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RESEARCH ARTICLE

Biochemical studies on *Xanthomonas axonopodis* pv. punicae causing bacterial blight of pomegranate

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ABSTRACT

Bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* is the devastating disease of pomegranate. The pathogen strains were collected from predominant pomegranate growing areas of Karnataka to understand the existence of biochemical variability among the different isolates. Among 3 strains (Xap1-Raichur , Xap2- Bellary, Xap3-Koppal) Xap1 showed maximum starch hydrolysis, Xap3 liquified maximum gelatin and all the strains showed positive reaction for ammonia production , H_2S production, protein digestion. All the strains showed negative reactions for indole production, voges-poskaure test, nitrate reduction and methyl red test.

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INTRODUCTION

Pomegranate (*Punica granatum* L.) is a favourite table fruit in tropical and sub tropical regions of the world which belongs to family Punicaceae. India is the second largest producer of pomegranate with a production of 7.92 lakh tons (Anonymous, 2007). In India, pomegranate is commercially cultivated in Maharashtra and small scale plantations are seen in Gujarat, Rajasthan, Karnataka, Tamil Nadu, Andhra Pradesh, Uttar Pradesh, Punjab and Hariyana (Chadha, 2001). Bacterial blight incited by *Xanthomonas axonopodis* pv. *punicae* (Hingorani and Singh, 1959) is an economically important and widely distributed disease in pomegranate growing areas of Karnataka and Andhra Pradesh. The disease accounted up to 70-100 per cent yield loss during 2006 in Karnataka and Maharashtra and total resulting pomegranate production in India was down by 60 per cent in the year 2007 (Raghavan, 2007). During 2008-09, the disease had reached at its alarming stage, resulting in substantial damage to the crop and heavy loss to the farmers.The production of pomegranate has declined from a high of 1.8 lakh metric tonnes per annum four years ago to less than 10,000 metric tonnes in 2007 – 08, thereby causing a revenue loss of about Rs. 200 crore (at an average price of Rs. 50,000 per tonne) in Karnataka (Giridhar, 2008). However, the magnitude of the disease severity has raised several questions to researchers, farm managers, administrators, private firms and above all the farmers to safeguard the cultivation of this dollar earning crop. Knowledge on biochemical nature of the pathogen helps to trace back the origin of different isolates. In this context, the present study was proposed to understand the biochemical characteristics of *Xanthomonas axonopodis* pv. *punicae* (Hingorani and Singh, 1959).

MATERIAL AND METHODS

Isolation of bacterial cells :

Different parts of plant affected by the disease *viz.*, infected leaves, twigs and fruits were collected from the farmer's field from Koppal, Raichur and Bellary districts which are the predominant pomegranate growing areas of the state. The suspected bacterial colonies were picked up with the help of sterilized inoculation loop and streaked on to the surface of Yeast extract dextrose calcium carbonate agar (Schaad, 1992)

contained in sterilized Petriplates. The plates were incubated at 28^{0}C for the 48-72 hours and the observations were made for the development of well separated typical light yellow coloured bacterial colonies, such pure colonies were further streaked onto the agar slants containing the Nutrient agar medium and incubated at 30^{0}C for 72 hours, then cultures were stored in the refrigerator at 5^0 C.

Biochemical variability :

Starch hydrolysis:

Iodine solution is used to detect the hydrolysis of starch. It gives a blue colour with starch, brown with erythrodextrins and no colour with maltose.The medium employed is referred to as starch hydrolysis medium (Peptone- 10.0 g; Beef extract - 5.0 g; Starch (soluble)- 2.0 g; Agar - 20.0 g; Water - 1000 ml; pH - 7.0).The medium was sterilized by autoclaving and poured into sterilized Petri plates. After the solidification of the medium, the test culture was spot inoculated in plates. The plates were incubated at 25^{0}C. After 2 days the agar surface was flooded with Lugol's iodine and allowed to act for a few minutes.

Lugol's iodine :

(Iodine -5.0g / 100 ml; Potassium iodide -10.0 g / 100 ml**).** Dissolve iodine and KI in 10 ml of H_2O.Adjust to volume with distilled water. For use, dilute 1/5 with distilled water.

Gelatin liquefaction :

Gelatin is decomposed by many bacteria which produce an extra-cellular enzyme gelatinize. As a result of decompositions, the gelatin loses its gel forming property. The medium (Peptone - 10.0 g; Beef extract - 5.0 g; Gelatin - 20.0 g; Water - 1000 ml; pH - 7.0) was autoclaved and cooled at 45^{0}C. It was then poured into Petriplates and allowed to solidify. The medium was spot inoculated by a 48 hr growth of the test bacterium. After incubation of 48 hr at 25^{0}C, agar surface was flooded with 0.2% mercuric chloride solution which was diluted (20%) in hydrochloric acid and allowed to act for a few minutes.

Protein digestion :

For liquid test, reconstituted powdered skim milk containing 0.004 per cent bromocresol purple (w/v) and sterilized by steaming for 30 min on three successive days. Inoculate milk solution in test tubes with a loop full of cells and incubate at 20^{0}C and observed for a clearing reaction (digestion of casein).For the agar plates test, reconstituted powdered skim milk at 48^0 C with sterile melted yeast extract nutrient agar (YNA) to obtain a 10 per cent v/v concentration and poured over the surface of a thin layer of Nutrient agar in Petriplates. The plates were dried, spot inoculated, and observed for a clear zone around the colonies after 3, 5 and 7 days.

Production of hydrogen sulfide :

This test reveals the ability of bacterium to liberate H_2S by dissimilation of sulphur containing amino acids like cystine and methionine. The usual bacteriological peptone contains cystine in enough concentration and it is used as a substrate. The medium (Peptone -10.0 g: NaCl - 5.0 g; Water -1000 ml; pH-7 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 x 50 mm strips which were then soaked in warm saturated solution of lead acetate. The strips were then dried, autoclaved and again dried at 60^0 C.The medium in each tube was inoculated with a loopful of 48 hr. slant growth of the bacterium. After inoculation, a test strip was inserted in between the plug and inner wall of the tube, so that it hangs just above the broth but does not touch it. The tubes were incubated at 25^{0}C and observations were recorded at regular intervals up to 14 days.

Production of indole :

Indole is volatile and on reaction with oxalic acid it forms indole-oxalic acid, which is pink in colour. This reaction is utilized in detecting presence of indole.The medium (Tryptophan or Casein digest -10.0 g; NaCl - 5.0 g; Water – 1000 ml;) was dispensed in tubes and autoclaved. To detect indole production the oxalic acid test strips were prepared as follows. Whatman No.1 filter strips (5 x 50 mm) were soaked in warm saturated solution of oxalic acid. After cooling, the strips get covered with oxalic acid crystals.Then the strips were dried at room temperature and used without sterilizing.The test organism was inoculated into tryptophan broth tube and inserted an oxalic acid test strip. The tubes incubated at 25^{0}C and observed for colouration of oxalic acid crystals at regular intervals for 14 days.

Ammonia production :

Test tubes containing eight ml sterile Nutrient broth were inoculated with three isolates with one tube kept uninoculated as a control and incubated at 27^0 C for 48 hr, after incubation cotton stopper from the tubes were removed and strip of red litmus paper was inserted inside the wall of the tube and stopper was replaced as earlier to hold the strip of litmus paper in place. No change in the colour indicated the negative result.

Voges - Proskauer test :

Tests were performed in glucose phosphate broth (0.5% glucose, 0.5% K_2HPO_4 and 0.5% peptone). The VP reaction depends on variability of an ability of an organism to first produce acid from glucose. Cultures were shaken incubated at 27^0 C for five days. One ml culture was added to a tube containing 0.6 ml of naphthol and 0.2 ml of 40 per cent KOH and shaken vigorously. Change in colour indicated positive reaction.

Table 1 : Biochemical characteristics of the three isolates of *Xanthomonas axonopodis* pv. *punicae*

Sr. No.	Biochemical characters	Isolates		
		Xap1	Xap2	Xap3
1.	Starch hydrolysis	+	+ + +	+ +
2.	Gelatin liquefaction	+	+	+ + +
3.	Protein digestion	+ +	+ +	+ + +
4.	Production of ammonia	+	+	+
5.	Production of Indole	-	-	-
6.	Production of hydrogen sulphide	+	+	+
7.	The Voges Proskaure test	-	-	-
8.	Nitrate reduction	-	-	-
9.	Methyl red test	-	-	-

+++ = Highly positive, ++ = Positive (moderately), + = Positive, - = Negative.

Methyl red test **:**

Methyl red indicator (0.1 g methyl red dissolved in 3000 ml of 95% ethanol and made up to 500 ml with distilled water) was added test culture; change in colour was noted and recorded as positive.

Nitrate reduction test **:**

Eight ml media was taken in a tube (KNO_3 3 g; peptone 5 g; yeast extract 3 g; agar 3g; distilled water one liter and pH was adjusted to pH 7.0-7.2 with 40% NaOH), and sterilised for 15 min at 121^0C, tubes were stab inoculated and incubated for 4 days. To each tube, one ml of 0.6 per cent solution of dimethyl-naphthylamine and one ml of a 0.8 per cent solution of sulphanilic acid was added. False positive was indicated by no development of red colour.

RESULTS AND DISCUSSION

The results of the present investigations have been presented in Table 1.

Starch hydrolysis :

The isolates showed variation in their ability to hydrolyze starch, which was evident from the zones formed. The isolate Xap 3 showed maximum starch hydrolysis compared to Xap 1 and Xap 2.

Gelatin liquefaction :

Three isolates of Xap were found to liquefy the gelatin but at varying intensity. Isolate Xap2 exhibited the highest positive reaction; similarly Xap1 showed moderate positive reaction while Xap3 showed positive reaction.

Ammonia production :

All the three isolates were found positive for the ammonia production.

Production of indole :

All the isolates were negative for indole production.

Hydrogen sulfide production :

Positive reaction for H_2S production was noticed in all the isolates.

The Voges proskaure test :

None of the isolate showed the positive reaction.

Nitrate reduction :

All the isolates showed negative reaction.

Methyl red test :

All the isolates were found negative for methyl red test.

Protein digestion :

All the isolates were found positive for protein digestion.

The present study indicated that, the pomegranate bacterium can hydrolysed the starch, liquefied the gelatin and was positive for H_2S production (Table 1). Some of these biochemical characteristics identified in the present investigation were in accordance with the results obtained by Hingorani and Singh (1959). It readily hydrolysed the starch and liquefied the gelatin. The yellow colour of the growth on gelatin gradually changes from usual bright yellow to dark brown on yeast glucose chalk agar. Chand and Kishun (1991) reported that pomegranate bacterium was positive to Xanthomonadin, tween 80 hydrolysis gelatin liquefaction, milk proteolysis, H_2S production etc. most of these biochemical characteristics were found similar in the present investigation. The findings are in accordance with the reports of the Ravikumar (1997) and Manjula (2002).

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