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Molecular Characterization of *Xanthomonas citrii* Pathovars from Marathwada Region

A.R. Suryawanshi[1], S.M. More[2] and M.M.V. Baig*

*Department of Botany, [1]Department of Biotechnology, [2]Department of Microbiology, Yeshwant Mahavidyalaya, Nanded - 431 602, India.

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Of the 300 strains of *Xanthomonas citrii* were isolated from the infected parts of citrus plant from Marathwada region. Eighteen representative strains were selected for further studies. These strains were characterized by biochemical methods. These strains were subjected to Aesculin test, milk hydrolysis, tween 80 lipolysis, gelatin liquefaction. All strains were positive for Aesculin test, gelatin liquefaction. Strain Xcc1, Xcc6, Xcc8, Xcc14, and Xcc16, were positive for milk hydrolysis while except strains Xcc8, Xcc14, Xcc16. All strains were positive for tween 80 lipolysis. These strains were further used for pathogenicity test to confirm pathogenic nature of *Xanthomonas*. The study of whole cell protein profile was done for showing relationship among the isolated strains of *Xanthomonas* by UPGMA cluster analysis. Similarly index suggested the isolates were distinguished into four clusters representing different area or region.

Key words: *Xanthomonas*, SDS-PAGE, Pathogenicity.

Citrus belongs to family Rutaceae and Sub family Aurantiodeae[1]. In Marathwada members of rutaceae belongs into *Citrus aurentifolia, Citrus limon, Citrus sinensis, Citrus reticulata* are widely planted over an area of 87100 hectares in Marathwada region. The most common varieties of citrus in Marathwada are Premalinae, Vikram, Sai sharbati and Local. Citrus canker is one of the important diseases of citrus in Marathwada caused by *Xanthomonas campestris pv citrii* (Xcc).The incidence of canker in all these varieties varies with age of plant, location and season. Generally, the infection developed during early rainy season and virulence of pathogen varies depending varieties in symptoms and severity[2].

Many studies throughout the world have been carried on citrus Xcc pathosystem to understand the molecular basis of interaction[3]. One of the aspects in this pathosystem is considered as pathogen diversity and pathogen population[4]. However, host resistance is also considered to be one of the factors in this host –pathogen relationship. Different techniques were used to distinguish and distinguish strains of Xcc[5], as well as DNA based techniques are also used for detection of genetic variability among Xcc and relationship among different Xcc pathovars[6].

To find out relation among strains at species and subspecies level the whole cell protein profiling analysis were used. Protein can be separated using polyacrylamide gel electrophoresis[7]. The whole cell protein profile had been used to characterize *Xanthomonas spp*. from different

* To whom all correspondence should be addressed.
Mob.: +91-9422170641
E-mail: mmvb@indiatimes.com

region[8]. Thus molecular characterization by protein profiling of Xanthomonas and its phenotypic diversity[9] was carried out to distinguish and distinguish among 18 strains of Xcc isolated from Marathwada region in Maharashtra..

MATERIAL AND METHODS

Bacterial strains

Three hundreds strains of Xcc isolated from *Citrus lemona* from 16 different location within Marathwada region of Maharashtra were used in this study. The source and geographical origin of strains are given in Table 1. All the strains were isolated from infected plant parts collected from tree during midrainy season following standard method. All these strains were purified and maintain on YDC (Yeast extract -1gm, D-glucose -2gm, Calcium carbonate -2gm) slants.

Morphological characteristics

Morphological characteristics were recorded for all these strains includes colony characters like Gram staining , Cell morphology, Cell motility [10].

Biochemical test

Bacteriological characteristics of the isolated were examined by using the biochemical test described by Goszczynska *et al.*,[11], Aesculin test, Starch hydrolysis, Tween 80 lipolysis, H_2S production, Urease production, Milk proteolysis, Gelatin liquefication, Oxidase test. The results of these entire tests were recorded as either positive or negative were shown in Table 1.

Pathogenicity test

Strains tested for pathogenicity test by using leaf fruit samples and other sources, and all 18 strains were pathogenic to citrus under field condition[12] (Table 2). The bacterial suspension or the inoculants were prepared by using 48hr old cultures grown on nutrient agar. A loopful of the bacterial colony was suspended in luria broth and photometrically adjusted to an optical density corresponding to 1 to 3x 10^7 CFU/ml. Then the healthy plant were grown in laboratory, after growth the plant leaves, petiole and fruits were punctured at three to five different location with a sterile needle and 10ìl of the bacterial suspension was spread on each wound. Leaves were inoculated either along with the secondary veins or the laminae. Control plants were inoculated in the same way. Plants were checked for symptoms weekly or after 20-25 days after inoculation [13].

Isolation of protein from the Bacteria

Whole cell protein were isolated by using modified method described by Sujatha *et al*[14].

SDS-PAGE of whole cell protein

SDS-PAGE of whole cell protein was performed by using modified method described by Sujatha *et al*[14].

Phylogenetic Relationship

The strains were compared on the basis of presence or absence of protein bands in the gel. The presence or absence of band was used as 1 or 0 for preparing binary matrix and used for analysis using NTSYS. The cluster analysis was performed by outweighed pair group method using arthmatic averages (UPGMA). The dendrogram was generated with the NTSYS-PC to show the similarity coefficient between the genotype [15].

RESULTS AND DISCUSSION

The geographical distribution of the pathogen *Xanthomonas campestris pv citri* is very broad. The isolated strains were collected from the different area of Marathwada like from Nanded, Parbhani and Aurangabad, Beed and Osmanabad of Maharashtra state in India. In the biochemical characteristic, all Aesculin test were positive for all strains, Oxidase test shows positive by all strains except Xcc1. Starch hydrolysis test positive by strain number Xcc1, Xcc2, Xcc4, Xcc5, Xcc7, Xcc8, Xcc9, Xcc10, Xcc11, Xcc12, Xcc13, Xcc14, Xcc15, Xcc17, Xcc18 while strain Xcc3, & Xcc6 are negative. The Tween 80 lipolysis Xcc1, Xcc2, Xcc3, Xcc4, Xcc5, Xcc6, Xcc7, Xcc9, Xcc10, Xcc11, Xcc12, Xcc13, Xcc15, Xcc17, Xcc18 were positive while Xcc8, Xcc14 & Xcc16 were negative. Gelatin liquefaction were positive for all strains. Urease production Xcc1, Xcc2, Xcc3, Xcc4, Xcc5, Xcc6, Xcc7, Xcc8, Xcc9, Xcc12, Xcc13, Xcc14, Xcc16 were positive while Xcc10, Xcc11& Xcc18 were positive. For milk proteolysis Xcc1, Xcc6, Xcc8, Xcc14, Xcc16 were positive while Xcc1, Xcc2, Xcc3, Xcc4, Xcc7, Xcc9, Xcc10, Xcc11, Xcc12, Xcc13, Xcc15, Xcc17, & Xcc18 were negative. All strains shows positive test for H_2S production, Arabinose utilization Xcc1, Xcc6, Xcc8, Xcc10, Xcc11, Xcc14, Xcc16, Xcc18, were positive, while Xcc2, Xcc3, Xcc4,

Table 1. :Biochemical charactes of *Xanthomonas citrii* species used for differentiating various strains

Biochemical Characteristics	Xcc 1	Xcc 2	Xcc 3	Xcc 4	Xcc 5	Xcc 6	Xcc 7	Xcc 8	Xcc 9	Xcc 10	Xcc 11	Xcc 12	Xcc 13	Xcc 14	Xcc 15	Xcc 16	Xcc 17	Xcc 18
Aesculin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch Hydrolysis	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Tween 80 Lipolysis	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+
Gelatin Liquefaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease Production	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
Milk Hydrolysis	+	-	-	-	-	+	-	+	-	-	-	-	-	+	-	+	-	-
H_2S Production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose Utilization	+	-	-	-	-	+	-	+	-	+	+	-	-	+	-	+	-	+
Mannose Utilization	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Galactose Utilization	-	+	+	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+
Trehalose Utilization	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cellobiose Utilization	-	+	+	+	+	-	+	-	+	+	+	+	+	-	+	-	+	+
Fructose Utilization	+	-	-	-	-	+	-	+	-	+	+	-	-	+	-	+	-	+

Table 2. Strains of *Xanthomonas campestris pv citrii* used in this study

Strains	Location	Virulence	HR	Year
Xcc1	Beed -I	V	+	2009
Xcc2	Nanded –I	V	+	2009
Xcc3	Nanded-Basar	V	+	2010
Xcc4	Nanded-II	V	+	2010
Xcc5	Nanded-III	V	+	2009
Xcc6	Osmanabad-I	V	+	2010
Xcc7	Nanded-IV	V	+	2009
Xcc8	Parbhani-I	V	+	2009
Xcc9	Nanded-V	V	+	2009
Xcc10	Aurangabad-I	V	+	2010
Xcc11	Aurangabad-II	V	+	2010
Xcc12	Nanded-VI	V	+	2009
Xcc13	Nanded-VII	V	+	2010
Xcc14	Parbhani-II	V	+	2010
Xcc15	Nanded-VIII	V	+	2009
Xcc16	Parbhani-III	V	+	2009
Xcc17	Nanded-IX	V	+	2010
Xcc18	Aurangabad-III	V	+	2009

Xcc5, Xcc7, Xcc9, Xcc12, Xcc13, Xcc15, Xcc17 were negative. All strains shows mannose utilization positive except Xcc6. Galactose utilization Xcc2, Xcc3, Xcc4, Xcc5, Xcc6, Xcc7, Xcc9, Xcc10, Xcc11, Xcc12, Xcc13, Xcc15, Xcc17, Xcc18 were positive while Xcc1, Xcc8, Xcc14, Xcc16 were positive. Trehalose utilization positive for all strains, cellobiose utilization Xcc2, Xcc3, Xcc4, Xcc5, Xcc7, Xcc9, Xcc10, Xcc11, Xcc12, Xcc13, Xcc15, Xcc17, Xcc18 were positive while Xcc1, Xcc6, Xcc8, Xcc14, Xcc16 were negative. For fructose utilization Xcc1, Xcc6, Xcc8, Xcc10, Xcc11, Xcc14, Xcc16, Xcc18 were positive, while Xcc3, Xcc4, Xcc5, Xcc7, Xcc9, Xcc12, Xcc13, Xcc15, Xcc17 were negative.

Citrus canker distribution and occurrence in different geographical areas in Marathwada region varies from location to location and this depends on the interaction with host plants.

On fruit e.g. healthy citrus, necrosis developed around (i.e.1-2mm) the inoculation wound after 10-15 days. Sometimes the necrotic spots tended to enlarge and some fruit fell prematurely. The inoculated leaf lamina and veins

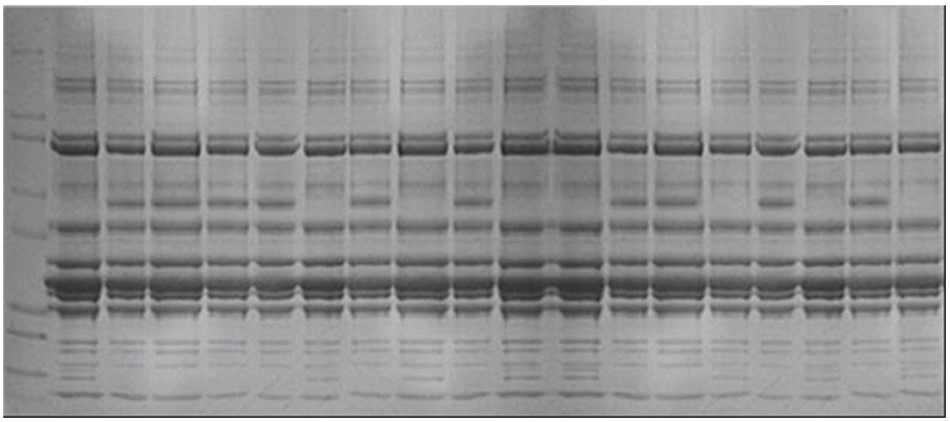

Fig. 1. SDS Gel electrophoregram of Total cell protein analysis of Various *Xanthomonas* strains

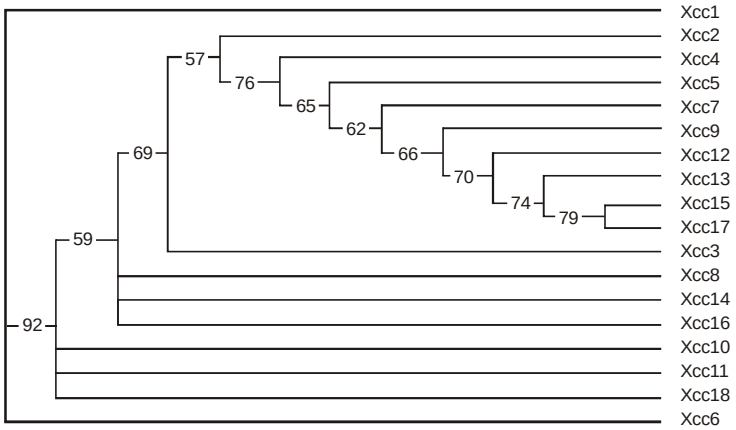

Fig. 2. Phylogram showing analysis of Various *Xanthomonas* strains based on total cell proteins

started to develops necrotic areas 7-11 days after inoculation. Some strains are more aggressive in the leaf lamina then veins, where large necrotic spots were developed. In control no any symptoms were developed.

All Xcc test strains were identified as Xanthomonas by UPGMA analysis. These strains divided into four cluster I, cluster II, cluster III, cluster IV. Cluster I with isolates Xcc3, Xcc17,Xcc15,Xcc13,Xcc12,Xcc9,Xcc7,Xcc5,Xcc4,Xcc2,Xcc1. Although with that analysis we separate in 9 group. Group I contain Xcc15 and Xcc17 which 79% correlate with group II Xcc13, these three isolates 74% correlate with group III Xcc12, like that remaining six group were 70% to 57% are correlate with each other. Cluster II contain xcc16 and Xcc14 and Xcc8, all these isolates 69% correlate with cluster I depending on the results of the biochemical test. Cluster III contain Xcc 10 and Xcc11 and Xcc 18 which 59% correlate with cluster II. Cluster IV contain Xcc6 and Xcc1 and this cluster 92% correlated with cluster III.

From this dendrogram it is clear that the isolates which are coming in cluster I are very similar with each other and they are belongs to same region i. e. they are from Nanded region. In cluster II isolates which are present some close to the cluster I and they are very close to Nanded i.e. from Parbhani region. In cluster III isolates vary from those present in cluster I and II and they are from different region like Beed, Aurangabad, Osmanabad and Latur. In cluster IV those isolates are present they are related with cluster III.

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