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Bio chemical profiling of isolates of *Xanthomonas vasicola pv. arecae.* causing bacterial leaf stripe disease of arecanut

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Abstract
Bacterial lea stripe disease of arecanut caused by *Xanthomonas vasicola* pv. *arecae* is one of the most destructive disease of arecanut (*Areca Catechu*). *Xanthomonas vasicola* pv. *arecae* was isolated from major arecanut growing districts of Karnataka. The biochemical studies indicated that, the pathogen isolates were positive for KOH, catalase, gelatin liquefaction, starch hydrolysis, methyl red, H_2S production and ammonia production tests. Whereas, pathogen isolates were found to be negative for negative for Gram's reaction and fluorescent test.

Keywords: Bacterial leaf stripe, biochemical test, *Xanthomonas vasicola* Pv. *arecae*

Introduction
Arecanut (*Areca catechu* L.) is a palm that belongs to the family Arecaceae, which is grown in most parts of the tropical Asia and East Africa. The term Areca is derived from the Malayan language, which means "cluster of nuts." Arecanut is an important plantation crop that plays an important role in supplying livelihood and nutritional security to millions of people in India and contributes significantly to the national economy and foreign exchange. It also provides direct and indirect employment to millions of people and supplies a wide range of raw materials for various rural and small-scale industries (Chowdappa *et al.,* 2014) [2]. Arecanut is popularly known as betel nut. The raw fruits and their value added industrial products are used for mastigatory purpose along with betel leaves. Since arecanut is a perennial crop, it is exposed to various pests and diseases around the year at all crop growth and development stages. Climate change and shift in arecanut cultivation from traditional arecanut cultivation regions to nontraditional localities, have lead to emergence of new diseases. Among the diseases that infects arecanut, bacterial leaf stripe of arecanut is an emerging and serious disease that infects during the early stage of arecanut crop. Under severe infection it leads to death of the palm. Considering the serious threat posed by the disease and meager work done, the present investigation was carried out to understand the biochemical characters that are necessary for detection, diagnosis and to study the variability among its isolates.

Material and Methods
Isolation of the pathogen
Infected plant parts showing typical symptoms of bacterial leaf stripe disease were collected from different arecanut growing districts of Karnataka. The pure culture of X. vasicola pv. Arecae was isolated (Schaad, 1992) [8] from infected leaf and designated as Xva-1 to Xva-12 (Table 1). The isolated cultures were stored in refrigerator at 5°C and renewed by sub culturing once in a fortnight on Nutrient Glucose Agar media (NGA).

Biochemical Characterization of isolates of *Xanthomonas vasicola* pv. *arecae*
Gelatin liquefaction
Fifteen ml of freshly prepared and autoclaved nutrient agar was added with 0.4 per cent (4 g / 1000 ml) gelatin and poured into sterilized petriplates. After the solidification of the media, spot inoculation of the isolates using sterilized tooth prick on the surface of the medium was made and the plates were incubated at 27 ± 1 ºC for three days. After the incubation period, plates were flooded with 10 ml of acid mercuric chloride solution

($HgCl_2$, 12 g, Distilled water, 80 ml, Concentrated HCl, 16 ml). And observations were made for the formation of a clear zone around the bacterial colonies.

Catalase test

A loopful of 72 to 96 hours old active culture slant growth of the test bacterium was smeared on a clean glass slide and flooded with a few drops of 20 per cent hydrogen peroxide. And the observations were made for the formation of gas bubbles as per the procedure.

Hydrogen sulphide (H2S) production

The peptone water medium comprising of Peptone 10 g, NaCl 5.0 g, water 1000 ml and pH 7.0 was dispensed in 5 ml quantities in tubes and autoclaved. To detect H_2S, the lead acetate test strips were prepared as follows. Whatman No. 1 filter paper was cut into 5 × 50 mm strips which were then soaked in warm saturated solution of lead acetate. The strips were then dried, autoclaved and again dried at 60 °C.

The medium in each tube was inoculated with a loopful of 72 to 96 hours slant grown test bacterium. After inoculation, the test strip was inserted in between the plug and inner wall of the tube, so that it hangs just above the broth. The tubes were incubated at 25 °C and the observations were recorded at regular intervals up to 14 days. The blackening of the test strip indicates the liberation of H_2S.

Starch hydrolysis

The medium employed is referred to as starch broth medium which contains, Peptone (10.0 g), beef extract (5.0 g), starch soluble (2.0 g), agar (20.0 g), water (1000 ml) and pH (7.0). The medium was sterilized by autoclaving and poured into sterilized petri plates. The medium was allowed to solidify and spot inoculated or streaked with the test culture and the plates were incubated at 25 °C. After four days of incubation, plates were flooded with Lugol's iodine and allowed to react for few minutes to observe the development of a colourless zone around the bacterial colonies.

Methyl red test

Methyl red indicator (0.1 g methyl red dissolved in 300 ml of 95 per cent ethanol and the volume was made up to 500 ml by adding distilled water) was added to test culture and observations were made with respect to change in colour.

Ammonia production

A test tube containing eight ml of sterile nutrient broth was inoculated with the test culture and one tube was kept uninoculated as a control and were incubated at 27 °C for 72 to 96 hours. After the inoculation, the cotton stopper from the tubes were removed and the strip of red litmus paper was inserted inside the wall of the tube and stopper was replaced as earlier to hold the stripe of litmus paper in place. Observations were made after four days of inoculation.

KOH test

The bacterial culture was taken with a bacterial loop which was vigorously stirred in a drop of 3 per cent KOH solution. Upon lifting, the loop thread-like slime formation appears that indicates a positive reaction.

Fluorescent test

Test bacterium was streaked on a petri plate containing King's B medium and two days after incubation, plates were observed under a fluorescent microscope for further studies.

Composition of Kings-B agar

Peptone	:	16.0 g
K_2HPO_4	:	1.60 g
$MgSO_4$	:	1.60 g
Glycerol	:	10.0 ml
Agar	:	20.0 g
Distilled water	:	1000 ml

Gram reaction

Test culture was smeared over a clean slide and then heat fixed and followed by transfer of one drop of crystal violet stain on the smear and allowed for two minutes. Then the slide was gently washed with tap water and air dried for few seconds. Then Gram's iodine gently flooded and allowed for 1 minute. Then the slide was again washed gently in tap water and air dried. Upon drying, 95 per cent ethyl alcohol, a decolorizing agent, was added and allowed for two minutes. Later secondary stain, safranin was added and allowed for one minute, then washed gently and air dried. Then the slide was observed under a microscope for gram reaction.

Table 1: Isolation and designation of *Xanthomonas vasicola* pv. *Arecae* isolates

Districts	Location	Designation	Latitude	Longitude
Shivamogga	Melina Kunchenahalli	Xva - 1	13.99430	75.58691
	Hirejambur	Xva - 2	14.31092	75.38364
	Aladalli	Xva - 3	14.02561	75.61987
	Tumari Hosur	Xva - 4	14.32645	75.35102
Davanagere	Gangagondanahalli	Xva - 5	13.02036	77.46721
	Gedlatti	Xva - 6	14.20221	76.00118
	Belagutti	Xva - 7	14.08449	75.34158
	Salabalu	Xva - 8	14.12635	75.54961
Chikkamagalur	M. C. Halli	Xva - 9	13.76723	75.76453
	Emme Doddi	Xva - 10	13.51205	75.90147
	Gondedahalli	Xva - 11	13.70401	75.98633
Chitradurga	Gunderi	Xva - 12	14.02934	76.19758

Results and Discussion

The biochemical properties of the twelve isolates of *Xanthomonas vasicola* pv. *arecae* with respect to KOH test, starch hydrolysis, catalase test, methyl red test, gelatin liquefaction test, hydrogen sulphide production test, ammonia production test, gram reaction and fluorescence test were studied following the procedures given in material and methods.

The results obtained on various biochemical characteristics of the pathogen are presented in Table 2. revealed that the bacterium was positive for KOH test, catalase test, gelatin liquefaction, starch hydrolysis, H_2S production test, methyl

red test and ammonia production test and negative for Gram's reaction and fluorescent test (Plate 1, 2 and 3).

Hydrogen sulphide production

All the isolates showed positive reaction to the H_2S production test (Table 3 and plate 4). Based on H_2S production 14 days after inoculation, isolates were categorized into three Groups *viz.,* Strong H_2S production (Xva-2, Xva-5, Xva-6, Xva- 8 and Xva-10), Medium H_2S production (Xva-3, Xva-9 and Xva-12). And weak H_2S production (Xva-1, Xva- 4, Xva-7 and Xva-11) (Table 23).

The results of the present study revealed that the arecanut leaf stripe disease causing bacterium could hydrolyze the starch, liquefied the gelatin and it was positive for ammonia production, H_2S production, methyl red test, catalase test, KOH test and negative for Grams reaction and fluorescent test.

The bacterium hydrolyzes the starch because of the ability to produce higher quantity of extracellular enzyme amylase, which is involved in the hydrolysis of starch and bacteria produced clear colour less zone around the bacterial growth on starch agar medium when Lugol's iodine solution is applied on the medium after 72 to 96 hours of incubation. (Lammert, 2007) [5].

All the isolates were positive for H_2S production. However, Strong H_2S production was noticed in the isolates *viz*., Xva-2, Xva - 5, Xva-6, Xva- 8 and Xva-10. Whereas, the isolates Xva-1, Xva- 4, Xva-7 and Xva-11 were weak in H_2S production. Higher H_2S production may be due to the ability of these isolates to liberate higher quantity of H_2S by dissimilation of sulphur containing amino acids like cysteine and methionine present in peptone (Lammert, 2007) [5]. Thus, the isolates showed variation in their ability to produce H_2S.

The catalase enzyme converts hydrogen peroxide (H_2O_2) into water and O_2, resulting in the liberation of O_2 gas bubbles and are positive for catalase reaction.

Due to the production of extracellular proteolytic enzyme gelatinases, *Xanthomonas* could easily liquefy by hydrolyzing the gelatin in the medium and shown positive reaction for the gelatin liquefaction test.

The bacterium utilizes the peptone present in the medium and results in the production of ammonia and CO_2. The formation of ammonia alkalinizes the medium and pH shift is detected when red litmus paper turns blue color.

KOH is a primary test performed to differentiate gram +ve and gram -ve bacteria within a short period of time. The cell wall of gram –ve bacteria is very much sensitive to KOH. When bacterial cells are subjected to KOH reaction, it leads to the destruction of the cell wall and subsequent liberation of DNA, which is very viscid in water and produces the string of slime. However, the gram + ve bacterial cell wall is more resistant to KOH. Hence the bacterial slime is not produced in gram +ve bacteria. Xanthomonads are gram negative bacteria and thereby showing positive reaction to the KOH test.

Methyl red test detects the production of sufficient acid like lactic acid or acetic acid or formic acid during the fermentation of glucose. The production of acids leads to decrease in the pH to 4.5 or below, which is indicated by the change in colour from yellow to red.

Gram's staining is an empirical method for differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell walls. Since the cell envelope in gram –ve bacteria consists of the outer membrane, cell wall and cytoplasmic membrane, while in gram +ve bacteria, the outer membrane is absent. Hence gram +ve bacteria easily retains the crystal violet stain and are gram positive. Xanthomonads are gram negative. They showed a negative reaction to gram staining.

Fluorescent pseudomonads produces fluorescence pigment when observed under UV transilluminator. However, fluorescence pigment was not produced by *Xanthomonas vasicola* pv. *arecae.* Hence, they have shown negative reaction for the fluorescent test when observed under UV transilluminator.

Some of the biochemical characteristics identified in the present investigation were in accordance with results obtained by Hingorani and Singh (1959) [4], Naik (2017) [6] Thammaiah, (1991) [9] Ravikumar, (1997) [7] and Chand and Kishun (1991) [1]. Similar results were also obtained by Yenjerappa (2009) [10] and Giri M. S. (2015) who observed that, the *Xanthomonas axonopodis* pv. *punicae* hydrolyzed the starch, liquefied the gelatin and was positive for H_2S production, catalase and oxidase enzyme activity. The organism utilized various carbon sources *viz.,* glucose, fructose, dextrose and produced mild acid from these carbon sources, but failed to use lactose, mannose and mannitol.

Table 2: Biochemical profiling of the isolates of *Xanthomonas vasicola* pv. *Arecae*

Isolates	Biochemical tests							
	Gelatin liquefaction	Catalase test	H_2S production	Starch hydrolysis	Methyl red test	KOH test	Fluorescent test	Gram reaction
Xva - 1	+	+	+	+	+	+	-	Gram –ve
Xva - 2	+	+	+	+	+	+	-	Gram –ve
Xva - 3	+	+	+	+	+	+	-	Gram –ve
Xva - 4	+	+	+	+	+	+	-	Gram –ve
Xva - 5	+	+	+	+	+	+	-	Gram –ve
Xva - 6	+	+	+	+	+	+	-	Gram –ve
Xva - 7	+	+	+	+	+	+	-	Gram –ve
Xva - 8	+	+	+	+	+	+	-	Gram –ve
Xva - 9	+	+	+	+	+	+	-	Gram –ve
Xva - 10	+	+	+	+	+	+	-	Gram –ve
Xva - 11	+	+	+	+	+	+	-	Gram –ve
Xva - 12	+	+	+	+	+	+	-	Gram –ve

(-) Fluorescence is not observed under UV transilluminator.

(+) All the isolates shown positive reaction to different tests.

Table 3: Production of Hydrogen sulphide by different isolates of *Xanthomonas vasicola* pv. *Arecae*

Isolates	Number of days after inoculation													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Xva - 1	-	-	-	+	+	+	+	+	+	+	+	+	+	+
Xva - 2	-	-	+	+	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Xva - 3	-	-	-	+	+	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Xva - 4	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Xva - 5	-	-	+	+	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Xva - 6	-	-	-	+	++	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Xva - 7	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Xva - 8	-	-	+	+	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Xva - 9	-	-	-	+	+	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Xva - 10	-	-	+	+	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Xva - 11	-	-	-	+	+	+	+	+	+	+	+	+	+	+
Xva - 12	-	-	-	-	+	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-

‘ – ’ absent, ‘+’ weak, ‘++’ medium, ‘+++’ strong

Table 4: Grouping of *Xanthomonas vasicola* pv. *arecae* isolates based on hydrogen sulphide production

Group	Category	Isolates
A	Strong H_2S production	Xva – 2, Xva - 5, Xva-6, Xva- 8, Xva-10,
B	Medium H_2S production	Xva – 3, Xva – 9 Xva - 12
C	Weak H_2S production	Xva – 1, Xva- 4, Xva – 7, Xva - 11

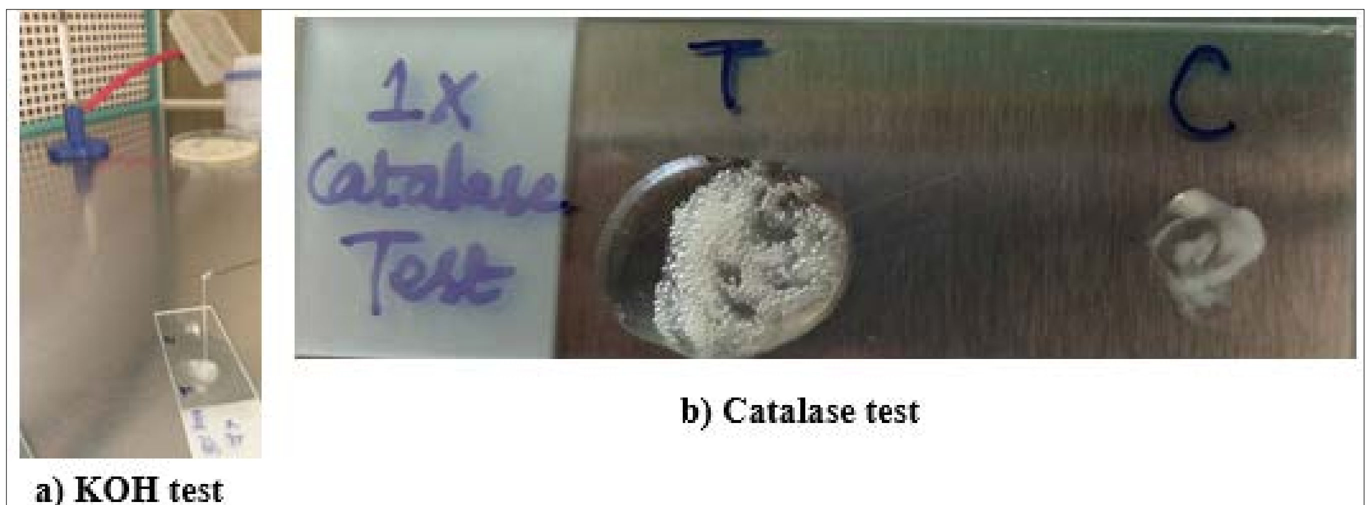
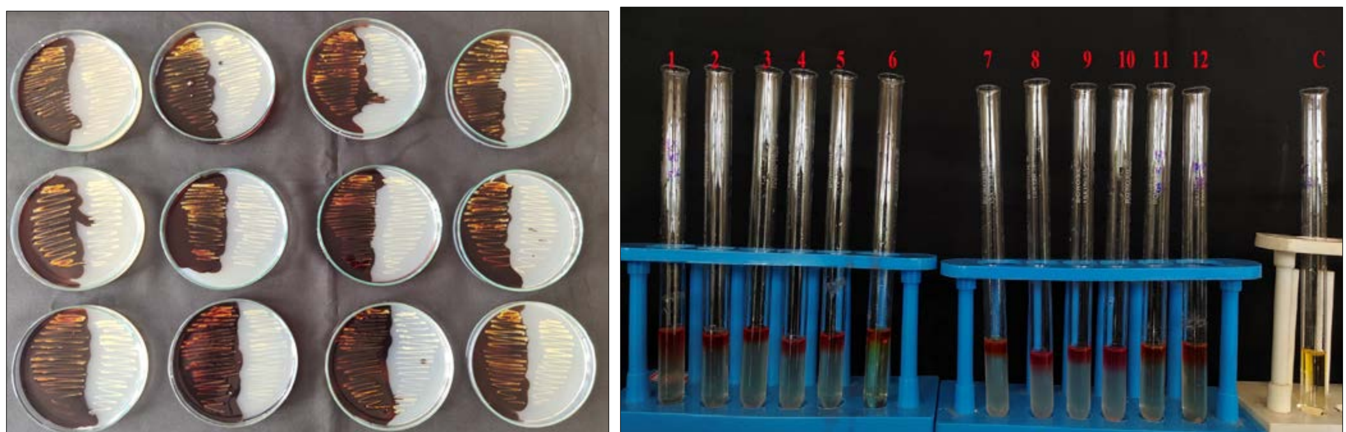
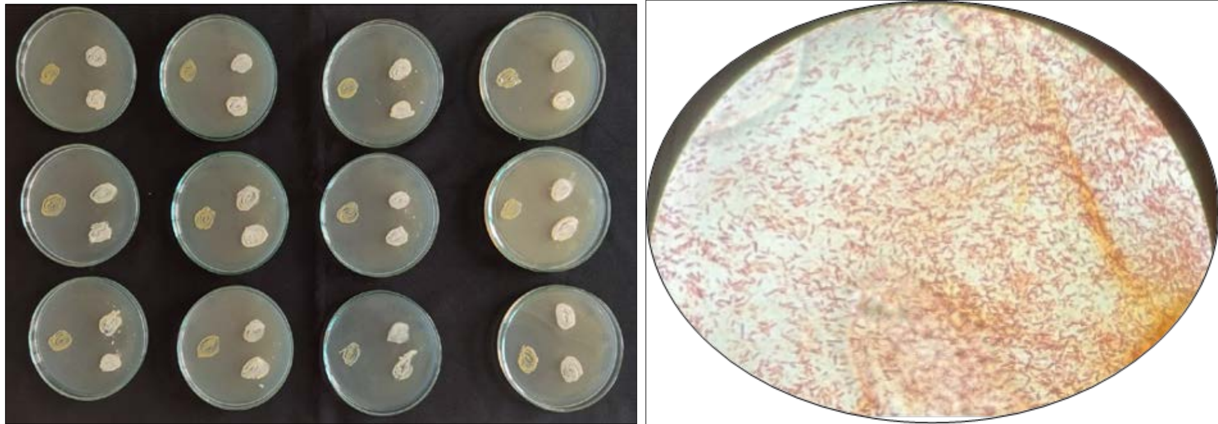


Plate 1: KOH and catalase test for *Xanthomonas vasicola* Pv. *arecae*

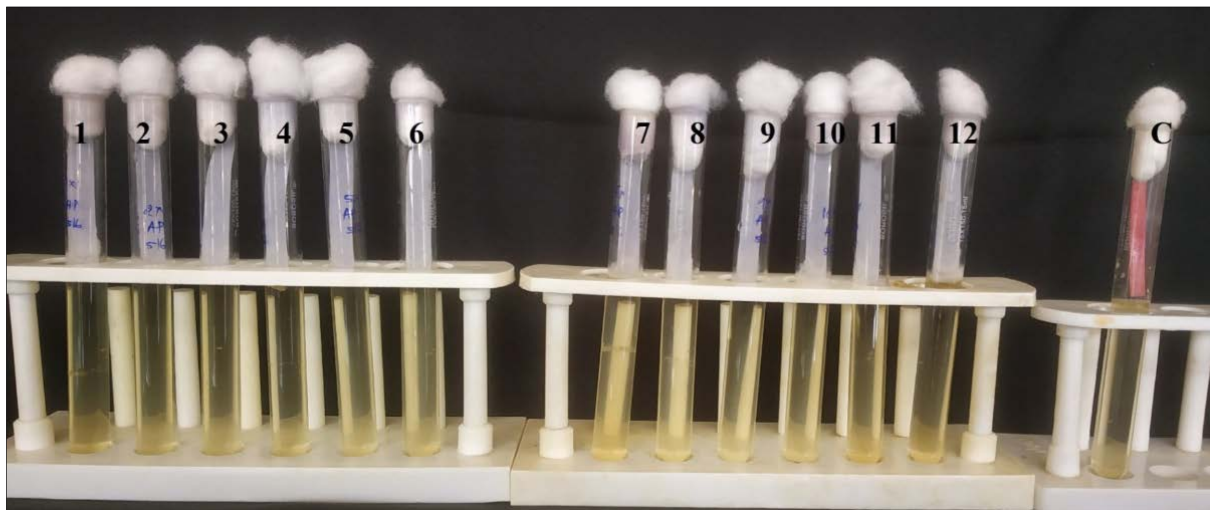


a) Starch hydrolysis b) Methyl red test

Plate 2: Starch hydrolysis and Methyl red test for *Xanthomonas vasicola* pv. *Arecae*

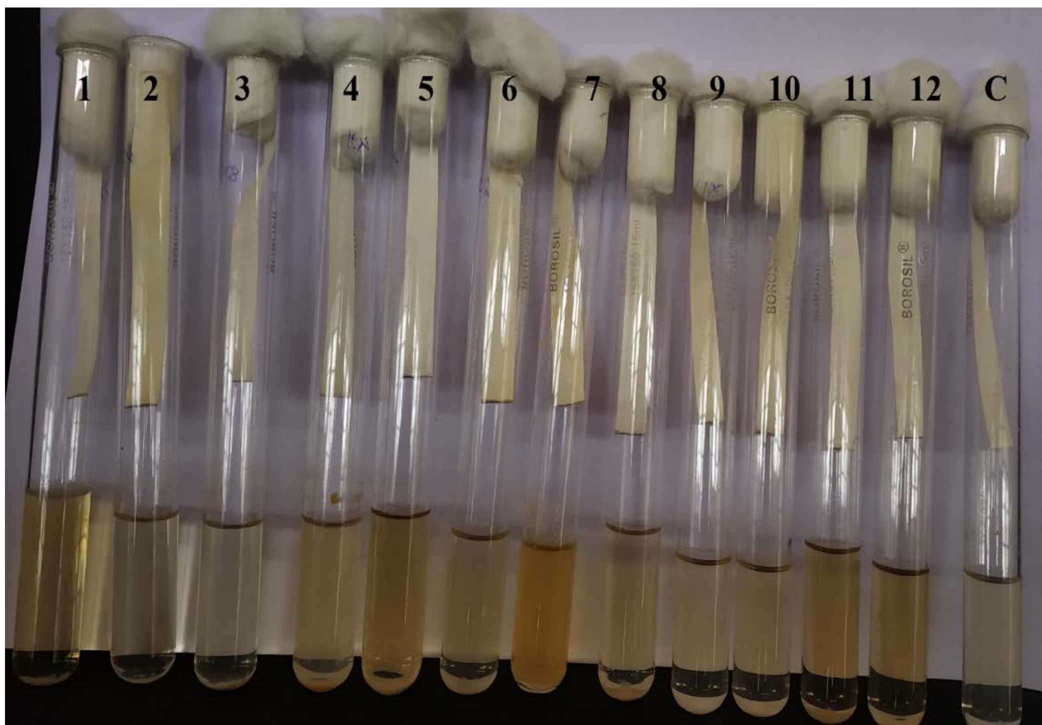


a) Gelatin liquefaction test b) Gram -ve



c) Ammonia production

Plate 3: Gelatin liquefaction, Gram –ve and Ammonia production test for *Xanthomonas vasicola* pv. Arecae



Melina Kunchenahalli	Xva - 1	Gangagondanahalli	Xva - 5	M. C. Halli	Xva - 9
Hirejambur	Xva - 2	Gedlatti	Xva - 6	Emme Doddi	Xva - 10
Aladalli	Xva - 3	Belagutti	Xva - 7	Gondedahalli	Xva - 11
Tumari Hosur	Xva - 4	Salabalu	Xva - 8	Gunderi	Xva - 12

Plate 4: Hydrogen Sulphide production by different isolates of *Xanthomonas vasicola* pv. *Arecae*

Conclusion

Present studies indicated that, the pathogen isolates were positive for KOH, catalase, gelatin liquefaction, starch hydrolysis, methyl red and ammonia production tests. And isolates showed variability in H_2S production. Whereas, pathogen isolates were found to be negative for Gram's reaction and fluorescent test.

References

1. Chand R, Kishun R. Studies on bacterial blight (*Xanthomonas campestris* pv. *punicae* of pomegranate. Indian Phytopathol 1991;44:370-371.
2. Chowdappa P, Sharma P, Anandaraj M, Khetarpal RK. Diseases of Plantation crops. *Indian Phytopathol Soc*, New Delhi 2014.
3. Giri MS, Prashanthi SK, Kulkarni S, Benagi VI, Hegde YR. Biochemical and molecular variability among *Xanthomonas axonopodis* pv. *punicae* strains, the pathogen of pomegranate bacterial blight. Indian Phytopathol 2011;64(1):2-6.
4. Hingorani MK, Singh NJ. *Xanthomonas punicae* sp. Nov. on pomegranate (*Punica granatum* L.). Indian J. Agric. Sci 1959;29:45-48.
5. Lammert JM. Techniques in microbiology: A student handbook. Pearson Education Inc. San Francisco 2007.
6. Naik S. studies on bacterial leaf stripe of arecanut caused by *Xanthomonas campestris* pv. arecae. M.sc (Agri.) Thesis, Univ. of Agric. and Hortic. Sci., Shivamogga (India) 2017.
7. Ravikumar MR. Studies on races ecology, detection and control of *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye causing bacterial spot of Tomato (*Lycopersicon esculentum* Mill.). *M.Sc. (Agri.) Thesis*, Univ. Agric. Sc*i*., Bangalore (India) 1997.
8. Schaad NW. *Xanthomonas*. In: *Laboratory Guide for Identification of Plant Pathogenic Bacteria*, II Ed. International Book Distributing Co. Lucknow 1992, 165.
9. Thammaiah N. Studies on the detection of *Xanthomonas campestris* pv. *vignicola* (Burkh.) Dye in cowpea seeds and its control. *M. Sc. (Agric.). Thesis*, Univ. Agirc. Sci. Bangalore (India) 1991.
10. Yenjerappa ST. Epidemiology and management of bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae* (Hingorani and Singh) Vauterin *et al., Ph. D. Thesis* submitted to *Univ. Agric. Sci., Dharwad*, Karnataka, India 2009.