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Isolation and Characterization of *Xanthomonas oryzae* isolates from different regions of Midnapore district of West Bengal and their ecofriendly management by some medicinal plant extracts

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Abstract

The potential of indigenous medicinal plants as alternative chemical pesticides for controlling bacterial leaf blight (BLB) of rice was tested in the present study. Infected plant samples were collected from five different fields of Purba and Paschim Medinipur, West Bengal. The obtained isolates were subjected to biochemical tests which showed similar results. *Xanthomonas oryzae* pv. Oryzae, the causal agent of the bacterial leaf blight of rice was characterized through different biochemical tests and assays. Isolates gave dome shaped, yellow colonies on nutrient agar (NA) plates. Gram staining showed that the pathogen is Gram negative and rod shaped, also potassium hydroxide (KOH) test was performed in order to support the results of Gram staining. Endospore staining showed that the isolated pathogen is a non-spore forming bacterium. Starch hydrolysis test, egg yolk reaction, gelatin hydrolysis test and oxidase test gave negative results against the pathogen. Four different and easily available medicinal plant species such as Adhatoda vasica, Lantana camera, Allium sativum and Citrus limon were screened for antibacterial activity against the isolated pathogen. Based on the biochemical responses and also the activity against different plant extracts, it may be concluded that the causal agent is the same for the bacterial leaf blight of rice in the chosen fields of Purba and Paschim Medinipur and there is no significant difference between the pathogenicity and activity of the isolates from the different fields put to test.

Keywords: BLB, Xanthomonas oryzae, Citrus limon, Adhatoda vasica

Introduction

Rice (Oryzae sativa) is life for millions of people and possibly the oldest domesticated food grain [1]. Over 90% of the world rice id grown and consumed in Asia, where 60% of the world's population lives. Rice accounts for up to 60% of the energy intake of around 3 billion Asians [2]. India has the largest area under rice cultivation (43 million hectares) and with production of 87.80 million tons, next to China. The major rice growing states of India are West Bengal, Uttar Pradesh, Madhya Pradesh, Andhra Pradesh, Karnataka, Kerala, Haryana, Tamil Nadu etc.

Rice crop is prone to number of bacterial diseases among which bacterial leaf blight or BLB caused by *Xanthomonas oryzae* pv. oryzae is a serious problem and threat to rice production in both tropical and temperate rice growing regions due to its high epidemic potential [3]. The disease occurs in the host plant at the seedlings, vegetative and reproductive stages but bacterial leaf blight at the tillering stage causes severe blighting of leaves resulting in yield loss up to 75% depending on weather, location and particular rice cultivar used [4].

The pathogen is seed-borne [5,6] and has been considered as an important quarantine organism in many countries. Sowing infected seeds can lead to reduced germination, vigor and yield. Thus, seed-borne bacteria act as a primary source of inoculums and may lead to extremely high field incidence. A seed infection usually occurs during the three distinct phases of seed production, seed development and seed maturation. The pathogen can infect the seed and developing plant leading to systemic infection [7]. More complete knowledge of the mechanism of transmission may lead to better method of controlling the disease.

Thus, one of the aims of the present study is to find out bacterial leaf blight incidence across rice growing regions of Purba and Paschim Medinipur of West Bengal and to characterize *Xanthomonas oryzae* pv. oryzae biochemically from the isolates collected from different agro-climatic regions of the state

Unsuccessful attempts have been made to manage this disease using chemotherapeutics, which prompted us to develop alternative management strategies. Several broad spectrum bactericides have been recommended as prevention against the bacterial leaf blight. However, the chemicals are expensive and they also affect the beneficial microorganisms present. Plant origin 'bioside' are non-phytotoxic, systemic and easily biodegradable [8-11]. The active compounds from plant act on the pathogen directly [12,13] and induce the systemic resistance in growing plants, which

in turn reduce the disease development [14,15]. Induced systemic resistance activates the multiple defence mechanisms which include increased pathogenesis related (PR) proteins (Peroxidase, chitinase etc.). Phenylalanine ammonia lyase (PAL) and peroxidase are the main enzymes involved in the phenyl-propanoid metabolism[16].

Therefore, in the present study, an attempt has been made to use plant extracts in place of synthetic chemicals not only to reduce the *Xanthomonas oryzae* pv. oryzae incidence, but also to improve the seed quality, evaluate the antibacterial activity of the plants under in vitro and greenhouse conditions and to study the potential induction of systemic resistance in rice by some easily available medicinal plant extracts.

Materials and Methods

Identification of the Diseased Leaves

The disease can be identified by the drying of the leaf tip and inward rolling and twisting of the leaf blade. Linear yellowish strips might be seen on the blade together with marginal necrosis at times. The disease might also extend to the leaf sheaths and culms, killing single tillers or the whole plant. This identification of the BLB infected leaves was done on the basis of the studies of Srivastava and Rao [17].

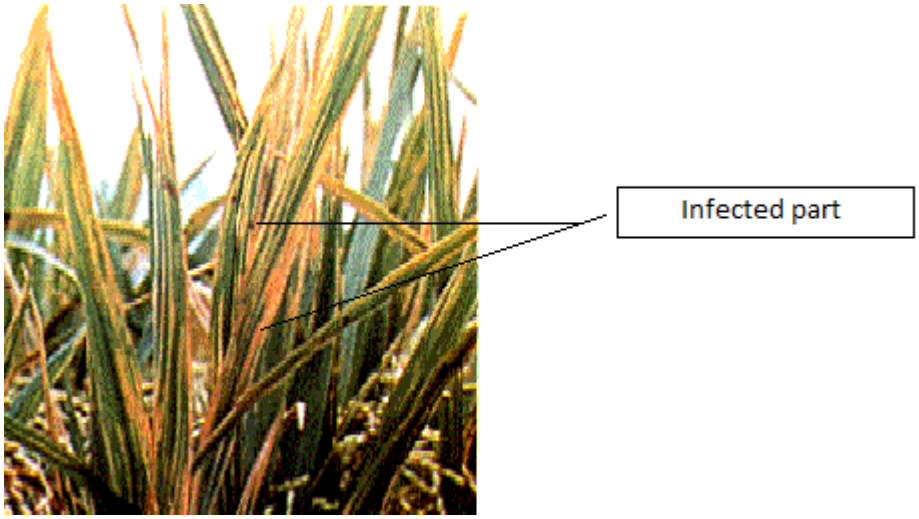


Fig 1: Field infected by *Xanthomonas oryzae*

Collection of diseased leaves

A survey of various rice fields of undivided Midnapore district of West Bengal was done for the collection of leaf samples of rice that are infected with bacterial leaf blight. Five samples of such diseased rice leaves were collected from the fields visited during the harvesting season. Samples were collected from all the areas visited randomly. Diseased leaves were cut and taken in paper envelopes that were labeled explaining the variety, location, and sampling data, and were then put into the plastic boxes. The samples were taken into the laboratory and stored at 4 C for further use. Out of the various collected samples we took just one sample randomly from the collected ones for easy handling.

Isolation of Xanthomonas oryzae

Infected leaves of rice plants were cut into small pieces of about 28 x 7 mm and were cleaned in tap water, and were air dried. These leaf pieces were then sterilized with 1% Sodium hypo chloride solution for three minutes, followed by sterilized distilled water. Now the leaf pieces were dried on sterile blotting paper and were transferred to Nutrient agar medium (NA media) 6-7 pieces in each plate and were incubated at 28-30 C for 24 hr. Some leaf pieces were cut into still smaller pieces of about 5 x 5 mm in size, sterilized in the similar way and were put into 1 ml of sterilized distilled water for about 5 to 10 minutes to allow the bacteria to ooze out from the leaf tissue. By using the sterilized loop needle, the bacterial suspension was streaked onto the Petri dishes containing NA medium. These plates were also incubated in 28-30 C for 24 hr. Single, yellow, round and smooth margined, non flat, mucous colonies developed on the plates. Such colonies were then selected and transferred into slant NA medium as pure culture; for maintenance, and then colonies were also used to spread on NA plates for further use. The single colony was selected as a representative strain for this study. These strains

were maintained at 4 C for further evaluation. Fresh nutrient broths were prepared and each experiment was then performed.

Pathotype Evaluation

The leaves were preserved for further use in sterile distilled water for short time preservation; and for long term preservation, silica gel has been used in the leaf cutting method [18]. For still longer duration of preservation, for further pathotype evaluation, the diseased seedlings were planted into pots containing natural paddy soil. Rice plants have been grown under greenhouse conditions. The disease inoculation was done when rice plants were 40 days old. The bacterial suspensions for inoculation were prepared using 2 days old culture of the isolate in 10 ml of sterilized distilled water. To test the virulence of the strains, the fully expanded healthy leaves of rice plants were inoculated by injecting the overnight culture of the isolated strain.

Diagnostic Tests

Hypersensitivity Reaction

To determine the pathogenic nature of the isolates, hypersensitivity reaction was studied on healthy rice plants. Approximately 24 hours old culture of the isolated strain was injected with a hypodermic syringe into the surface of leaf [19]. Sterile distilled water was used as control.

Biochemical Characterization of *Xanthomonas oryzae*

Gram Staining

The Gram staining is the most widely employed staining method in bacteriology, and it is a differential staining procedure as it differentiates between the two principal groups of bacteria: Gram positive and Gram negative according to their morphology.

A thinly spread air-dried bacterial film was heat fixed on a clean, grease-free glass slide. The specimen was gently treated with Crystal Violet solution for 1 minute, and after that washed with running tap water. Then this specimen was treated with Lugol's iodine as mordant for 1 minute, and rinsed with tap water and then decolorized with 95% ethanol. The specimen was then counter-stained with Safranine for approximately 20 seconds. It was eventually washed with water, blotted dry with a tissue paper, and examined under microscope at 100 X oil immersion[20].

Endospore Staining

A thick spread air-dried bacterial film was heat fixed on a clean, grease free glass slide. The specimen was gently treated with Malachite Green and placed on a warm hot plate, allowing the preparation to steam for 2 to 3 minutes. After the removal of slide from the hot plate, the slide was cooled and rinsed with running tap water. Then the specimen was Counter-stained with Safranine for approximately 20 seconds. It was eventually washed with water, blotted dry with a tissue paper, and examined under microscope at 100 X oil immersion.

Potassium Hydroxide (KOH) Test

Gram staining results were confirmed by potassium hydroxide (KOH) test [21]. The bacteria were aseptically removed from Petri plates with a sterile loop needle, and placed on glass slide in a drop of KOH solution and stirred for 10 second using a quick circular motion of hand.

Starch Hydrolysis Test

Starch is a complex carbohydrate made of glucose which acts as source of carbon for microorganisms which have the ability to degrade them. Some bacteria possess the ability to produce amylase that breaks starch into maltose. The amylase is an extra cellular enzyme which is released from the cell of microorganisms. Starch hydrolysis test is used to identify the bacterial species having the property of amylase production. Powdered nutrient agar was dissolved in water by extreme heating. Starch was then dissolved in distilled water separately and added to the molten agar with constant stirring. An aliquot of this basal medium was dispensed in conical flask and sterilized for 10 minutes. The medium was poured in Petri plates into which an overnight culture of the isolated strain was streaked properly and incubated at 37 C for 24 hours. The plates were then flooded with Lugol's iodine(i.e., prepared by dissolving iodine along with potassium iodide in distilled water, and stirred for several hours until dissolved completely [22]. It is known that a blue-black color appears due to formation of starch-iodine complex. Absence of the blue-black color indicates that starch is absent. If the areas around streaked culture remained clear it indicates the degradation of starch. And hence it would be due to the production of exoenzyme amylase, which cleaves the starch into di- and monosaccharides and vice versa.

Egg Yolk Reaction

Egg yolk agar is an enriched non-selective and differential medium that contains egg yolk for presumptive identification. The egg yolk suspension allows for the detection of lecithinase and lipase activity. The degradation of lecithin in the egg yolk results in an opaque colonies. The Lipase enzyme hydrolyzes the fats within the egg yolk, which results in a sheet on the colony surface. Another common reaction observed is proteolysis of the egg yolk as indicated by a clearing of the medium around the colonies.

Egg yolk emulsion was prepared from a fresh egg, washed well in soap solution, rinsed and surface sterilized with 70% ethanol for 5 minutes. Then the egg was flamed, broken aseptically, yolk separated in to sterile graduated cylinder and diluted to 40 %v/v NaCl, both taken with an equal volume. An aliquot of egg yolk was incorporated into molten nutrient agar prior to pouring in to the plates. The medium was spotcultured and incubated for 24 hours at 37 C [23].

Gelatin Hydrolysis Test

Gelatin is a polymer of amino acids and the protein is used as nitrogen and carbon source for microorganisms. The gelatin is usually broken down into peptides of short amino acids polymers and amino acids can be transported into the cell. Use of gelatin is

accomplished by the enzyme gelatinase. When gelatin is used; the medium changes from semisolid to liquid and cannot be resolidified.

At first, nutrient broth is prepared, into which 12% gelatin was added converting it into a semisolid medium and then the medium was autoclaved. Then the media was poured into the sterile petri plates, and gently streaked with bacterial culture on the plates, after that it was incubated at 37 C for 24 hours. After incubation, the plates were then flooded with tannic acid. It is known that if tannic acid will precipitate the gelatin then the plate become opaque and hence, the test was said to be positive.

Tetrazolium Salt Tolerance Test

Nutrient agar (NA) was prepared and dispensed in flasks and sterilized at 121 C for 15 minutes. Aqueous 1% triphenyl tetrazoliumchloride (TTC) solution was prepared by adding 1 gm TTC reagent in 100 ml sterile distilled water. TTC of different concentrations like 0.1%, 0.01%, and 0.02 % were also prepared by adding 5 ml of TTC solution in the 20 ml of molten agar medium that gave a concentration of 0.1%, and similarly by adding 1 ml or 0.5 ml of TTC solution in the 20 ml molten agar medium of each plate that also gave a concentration of 0.02% and 0.01% respectively. The medium was poured into each plate, and inoculum was added to the medium held at three different concentrations, whereas, one plate of nutrient agar was used as control. Presence or absence of growth was recorded by notifying that, in which concentration (0.1%, 0.01%, and 0.02%) most *Xanthomonas oryzae* were partially or completely inhibited.

Oxidase Test

Nutrient agar was supplemented with 1% glucose, sterilized, dispensed, and poured in the plate with a loop full of bacterial suspension, and incubated for 24 hours at 37 C. Oxidase test needs 24 hours old culture. Then, upon a slide, a loop full of the inoculum from the plate was rubbed on filter paper that was impregnated with 15% (w/v) freshly prepared aqueous solution of Tetramethyl-p-phenylene diamine dihydrochloride [24]. It is known that, if the colonies first become pink and then change to dark red and finally turn to black, it indicates the bacterium to be oxidase positive. The pink color was generally obtained within 15-30 seconds of pouring of the reagent. Thus the organism would be gram-positive. If no color was formed, then the result was negative, so the organism would be Gram-negative.

Catalase Test

Most of the organisms show catalase activity. Organisms utilize oxygen to produce hydrogen peroxide. This hydrogen peroxide is toxic to their enzyme system. Hence these organisms produce an enzyme, called catalase, which converts the hydrogen peroxide to water and oxygen.

A colony of the isolated strain was picked from the plate and transferred on a glass slide in a drop of water and , a few drops of 3% hydrogen peroxide (dilute 30% commercial solution 1: 30)was added over the culture. If any bubble appears within 20 seconds, the organism is said to possess catalase activity. Absence of bubble shows negative catalase activity.

Conc. Sulfuric Acid Test

An overnight culture of the isolated strain was placed on the grease free slide, and then concentrated sulfuric acid was applied drop by drop on this specimen.

If the culture becomes transparent then the test would be said to be positive.

Physical Characterization of *Xanthomonas oryzae*

Growth Curve

Bacterial growth pattern can be studied in vitro. Depending upon nutritional status, bacteria exhibit different growth patterns. In a freshly inoculated nutrient broth, bacteria take time to adjust in the new environment. This gap of time is called lag phase. Thereafter, it uses the nutrients of the medium and multiplies very fast, showing exponential growth. This period is called the log phase. After that the growth becomes stagnant, this stage is called the stationary phase. After a few days nutrients in the medium starts diminishing; therefore, fresh medium containing nutrients should be added. If these are not added, growth rate declines, this stage is known as the decline phase. The density of cell suspension is expressed as absorbance or optical density (OD) which is directly proportional to the cell concentration and is measured in spectrophotometer. OD is a logarithmic value and is used to plot a graph of bacterial growth.

1 ml of fully grown culture of *Xanthomonas oryzae* from the broth culture prepared earlier is dispensed in 50 ml of nutrient broth medium taken in a 250 ml of Erlenmeyer flasks. Then the flasks were placed in the shaker at 150 rpm at 37 C. The OD values were measured in spectrophotometer at 630 nm wavelength at an interval of 12 hours. Un-inoculated growth medium was treated as blank. Finally growth curve was prepared by plotting in terms of OD against time (hours).

Transverse Leaf Section of Infected Paddy Leaf

The transverse sections of the infected leaves were done and observed under microscope at 10X.

Antimicrobial Activity of Selected Plant Extracts on *Xanthomonas oryzae*

Selection of Medicinal Plants

In the present work, Citrus limon, Adhatoda vasica, Lantana camara, and Allium sativum has been studied.

Plant Extracts

The extracts of all the selected medicinal plants were used to determine the titer value on (BLB). The medicinal plants have been collected from different regions of West Bengal.

Table 1: Selection of Plant material

Common Names of the Plants Studied	Scientific Name of the Plants Studied	Plant Parts that have been used	Prepared extracts of these plants parts
1. Basak	*Adhatoda vasica*	Leaf	Aqueous and Ethanolic extracts
2. Bhoot bhairabi	*Lantana camara*	Leaf	Aqueous and Ethanolic extracts
3. Garlic	*Allium sativum*	Bulb	Aqueous and Ethanolic extracts
4. Lemon	*Citrus limon*	Juice	Aqueous extract

Extraction Methods

Preparation of Extracts

Plant samples were collected in cotton bags, then all the plant parts were cleaned with tap water and surface sterilization was done with 95% ethanol. All plant parts were again washed with sterile distilled water for 20 minutes, and then there materials were air-dried. The materials were ground to fine powder with the help of a mixer grinder. These powdered materials were used for the preparation of aqueous and ethanolic extracts.

Preparation of Aqueous Extracts

At first, 15 gm of dry powder of plant samples were taken in an airtight container with 100 ml of sterile distilled water, and then it was kept for 4-5 days in a rotary shaker with occasional shaking. Plant extracts were filtered in muslin cloth and the filtrate was then centrifuged at 2000 rpm for 10 minutes. The supernatant was then collected and stored at 4 C.

Aqueous extracts of all the four plants were processed in the same way.

Preparation of Ethanolic Extracts

At first, 15 gm of dry powder of plant samples were taken in an airtight container with 100 ml of 95% ethanol, and then it was kept for 4-5 days in a rotary shaker with an occasional shaking. The plant extracts were then filtered by muslin cloth and the filtrate was then centrifuged at 2000 rpm for 10 minutes. The supernatant was collected and stored at 4 C.

Ethanolic extracts of the following three plants A. vasica, L. camara, A. sativum parts were also processed in the same way.

Antibacterial Assay

Determination of Titer Value by Minimum Inhibitory Concentration (MIC)

MIC of plant extracts would be determined depending on their inhibition of the growth of the organism Xanthomonas oryzae.

Nutrient broth of 2X was prepared. Nutrient broth of about 2ml was transferred in each test tube. All the test tubes were sterilized at 121 C for 15 minutes. Each plant extract was diluted in 1:1 ratio. Then 2 ml of plant extract was transferred into 2 ml of nutrient broth, marked as tube 1. Then subsequently 2 ml of nutrient broth was transferred in rest of the tubes. Also, blank (nutrient broth + plant extract) and control (nutrient broth + culture) for each extract was prepared. This process was repeated for all the plant extracts. Therefore, each test tube was inoculated with 2 ml of culture except the blank. Test tubes were then incubated at 37 C for 24 hours. The turbidity was measured in terms of optical density by spectrophotometer at 630 nm.

Agar- well Diffusion

The sensitivity against these plant extracts was tested on this organism. This helps in selecting the appropriate line of treatment on this disease. The effectiveness was based on size, inhibition zone. However, zone may vary due to diffusibility of extracts, size of inoculums, type of medium, etc.

At first nutrient agar was prepared and then sterilized at 121 C for 15 minutes, and after sterilization the plates were marked as aqueous, ethanolic extracts, and separate plate for lemon (aqueous extract). Then 1 ml culture dispensed into molten agar medium and poured into the plates (3 plates) and allowed to solidify. Therefore, wells were made with the help of a puncture in each solid agar plate. Then, on every plate 20 microlitre of each crude extract was poured respectively. Finally, all plates were incubated for 24 hours at 37 C.

Paper Disk Method

The effectiveness of each extract was determined accurately by disc diffusion method.

At first nutrient agar was prepared and then sterilized at 121 C for 15 minutes, and after sterilization the plates were marked as aqueous, ethanolic extracts, and separate plate for lemon (aqueous extract). Then 1 ml culture dispensed into molten agar medium and poured into the plates (3 plates) and allowed to solidify. Sterile filter paper was separately impregnated in each extract and placed on the agar surface and the disc was pressed slightly on the surface of the medium. Finally, all plates were incubated for 24 hours at 37 C. After incubation if the extracts were

more effective, a large sized clearing zone would surround the disc.

Results

Isolation of *Xanthomonas oryzae*

Five different samples were taken from five different regions of undivided Midnapore districts of West Bengal. The bacterial culture was grown in nutrient agar medium. They produced colonies that were yellow, convex, mucoid and shiny in texture. Such colonies were consistently observed and tentatively identified as the causal organism.

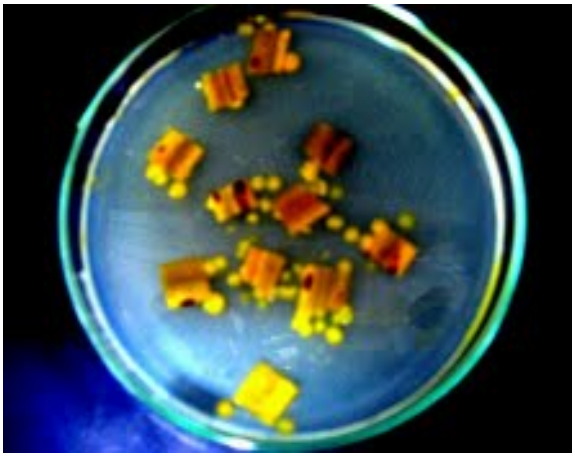


Fig 2: Isolation of *Xanthomonas oryzae*

Disease assessment

The length of the lesion of cut leaf tip was measured in centimeters (cm) on 18th day after inoculation. The lesion length of 0-6cm has been classified as resistant (R) and that which is more than 6cm has been classified as susceptible (S). [25].

Characterization of Xanthomonas oryzae

Physical characterization of *Xanthomonas oryzae*

a) Morphology of infected leaf

Infected leaf has yellow water soaked lesions at the margin of its leaf blade. The lesions run parallel along the leaf and when they join together may cover the whole leaf.

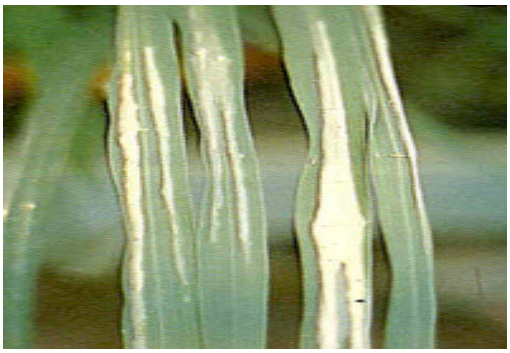


Fig 3: Showing the Bacterial leaf blight infected leaves of a rice plant

b) Transverse leaf section of the infected leaf

Below the hypodermal zone mycelial mat can be seen.

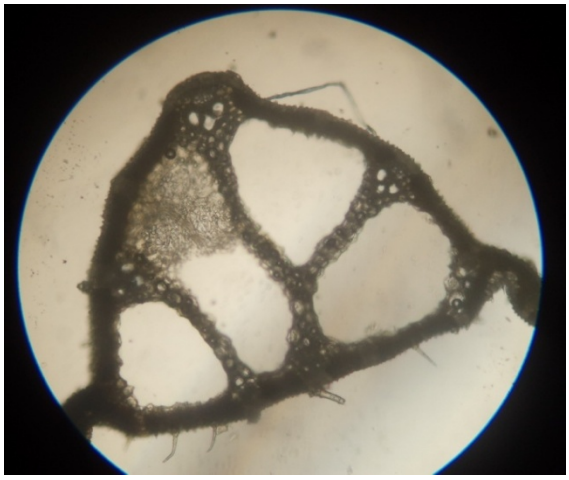


Fig 4: Showing the infected areas on the leaf blade of the studied rice plant.

c) Growth curve

Lag phase exists for 12hrs. Then it moves to log phase as shown in the graph. Then to stationary phase which exists for 18hrs. Reading was taken at an interval of 6hrs till decline phase was reached.

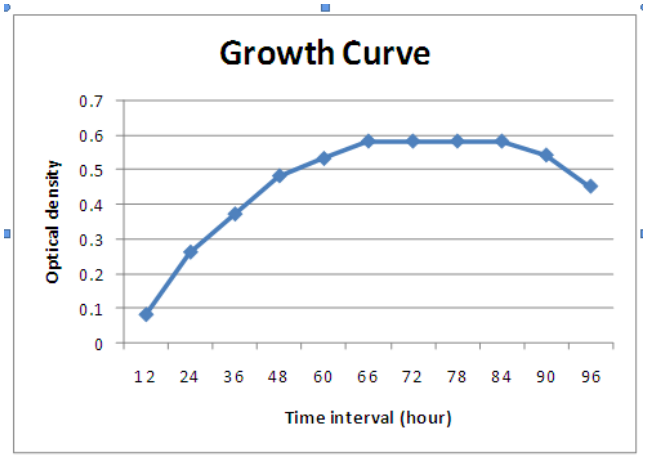
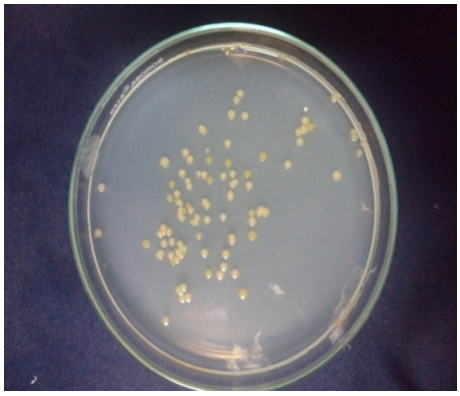


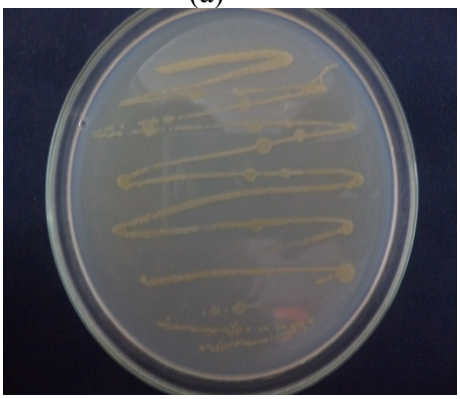
Fig 5: The growth curve of the isolated strain of *Xanthomonas oryzae*

d) Colony morphology

Colonies were found to be light yellow in color, slime in nature, circular, convex, and smooth in appearance.



(a)



(b)

Fig 6: Isolated strain of *Xanthomonas oryzae* A) Spread plate showing the single colonies (10^{-4} dilution); B) A streak plate from the overnight culture.

e) Hypersensitivity reaction test

The isolated strain of *Xanthomonas oryzae* showed typical hypersensitive reaction on a leaf. After 24 to 48 hr of injection the injected leaf area became necrotic and in 3-4 days, the treated tissue was entirely dry and yellow.

(a)

(b)

Fig 7: The hypersensitivity reaction on a leaf. (A) Before injection and (B) 48 hrs after injection.

Biochemical characterization

a) Gram staining

The overnight culture of isolated strain of *Xanthomonas oryzae* used for Gram staining which showed that the organism is Gram-negative, rod shaped bacterium with a polar flagellum.
Cells appear red, singly or in pairs, or sometimes even in chains as has been showed in the photograph below.

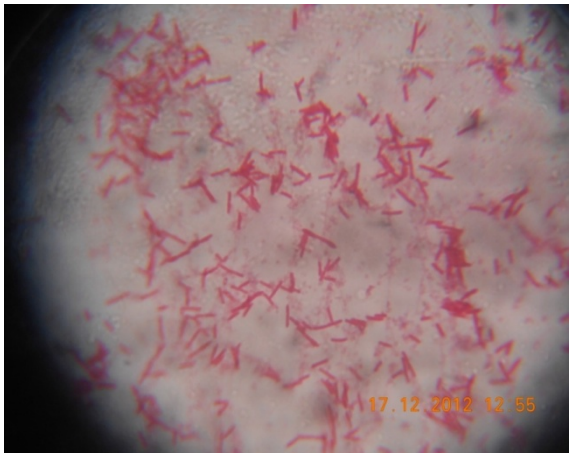


Fig 8: Gram stained slide of the isolated strain.

b) Endospore staining

The endospore staining of the isolated strain of *Xanthomonas oryzae* showed it to be a non-spore forming organism.

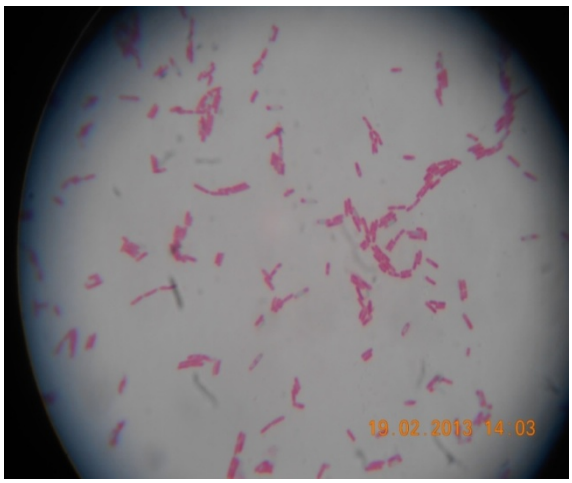


Fig 9: Endospore stained slide of the isolated strain (100X)

c) Most probable number (MPN)

The MPN test performed with the isolated strain of *Xanthomonas oryzae* showed that there was no production of gas in both single and double concentrations of lactose broth. Hence, it can be confirmed that *Xanthomonas oryzae* is not a coliform bacteria.

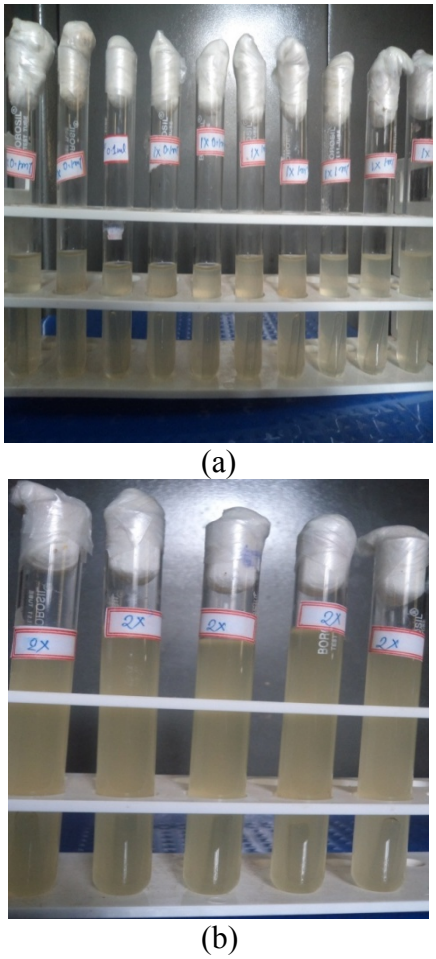


Fig 10: MPN done with different dilutions of the overnight culture of the isolated strain of Xanthomonas oryzae in (A) 1X lactose broth and (B) 2X lactose broth

d) Potassium Hydroxide (KOH) test

This test was performed to confirm the Gram stain result. When a drop of KOH was added to a single colony of an overnight culture it became viscous whereas when a drop of conc.H_2SO_4 was added to the same culture isolated from the overnight culture it became clear and transparent. This supported the fact that the isolated bacterium is Gram negative in nature as found in the Gram staining experiment.

e) Starch hydrolysis test

After the addition of iodine it was observed that the dark blue or black color of iodine surrounding the bacterial growth did not bleach indicates the absence of starch hydrolysis. Thus starch in the medium has not been hydrolyzed by the organism. This concluded that the isolated strain of *Xanthomonas oryzae* did not produce the extracellular enzymes i.e., alpha-amylase.

Fig 11: Starch hydrolysis test for the isolated strain of *Xanthomonas oryzae.*

f) Egg yolk reaction test

The bacterium *Xanthomonas oryzae* was put to egg yolk reaction test to see if it produces lecithinase. The test was positive as the bacteria formed white opaque colonies.

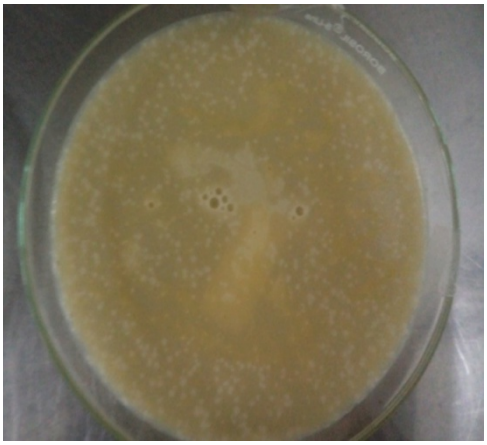


Fig 12: Egg yolk reaction test for the isolated strain of *Xanthomonas oryzae.*

g) Gelatin hydrolysis test

By this test it was seen that gelatin was not hydrolysed by the isolated organism probably because it does not possess gelatinase activity. Formation of white precipitate occurred within 5minutes when 1% tannic acid was added to the medium which showed that the test was negative.

Fig 13: Gelatine hydrolysis test of the isolated strain *Xanthomonas oryzae.*

h) Tetrazolium salt tolerance test

In this test presence or absence of growth was recorded and it was found that most Xanthomonads were inhibited at 0.01% and 0.02% triphenyl tetrazolium chloride (TTC) but completely inhibited at 0.1% concentration of the same.

i) Oxidase test

Oxidase test proved to be negative in case of the isolated strain of *Xanthomonas oryzae*. They failed to produce any color.

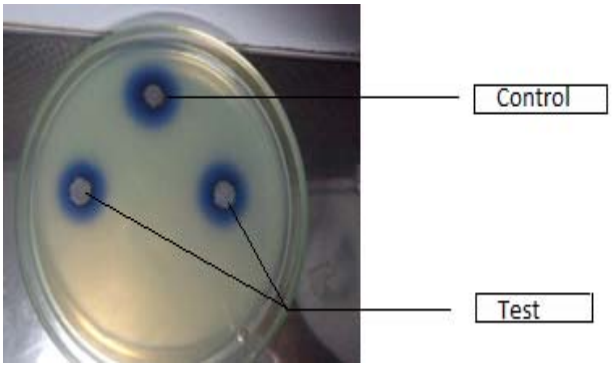


Fig 14: Plate showing oxidase test for the isolated strain of *Xanthomonas oryzae.*

j) Catalase test

The bacterium *Xanthomonas oryzae* gave a positive catalase test. When a drop of hydrogen peroxide was added to its culture, oxygen bubbles were released.

Hence, the results of the biochemical tests can be summarized as below:-

Table 2: Summary of the results of biochemical tests.

Biochemical tests	Results
Gram staining	Negative
Endospore staining	Non-spore forming
Most probable number(MPN)	No gas formation(they are non-coliform)
Potassium hydroxide test	Gram negative confirmed
Starch hydrolysis test	Negative
Egg yolk reaction test	Positive
Gelatine hydrolysis test	Negative
Tetrazolium salt hydrolysis test	Totally inhibited at 0.1% of triphenyl tetrezolium chloride(TTC)
Oxidase test	Negative
Catalase test	Positive

Table 3: Data of MIC with different extracts

Serial number	Dilution number	Optical density						
		Citrus limon	*Allium sativum* (Aq)	*Allium sativum* (Eth)	*Lantana camara* (Aq)	*Lantana camara* (Eth)	*Adhatoda vasica* (Aq)	*Adhatoda vasica* (Eth)
1	10^{-1}	0.09	0.09	0.02	0.21	0.01	0.22	0.01
2	10^{-2}	0.15	0.52	0.04	0.22	0.05	0.28	0.09
3	10^{-3}	0.20	0.46	0.24	0.23	0.06	0.32	0.14
4	10^{-4}	0.23	0.41	0.26	0.26	0.08	0.26	0.13
5	10^{-5}	0.21	0.39	0.29	0.24	0.15	0.16	0.12
6	10^{-6}	0.14	0.38	0.27	0.23	0.07	0.15	0.06
7	10^{-7}	0.11	0.36	0.24	0.20	0.05	0.13	0.03
8	10^{-8}	0.07	0.34	0.22	0.18	0.04	0.10	0.02
Titer value		0.09	0.09	0.02	0.21	0.01	0.22	0.01

Antimicrobial activity of selected plant extracts on *Xanthomonas oryzae*:-

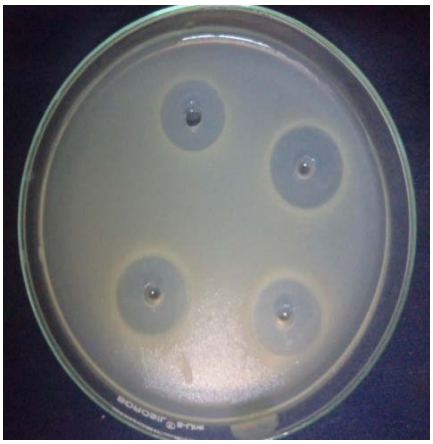
a) Determination of titre value by Minimum Inhibitory Concentration(MIC)

b) Agar well diffusion method

This test was done to see the formation of a clear zone around the agar well on the nutrient agar plate by the interaction of the plant extract in the well and the organism in the medium.

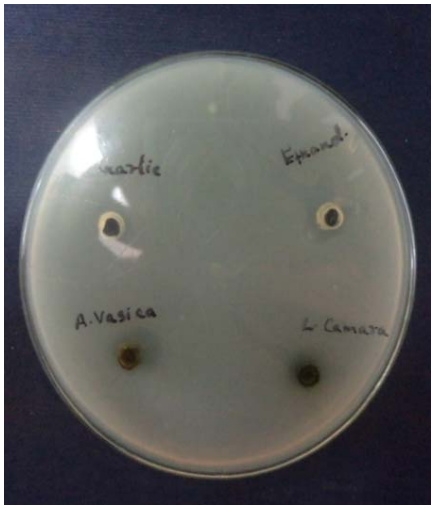
Table 4: The results obtained from the respective plant extracts.

Plant extracts put to test	Diameter of the obtained clear zone(cm)
***Citrus limon* (aqueous extract)**	1.9
***Adhatoda vasica* (aqueous extract)**	1.9
***Allium sativum* (aqueous extract)**	0.8
***Lantana camera* (ethanolic extract)**	0.2



(a)

(b)



(c)

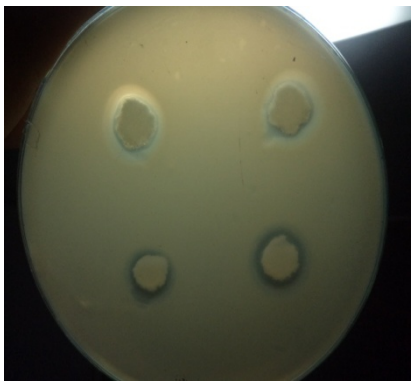
Fig 15: The plates show the results of agar well diffusion.
Activity of *Citrus limon* (aqueous extract); B) activity of aqueous extracts; C) activity of ethanolic extracts.

Hence, it can be concluded that *Citrus limon* (aqueous extract) and *Adhatoda vasica* (aqueous extract) showed maximum inhibitory activity and is more effective than the other two extracts put to test against the isolated strain of *Xanthomonas oryzae.*

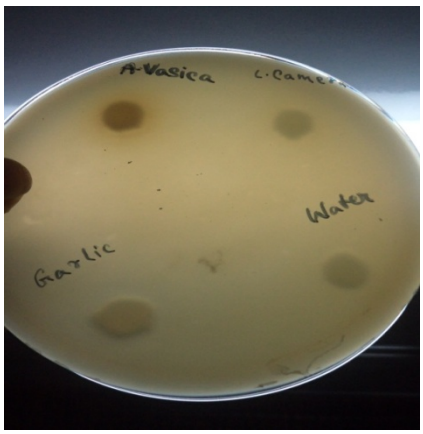
c) Paper disc method

Similar to the agar well diffusion, this experiment was done on the same principle the only difference being that the extracts were soaked in filter paper discs and kept on the plated medium with the organism in it. Formation of clear zone around the paper disc on the plate occurred.

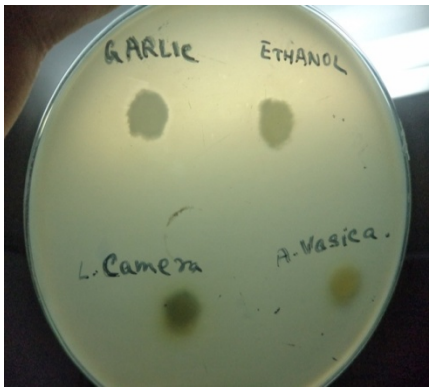
It also showed similar results as the agar well diffusion, i.e. *Citrus limon* (aqueous extract) showed maximum activity forming a clear zone of diameter about 1.2cm, followed by , *Allium sativum* (aqueous extract), 1cm and *Allium sativum* (ethanolic extract), 0.9cm.This was a bit different from what was found in the earlier case.



(a)



(b)



(c)

Fig 16: Plates show the results of paper disc method with the plant extracts on the isolated strain of *Xanthomonas oryzae.* A) *Citrus limon* (aqueous extract); B) aqueous extracts; C) ethanolic extracts.

Discussion

The causal bacterium *Xanthomonas oryzae* was isolated from green rice leaves with BLB lesion from five different regions of undivided Midnapore district. BLB of rice is a destructive, wide spread disease and is a threat to rice production in rice growing regions like Midnapore, West Bengal. The disease occurs in the host plants at the seedling, vegetative and reproductive stages, but BLB infection at the tillering stage causes severe yield loss of upto 75% depending on weather, location and particular rice cultivar [4]. In our study none of the field surveyed were free from disease (BLB).

In an attempt to screen and confirm different rice leaf samples for the incidence of *Xanthomonas oryzae*, we have used diagnostic tests like hypersensitivity reaction and biochemical tests like Gram staining, endospore staining, KOH test, starch hydrolysis test, egg yolk test, gelatin hydrolysis test, tetrazolium salt tolerance test, oxidase test, catalase test and conc. H_2SO_4 test. Physical characterization was also done using growth curve analysis and transverse leaf section of infected paddy leaf. These results suggest that the isolates obtained from different field do not differ in their degree of virulence. In the present study, we also tested four different plant species which belong to different families for antibacterial activity against bacterial leaf blight pathogen, *Xanthomonas oryzae pv. Oryzae* and they are commonly available throughout India.

Aqueous extract of *Citrus limon* and *Adhatoda vasica* was found to be highly effective in inhibiting the growth of *Xanthomonas oryzae* compounds to the aqueous extracts of *Allium sativum* and *Lantana camera.* Narasimham *et al.,* and Kagale *et al.,* have reported antibacterial activity and management of bacterial diseases with use of different plant extracts in other crops[11].

The present study clearly demonstrates that leaf blight disease is prevailing in all the surveyed regions of the Midnapore with varied degree of disease incidence.

In this study, we have demonstrated antibacterial activity of *Citrus limon* extract against Bacterial Leaf Blight disease caused by *Xanthomonas oryzae pv. Oryzae* in rice.

Hence, the present work suggests that use of *Citrus limon* and *Adhatoda vasica* extract is safe in the management of Bacterial Leaf Blight disease in rice.

Future studies are needed to understand the mode of infection of this pathogen and ecological behaviour of *Xanthomonas oryzae,* and its host cultivar is required to develop sound control strategies.

Conclusion

Xanthomonas oryzae pv oryzae has been isolated from green leaves of pady plants with Bacterial leaf blight lesion. The isolated organism has been studied both physically and biochemically. The botanical study of plant is important for modern day medicine but its usefulness cannot be overemphasized if methods are not standardized to obtain comparable and reproducible results. Plants contain thousands of constituents and are valuable sources that possess antimicrobial properties. It has been found that this infectious organism can be controlled by treatment with plant extracts. At present, many scientists are investigating for plant

products with antimicrobial properties. It would be advantageous to standardize methods for extraction and *in vitro* antimicrobial efficacy testing so that the search for new biologically active plant products could be more systematic and interpretation of results would be facilitated. Among all the plant extracts used in our study, two plant extracts such as *Citrus limon* (aqueous extract) and *Adhatoda vasica* (aqueous extract) showed maximum inhibitory effects on the casual organism of Bacterial leaf blight of rice.

Author's Contributions

TT participated in the design of the study and helped in the final drafting of the manuscript. PS maintained the isolated organisms and also helped in framing the manuscript. AD performed all the other tests and coordinated to draft the manuscript. All authors have read and approved the final manuscript.

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