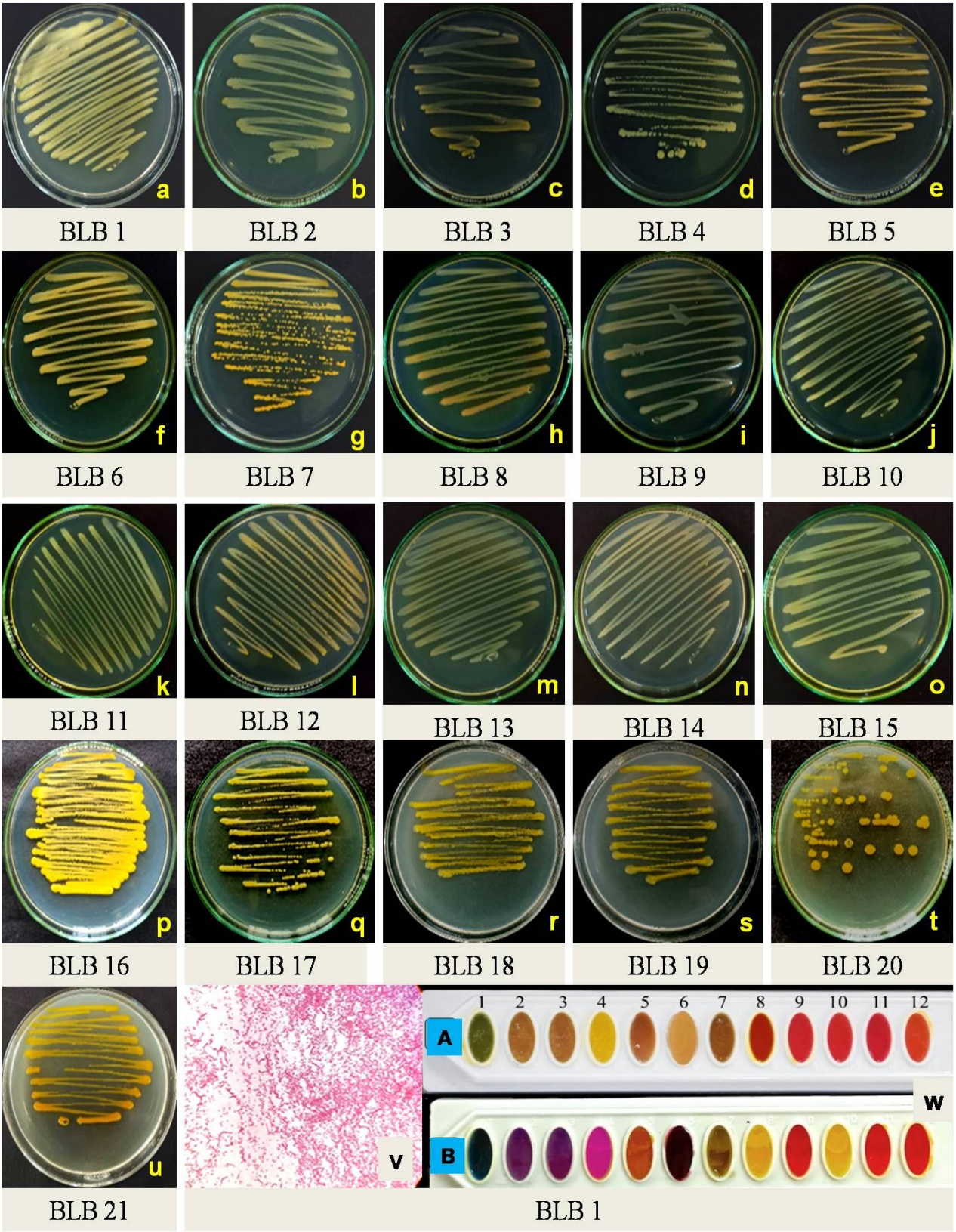

Fig. 2 (a-u) : Growth patterns of 21 BLB isolates on Nutrient Agar Medium after 24 h of incubation. Gram reaction (v) and biochemical tests (w) of one of the most virulent isolate BLB 1. Colour codes for biochemical test: (A-Control B- Bacterial isolate BLB 1) [1. Citrate utilization, 2. Lysine utilization, 3. Ornithine utilization, 4. Urease Test, 5. Phenylalanine Deamination Test, 6. Nitrate Reduction Test, 7. H_2S production, 8. Glucose fermentation Test, 9. Adonitol fermentation Test, 10. Lactose fermentation Test, 11. Arabinose fermentation Test,12. Sorbitol fermentation Test]

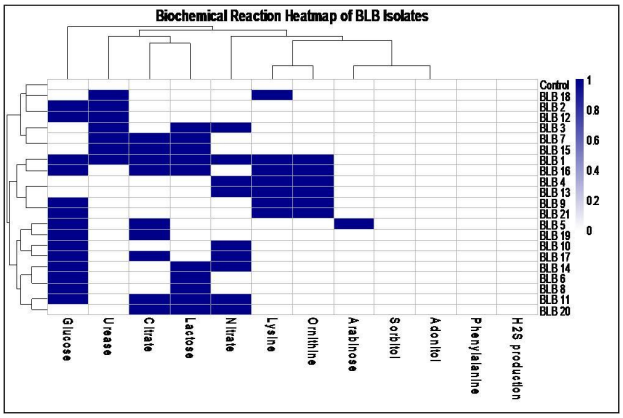

Fig. 3: Biochemical Reaction Heatmap of BLB isolates evaluated using hierarchical cluster analysis based on Biochemical Reactions of all the isolates. (Blue and white indicate positive and negative reactions, respectively)

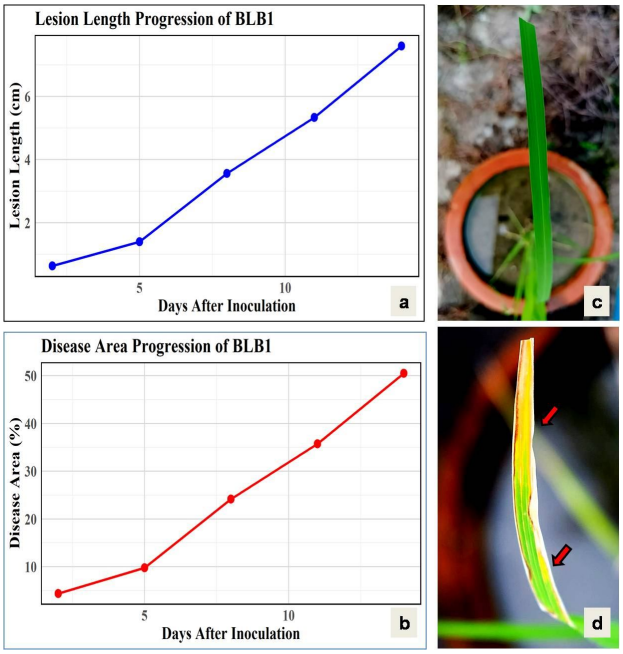

Fig. 4: Assessment of pathogenicity of isolate BLB 1 conducted on the susceptible rice cultivar Shampa Masuri (BPT 5204). Lesion Length Progression **(a)**; and diseased Area (%) progression **(b)**. Control leaf with no any symptoms **(c)**; leaf inoculated with BLB 1 showing bacterial leaf blight symptoms after 15 days of inoculation **(d)** in pot culture bioassays

biochemical reaction patterns of the bacterial leaf blight (BLB) isolates were further evaluated using hierarchical cluster analysis, as illustrated in Fig. 3. In the heatmap, blue and white indicate positive and negative reactions, respectively. The majority of isolates exhibited positive responses to carbohydrate utilization tests, including glucose, citrate, and lactose, along with nitrate reduction and lysine utilization, suggesting the presence of conserved metabolic characteristics. Nevertheless, differences in certain biochemical reactions led to the segregation of isolates into

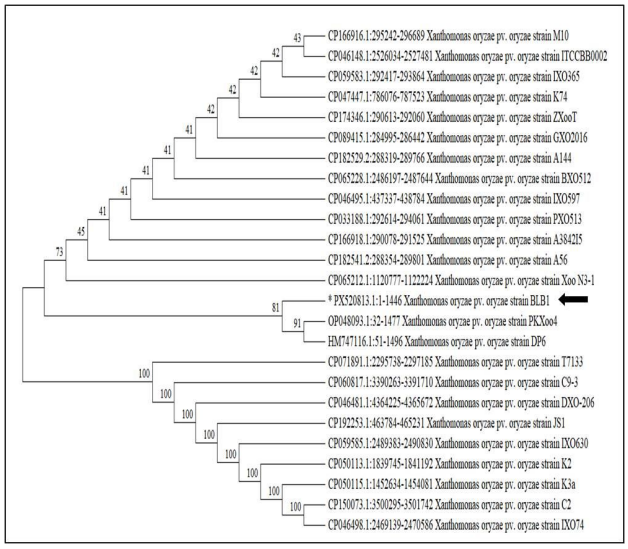

Fig. 5 : Phylogenetic tree of BLB 1 identified as *Xanthomonas oryzae* pv. *oryzae* with ex-type strains obtained fron NCBI GenBank database. Generated using the neighbor-joining method of MEGA 12 software

distinct clusters. This clustering pattern reflects both phenotypic similarity and biochemical variability among the BLB isolates. Similar observations were reported by Debnath *et al.* (2023), who recorded a Gram-negative reaction for all *Xanthomonas oryzae* pv. *oryzae* isolates along with positive reactions in citrate, ornithine, glucose, and lactose utilization tests using the KB002 biochemical kit, which is consistent with the biochemical characteristics observed in the present study. In agreement with the present study, Jonit *et al.* (2016) also reported Gram-negative staining and a positive KOH reaction in Xoo isolates, further supporting the biochemical characterization obtained in this investigation.

Pathogenicity test

Pathogenicity assessment revealed that among the twenty-one isolates evaluated, only one isolate, designated BLB 1, exhibited virulence to induce bacterial leaf blight symptoms in rice. Visible disease symptoms started at two days after inoculation, whereas bacterial ooze formation was observed seven days post-inoculation. Lesions initially developed at the site of clipping and progressively extended downward along one or both leaf margins in a characteristic wavy pattern, ultimately leading to necrosis of the affected tissues. By 14 days after inoculation, lesions had expanded to cover approximately

50.5% of the mean diseased leaf area, with an average lesion length of 7.6 cm on the inoculated leaves (Fig. 4). In contrast, the control plants remained symptomless throughout the observation period. The pathogen was subsequently re-isolated from the infected tissues and the recovered colonies displayed morphological characteristics identical to those of the original isolate thereby confirming its pathogenic nature.

Notably, the virulent isolate BLB 1 was collected from Nazirpur, a location that recorded the highest disease incidence during the field survey. This observation suggests that local disease pressure and favourable agro-climatic conditions in certain locations may contribute to the persistence or emergence of virulent pathogen populations. Although the remaining isolates did not induce symptoms under the test conditions, the geographic association of BLB 1 highlights the possible influence of location-specific factors on pathogenic behaviour of *Xanthomonas oryzae* pv. *oryzae*. Similar observations were also reported by Triplett *et al.* (2011) while evaluated the pathogenicity of U.S. *Xanthomonas oryzae* strains and recorded lesion lengths ranging from 2 to 10 cm over a 16-day observation period. Similarly, Yasmin *et al.* (2016) reported that Xoo strains induced typical bacterial leaf blight symptoms, including initial yellowing from the leaf tip followed by downward progression and wilting, with the most virulent strain causing up to 39.7% diseased leaf area.

Molecular characterization of the most virulent BLB isolate

Analysis of the 16S rRNA gene sequence confirmed that the isolate BLB 1 belonged to *Xanthomonas oryzae* pv. *oryzae*. The corresponding nucleotide sequence was deposited in the NCBI GenBank database under the accession number PX520813.1. Reference sequences of *Xanthomonas oryzae* pv. *oryzae* available in GenBank were retrieved, and a phylogenetic tree was generated using the neighbor-joining method implemented in MEGA 12 software to examine the evolutionary relationships among the strains (Fig. 5). The isolated bacterium (PX520813.1) exhibited 99.59% sequence similarity with the reference strains OP048093.1 and HM747116.1.The nearly full-length 16S rRNA gene sequence of the isolate BLB 1 (PX520813.1) clustered within the *Xanthomonas oryzae* pv. *oryzae* clade. BLB 1 formed a distinct sub-clade with strains PKXoo4 (OP048093.1) and DP6 (HM747116.1), supported by high bootstrap values (81–91%), indicating a close phylogenetic relationship. The analysis confirmed the taxonomic identity of BLB 1 as *Xanthomonas oryzae* pv. *oryzae*.

Effect of pH and temperature on growth of BLB isolate

The effect of different level of pH and temperature were carried out and is presented in Table 3 and fig. 6. The results indicated that the pathogen attained its highest colony population at pH 6, recording a mean count of 335.50 × 10^5 CFU mL, which was statistically at parwith the observation at pH 7 (299.75 × 10^5 CFU/ mL (Fig.11). In contrastno bacterial growth was found at pH 4 or pH 9. Similarly, maximum bacterial growth was observed at an incubation temperature of 30 °C, with a mean colony count of 332.75 × 10^5 CFU/ mL. This was found to be statistically at par with growth recorded at 25 °C (307.25 × 10^5 CFU /mL (Fig.12). However, the pathogen failed to grow at 40 °C.

The temperature and pH responses observed in our present study are similar with the observation of Suresh *et al.* (2014) who reported the highest population of *Xanthomonas oryzae* pv. *oryzae* at 30°C, with a marked decline in colony numbers at 40°C and optimum growth at neutral pH (pH 7). Similarly, Thimmegowda (2006) demonstrated that Xoo isolates exhibited maximum growth at temperatures ranging from 30°C to 35°C, while lower (10°C) and higher (40°C) temperatures supported poor growth. They also reported enhanced bacterial growth at pH 7.0–7.5 after 48 h of incubation.

CONCLUSION

In conclusion out of twenty-one surveyed Nazirpur (51.11 %) recorded highest and Paikpara (11.11%) recorded lowest disease incidence in the Dakshin

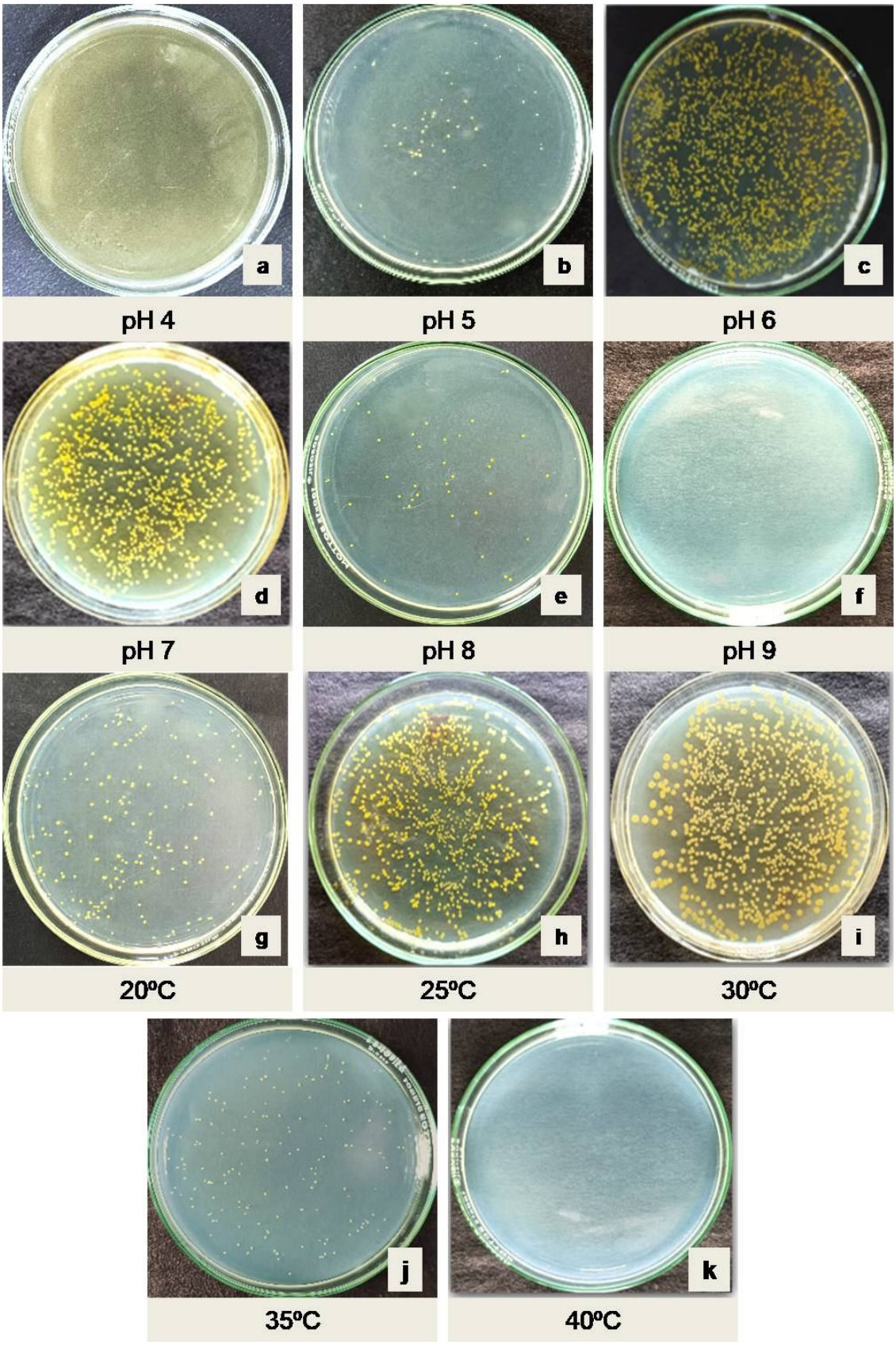

Fig. 6. Growth of isolate BLB 1 under two different conditions; Varying pH of 4-9 (**a-f**) and varying temperature of 20^0 C -40^0 C (**g-k**)

Dinajpur district. All the isolates were small, circular with smooth margins, flat or slightly raised elevations, Gram-negative and positive to potassium hydroxide (KOH) solubility test. Almost all the isolates exhibited positive responses to carbohydrate utilization tests, including glucose, citrate, lactose, nitrate reduction and lysine utilization. Pathogenicity test indicated 50.5% of the mean diseased leaf area, with an average lesion length of 7.6 cm on the inoculated leaves by 14 days of inoculation as compared to control where no symptoms was found. 16S rRNA gene sequence confirmed that the most virulent isolate (BLB 1) as *Xanthomonas oryzae* pv. *oryzae*. Also the pH 7, 30 °C temperature was found to be most congenial for the growth of the pathogen. Overall, the results of this study will enhance the understanding of the distribution, pathogenic potential, and growth characteristics of *Xanthomonas oryzae* pv. *oryzae* in North Bengal. The information generated can serve as a valuable baseline for developing targeted disease forecasting models, resistance breeding programmes, and integrated management strategies aimed at mitigating bacterial leaf blight in rice under similar agro-climatic conditions.

DECLARATION

Conflict of Interest. Authors declare no conflict of interest.

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