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OCCURRENCE OF BACTERIAL CANKER OF TOMATO IN HIMACHAL PRADESH, INDIA: IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF THE PATHOGEN

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ABSTRACT
Bacterial canker disease was widespread in all the tomato growing localities surveyed in Himachal Pradesh, India with an incidence and severity of 30.10 and 23.68 per cent, respectively. The disease symptoms appeared on stem, leaves, fruits and buds of infected tomato plants. Young plants showed poor growth and wilting. On older leaves dark brown spots appeared with yellow halo at leaf edges. Bird's eye spots appeared on fruits as brown, raised and surrounded by a persistent white halo. The bacterial colonies appeared as creamish to yellow in colour, roundish and semi fluidal on nutrient agar. Bacterium was identified to be *Clavibacter michiganensis* subsp. *michiganensis* on the basis of morphological characters, physiological and biochemical tests as well as pathogenicity tests. After molecular characterization of five isolates through direct polymerase chain reaction (PCR) with *C. michiganensis* specific primer pair CMR 16F1/R1 and nested PCR with *C. michiganensis* specific primer pair CMR 16F2/R2, the identity of three isolates viz., *Cmm 1, Cmm 2* and *Cmm 3* was confirmed to be *Clavibacter michiganensis* ssp. *michiganensis*. This is the first confirmed report on the bacterial canker in the tomato growing areas of Himachal Pradesh, India. The present investigation has firmly indicated the appearance of the disease in main tomato growing belt of the state.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is the most important vegetable crop grown word wide. Various fungi, bacteria, viruses and other pathogens are known to affect the crop in all tomato growing countries including India. Recently bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis*has emerged as a major disease of tomato in open as well as protected cultivation conditions in the southern parts of the country caused huge losses to the growers (Sarala and Shetty, 2005; Umesha, 2006).The pathogen is very destructive and causes wilting and stunting of plants, necrosis of leaves, necrotic lesions on fruit, discolouration of vascular tissues and death of severely infected plants. It requires relatively warm temperature and high relative humidity for infection and development. It is known to spread through contaminated seed to the new areas (Chang and Pataki, 1992). Due to seed transmission the disease spread to the areas where it was not reported earlier. In Himachal Pradesh also the disease was not reported earlier. The average temperature during most of the time period remains mild in this region. Therefore, tomato is grown during summer months unlike other parts of the country. During summers the congenial conditions for the development of bacterial canker do prevail in the month of June when the temperature is high and pre-monsoon showers provide humidity. The disease might, therefore, be present in the tomato growing areas of the state but it was not noticed earlier. The farmers usually applied control measures meant for fungal diseases due to ignorance. Under present investigation the attempt was therefore, made for the first time to know the status of this disease in the main tomato growing areas of the state and to identify the bacterial pathogen on the basis of various parameters including molecular characterization.

MATERIALS AND METHODS

Disease occurrence

Periodic surveys of tomato growing localities in Solan and Sirmour districts of Himachal Pradesh were undertaken to record the occurrence of bacterial canker during crop season of 2013. Disease incidence was recorded after observing tomato plants showing typical symptoms of the disease as discussed by Umesha (2006) and Sahu *et al*. (2013). Disease severity was recorded with the help of 0-5 scale, developed with slight modification in the scale of Bogo and Takatsa (1997) and Shukla and Gupta (2005) and the per cent disease severity was calculated as per the method of McKinney (1923).

Isolation, identification and characterization of the bacterium

The bacterial canker pathogen was isolated from infected tomato plants on nutrient agar (NA) medium as per the method described by Schaad *et al*. (2001) and Shivalingaiah and

Umesha (2011). Different isolates of the bacterial pathogen were isolated and purified by streak plate method and maintained on NA at 4°C for further studies. The colony characters of the bacteria viz., colour, size, shape etc. were recorded as per Schaad *et al*. (2001). The physiological and biochemical tests viz., Gram reaction, growth on TTC, gelatin liquefaction, casein hydrolysis, soluble starch hydrolysis, esculin hydrolysis, presence of catalase and oxidase, growth at 40°C and levan production from sucrose were performed as per the methods given by Holt *et al*. (1994), Bradbury (1986) and Schaad *et al*. (2001).

Pathogenicity test

For pathogenicity tests, 25 to 30 days old tomato plants of susceptible cultivar ArkaVikas were grown in earthen pots containing sterilized soil under glasshouse condition. The plants were inoculated with bacterial culture and evaluated for appearance of disease symptoms as per the method followed by Foster and Chandi (1973).

Molecular characterization

Extraction of genomic DNA: Total genomic DNA of bacterial isolates was extracted using CTAB method of Doyle and Doyle (1987). The extracted DNA was stored at -20°C in deep freezer for further studies and the quality and quantity of DNA was checked on 0.8 per cent agarose gel (Mills *et al.*, 1997).

PCR reaction conditions

Amplification of genomic DNA of bacterial isolates was performed by polymerase chain reaction (PCR) in Applied Biosystem Thermal Cycler as per the procedure followed by Lee *et al*. (1997). First direct PCR was carried out with a set of two specific primer pairs for *Clavibacter michiganensis*, CMR 16F1 (5′ - GTG ATG TCA GAG CTT GCT CTG GCG GAT C GTA-3′) and CMR 16R1 (5′ - CGG CTA CCT TGT TAC GAC TTA GT-3′); CMR 16F2 5′ - CCC CGA CTC TGG GAT AAC TGC TA -3′) and CMR 16R2 (5′ - CGG TTA GGC CAC TGG CTT CGG GTG TTA CCG A -3′) designed from the 16S rDNA region.The reaction was performed in final volume of 25 ìl containing 1 × PCR Buffer, dNTP mix (0.2 mm each of dCTP, dGTP, dATP and dTTP), 0.5 µm each forward (CMR F1) and reverse primer (CMR R1), 1 unit of *Taq* DNA polymerase (3U/µl), 25-30 ng template DNA. These tubes were placed on thermo-cycler for cyclic amplification. Conditions for amplifications were programmed as initial denaturation (94°C, 5 min), denaturation (94°C, 1 min), annealing (62°C, 2 min), extension (72°C, 3 min) and final extension (72°C, 10 min) for 35 cycles. The nested PCR with the primer pair CMRF1, R1 and CMRF2, R2 was then performed. The amplified products were separated on 1% agarose gel for 1 hour at 60V constant voltage. Gels were stained with 0.5 µg ml^{-1} ethidium bromide solution and photographed under ultraviolet light using Alpha imager Gel doc system (Sambrook and Russell, 2001).

Table 1: Incidence and severity of tomato bacterial canker in Solan and Sirmour districts of Himachal Pradesh

Location	Disease incidence (%)	Disease severity (%)
Solan:		
Bergaon	37.00	31.60
Deothi	35.48	27.73
Salogda	20.00	16.56
Galanag	27.48	13.50
Kyar	33.00	20.83
Nauni	18.50	17.64
Khaltu	29.53	21.40
Basal	34.00	19.50
Sirmour :		
Kalaghat	18.50	16.96
KotlaPanjola	28.50	24.36
Nandal	20.00	18.70
Deothal	47.50	43.50
Nauhra	27.84	23.72
Narag	34.00	27.00
Sanoura	39.50	29.53
Mean	30.10	23.68

RESULTS AND DISCUSSION

Disease occurrence

The bacterial canker disease was observed in all the tomato growing localities surveyed in Solan and Sirmour districts of Himachal Pradesh, India during 2013 (Table 1). The overall incidence of the disease was observed up to 30.10 per cent with a severity of 23.68 per cent. Amongst various localities surveyed, the highest incidence of 47.50 per cent with a severity of 43.50 per cent was recorded atDeothalarea of Sirmour district. In other localities the incidence varied between 18.50 to 39.50 per cent with a severity of 13.50 to 31.60 per cent. Previously no such surveys were conducted for recording bacterial canker of tomato in Himachal Pradesh. Hence, present investigation is the first attempt to record the occurrence of this disease in the state. However, Sarala and Shetty (2005) and Umesha (2006) have reported the occurrence of bacterial canker of tomato in the southern part of India like Karnataka state with an average incidence of 48 per cent. Earlier many workers have described the bacterial canker disease of tomato to be seed borne in nature (Chang *et al*., 1989; Gitaitis *et al*., 1991 and Fatmi *et al*., 1991). The disease might have appeared in this state through contaminated seed. The seed production farms of most of the seed companies in India are situated in south Indian states like Karnataka and Andhra Pradesh where

Table 2: Colony characteristics of different isolates of bacterial canker pathogen on nutrient agar medium

Isolates	Colony characteristics Colour	Shape	Size (mm in dia.)	Other characteristic	Colour
*Cmm*1	Creamish to yellow	Round	1-2	Round and semifluidal	Creamish to yellow
*Cmm*2	Yellow	Round	2-3	Glistening with longer incubation (>7 days)	Yellow
*Cmm*3	Creamish to yellow	Round , mucoid	2-3	Mucoid, circular, convex with entire margin	Creamish to yellow
*Cmm*4	Creamish to yellow	Round, mucoid	2-3	Mucoid, circular, convex with entire margin	Creamish to yellow
*Cmm*5	Orange	Round	2-3	Flat	Orange

Table 3: Physiological and biochemical characters of different strains of tomato bacterial canker pathogen

Character	Reaction as per Bergey's Manual, 1994	Reaction of diffrent strains *Cmm*1	*Cmm*2	*Cmm*3	*Cmm*4	*Cmm*5
Gram reaction	+	+	+	+	+	+
Esculin hydrolysis	+	+	+	+	+ w	+ w
Starch hydrolysis	D	-	-	-	-	-
Gelatin liquefaction	+ w	+	+	+	+	+ w
Presence of catalase	+	+	+	+	+	+
Presence of Oxidase	-	-	-	-	+	+
Growth on TTC		+	+	+	+	+
Levan production	-	-	-	-	+	-
Casein hydrolysis	-	-	-	-	-	+
Growth at 40°C		+ w	+ w	+ w	+ w	+ w

+ = 90% or more strains positive, – = 90% or more strains negative, D = 11–89% of strains positive, + w = weak reaction.

Table 4: Pathogenicity of different strains of tomato bacterial canker pathogen on seedlings

Strain	Symptoms development (days after inoculation)														
	0	6	9	12	15	18	21	24	27	30	33	36	39	42	45
*Cmm*1	0	1	1	1	2	2	2	3	3	3	4	4	4	4	4
*Cmm*2	0	0	1	1	2	2	2	3	3	3	3	4	4	4	4
Cmm3	0	0	1	1	2	2	2	3	3	3	3	4	4	4	4
*Cmm*4	0	0	1	1	2	2	2	3	3	3	3	3	4	4	4
*Cmm*5	0	0	0	0	0	0	0	0	0	1	1	1	1	1	2

0 = no symptom, 1 = up to 1/3 of the leaves showed marginal necrosis and yellowing, 2 = marginal necrosis and up to 2/3 of the leaves showed yellowing and wilting, 3 = more than 2/3 of leaves and leaflets shrivelled and wilted but the terminal leaves on the main shoot showed no wilt, 4 = terminal leaves of the main shoot and most leaves wilted, 5 = plant died

the disease is of common occurrence. Farmers mostly procure seed from private companies (Sarala and Shetty, 2005; Umesha, 2006).

Symptomatology

The disease symptoms appeared on almost all the above ground plant parts viz., leaves, petioles, stem, branches and fruits (Fig. 1 and Fig. 2). Young plants showed poor growth and wilting of branches. The lower leaves of affected plants were yellow, shriveled with dark brown spots usually at the edges and surrounded by yellow halo. In some cases one half of the affected plant showed wilt symptoms while the other half appeared healthy (Fig. 1A & B). The stem of the affected plant developed with scars or cavities at some locations (Fig. 1C). Sometimes splitting of the affected stem was also noticed with longitudinal cankers (Fig. 1D).The pith of affected stem appeared reddish brown, especially at the nodes (Fig. 1E). The pith later turned somewhat mealy appearance and hollow. Fruit symptoms as bird's eye lesions were noticed on severely affected plants. The lesions on fruits were yellow to brown, slightly raised, about 3 mm in diameter and surrounded by a persistent white halo (Fig. 2 B). Similar symptoms of bacterial canker disease on tomato plants have been described by earlier workers from India and abroad (Gitaitis, 1991; Umesha, 2006; Ftayeh *et al.*, 2011).

Identification and characterization of the pathogen

Morphological characters

Five different isolates of bacterial canker pathogen (*Clavibacter michiganensis* subsp. *michiganensis*), *Cmm1*, *Cmm2*, *Cmm3*, *Cmm4* and *Cmm5* were isolated and purified. The colony characteristics of these isolates were recorded and are presented in Table 2. The colonies of all the isolates were 1-3 mm in diameter and developed within 3 days of inoculation. The colonies were mostly creamish to yellow in colour, roundish and semi fluidal. The colonies became deeper yellow and glistering with the period of incubation. Umesha (2006) has also observed colonies of tomato bacterial canker pathogen as smooth, yellow, mucoid, circular and convex. Similar characteristics of bacterial canker pathogen affecting tomato have also been described by earlier workers (Fatmi and Schaad, 1988; Shirakawa and Sasaki, 1988; Ftayeh *et al.*, 2011).

Physiological and biochemical characters

All the bacterial isolates were found positive for Gram stain, esculin hydrolysis, gelatin liquefaction, catalase and 2,3,5-triphenyl tetrazolium chloride (TTC), and were negative for starch hydrolysis, oxidase, levan production and casein hydrolysis (Table 3). The results of present investigation with regard to physiological and biochemical characters of tomato bacterial canker pathogen were in accordance with that described in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) and Guide to Plant Pathogenic Bacteria (Bradbury, 1986). In present study, the bacterial canker pathogen strains *Cmm*1, *Cmm*2 and *Cmm*3 provided accurate results with respect to various biochemical tests, while *Cmm*4 and *Cmm*5 had weak reaction with some of the tests. The weak reaction of these strains might be due to the prolonged sub-culturing of the bacterial isolates on the artificial medium. Burokiene *et al.* (2005) and Milijasevic *et al.* (2012) have also conducted such tests against various strains of bacterial canker pathogen and found similar results.

Pathogenicity test

Most of the strains of *Cmm* showed the incubation period between 6 to 9 days when artificially inoculated to the tomato seedlings of cv. ArkaVikas (Table 4). The disease symptoms

Figure 1: Symptoms of bacterial canker on tomato leaves and stem: infected tomato plant (A), marginal necrosis of leaf (B), stem splitting (C), hollow pith (D), browning of internal tissues (E).

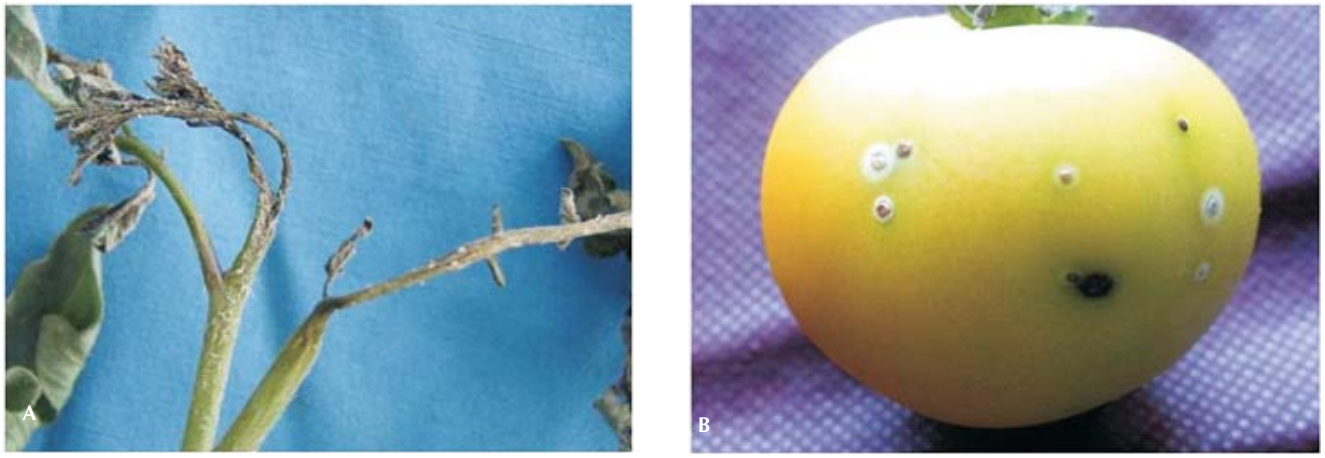

Figure 2: Symptoms of tomato bacterial canker on young bud and fruit: infected bud of tomato, bird's eye spots on young fruit

were noticed as marginal necrosis with hallow on leaves. Similar results have been observed with various strains of bacterial canker pathogen in tomato by different workers upon artificial inoculation (Chang *et al*., 1989; Bogo and Takatsa, 1997; Burokiene *et al*., 2005).

Molecular characterization

Direct PCR with *Clavibacter michiganensis* specific primer pair CMR 16F1/R1 and nested PCR with *C. michiganensis* specific primer pair CMR 16F2/R2 revealed that CMR 16F1/R1 and CMR 16F2/R2 amplified (614bp) the genomic DNA of 3 tomato bacterial canker isolates, *Cmm*1, *Cmm*2 and *Cmm*3 (Fig. 3). However, other two isolates,*Cmm*4 and *Cmm*5 did not show any amplification. Hence, the isolates,*Cmm*1, *Cmm*2 and *Cmm*3 were confirmed to be *Clavibacter michiganensis*. As these isolates produced characteristic symptoms of bacterial canker on tomato seedlings after artificial inoculation, these were identified as *Clavibacter michiganensis* ssp. *michiganensis* (Smith) Davis *et al*. Nested PCR for detection of *Clavibacter michiganensis* with these primer pair were also used by Lee *et al*. (1997) and Kyu *et al*. (2012).

Previously no such attempt to isolate and detect the bacterial canker pathogen using molecular tools has been carried out in Himachal Pradesh. Hence, the present study provided the first reliable detection of the bacterial canker pathogen *Clavibacter michiganensis* subsp. *michiganensis* in the tomato

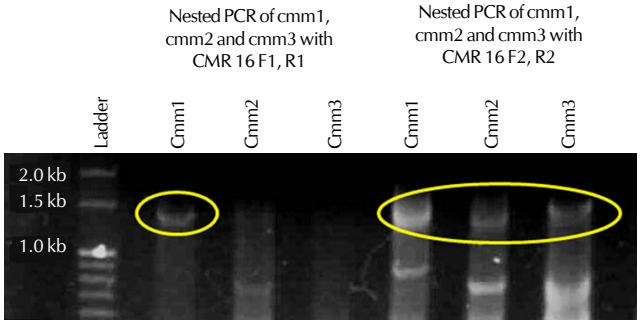

Figure 3: PCR amplification of three strains of *Clavibacter michiganensis* subsp. *michiganensis* using nested primer pairs (CMR16F1, R1and CMR16F2, R2)

growing areas of Himachal Pradesh, India. The present investigation has firmly indicated the occurrence of the disease in main tomato growing belt of the state.Hence, there is urgent need to develop proper management practices for this disease. So that the losses caused by the disease to the farmers be minimized.

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