



## Identification and Functional Characterization of *Xanthomonas oryzae* Pv. *oryzae* Isolates

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### Authors' contributions

This work was carried out in collaboration among all authors. Authors DK and BDP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. All authors managed the analyses of the study and literature searches, read and approved the final manuscript.

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### ABSTRACT

Rice is a model crop for studying host - pathogen interaction with one of the most devastating pathogens viz. *Xanthomonas oryzae* pv. *oryzae* (Xoo). In the present investigation, an attempt was made to isolate a virulent strain of *Xanthomonas oryzae* from infected rice leaves and production of antioxidant enzymes, which are widely used in studying host - pathogen interactions. Among five isolates of *X. oryzae* pv. *oryzae*, SboBLB3 showed greater virulence as it showed susceptibility symptoms in infected rice leaves. The NCBI accession number of SboBLB3 was MH986180, which was obtained by sequencing 16s rDNA. The increased activity of antioxidant enzymes after SboBLB3 further confirms its virulence. Induction of antioxidant enzymes showed that SboBLB3 is a virulent strain of *X. oryzae* and can be used in host - pathogen interaction at molecular level.

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## 1. INTRODUCTION

Rice is the world's most important staple food of more than half of the world population. The tremendous increase in human population imparts pressures on farming community for increasing rice production in India. The diminishing area under cultivation and various environment stresses makes the worst situation. Continuous breeding for specific trait predominated by few varieties led to narrow genetic base making the rice ecosystem highly vulnerable to different environmental stress.

Rice is the model system for monocotyledons due to its relatively small and completely sequenced genome (International Rice Genome Sequencing Project, 2005; 430 Mb), the availability of high-density genetic maps, genome-wide expression data [1], and genetic transformation methods [2,3]. Several biotic and abiotic stresses affect rice growth, production and productivity. Around 70 diseases of rice are known worldwide, including fungal blast, sheath blight and bacterial leaf blight disease [4]. The average yield loss due to diseases ranges from 12-15% in rice [5].

Among several biotic stresses, infection by *Xoo* which causes rice leaf blight (BLB), resulting 20-50% and as high upto 50% to 80% and even 100% losses under very severe conditions [6,7]. BLB also affect grain quality by interfering with maturation in rice [6]. Chemicals including antibiotics are extensively used in controlling this disease. However, extensive use of these chemical causes harmful impacts on human and environment. Developing new resistant genotypes against virulent strain of BLB might be an alternative approach to minimize the yield losses. A plethora of report indicates the use of well characterized *Xoo* for screening breeding materials against BLB resistance [4]. However, very limited study has been performed to isolate and characterize locally infective virulent strain of BLB to use in rice improvement program. In present investigation was focused to identify native strain of BLB and characterize it to use in breeding and genetic engineering program. The activities of defence enzyme viz. peroxidase and SOD were also analyzed in rice at different time point after forced inoculation of BLB in rice plants.

## 2. MATERIALS AND METHODS

### 2.1 Isolation of Rice BLB Pathogen

Rice BLB pathogen, *Xoo*, was isolated from rice plant showing typical BLB symptom established at different fields of Bihar Agricultural College, Sabour. The isolation of BLB pathogen was carried out as described previously [8,9] and cultures were maintained on nutrient agar medium at 4°C in a refrigerator.

### 2.2 DNA Isolation and PCR Amplification

BLB culture was grown in 5 ml Luria Berteni (LB) broth for overnight at 28°C and DNA isolation was done by following the protocols of Carpenter et al. [10]. For identification of BLB, extracted bacterial genomic DNA was amplified using 16s universal primers (16sF: 5'AGAGTTTGATCCTGGCTCAG3' & 16sR: 5'GGTTACCTTGTTCAGACTT3'). Reaction mixture used for PCR amplification include 50 ng/µl DNA, dNTP mixture (10 mM), 10X buffer, 10 µM (Forward), 10 µM (Reverse), Pfu DNA polymerase in a Thermalcycler (Applied Biosystems, USA) for cyclic amplification and the conditions for amplification was programmed as step 1 (94°C for 5 min), step 2 (94°C for 1 min), step 3 (51°C for 30 sec), step 4 (72°C for 1 min), step 5 (72°C for 5 min for final extension). The step 2 to 4 were repeated for 30 times.

The amplified PCR product was eluted from agarose gel followed by gel electrophoresis as per the instruction of manufacture protocol (Xcelris). The eluted band was purified using gel elution kit as per the instruction of manufacturer (Xcelris).

### 2.3 Cloning and Sequencing of Amplified Fragments

The purified DNA was cloned into pJET 1.2/blunt (linearized cloning vector) vector using cloning kit (Invitrogen) by following manufacturer instructions. The ligated mixture was transformed into competent cells made from *E. coli* strain DH5α. as described previously [11].

Plasmid was isolated from transformed bacteria using plasmid Purification Kits by following manufacture protocol (Qiagen, Hilden,

Germany). For the confirmation of insert restriction enzyme based double digestion was performed using restriction enzyme *XhoI* and *XbaI*. The recombinant clone was further confirmed using sequencing vector specific primers (pJET1.2 F: 5'-CGACTCACTATAGGGAGAGCGGC-3' and pJET1.2 R: 5'-AAGAACATCGATTTTCCATGGCAG-3').

## 2.4 Identification of Bacteria

The nucleotide sequences were used for BLASTN searches (<https://blast.ncbi.nlm.nih.gov/>). Further, alignment of the sequenced amplicon showed maximum identity to the known 16S gene sequence in NCBI database which was used for genus and species identification of bacterium.

## 2.5 Pathogenicity Test of the Isolates

In an *in-planta* study, *X. oryzae* pv. *oryzae* (Xoo) inoculation was done by the scissors clip-inoculation method [12]. Five BLB isolates were tested to understand the pathogenicity of the isolates. Under glasshouse condition, 40-day-old plants (Rajendra Kasturi, susceptible variety) were inoculated using bacterial suspension ( $1 \times 10^{8-9}$  cfu/ml) developed from 3-day-old colonies. The inoculated plants were covered with transparent polythene bag and incubated at 28°C with 12 h light cycle. For mock treatment, leaf tips clipped using a pair of scissors dipped only into 0.9% saline. After 14 days of inoculation, the lesion length was measured in centimeters (cm) from the tip which was cut before inoculation. The reaction of the isolates was recorded according to the scale suggested by Cottyn and Mew [13].

## 2.6 SOD Enzymatic Activity

Total SOD (EC 1.15.1.1) activity was measured spectrophotometrically based on inhibition in the photochemical reduction of nitrobluete-trazolium (NBT). Superoxide dismutase (SOD) activity set up was prepared as described by Dhindsa et al. [14]. The specific activity was measured in terms of unit/g of protein and by formula given by Zang et al. [15].

## 2.7 Peroxidase Enzymatic Activity

The enzymatic assessment of Peroxidase (EC 1.11.1.7) was measured through increase in

absorption due to the formation of Purpurogallin (oxidized donor) [16].

## 2.8 Catalase Enzymatic Activity

Hydrogen Peroxide oxidoreductase (EC 1.11.1.6) determined in homogenate by measuring decrease in absorption at 240 nm as describe by Luck 1974 [17] for 180 sec.

## 3. RESULTS

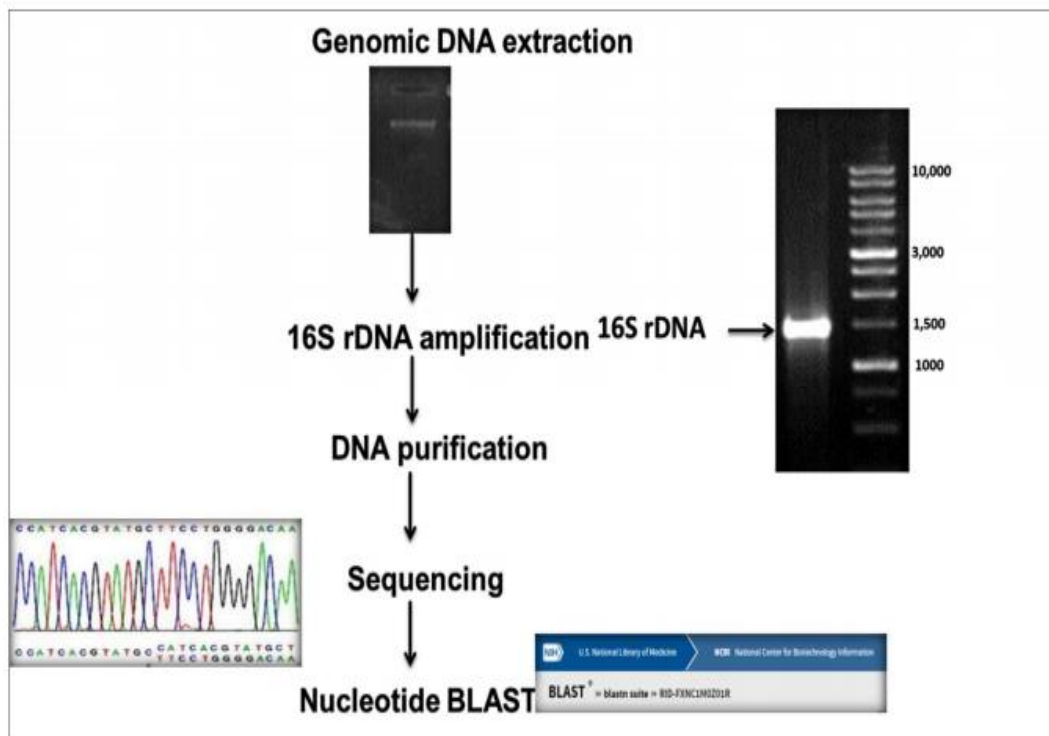
*Xanthomonas oryzae* pv. *oryzae* (Xoo) was isolated from blight infected rice field at Bihar Agricultural College, Sabour. Among five isolates of Xoo SboBLB3 showed greater virulence. Fully expanded leaves of four plants in each row were clip inoculated with individual isolates. Lesion length from the cut leaf tip was measured in centimetres (cm) after 14 days of inoculation. Resistance or susceptibility was assessed from the mean lesion length of the inoculated leaves of each differential line per treatment. Lesion length (cm) on at-least 10 inoculated leaves for each bacterial isolate and host line combination was recorded 14 days after inoculation and compared with mock treated sample. The disease reaction on the basis of lesion length (cm) measured as classified by Cottyn and Mew [13].

On the basis of reaction type SboBLB3 chosen for further study (Table 1). The 16S rRNA gene, a molecular marker for identification of bacterial species, was amplified from total genomic DNA using 16s universal primers and cloned in cloning vector (Fig. 1). The recombinant clone was sequenced using vector specific primers by out sourcing to Xcelris Genomics Services; Gujarat. The BLASTN (<https://blast.ncbi.nlm.nih.gov/Blast>) searches showed 82% sequence identity with Xoo (Fig. 2). Isolated strain having accession ID: BAU 1.

The SOD and Peroxidase activity studied in Xoo treated rice seedling at 24 h and 48 h dpi respectively. There was two times reduction in SOD activity compare to 24h mock to treatment and fifteen times reduction in response to 48h control (mock) to treatment (Fig. 3). Peroxidase activity reduced at 24h treatment compare to control and increased after 48h treatment compare to mock (Fig. 4). Catalase activity found to be increasing 48h after Xoo treatment compares to 48h mock (Fig. 5).

**Table 1. Disease reaction on the basis of lesion length (cm) from isolates of sabour field**

Isolates	Lesion length (cm)	Reaction
SboBLB1	11.4 cm	Moderately Susceptible
SboBLB2	12.3 cm	Moderately Susceptible
SboBLB3	16.5 cm	Susceptible
SboBLB4	13 cm	Moderately Susceptible
SboBLB5	13.4 cm	Moderately Susceptible



**Fig. 1. Identification of new strain of *X.oryzae* pv. *oryzae***

#### 4. DISCUSSION

The authors identified five strains of *Xoo* from the infected rice leaf. SboBLB3 was appeared to be highly virulent among the different strains of *Xoo* studied.

Defense enzymes like superoxide dismutase (SOD) are a major scavenger of superoxide ( $O_2^-$ ) and its enzymatic action results in the formation of  $H_2O_2$  and  $O_2$ . The reduction the specific activity of SOD confirms the onset of defence responses against *Xoo* infection. The rate of reduction of SOD is not very stiff which might be due to susceptible nature of rice variety used in this

study. Peroxidase is related to diverse responses to plant stress. We have noticed the decrease in the peroxidase activity at 24h treatment which may be due to dominance of pathogen. However, its activity increases after 48h of treatment which might be due to onset of plant defense responses. Previous reports showed the Induction of peroxidase activities which had been correlated with onset of defence responses in rice against *Xoo* [18,19]. Taken together our analysis showed the isolate SboBLB3 is a virulent strain of *Xoo* and able to induced disease resistance responses in rice which can be used in studying plant pathogen interaction studies at molecular level.

Xanthomonas oryzae pv. oryzae strain GZ0008 16S ribosomal RNA gene, partial sequence  
 Sequence ID: [MH158541.1](#) Length: 1423 Number of Matches: 1

Range 1: 812 to 1389 [GenBank](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
484 bits(262)	1e-135	480/585(82%)	16/585(2%)	Plus/Minus
Query 40	CTTCTTTT	GCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTA	99	
Sbjct 1389	CTTCTGGT	GCAACAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTA	1330	
Query 100	TTACCCGC	GGCA-TGCTGATCCACGATTACTAGCGATTCCGACTTCACGGAGGCGAGTTG	158	
Sbjct 1329	TTACCCGC	AGCAATGCTGATCTGCGATTACTAGCGATTCCGACTTCATGGAGTTCGAGTTG	1270	
Query 159	CAGACTGC	GATCCGAACTAAGA-AGGACTTTATGAGGTCGGCTTGCTCTCGCGAGGTCGC	217	
Sbjct 1269	CAGACTCCA	ATCCGGACTGAGATAGGG-TTCTGGGATTGGCTTGCCCTCGCGGGTTTGC	1211	
Query 218	TTCTCTTT	GTATGCT-CCATTGTAGCACGTGTGTAGCCCTAGTCGTAAGGGCCATGATGA	276	
Sbjct 1210	AGCCCTCT	GTCC-CTACCATGTAGTACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGA	1152	
Query 277	CTTGACGT	CATCCCCACCTTCTCCGGTTTGTACCCGGCAGTCTCCTTTGAGTTCCTCGAC	336	
Sbjct 1151	CTTGACGT	CATCCCCACCTTCTCCGGTTTGTACCCGGGCGTCTCCTTAGAGTTCCTC-AC	1093	
Query 337	CGA-A--	TGCTGGSCAACAAAAGATAAGGGTGGGCTCGTTGCGGGACTTACCCACC-T	392	
Sbjct 1092	CATTACGT	GCT-GGCAACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACAT	1034	
Query 393	CTTCCCAC	CACAAGCGGACAACACCCATGCA-CAACTGCTTCTGGTTCCCAGGGCAC	451	
Sbjct 1033	CT-CACGAC	CAGGCTGACGACAGCCATGCAGCACCTG-TCTCACGGTTCCCAGGGCAC	976	
Query 452	AAAGGCAT	CTCTGCCGAATCCGTAAGAGGCCAGAACATGTAAGGTTCTTCCCGTTGCCT	511	
Sbjct 975	CAATCCAT	CTCTGSAAGTTCCGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCAT	916	
Query 512	CGAATTA	AACCACATGGTCCACCGCTTTGGGGGGCCCCCGGCCATTCAATTTGAATTTAA	571	
Sbjct 915	CGAATTA	AACCACATACTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAG	856	
Query 572	CCTTGC	GGCGTACTCCCCAAGGGGGAGAGTTTA-CGGGTTAGCT 615		
Sbjct 855	TCTTGC	GACCGTACTCCCCA-GGCGGCGAACTTAACGCGTTAGCT 812		

Fig. 2. BLASTN analysis of 16s rDNA sequence

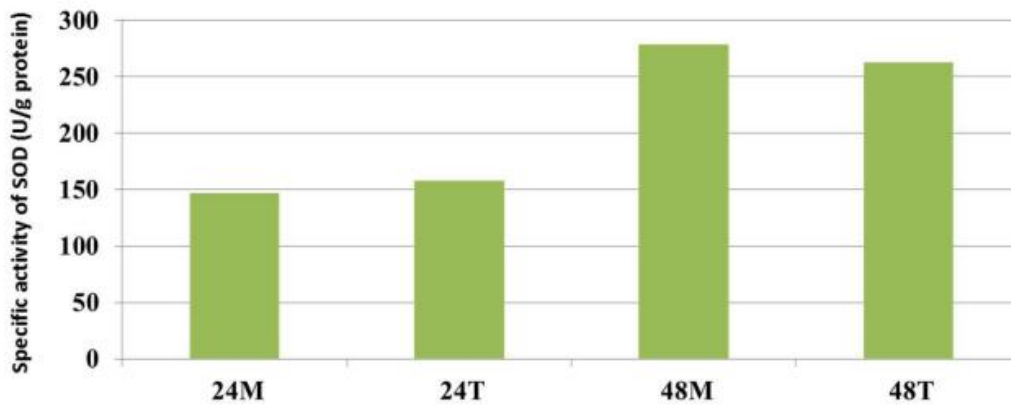
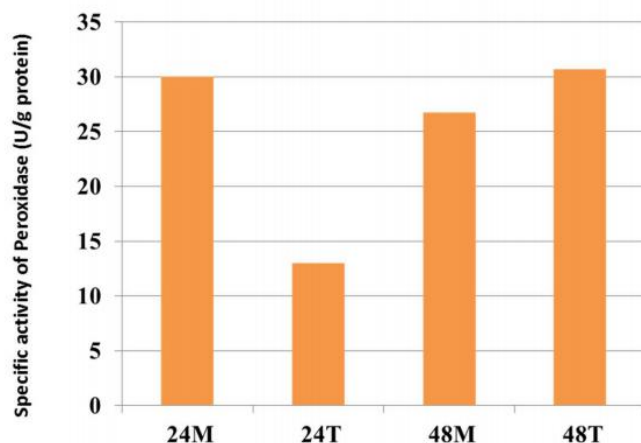
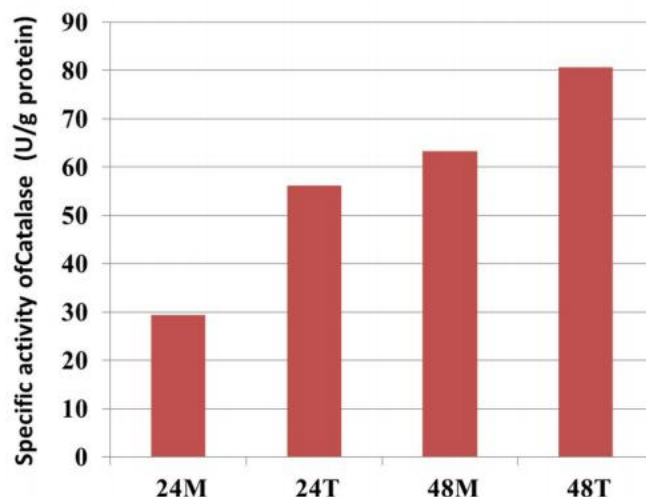


Fig. 3. SOD activity at different time point  
 SOD activity increases at 24h treatment and decreases after 48h treatment X. oryzae



**Fig. 4. Peroxidase activity at different time point**

Enzymatic activity decrease after 24h treatment and subsequently increases after 48h treatment of *X. oryzae*



**Fig. 5. Catalase activity at different time point**

Enzymatic activity increases after 24h and 48h treatment *X. oryzae*

## 5. CONCLUSION

This study was carried out to find out interaction between virulent strain and plant. Among various isolates SboBLB3 is a virulent strain of *Xoo* and able to induced disease resistance responses in rice. Enzymatic assay viz. catalase, peroxidase, SOD shows interaction and resistance response of plant at different time points.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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