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Morphological and Biochemical Characterization of *Xanthomonas oryzae* pv. *oryzae* Isolates from different Regions of India

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Swathi, S. Patil : Performed the experiment, analyzed the data and wrote the manuscript;
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Abstract

Rice is among the oldest crops of the world which feeds about half of the world's population. The most significant bacterial disease impacting the rice production is bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Ten *Xoo* isolates collected from different rice growing regions of India were characterized morphologically and their reaction to different biochemical tests. *Xoo* isolates were gram negative, showed positive reaction for potassium hydroxide test and catalase production. The isolates TLG2 and AP showed strong oxidase activity. All the isolates except those from Telangana state showed tolerance to 5 per cent sodium chloride. None of the isolates were able to utilize sorbitol, mannitol and malonate as carbohydrate source for their growth whereas all of them were able to grow at 37 °C. Among all the isolates, KA showed highest swarming ability along with exopolysaccharide and xanthomonadin production. The results indicate that there exists variation in physiological characters among the *Xoo* isolates and this may have impact on their virulence and survival.

Keywords : Bacterial leaf blight, Xanthomonas oryzae pv. oryzae, Biochemical characterisation, Morphological characterisation, Virulence

Rice (*Oryza sativa*) is a staple food for more than half of the world's population, providing more than 20 per cent of the calories consumed world wide, especially in East and South Asia, the Middle East, the West Indies and South America (Sharif *et al*., 2014). Rice is cultivated in more than 100 countries worldwide and 90 per cent of the total global production of rice is from Asian countries. It provides 27 per cent of the calories for the global population and remains a major source of carbohydrate in developing and underdeveloped countries.

Bacterial leaf blight (BLB) of rice is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Swings *et al*., 1990) and remains a major production obstacle in rice cultivation, by and large in irrigated and rainfed lowland ecosystems of India. It occurs worldwide and can reduce the yield of rice by 60-70 per cent (Nino-Liu *et al*., 2006). In India, the disease is endemic in Punjab, Haryana, eastern and western Uttar Pradesh, Bihar, West Bengal, Tripura, Odisha, Tamil Nadu, Kerala, coastal parts of Andhra Pradesh, parts of Chhattisgarh and Assam (Laha *et al.,* 2009; POS, 1975-2016).

X. oryzae pv. *oryzae* enters the plant through wounds or hydathodes on the leaf margin and subsequently produces systemic infection (Mew, 1987). Small, water soaked lesions are formed on the leaves which later coalesce and become chlorotic and necrotic. Later these lesions turn to grey in colour along the veins of the plant. Symptoms start appearing from tillering stage of the crop and the severity progresses as the plant matures, reaching an apex at the flowering stage. In case of early infection, it produces *Kresek* symptom where in the plants wilt, ultimately leading death (Mizukami & Wakimoto, 1969; Sharma *et al*., 2017).

X. oryzae pv. *oryzae* is highly dynamic in nature and there is a need to understand the pathotype composition among the *X. oryzae* pv. *oryzae* population to design scientific resistance breeding program (Yugander *et al*., 2017) because variation exists among the pathogen populations (Lore *et al.,* 2011; Mishra *et al*., 2013 and Yugander *et al.,* 2017). However, information regarding biochemical variation among *X. oryzae* pv. *oryzae* isolates is lacking. Therefore, an attempt was made to study the profile of different biochemical characters among the *X. oryzae* pv. *oryzae* isolates from different parts of India. In the present study, isolates were collected from different rice growing zones of India and subjected them for pathogenicity test, morphological and biochemical characterization.

Material and Methods

Plant Varieties and Growth Conditions

The seeds of the susceptible variety of rice TN-1 were surface sterilized in 70 per cent ethanol for 5 min, washed thrice under running water and were soaked in sterile distilled water overnight. Soaked seeds were sown in the glasshouse and germinated seedlings were transplanted to pots 15 days after sowing. The plants were maintained in greenhouse conditions with the maximum and the minimum temperatures of 29 and 21 °C, respectively and the relative humidity was 75 per cent.

Bacterial Strains used in the Study

A total of ten isolates were collected from different regions of India (Table 1) and all the isolates were sub-cultured periodically. In this study, all isolates were tested for pathogenicity on susceptible rice variety TN-1. The cultures obtained from re-isolation of the infected tissues were used in all the experiments.

Morphological Characterization

Colonial morphology of 10 *Xoo* isolates was studied using the standard procedure described by Schaad (1992) with emphasis to colour, size of colonies and their outline - whether circular and entire or indented or wavy or rhizoid. Their elevations were recorded as convex or flat. A loopful of culture was taken from 48 hours old culture and streaked on Nutrient Agar medium. The plates were then incubated at 28 °C for 48 hours and were examined for appearance of the colonies.

Table 1

Xoo isolates used in the study

Area	*Xoo* isolates
KA	Karnataka
KL	Kerala
TN	Tamilnadu
AP	Andhra Pradesh
TLG1	Telangana
TLG2	Telangana
TLG3	Telangana
OD	Odisha
MH	Maharashtra
PB	Punjab

Biochemical Characterization

Gram Staining

Gram staining procedure was performed as described by Gerhardt *et al*., (1981). Bacteria were heat fixed on a glass slide treated with (0.5%) crystal violet for 30 seconds and then washed with tap water. After that, iodine was added for 1 min, washed again and decolorized with (95%) ethanol for 30 seconds, washed again and counter-stained with safranin. Magnifications of 40X was used for microscopic observation. Gram negative bacteria stained red whereas Gram positive retained the colour of crystal violet.

Potassium Hydroxide Test

Potassium hydroxide (KOH) test is an excellent validation assay for Gram staining (Suslow *et al*., 1982). The culture of each *Xoo* isolate (48 hours old) was taken with sterilised tooth pick and was mixed with a drop of 3 per cent KOH solution on sterilised glass slide under aseptic conditions. Formation of thread-like slime indicated negative Gram reaction.

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Catalase

A loopful of 24 hours old colony was smeared on a slide and was covered with a few drops of hydrogen peroxide. The reaction was positive if gas bubbles were produced (Taylor and Achanzar, 1972).

Oxidase

Oxidase reaction was carried out by spreading a well-isolated colony on the oxidase disc (DD018, HiMedia, Mumbai) with an inoculation loop. The reaction is observed within 5-10 seconds at 25-30 °C. The isolate was rated oxidase-positive if a purple colour developed within 10 seconds, delayed positive if coloration developed within 10-60 seconds and negative if no colour developed after 60 seconds (Gaby and Hadley, 1957).

Tolerance to 5 per cent NaCl

The NA medium was prepared with five per cent NaCl, poured onto Petri plates and allowed for solidification. A 24 hours old bacterial culture was streaked on the media and incubated for 48 hours at 28 °C. The growth on the medium indicated tolerance to high salt concentration (Shankara *et al*., 2017).

Growth at 37 °C

The 24 hours old bacterial culture was streaked on Petri plates containing NA media and incubated at 37 °C for 48 hours. The growth of culture on media indicated positive for survival under high temperature.

Mucoid Growth

Luria Bertani agar (M1151, HiMedia, Mumbai) was prepared and poured onto Petri plate for solidification. The 48 hours bacterial colony was streaked on the medium and incubated for 48 hours at 28 °C. The mucoidness on media was observed (Shankara *et al*., 2017).

Carbohydrate Utilization Test

Nutrient broth was prepared and a pinch of Bromothymol blue indicator was added which is responsible for the colour change during the growth of bacteria in the nutrient broth. 10ml of nutrient broth was poured into the test tubes and autoclaved. After autoclaving, the test tubes were added with different carbon sources like sorbitol, mannitol and malonate. To this test tubes 1ml of 48 hours old culture of each isolate was added and observed for culture change in the test tubes.

Motility Test on Agar Plates

Motility tests were performed on a semi-solid Luria Bertani agar medium supplemented with 0.3 per cent agar. A loopful of 24 hours old culture was stabbed in the exact centre of Petri plates and incubated at 28 °C. Motility was compared after 24 and 48 hours of incubation (Lee *et al*., 2013), photographs were taken and diameter of the movement was measured at 48 hours.

EPS Quantification

Xoo isolates were grown in NB at 28 °C for 72 hours. Subsequently, 10 ml portions of the each cultures were collected and the cells were removed by centrifugation at 8000 g for 20 minutes (Guo *et al*., 2010). Finally, three volumes of ethyl alcohol were added to the supernatants. The precipitated EPS were pelleted *via.,* centrifugation, dried and weighed. The test was performed three times independently (Vojnov *et al*., 1998).

Xanthomonadin Quantification

The cells of *Xoo* isolates were collected by centrifuging 4 ml broth suspension and was mixed with 1 ml 100 per cent methanol. The mixtures were further incubated in darkness for 10 minutes kept on rotating shaker followed by centrifugation at 12,000 g for 8 minutes to collect the supernatant. The xanthomonadin pigment was estimated by measuring the absorbance at OD_{445} (Wang *et al*., 2015).

Pathogenicity Test

The bacterial inoculum of each isolate was prepared by dissolving one loopful of the pure bacterial culture in 10 ml of sterilized distilled water, so as to get the 10^8CFU/ml. 45 days old plants of TN-1 grown in glass house condition were artificially inoculated

with the bacterial suspension by leaf clipping method (Ke *et al*., 2017). The inoculated plants were observed for the development of symptoms. After the symptom development, the bacterium was re-isolated from the artificially inoculated seedlings to prove the Koch's postulates and compared with the original culture.

Results and Discussion

Morphological Characterization

Xoo isolates were morphologically characterized based on average size, shape, colour and appearance of the colony on Nutrient agar plate. The colonies of *Xoo* isolates appeared as circular/irregular, raised or flattened and yellow to creamy yellow coloured with slimy appearance (Table 2). The maximum colony size of 5.0 mm was observed in KA and AP isolates and the minimum colony size of 1-2 mm was observed in isolates TN and TLG1 (Fig. 1).

All isolates exhibited yellow colour colonies whereas the isolates KA from Karnataka and AP from Andhra Pradesh produced creamy yellow coloured colonies on nutrient agar medium. The isolate TN appeared

Table 2

Morphological characterization of *Xoo* isolates

Xoo isolates	Average colony size (mm)	Colony color	Colony shape	Appearance
KA	4-5	Creamy yellow	Circular, Irregular	Raised and slimy
KL	2-3	Yellow	Circular	Flattened and slimy
TN	1-2	Light yellow	Circular	Flattened and slimy
AP	4-5	Creamy yellow	Circular	Raised and slimy
TLG1	1-2	Yellow	Circular, Irregular	Raised and slimy
TLG2	3-4	Yellow	Circular	Raised and slimy
TLG3	2-4	Yellow	Irregular	Raised and slimy
OD	1-3	Yellow	Circular	Raised and slimy
MH	2-4	Yellow	Circular	Raised and slimy
PB	2-3	Yellow	Circular	Flattened and slimy

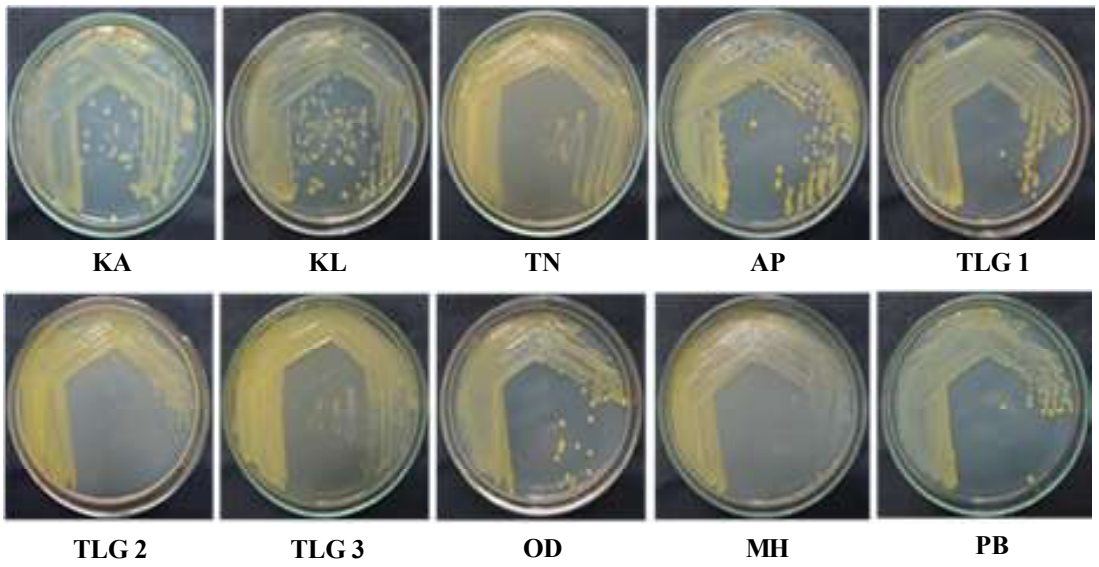

Fig. 1 : Growth of *Xoo* isolates on Nutrient Agar

light yellow compared to other isolates. The colony shape of most of the isolates was circular whereas two isolates KA and TLG1 appeared circular to irregular in shape and TLG3 exhibited irregular shape on NA. Most of the isolates exhibited raised and slimy colonies whereas some isolates produced flattened and slimy colonies (PB, KL and TN) (Fig. 2). Present results are supported with similar results obtained by Han *et al*. (2005), wherein *Xoo* colonies were slightly convex, smooth, with regular to irregular diffused edges. Colonies of the bacterium appeared as circular, convex, yellow to straw yellow coloured with smooth surface on the nutrient agar medium and were opaque against the transmitted light on 48 hours old culture.

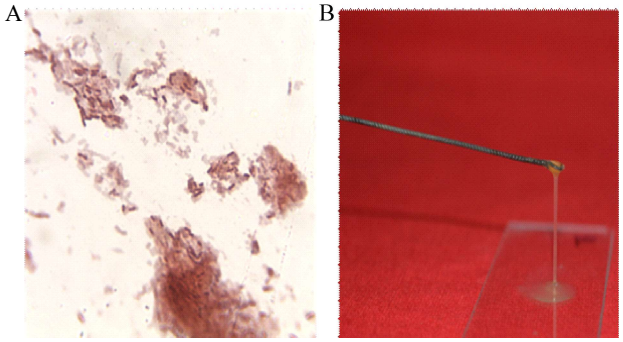

Fig. 2 : Reaction of Xoo to Gram staining and KOH test

Biochemical Characterization of *Xoo* Isolates

The detailed reactions of the ten *Xoo* isolates to different biochemical tests are presented in Table 3. All isolates exhibited Gram negative reaction with red colour when studied under light microscope (Fig. 2a). Since Gram negative bacteria have thin peptidoglycan layer, they do not retain purple colour and are counter stained by safranin giving red colour appearance. Potassium hydroxide (KOH) test is an excellent validation assay for Gram staining (Suslow *et al*., 1982). The cell walls of Gram negative bacteria are broken down in the presence of KOH and this results in releasing of the viscid chromosomal material which causes the bacterial suspension to become thick and stringy. The ten *Xoo* isolates produced characteristic slimy thread like structure when treated with 3 per cent KOH confirming Gram-negative nature of *Xoo* (Fig. 2b). Rukhsana *et al*. (2012), reported that *Xoo* was Gram negative, rod shaped and produced red colour when counter stained with safranin.

The Xoo isolates used in the present study were found to be positive for catalase. All the isolates formed bubbles when mixed with few drops of hydrogen peroxide (Fig. 3). Catalase enzyme decomposes hydrogen peroxide to water and oxygen and *Xanthomonas* spp. are reported to be positive for catalase (Anonymous, 2001).

TABLE 3

Biochemical characteristics of *Xoo* isolates from different rice growing regions of India

Isolates	Oxidase test	Catalase test	3 per cent KOH test	Tolerance to 5 per cent NaCl	Growth at 37°C	Mucoidness
KA	+	+	+	+	+	++
KL	+	+	+	+	+	+
TN	+	+	+	+	+	+
AP	++	+	+	+	+	+
TLG1	+	+	+	-	+	+
TLG2	++	+	+	-	+	+
TLG3	+	+	+	-	++	+
OD	+	+	+	-	+	+
MH	+	+	+	++	+	+
PB	+	+	+	+	+	+

'+' denotes positive reaction and '-' denotes negative reaction

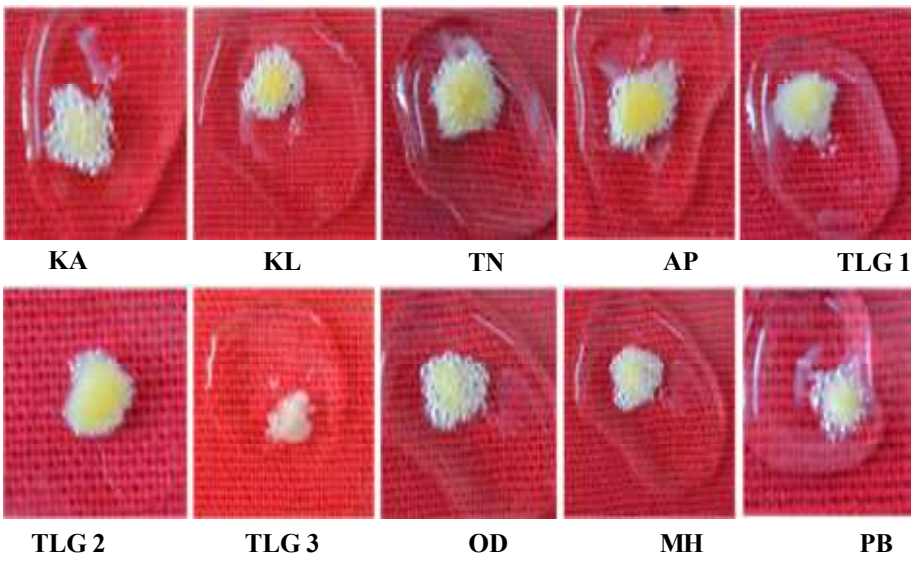

Fig. 3 : Reaction of Xoo isolates to catalase test

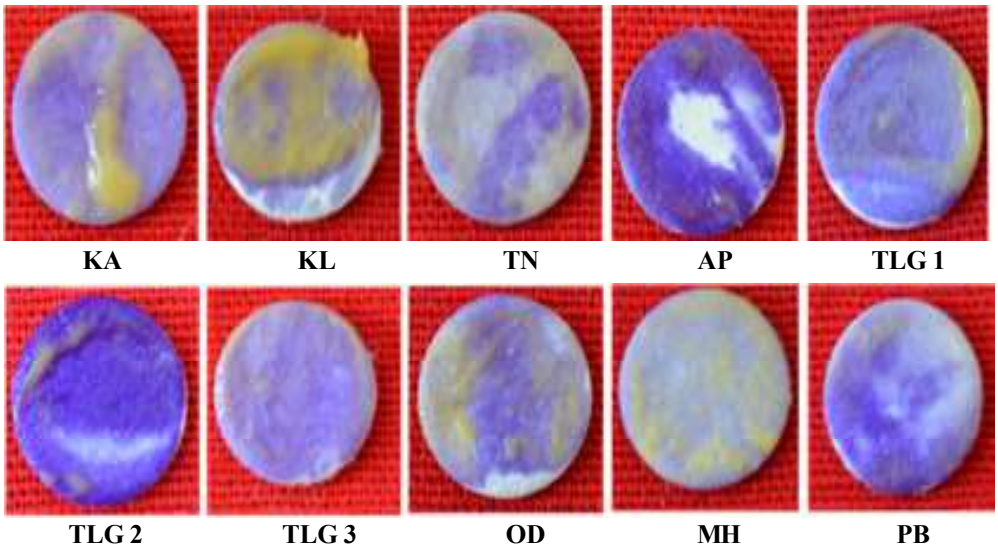

Fig. 4 : Reaction of Xoo isolates to oxidase test

Oxidase test was performed to determine presence of cytochrome oxidase in bacteria. Dark purple colour was produced when a loop of actively growing bacterial cells was rubbed on filter paper disc impregnated with 1 per cent tetra-methyl-p-phenylenediamine dihydrochloride indicated positive reaction. The isolates KA, KL, TN, TLG1, TLG3, OD, MH and PB showed delayed positive reaction for oxidase since the colour change occurred after 20 seconds. A strong oxidase reaction was found in the isolates TLG2 and AP (Fig. 4).

Salt acts as a selective agent for bacteria and interferes with membrane permeability and osmotic equilibrium. A high salt concentration thus inhibits a range of bacteria but allows salt-tolerant organisms to grow. The tolerance of *Xoo* isolates to 5 per cent NaCl varied among the isolates. The isolates KA, KL, TN, AP, MH and PB showed growth on NA containing 5 per cent NaCl. No growth was observed in the isolates TLG1, TLG2, TLG3 and OD (Fig. 5). None of the isolates from Telangana state grew on NA with 5 per cent NaCl.

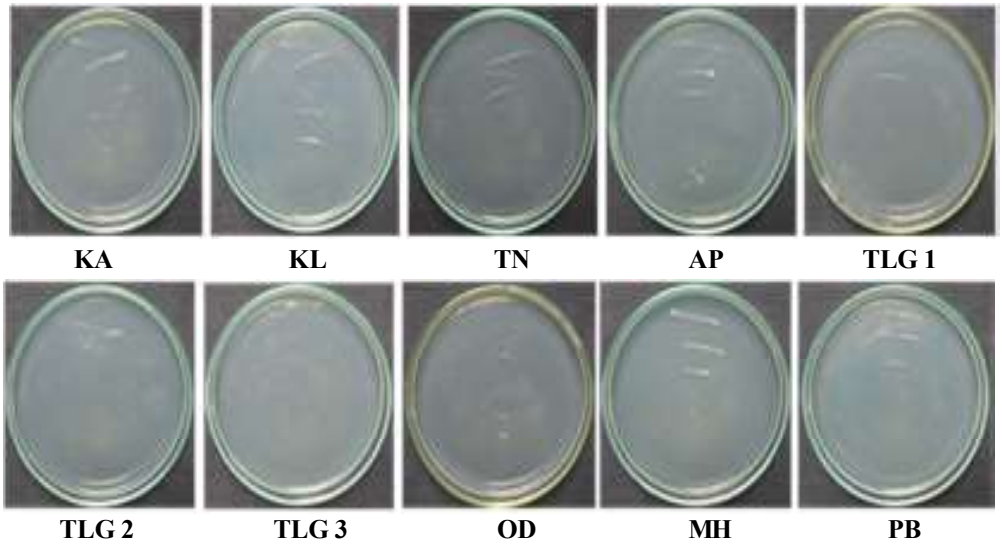

Fig. 5 : Growth of Xoo isolates on nutrient agar containing 5 per cent NaCl

The *Xoo* isolates showed growth when placed @ 37°C and among them more growth was observed in TLG3 (Fig. 6). LB media is considered as nutrient-rich medium and all the isolates produced mucoid and slimy colonies. Amongst them KA isolate showed highest mucoidness (Fig. 7). The isolate TN showed least mucoidness.

The bacteria use carbohydrate differently depending upon their enzyme complement. The pattern of fermentation is characteristics of certain species, genera or groups of organisms and this property has been extensively used as a method for biochemical differentiation of microbes. All the 10 *Xoo* isolates showed negative reaction for utilization of sorbitol, mannitol and malonate. Pradhan *et al*. (2018) characterized 8 isolates from Chattisgarh, India for carbon utilization and all the isolates did not utilize sorbitol, mannitol and mannitol (Fig. 8).

The ability to move on solid surfaces provides ecological advantages for bacteria (Hagai *et al*., 2015).

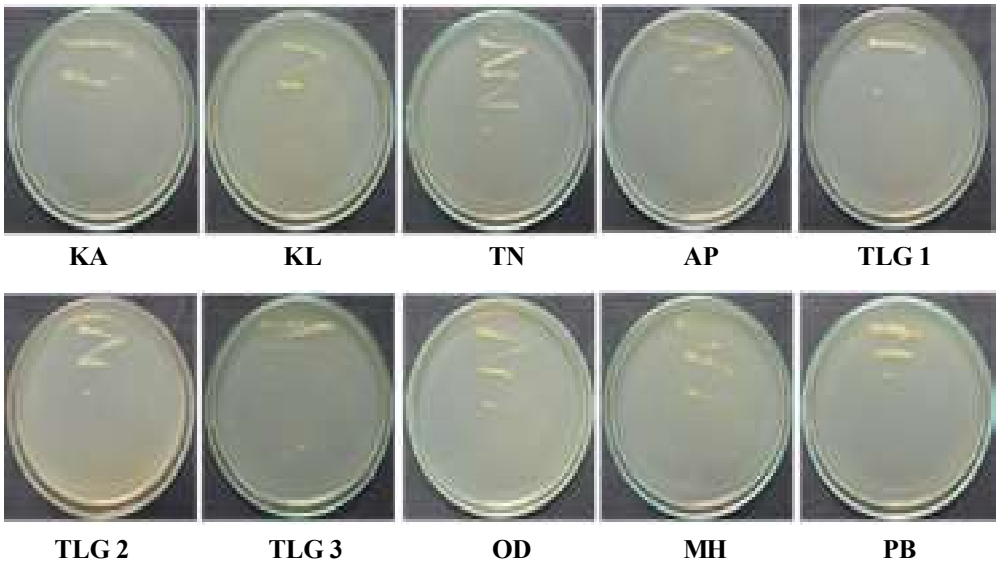

Fig. 6 : Growth of Xoo isolates at 37 °C

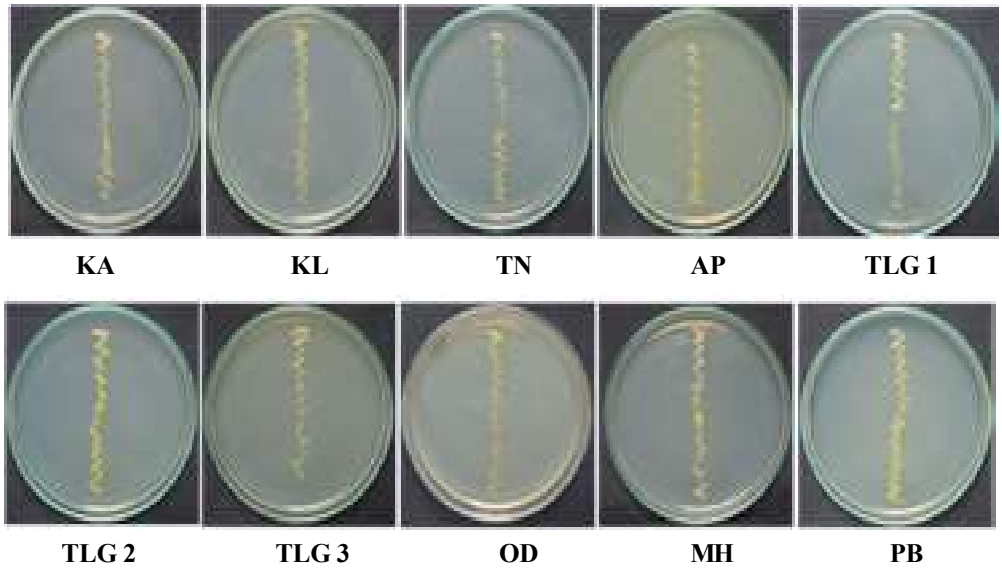

Fig. 7 : Growth of Xoo isolates on Luria Bertani agar medium

Fig. 8 : Reaction of Xoo isolates to different carbohydrates

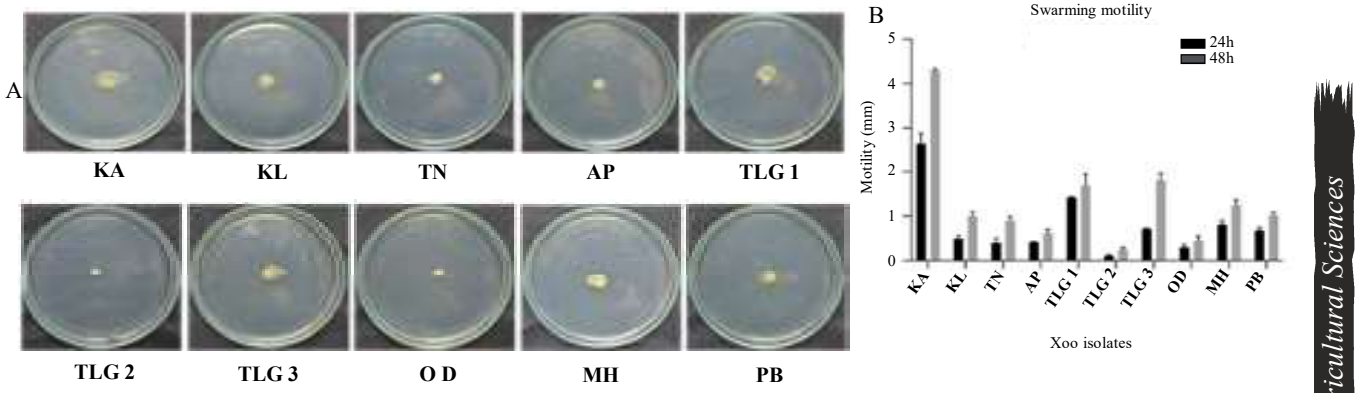

Fig. 9 : Swarming motility assay for Xoo isolates. A: Movement of Xoo isolated on modified NA medium at 48 hours; B: Motility of Xoo isolates measured in mm at 24 and 48 hours

Bacteria colonize surfaces of various environments and are often dependent on surface motility for survival. Such a mode of motility allows bacteria to escape local stresses and translocate to a better nutritional environment and efficiently invade host tissue (Harshey, 2003). Among the ten isolates, KA was found to show significantly higher motility at 48 hours on the modified NA plate. KA isolate

swarmed approximately 4.2 cm^2. The least motility was observed in TLG2 isolate which showed 0.25 cm^2 at 48 hours (Fig. 9).

Bacterial exopolysaccharides (EPS) play role in pathogenesis (Sutton and Williams, 1970; Coplin and Cook, 1990; Dharmapuri and Sonti, 1999). Loss of EPS production has been correlated with loss of virulence in plant pathogens (Coplin and Cook, 1990). EPS provide strength to the interaction of the microorganisms in the biofilm and format in the attachment stage of a biofilm to the surface (Jamal *et al*., 2018) and it is one of the most important virulence factors of *Xoo* (Jansson *et al*., 1975 and Dharmapuri and Sonti, 1999). In our study, KA produced significantly higher amount of EPS (73mg) and the least amount of EPS was produced by TN weighing about 13mg (Fig. 10). Rathna and Prasannakumar (2022) reported EPS production among the nine isolates of *Xanthomonas axonopodis* pv. *punicae* and it varied between 20 mg to 106 mg. The highest EPS was produced in the isolate Xap1 which proved to be more virulent and lowest in Xap9 which showed the least necrotic lesions on pomegranate.

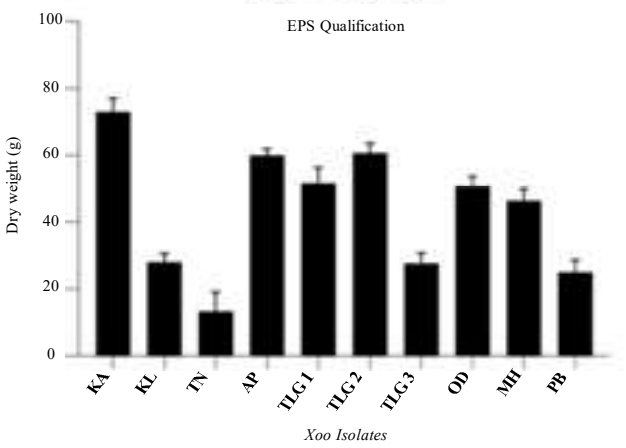

Fig. 10 : Exopolysaccharide production by different Xoo isolates

Xanthomonadins are the yellow membrane-bound pigments which are involved in biofilm formation (Poplawsky *et al*., 2000) and has been linked to *Xoo* virulence (Yu *et al*., 2019). The isolate KA produced more xanthomonadin pigment followed by isolates MH, TLG3, OD, KL,PN, TLG1, TLG2 and AP. TN produced the least xanthomonadin compared to all the isolates (Fig. 11).

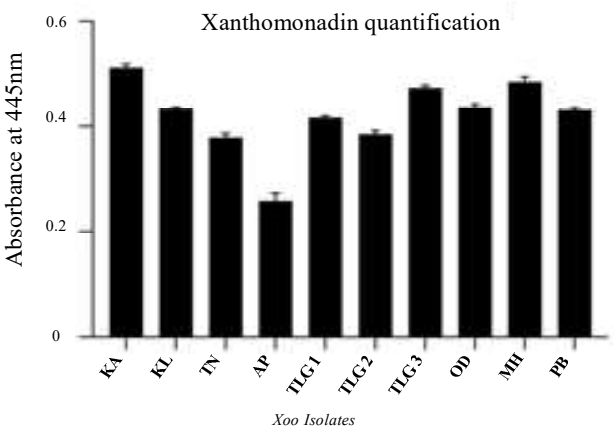

Fig. 11 : Xanthomonadin production by different Xoo isolates

The ten *Xoo* isolates produced typical bacterial leaf blight symptoms of pale-green to grey-green water-soaked region near the cut end of the leaf tip and margin. These lesions coalesced and turned into chlorotic and necrotic with wavy edges. When inoculated on susceptible check variety TN1 (Taichung Native1) the lesion length produced by different varieties ranged from 2.45 cm to 6.97 cm at 10 days post inoculation (dpi). The most virulent isolate was KA from Gangavathi region of Karnataka which produced a lesion length of 6.97 cm at 10 dpi followed by TLG3 from Telangana and AP from Andhra Pradesh. The least virulent isolate was TN isolate from Tamil Nadu displaying lesion length of 2cm at 10dpi (Fig. 12). Bakade and Prasannakumar (2020) studied virulence of ten isolates from

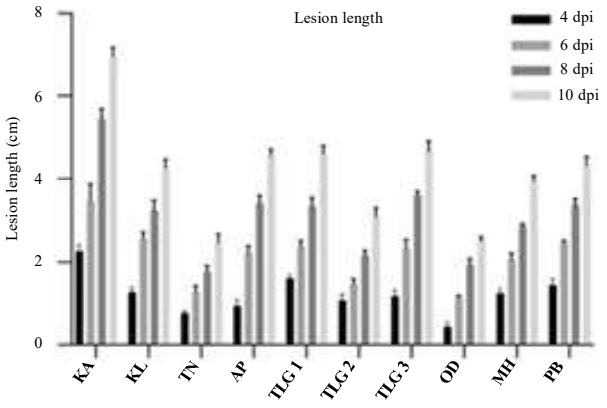

Fig. 12 : Lesion length produced by Xoo isolates on TN-1 at different time intervals

different regions of Southern India and revealed that isolate *Xoo5* from Gangavathi, Karnataka showed higher virulence on TN-1 producing a lesion length of 25cm at 21 dpi thereby indicating aggressive nature of the isolate.

The virulence of KA isolate from Karnataka in causing highest lesion length may be attributed to its capacity of producing highest amount of EPS, xanthomonadin as well as its ability to produce 4-5 mm of colony compared to all other isolates. In isolate TLG3, virulence is contributed by its production of EPS, xanthomonadin as well as its swarming motility. While in the isolate AP, virulence may be assisted by its ability to produce higher amount of EPS and colony size of 5mm, but not by its ability to swarm since the movement of AP is only about 0.4cm^2 on modified nutrient agar which was least among all the isolates. The other isolates showed varied characteristics with respect to lesion length and their potentiality in producing EPS and xanthomonadin. The isolate TN from Tamil Nadu was the least virulent among all the isolates and its production of EPS and xanthomonadin was also minimum.

Variability is an essential requirement for the survival and perpetuation of plant pathogenic bacteria in various environmental conditions in different ecosystems. Variations in the genetic makeup of bacteria are reflected in their cultural and biochemical characteristics that may or may not have an impact on their pathogenic potential (Han *et al*., 2005). Biochemical tests are important parameters in distinguishing the isolates of a particular pathogen. Variation occurs in plant pathogenic bacteria mainly because of spontaneous mutation (Agrios, 2005) and could be influenced by the environmental conditions and varietal profile in a particular region. There are many reports of occurrence of variation within a field or geographical region in *Xoo* (Ardales *et al*., 1996; Vera Cruz *et al*., 1996) and other plant pathogenic bacteria (Davis *et al*., 1997; Restrepo *et al*., 2004). In the present study, based on differential reaction in some biochemical tests, it was established that variability was present in *Xoo* isolates. This reveals existence of variation with respect to different biochemical characters among the *Xoo* isolates collected from different regions of India and this may have impact on their virulence and survival.

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