

DETECTION AND IDENTIFICATION OF *XANTHOMONAS CAMPESTRIS* PV. *CENTELLAE* ON LEAVES OF *CENTELLA ASIATICA* COLLECTED IN TAMILNADU

D. HERIN SHEEBA GRACELIN*, A. JOHN DE BRITTO AND P. BENJAMIN JEYA RATHNA KUMAR

Plant Molecular Biology Research Unit, Post Graduate and Research Department of Plant Biology and Biotechnology, St. Xavier's College (Autonomous), Palayamkottai - 627 002, Tamil Nadu, India , Email:herin.gracelin@gmail.com

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ABSTRACT

A bacterial leaf spot disease has been the major problem in cultivation of *C. asiatica* in Tirunelveli districts (Tamilnadu) in India. Hence laboratory experiments were conducted to detect and identify the causative pathogen on leaves of *Centella asiatica* collected from naturally contaminated commercial plants. Two modified semi-selective media namely mTBM and mMD5A were used to detect the bacterium from the infected leaves. Identification of the strain was proved by using morphology, physiology and biochemical test. The results showed the presence of strain i.e. *Xanthomonas campestris* pv. *centellae* (*X.c.pv.c*) in all the assayed plants.

Key words: Detection, *Centella asiatica* and *Xanthomonas campestris* pv. *centellae*.

INTRODUCTION

Centella asiatica L. Urban (syn. *Hydrocotyle asiatica* L.) belongs to the family of Apiaceae (Umbelliferae) has a historical reputation for boosting mental activity and for helping a variety of systemic illness, such as high blood pressure, rheumatism etc¹. Due to high demand, this plant is being cultivated in many places of Tamilnadu including Tirunelveli district both for use within India and for exporting purposes.

But a bacterial leaf spot disease has been the major problem in cultivation of *C. asiatica*. The quality of the plant is very much affected by this and it affects marketing and income. The disease causes heavy yield losses. *Centella* suffers from many diseases like powdery mildew, downy mildew, bacterial leaf spot etc. Among them, bacterial leaf spot is an important disease. It has become more serious in major *centella* growing areas of Tirunelveli districts. Hence efforts were made to identify and isolate the harmful pathogen. Finally the pathogen was identified as *Xanthomonas campestris* pv. *centellae* (*X.c.pv.c*).

Hence the present investigation deals with the detection and identification of the strain *Xanthomonas campestris* pv. *centellae* from the infected *C. asiatica* plant.

MATERIALS AND METHODS

Sample collection

The infected leaves showing the typical symptoms of bacterial spot were collected during 2010-2011 from farmer's fields of major centella growing areas of Tirunelveli district in Tamilnadu. The collected samples were transported to Plant Molecular Biology Research Unit (PMBRU) in fresh condition in plastic bags and stored at 27°C for 24 h for further analysis. All the leaves showed typical external symptoms like yellowish brown spots, decolouration, minute water soaked lesions, cankers lesions and irregular yellow and brown patches (Fig.1).

Isolation of *Xanthomonas campestris* pv. *centellae* from infected *Centella* leaves

Leaves of *Centella* showing typical symptoms of bacterial spot caused by *Xanthomonas campestris* pv. *centellae* were collected during 2010-11 from major centella growing areas of Tirunelveli district. The bacterium was isolated by extracting the ooze in sterile distilled water taken in test tubes followed by dilution plate technique on nutrient agar.

Small pieces of infected leaves were cut aseptically from the edge of typical spots along with a little portion of healthy tissue. The infected leaf bits were surface sterilized in 70 % alcohol or 1 % sodium hypochlorite and washed in three series of sterile water to remove traces of alcohol. The infected leaf bits were then suspended

in a small test tube containing 3 ml sterilized distilled water for 10 min. When water became slightly turbid due to oozing of bacterial cells from the cut ends of the diseased tissue, the bacterial suspension was serially diluted in 9 ml sterile distilled water. Then one ml of the diluted bacterial cell suspension was poured into sterilized petriplates containing nutrient agar. The plates were rotated gently in clockwise and anti-clockwise direction, so as to distribute the bacterial cell suspension uniformly in the plates and to obtain well separated bacterial colonies. The inoculated plates were incubated at 28°C for 72 hours. Observations were made for development of well separated yellow, convex, small bacterial colonies on the nutrient agar medium².

Isolation of the bacteria on modified semi- selective media

Then one ml of the diluted bacterial cell suspension was poured into sterilized separated petriplates containing two modified selective media namely mTBM and mMD5A. The plates were rotated gently in clockwise and anti-clockwise direction, so as to distribute the bacterial cell suspension uniformly in the plates and to obtain well separated bacterial colonies. The inoculated plates were incubated at 28°C for 4-7 days. Observations were made for development of well separated light yellow, convex, mucoid, bacterial colonies on the two semi- selective media.

Purification of bacterial culture

The suspected bacterial colonies were picked up with the help of sterilized inoculated loop and streaked onto the surface of yeast extract dextrose calcium carbonate agar (YDCA)². The inoculated plates were incubated at 28°C for 48 to 72 hours and the observations were made for the development of well separated light yellow coloured bacterial colonies. The purified bacterial colonies were streaked on nutrient agar slants and stored at 5°C in refrigerator and also in sterile distilled water taken in small culture tubes, by suspending 2-3 loops full of the bacterial culture for future use.

Identification of causal organism

The morphological characteristics such as cell shape, gram reaction, capsule and spore staining characters of the isolate was studied as described by society of American Bacteriologists^{3,2}.

Physiological and biochemical characters

The physiological and biochemical characters of the isolate of the bacterium was studied for hydrolysis of starch, gelatin liquefaction, indole production, hydrogen sulphide production, urease production and acid from different sugars viz., glucose, mannose, arabinose, galactose, fructose, lactose, maltose, sucrose and alcohol^{3,2}.

Pathogenicity on *Centella*

Pathogenicity test was carried out to find out whether the isolated bacteria was capable of producing typical symptoms of bacterial spot under artificial inoculation condition on centella seedlings or not. The leaves of plants were slightly injured by insect pin and sprayed with bacterial suspension. The inoculated plants were kept in the plastic tent for two days in which high humidity was maintained by spraying sterile water inside the tent at 25 to 30°C. The plants were taken out from the plastic tent and kept in glasshouse. Observations were made for the development of symptoms of bacterial spot. Plants similarly, injured and sprayed with sterile water constituted control. The isolated and reisolated bacterium was compared with the original culture of *X. campestris* (MTCC 2286) by studying the colony characters and staining and morphology. Vein inoculation and pin pricking methods were used for bacterial inoculation.

RESULTS

Isolation of *Xanthomonas campestris* pv. *centellae* from infected centella leaves

The ooze test was conducted by cutting a small bit of the leaf tissue from the infected leaves and suspending it in a drop of sterile water taken on a microscopic glass slide and observing under low power objective of the microscope. Within few minutes the bacterial ooze started jetting out from the cut ends of the infected tissue, thus revealing the association of bacteria with the disease.

Isolation was made from the bacterial ooze obtained from the infected leaf tissue in sterile distilled water followed by dilution plate technique on nutrient agar produced typical *Xanthomonas*

colonies in 48 hours. The colonies were yellow, slimy, glistening and round in shape (Plate 1).

Isolation of the bacteria on modified semi - selective media

Isolation was made from the bacterial ooze obtained from the infected leaf tissue in sterile distilled water followed by dilution plate technique on two semi-selective media namely mTBM and mMD5A produced typical *Xanthomonas* colonies in 4 - 7 days. The colonies were pale yellow, mucoid, slimy, glistening and round in shape (Plate 1).

Purification of bacterial culture

Well separated out colonies isolated from the infected leaves were purified by streaking on the surface of YDCA medium. The culture was stored on nutrient agar slants at 5°C and also suspended few loopful of culture in sterile distilled water contained in ampules. These were kept as the stock cultures for further studies. The bacterial colonies on YDCA medium were deep yellow, slimy, highly viscous and irregular to round in shape.

Identification of causal organism

Morphological, physiological and biochemical characteristics

The results of the various morphological, physiological and biochemical tests are given in Table 1 and Plate 1. The isolate was found to be rod shaped, obligately aerobic, gram negative, oxidase negative and monotrichously flagellated, but not utilized asparagines as a sole source of carbon and nitrogen. It was positive for catalase reaction utilized glucose, fructose, sucrose for acid production, liquefaction of gelatin and produced hydrogen sulphide and did not produce indole, in addition, the strain failed to reduce nitrate to nitrites.

Plate 1: Isolation of *Xanthomonas campestris* pv. *centellae* from infected *Centella* plant



Collection of *Centella asiatica*



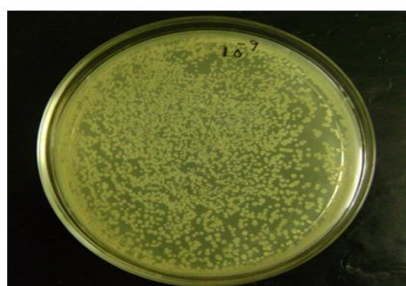
Leaf spot disease in infected leaf



***X.c.pv.c* on nutrient agar medium (yellow colonies)**



Biochemical test for *X.c.pv.c*



***Xc.pvc* on selective media (mMD5A and mTBM)**



***Xc.pvc* on selective media (mMD5A and mTBM)**

Table 1: Show the morphological and biochemical characteristics of isolated *Xanthomonas campestris* pv. *centellae* compared with the reference strain (MTCC 2286).

Characters	Isolated strain	Reference strain
I. Morphology :		
a. shape	Small rods	Small rods
b. Occurrence	In single	In single
c. Flagellation	Monotrichous	Monotrichous
II. staining		
a. Gram reaction	G -ve	G -ve
b. Capsule staining	+	+
c. Spore staining	-	-
d. Acid fast	-	-
III. Biochemical characters		
a. Utilization of glucose, sucrose, fructose for acid production	+	+
b. Utilization of Asparagine as sole source of carbon and nitrogen	-	-
c. Catalase reaction	+	+
d. Gelatin liquefaction	+	+
e. Casein hydrolysis	+	+
f. Methyl red reaction	-	-
g. Reduction of nitrate to nitrite	-	-
h. Urease reaction	-	-
i. Indole production	-	-
j. Hydrogen sulphide production	+	+
k. Oxidase reaction	-	-
l. Starch hydrolysis	+	+

Pathogenicity on *Centella*

The strain of *X. c. pv. c* artificially inoculated to 30 days old centella seedlings, by spraying the bacterial culture to pre-injured leaves. The plants started producing small water soaked lesions on the leaves. The lesions became necrotic leading to blighting of infected leaves. The first symptoms of the disease observed 15 days after inoculation of the plants. Re-isolations of the bacterium made from artificially inoculated plants yielded yellow coloured colonies on YDCA and the strain was confirmed as *X.c.pv.c*.

DISCUSSION

The selected medicinal plant *Centella asiatica* is severely affected by *Xanthomonas* Pathovars. It has become more serious in *centella* growing areas of Tamilnadu. ⁴ studied the taxonomy and biodiversity of micro fungi in the leaves of *Centella asiatica* showed spots with chestnut brown margins and a pale central region. They identified the pathogen as *Cercospora* sp. ⁵ described a new leaf spot disease of *Centella asiatica* L. was encountered during a survey for diseases of medicinal plants in nurseries and gardens of Jabalpur. The incidence of disease was during monsoon season and damaged 60-70% of the plants. The pathogen responsible for the disease was identified as a new species of *Pseudocercospora* namely *P. centelli* sp. nov. A critical problem in the study of bacterial pathogens of the plants is the correct identification of the infectious agent. The study of diseases in plants is an important aspect of assessing plant health. Hence in the present investigation the identification and isolation of pathogenic bacteria from the infected plants of *C. asiatica* has been demonstrated for the first time in Tamilnadu.

CONCLUSIONS

The experiments confirmed the presence of *X.campestris* pv. *centellae* as causative pathogen for leaf spot disease in the infected leaves of *C. asiatica*. These results also indicate that the *Centella* plants grown in Tirunelveli district is highly infected by the bacterium.

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