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Morphological And Bio-chemical Characterization of *Xanthomonas Campestris Pv.vesicatoria:* A Gram Negative Bacterium Causing Bacterial Spot

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ABSTRACT

Bacterial spot is one of the major causes of yield losses in tomato growing areas of the world. *Xanthomonas campestris pv.vesicatoria* is the cause of this disease, which is a gram negative bacterium. Biochemical analysis helps to differentiate between gram positive and gram negative. Gram staining, KOH test, Kovacs' Oxidase, H_2S production test and Catalase tests were performed to characterize the *Xcv.* The results of all biochemical tests confirmed the *Xcv* a gram negative bacterium.

Key- words- *Xanthomonas campestris pv.vesicatoria,* bacterial spot, Biochemical characterization

INTRODUCTION

Tomato bacterial spot, caused by *Xanthomonas campestris pv. vesicatoria* [Applied Environment Microbiology71 (2005) 3581], occurs worldwide, wherever tomato is cultivated. Tomato (*Lycopersicon esculentum* Mill.), proposed as *Solanum lycopersicum* L. in a new classification (Peralta *et al.*, 2005), is one of the most commonly used vegetables and plays an important role in economics and social aspects.

Tomato (*Lycopersicon esculentum*) belongs to the genus Lycopersicon under Solanaceae family. Tomato is a herbaceous sprawling plant growing to 1-3 m in height with weak woody stem. The flowers are yellow in colour and the fruits of cultivated varieties vary in size from cherry tomatoes, about 1–2 cm in size to beefsteak tomatoes, about 10 cm or more in diameter. Most cultivars produce red fruits when ripe. Tomato is a native to Peruvian and Mexican region. Though there are no definite records of when and how it came to India , the Portuguese perhaps introduced it to India. Tomato is one of the most important "protective foods" because of its special nutritive value. It is one of the most versatile vegetable with wide usage in Indian culinary tradition. Tomatoes are used for soup, salad, pickles, ketchup, puree, sauces and in many other ways It is also used as a salad vegetable. Tomato has very few competitors in the value addition chain of processing. The crop is however vulnerable to a number of diseases that affect fruit production and quality including bacterial spot, caused by *Xanthomonas spp.* (Schaad and Stall, 1988; Jones et al., 1995, 1998, 2004; Hert et al.,2005), one of the main diseases of tomato. It can affect all above-ground plant organs at any development stage while the field control is difficult (Lobo *et al*.,2005; Silva *et al.*, 2006).

The major tomato growing states in India are Andhra Pradesh, Bihar, and Chattisgarh. In Jharkhand, tomato is extensively cultivated in the vegetable belts covering districts like Ranchi, Lohardaga, Hazaribagh and Godda and covers approximately 13.9% of the area under vegetable cultivation. At present, the estimated area and production under tomato is 7290 ha and 131220 t respectively.

This study therefore, aimed at isolating and characterizing the causative agent of bacterial leaf spot using morphological and biochemical characteristics.

Materials and Methods

Isolation of *Xanthomonas campestris pv.* vesicatoria from diseased leaves of *Lycopersicon esculentum*

Infected leaves of *Lycopersicon esculentum* showing

water-soaked and necrotic areas were collected from farmer's fields around and taken to the Plant Pathology and Microbiology lab of Ranchi University, Ranchi for isolation and characterization. The infected leaves were washed with sterile water, surface-sterilized in 70% ethanol and rinsed in several exchanges of sterile water. Small pieces of the infected portions were cut from advancing margin of the lesion on leaves using sterile scalpel then teased apart with sterile dissecting needle in 1ml sterile water and left to stand for 30 minutes. The suspension was then streaked onto the surface of YDCA media. Also the pure sample of the bacterium was procured from ITCC, New Delhi and streaked on to the plates of YDCA media.

Media preparation and bacterial inoculation

The media for isolation of *X. campestris pv.vesicatoria* was Yeast Dextrose Chalk Agar (YDCA) media which was prepared from Yeast extract 10.0g, Dextrose 10.0g, Calcium carbonate 20.0g and Agar 20.00g. The chemicals were weighed and then dissolved in 1000ml of sterile water containing conical flask.

The mixture was melted on a hot plate for complete dissolution then autoclaved at 121ºC for 15 minutes at a pressure of 15psi. The media was then cooled to about 45-50°C . About 20ml of the media was dispensed in Petri-plates in a laminar flow hood. The media was allowed to set. The surface of the media was then inoculated by a loop full of bacterial suspension using a sterile wire loop and incubated for 48 hours at 30^0C.

Morphological and Biochemical identification of the bacteria

Morphological characterization

Characteristic colonies based on size and colour were identified, isolated and purified on yeast dextrose calcium carbonate (YDC) to obtain pure culture. Thin smear was prepared from the colonies, heat fixed by passing the slide over the flame, then methylene blue stained and observed microscopically under oil of emulsion objective lens to reveal the shape and arrangement of the bacterial cells. The cells could be either cocci or bacilli.

Biochemical characterization

Gram's staining

A thin fixed smear was prepared using characteristic colonies and stained using Gram's reagents. The reagents were applied in the following order. Crystal violet for 2 minutes, gram's iodine for 1 minute, absolute ethanol for 30 seconds and lastly safranin for 1 minute. Washing with tap water was after every step as described by Rafi *et al.* (2013). The smear was then allowed to dry in air and observed microscopically using oil of emulsion objective lens. Purple coloured cells would indicate Gram positive while red or pink coloured cells would be Gram negative.

Oxidase test

On a piece of Whatman No. 2 filter paper placed in a Petri dish, several drops of oxidase test reagent (1% solution of dimethyl p- phenylenediamine hydrochloride) were added then a loop full of the bacteria from the colonies smeared on the paper and the reaction observed. The isolates were rated as oxidase-positive if a purple color developed within 10-60 seconds, and negative if no color developed within 60 seconds (Rafi *et al.*, 2013).

KOH solubility test

Two drops of 3% KOH were put on a glass slide then a colony of the test bacterium picked from the surface of a solid medium with an inoculating loop and mixed vigorously in the KOH for 5-10 seconds. Thread-like slime formation when picked by wire loop indicated presence of G- bacterium but no slime or thread formation indicated G+ bacterium (AL-Saleh,2011).

Catalase test

A few drops of 3% hydrogen peroxide were placed on a clean slide and two colonies mixed in it to observe the reaction. Presence of bubbles indicated positive results while no bubbles indicated negative results (Gracelin et al., 2012).

Hydrogen sulphide production.

The method developed by Gupta *et al.* (2007) with slight modification of not incorporating bile salt was used. The medium was formulated by combining 20.0g peptone, 1.5g dipotassium hydrogen phosphate, 0.75g ferric ammonium citrate, 1.0g sodium thiosulfate 3.5g, agar and 5.0 yeast extract.

30g of the mixture was suspended in distilled water, mixed thoroughly by stirring and then heated for complete dissolution. The media was then dispensed into universal bottles and autoclaved at 121ºC for 15 minutes. After cooling, the media was inoculated with the test organisms by stab method using inoculating wire. The bottles were then incubated with loose caps at 28ºC for 10-14 days and observed for hydrogen sulphide gas production. Colour change to black indicated H2S production by the bacteria while no colour change indicated inability of the bacteria to produce H2S from media.

Table1. Biochemical characteristics of *X.c pv. vesicatoria* isolated from diseased *Lycopersicon esculentum* leaves

Test	Result
KOH solubility	+
Catalase test	+
Hydrogen sulphide production	+
Oxidase	-
Gram stain	Gram negative

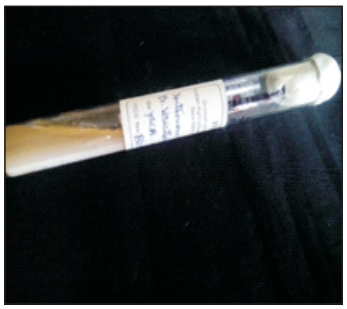

Fig.-1

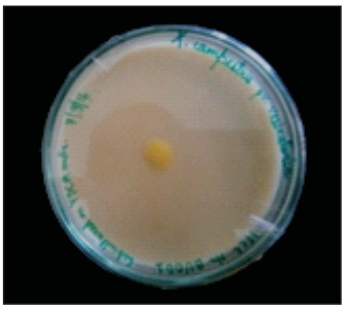

Fig.-2

Fig.-3

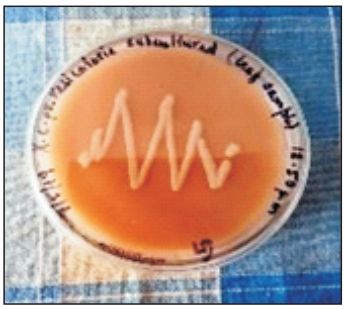

Fig.-4

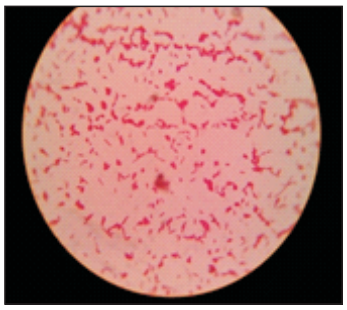

Fig.-5

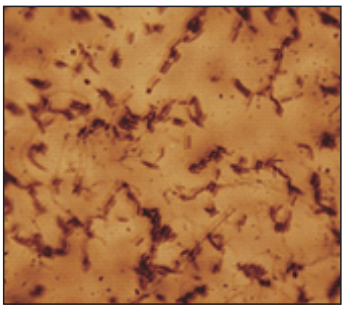

Fig.-6

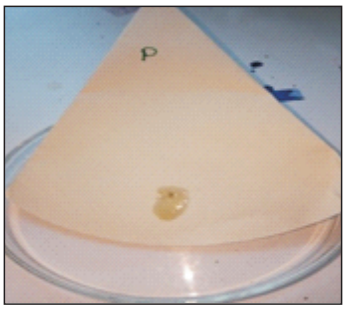

Fig.-7

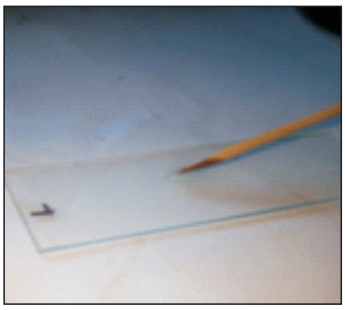

Fig.-8

Pure culture of Xanthomonas campestris pv vesicatoria procured from ITCC, New Delhi and cultured in lab(Fig1-2)
Pure culture of Xanthomonas campestris pv vesicatoria cultured from leaf samples (Fig3-4)
Gram stained pinkish colour of bacterial cuture (Fig5)
Microphotograph of gram stained X.cv(Fig-6)
Oxidase test (negative) (Fig 7), KOH string test (positive) (Fig-8)

RESULTS AND DISCUSSION

Morphological characteristics

Colonies of the three isolates on media of yeast dextrose calcium carbonate (YDC) were deep yellow, highly viscous, slimy and round in shape. Microscopic analysis of the bacterial isolates showed that they were rod shaped occurring in singles with polar flagella. These growth pattern and microscopic observation provided preliminary information on the identification of the Xc.pv vesicatoria as similar characteristics had been described by Lee *et al.* (2009) and Gracelin et al., (2012).

The best growth was observed on YDCA and NA at 25-27ºC and 26ºC respectively. These were yellow on NA at 28ºC, smooth and shiny yellow on YDCA at 27-30ºC

KOH-String test: All isolates responded positively to loop test by forming a thread when uplifted gently. The loop formation is confirmation of gram negative bacteria and the above mentioned isolates were Gram negative (Halebian *et al.*, 1981; Suslow et al., 1982). Each isolate was tested twice and same results were revealed..

Gram Staining: The isolates marked as Gram negative from the loop test were tested using Gram staining. All isolates retained a pinkish colour thus confirming that they were gram negative. The test was executed twice and similar results were obtained.

For the observation of cell size and shape, the counterstained slides were observed using a microscope at 100X magnification. Each slide, representing one isolate, was examined and the shape of the bacteria found to be elongated rods.

H2S production: All isolates tested positive for H2S production giving a black discoloration on lead acetate paper strips.

Catalase test: All the isolates of Xcv were found catalase positive and gave off H2O2 bubbles in the test petri plates.

The results of the different biochemical tests carried out on the pathogenic isolates indicated that the isolates are likely Xanthomonas spp. (Abdul-Rahim and Adam, 1990). The fact that they were Gram negative rod shaped bacteria as observed by Kottle (1985). The size, growth characteristics on various media and yellow, smooth and shiny colonies also supported the isolates as Xanthomonas species (Dye, 1962; Kado and Heskett, 1970; Schaad and White, 1974; Kottle, 1985). The genus Xanthomonas was separated from other plant pathogenic bacteria on the basis of the motile, yellow coloured cells, with a single polar flagellum, mostly producing acids in media containing lactose. In naming the genus, the yellow colour of the bacteria, which is one of the most significant characteristics of the genus (Sabet and Dowson, 1960). Since then the yellow colour has been an invaluable aid in the identification of *Xanthomonas* (Nancy *et al.*, 1988).

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