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Isolation, characterization and chemical management of bacterial leaf blight pathogen of rice

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Abstract
Bacterial leaf blight pathogen, *Xanthomonas oryzae* pv. *oryzae* (Xoo) being the one of the most destructive organism that constraints the rice cultivation in tropical parts of Asia. The pathogen causing leaf blight in rice was isolated from Coimbatore, Aduthurai, Gudalur, Theni, Krishnagiri, Vellur, Wayanad, Hyderabad and New Delhi were subjected to molecular characterization and the highly virulent isolate assessed through screening each isolates in bacterial leaf blight susceptible, ADT 38 variety. Coimbatore isolate (MH464904.1) has supervened over the other isolates by showing maximum lesion length. Also, the Coimbatore isolate showed genetic lineage to the other strains of *Xoo* characterized from China and parts of Indian subcontinent showing less divergence from the strains prevalent in those regions. Efficacy of chemicals *viz.*, bionol, bactrinashak, niclosamide, chloramphenicol, ciprofloxacin, copper oxychloride, streptomycin sulphate were assessed against bacterial leaf blight pathogen where ciprofloxacin and niclosamide at all the concentration has showed better efficacy than the other chemicals.

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

Introduction
Rice plays a crucial role in agricultural system by serving as caloric intake to half of the world population. In India, rice cultivation is prospered in the river basins, estuaries and lowland coastal areas of north eastern and southern India, particularly in the states of Andhra Pradesh, Assam, Bihar, Chhattisgarh, Karnataka, Kerala, Maharashtra, Orissa, Tamil Nadu, Uttar Pradesh and West Bengal, which altogether contribute about 97% of the Country's rice production (Randhawa *et al*., 2006; Jayaprakashvel and Mathivanan, 2009) [8, 3]. Any disease that withhold the productivity of rice may distress the food security of the global population because the contribution of calories consumed globally by human population through rice products comes upto 23 per cent (Wilson and Talbot, 2009) [11]. Rice production has attained new records during 2016-17 (271.98 million tonnes), an additional of 1.5 – 2 million tonnes of rice every year has to be achieved to combat the pests, diseases and other abiotic stresses (DRR, 2017). Bacterial leaf blight of rice is a detrimental disease caused due to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) has wide spread occurrence throughout Asia, northern Australia, Africa and USA (Adhikari *et al.*, 1995; Sere *et al.*, 2005) [1, 9]. Crop loss assessment in all these epidemic prone locations estimated up to 50% which is favored by the stage of the crop followed by degree of cultivar susceptibility and conducive nature of environment (Sere *et al.*, 2005) [9]. In integrated disease management (IDM) system, application of chemicals being the effective remedial method to contain the disease. Use of chemicals in BLB disease management has been in progress since time immemorial itself but on due course of time, efficacy of chemicals were diminished as the pathogen became more virulent to overcome the chemical. Hence effectiveness of chemicals in reduction of disease is highly imperative to study and thus in the present study novel chemicals along with most common chemicals were tested against the bacterial leaf blight pathogen under *in vitro* and *in vivo* condition thereby to assess their efficacy.

Materials and methods
Collection of diseased samples of bacterial leaf blight of rice
A sampling survey was carried out for the collection of diseased samples of bacterial leaf blight infected rice plants from the following locations *viz.*, Coimbatore, Aduthurai, Gudalur, Theni, Krishnagiri, Vellur, Wayanad, Hyderabad and New Delhi. Bacterial leaf blight suspected leaf samples were collected, bagged, labelled and brought into the laboratory and store at 4ºC for isolation of the pathogen and for further studies.

Isolation and purification of the pathogen

Bacterial leaf blight symptom of rice was collected during the survey were subjected to isolation of bacterial leaf blight pathogen. Under *in vitro* condition, the diseased samples collected were sliced off into leaf bits containing both the healthy and infected portions were transferred into an Eppendorf tube containing sterile water. Later, the leaf bits were crushed using sterile rod to release bacterial colonies as ooze and the loopful of suspension was streaked onto the autoclaved, solidified Peptone sucrose agar media ($CaNO_3$-0.5g, $FeSO_4$. $7H_2O$- 0.5g, sucrose-15g, peptone-5g, Na_2HPO4. 7H2O-2g, agar-15g, distilled water-1000ml) poured into the Petri dish. Triplicates of Petri plates were maintained for each isolates and were incubated at 25ºC for the growth of bacterial colonies. Virulent bacterial colonies were with formation of yellow, mucoid, doom shaped colonies with entire margins were developed after 3rd of plating. Loopful of single colony of bacterial isolate was collected separately from each isolate using sterilized bacterial loop and sub cultured into PSA mediated slants and stored in 4ºC for further studies. Single colony of each isolate was also stored in 70 per cent double autoclaved glycerol stock in -70ºC for long term storage.

Pathogenicity test

Pathogenicity of the isolate was proved in bacterial leaf blight susceptible varieties *viz.,* ADT 38 and TN1. Seeds of the susceptible varieties were collected in a jute bag and immersed overnight in water and on next day, the soaked seeds in jute bag were kept overnight inside dried hay to facilitate seeds sprouting. Sprouted seeds were collected on the next day for sowing in pots (diameter 13cm) filled with clay loamy soil. Seedlings emerged from the pots were transplanted to another one after 20 days of sowing which were maintained in a glass house with an optimum temperature of 25ºC and 85-90% relative humidity. Single bacterial colony was multiplied in nutrient broth (100ml) and incubated at 28ºC and 180 rpm speed for 48 hr which grown to an optical density of 1.0 at 600nm. Clip inoculation method (Kauffman, 1973) [4] was followed for artificial inoculation of bacterial isolate into rice seedlings. Clip inoculation method was performed in the rice seedlings which were grown upto its maximum tillering stage (40 days of transplantation). Surface sterilized scissors were dipped into the bacterial suspension multiplied in the nutrient broth and nicked out top 5cm of leaves of the seedlings. Remaining bacterial suspension was sprayed onto the cut ends and margins of leaves to permeate the entry of bacterial colonies into the leaves and cause the infection. Observation for symptom appearance from the day of inoculation was recorded and the organism was re-isolated from the lesion and compared with that of the original isolate to prove the pathogenicity of the isolate.

Molecular characterization of *X. o. oryzae* isolates

Bacterial leaf blight pathogen, *Xanthomonas oryzae* pv. *oryzae* is highly variable and there are several reported bacterial species with similar cultural, morphological, biochemical characteristics and could initiate infection in rice. Cultural, morphological and biochemical characterization for identification of an organism is not highly suitable since it may mislead the identity. Hence, molecular characterization with 16s rRNA primers were performed for identification.

Isolation of total genomic DNA

Characterization of bacterial leaf blight pathogen was instigated by isolation of total genomic DNA from the bacteria which was performed using lysis buffer method (Chen and Kuo, 1993) [2] where the bacterial culture was multiplied in 100 ml nutrient broth kept for 48 hour in rotary shaker at 180rpm. Saturated culture was harvested in 1.5 ml of Eppendorf tube and allowed for centrifugation for 3 min at 12,000 rpm. 200 µl of lysis buffer (40 mM Tris-acetate pH 7.8, 20 mM sodium-acetate, 1 mM EDTA, 1% SDS) was suspended in the cell pellet and lysed by vigorous pipetting. 66 µl of 5M NaCl was added and mixed well which remove most proteins and cell debris and then the viscous mixture was centrifuged at 12,000 rpm for 10 min at 4°C. Clear supernatant obtained after centrifugation was transferred into new vial and an equal volume of chloroform was added followed by vortex until the solution turned milky. Subsequently, centrifuged the solution at 12,000 rpm for 3 minutes, and the supernatant extracted was transferred to another vial and the DNA was precipitated with 100% ethanol, washed twice with 70% ethanol, dried, and redissolved in 50 µl of 1 x TE buffer.

PCR amplification of 16s rRNA region of bacterial leaf blight pathogen

To assess the genetic identity of the pathogen, PCR was performed using 16s rRNA primers (27F: 5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The reaction mixture for PCR amplification of the rDNA consisted of 25 µL reaction volume containing PCR buffer 1x, 1.5 mM $MgCl_2$, 0.4 mM each dNTP, 30 ng primer, 3 U Taq DNA polymerase and 30 ng of genomic DNA. Initial denaturation of 5 min at 94°C followed by 40 cycles of 1 minute of denaturation at 94°C, 45s of annealing at 55°C, and 1 minute of extension at 72°C. Final extension was 10 min at 72°C. The DNA products were separated by gel electrophoresis on agarose (1.2%) and 1x Tris-acetate (TAE) buffer for one and half hours at 90 Volts. Molecular (DNA) marker of size 100 bp (GeNei, Bangalore) was loaded along with the samples for marking the bands. After applying 20 µL of reaction and 5 µL of bromophenol blue stain, the gels were treated with ethidium bromide. The gels were later photographed in the gel documentation system (Alpha Imager EC, USA). The PCR product was outsourced for sequencing to identify upto the species level of bacteria.

Virulence study of bacterial leaf blight pathogen

Isolates of *X.o.*pv. *oryzae* isolated from different location were compared for their virulent nature in bacterial leaf blight susceptibility variety, ADT 38. The rice seedlings were maintained for virulence study in glass house at 25 ºC of 85-90 per cent relative humidity. Bacterial pathogen multiplied in nutrient broth which attained 10^8 cfu/ml was inoculated into the plants at maximum tillering stage. Observation for lesion length was recorded at 7 and 14 days of inoculation and the virulence nature of isolates of *Xoo* were compared. Genetic diversity of Coimbatore isolate of *Xoo* was compared to that of *Xoo* strains deposited in NCBI (www.ncbi.com). Mega 7 version software was used to depict the phylogenic tree of collected strains of *Xoo* along with the Coimbatore isolate used in this study.

Management of bacterial leaf blight of rice through chemicals

Management of bacterial leaf blight pathogen was performed under *in vitro* condition using following chemicals *viz.*, Bionol (100, 300 and 500 ppm), Bactrinashak (100, 300 and 500 ppm), Niclosamide (300, 500 and 1000ppm), Chloramphenicol (300, 500 and 1000 ppm), Ciprofloxacin (300, 500 and 1000 ppm), Copper oxychloride (0.1, 0.3, 0.5%), Streptomycin sulphate (100, 300 and 500 ppm). Poison food technique, measurement of inhibition zone by disc method and reduction of optical density of bacterial suspension were employed to test the efficacy of above chemicals against bacterial leaf blight pathogen. By poison food technique, peptone sucrose agar media was prepared and sterilized at 121ºC for 20 minutes. An appropriate quantity of chemical was added to 100ml sterilized molten PSA medium so as to get the final required concentrations of poisoned medium. The medium was mixed thoroughly before plating. Bacterial leaf blight pathogen, *X. o.* pv. *oryzae* was multiplied in nutrient broth until it obtained an optical of 1-2 at 600 nm. One milliliter of bacterial suspension was poured into sterilized Petri plate into which poisoned medium was poured and incubated at 26±1ºC. Non toxicated media was poured into Petri plates and kept as control. Observations on growth of bacterial colonies were recorded. In disc method, *X. o.* pv. *oryzae* was multiplied in nutrient broth medium until it attained 10^8 cfu/ml and 1 ml of culture was poured into sterilized Petri plate under *in vitro* condition. Later, autoclaved, molten and cooled PSA medium was poured into those Petriplate and kept for solidification for 15-20 minutes. Chemicals prepared at desired concentration where sterilized 8 mm filter paper was dipped and placed over the solidified PSA in Petri plate. Triplicates of each chemicals were allowed to incubate at 28 ºC for 48 hr. Inhibition zone formed around the disc were recorded for each chemicals separately. Efficacy of chemicals was also tested by measuring the reduction in optical density of bacterial growth in nutrient broth. *X. o.* pv. *oryzae* colony was inoculated into 100 ml of autoclaved nutrient broth and kept for multiplication at 28 ºC for 48hr in BOD incubator at 180 rpm. Chemicals were weighed and added into 100 ml of nutrient broth to attain desired concentration and correspondingly control was maintained. Optical density was checked in Spectrophotometer at 600 nm at 24, 48 and 72 hr after incubation and the data was recorded.

Results and discussion

Nine isolates of bacterial leaf blight pathogen were isolated from diseased samples collected from Coimbatore, Aduthurai, Gudalur, Theni, Krishnagiri, Vellur, Wayanad, Hyderabad and New Delhi. Round, yellow, mucoid with convex shaped colonies which were observed after 72 hr of incubation were the virulent colonies which was in accordance with the description of Webster and Gunnel (1992) [10]. The isolates were subjected to pathogenicity test in bacterial leaf blight susceptible variety, ADT 38 and all the nine isolates were ascertained Koch postulates of pathogenicity test. Inoculation of bacterial suspension into rice seedlings resulted in Molecular characterization of nine isolates revealed that 16s rRNA primers showed amplification in all the isolates at 1200 bp and partial sequencing of Coimbatore isolate confirmed the isolate as *Xanthomonas oryzae* pv. *oryzae* after Blast analysis of the nucleotide (NCBI Accession No. MH464904.1). Virulence nature of nine isolates bacterial leaf blight pathogens was carried out in ADT 38 variety which revealed that Coimbatore isolate showed highest virulence nature than rest of the isolates. Genotype of Coimbatore isolate obtained in present study was compared to that of other *Xoo* isolates and the phylogeny diagram showed that the strain of *Xoo* used in present study was lying in same lineage with the other strains obtained from China (MH158537.1) and parts of India (Andaman & Nicobar Islands- KX088300.1, Andhra Pradesh- HM747119.1, New Delhi- KM589047.1, Mau- JQ081066.1) whereas the strain collected from Chennai (KJ740440.1) showed different lineage from the rest of them. Similar study of genetic diversity of virulent strains of *Xoo* was carried out by Nayak *et al*. (2008) [6] where they estimated genetic distance by multivariate analysis *Xoo* isolates which could be classified into 13 clusters and five broad groups. *In vitro* assessment of efficacy of chemicals against bacterial leaf blight pathogen, ciprofloxacin antibiotic at 300, 500 and 1000 ppm showed largest inhibition zone of 5.3, 5.7 and 6.4 respectively where niclosamide also showed 2.5, 2.9 and 3.2 cm of inhibition zone at the same concentration of chemicals. Diameter of inhibition zone of bactrinashak chemical was on par with that of bionol. Pramesh *et al*. (2017) [7] has reported that Bionol at 300 ppm has effectively contained the growth of *Xoo* and which was on par with the results obtained with the use of Streptocycline at 100 ppm, tank mixture of Streptocycline at 0.1 g/l + Copper oxychloride at 0.1 g/l. In accordance with the above study, present study also showed efficacy of bionol against bacterial leaf blight pathogen. In the case of measurement of reduction in optical density, highest per cent of reduction was noticed when ciprofloxacin was applied into the broth culture where it showed 36.6 per cent of reduction which directly relating to the reduction of bacterial colonies. Hence, both the experiments proved the efficacy of ciprofloxacin in reducing the multiplication of bacterial colonies. The experiment also revealed the effectiveness of niclosamide in containing the pathogen growth as well. Kim *et al.* (2015) [5] has also revealed the efficacy of niclosamide, an oral antihelminthic drug and molluscicide in management of bacterial leaf blight of rice.

Table 1: Assessment of efficacy of chemicals against bacterial leaf blight pathogen by disc method

Sl. No.	Chemical	Concentration	Inhibition zone (cm)
1.	Bionol	100 ppm	2.6
		300 ppm	3.2
		500 ppm	3.5
2.	Bactrinashak	100 ppm	2.3
		300 ppm	2.9
		500 ppm	3.2
3.	Niclosamide	300 ppm	2.5
		500 ppm	2.9
		1000 ppm	3.2
4.	Ciproflaxacin	300 ppm	5.3
		500 ppm	5.7
		1000 ppm	6.4
5.	Chloramphenicol	300 ppm	1.0
		500 ppm	2.6
		1000 ppm	3.0
6.	Copper oxychloride	0.1 %	2.1
		0.5 %	3.2
		1 %	4.4
7.	Streptomycin sulpate	100 ppm	2.4
		300 ppm	3.5
		500 ppm	5.3
8.	Control	-	-

Table 2: Assessment of efficacy of chemicals against bacterial leaf blight pathogen by measurement of optical density

S. No.	Chemical	Concentration (ppm)	Optical density at 600 nm				Per cent reduction (%)
			0th day	1st day	2nd day	3rd day	
1.	Bionol	100	0.552	0.501	0.471	0.385	16.7
2.	Bactrinashak	100	0.532	0.481	0.428	0.376	15.6
3.	Niclosamide	300	0.736	0.607	0.523	0.423	31.3
4.	Ciproflaxacin	300	0.687	0.536	0.394	0.321	36.6
5.	Chloramphenicol	300	0.579	0.513	0.497	0.418	16.1
6.	Copper oxychloride	1000	0.752	0.674	0.524	0.461	29.1
7.	Streptomycin sulphate	100	0.687	0.535	0.498	0.468	21.9
8.	Control	-	0.766	0.895	1.52	1.65	-

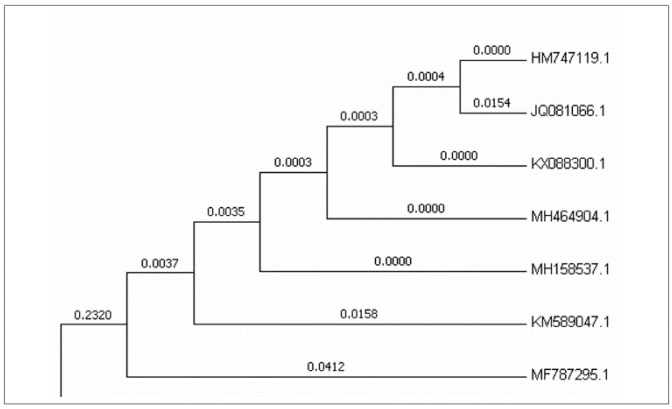

Fig 1: Comparison of 16s rRNA sequences of *Xanthomonas oryzae* pv. *oryzae* (MH464904.1) with the other strains using Neighbour joining method

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