



Prevalence of Citrus Canker Caused by *Xanthomonas axonopodis* pv. *citri* in Subtropical Zone of Himachal Pradesh

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Abstract

Citrus canker is most important disease of citrus causing extensive losses all over the world including India as well as in Himachal Pradesh. To record the prevalence of disease on different *Citrus* spp. a survey was conducted in different citrus growing areas of Himachal Pradesh during 2018 and data in terms of disease incidence (%) as well as disease severity (%) was recorded. During the survey the disease incidence was found to be ranging from 40 to 100% and disease severity ranged between 2.78% to 50.55% having maximum mean disease incidence (87.77%) as well as mean disease severity (29.07%) in district Kangra and minimum mean disease incidence (69.74%) as well as severity (11.21%) in Hamirpur district of Himachal Pradesh. The areas in which disease severity was found to be more than 15.00 %, diseased samples from those areas were collected and studied *in vitro*. Total nine isolates were collected and these different isolates produced yellow, pale and light yellow circular colonies with colony diameter ranging between 1.45 mm to 3.15 mm and tested positive both for protein digestion as well as esculin hydrolysis and exhibited negative reaction towards Gram's staining. During pathogenicity test of all isolates on K. lime host as detached leaf assay, the symptoms appeared in 3.00 to 5.00 days while, 14.00 to 16.00 days time was recorded for complete symptom development under *in vitro* conditions.

Keywords: Citrus canker, *Xanthomonas axonopodis* pv. *citri*, intensity

1. Introduction

Citrus is a very important fruit crop of India as well as Himachal Pradesh. In India citrus is cultivated on an area of 1055 thousand hectares with a production of about 12746 thousand tonnes. (Anonymous, 2017). In Himachal Pradesh, total production of citrus is about 26853 tonnes from an area of 24649 ha having maximum area under K. lime (Anonymous, 2018). Citrus crop is threatened by number of diseases and among them citrus canker caused by *Xanthomonas axonopodis* pv. *citri* (Hasse) Vauterin et al. (1995) is one of the most devastating diseases in nursery and young orchards. So, the disease is of great economic importance all over the citrus growing area of the world including India. The disease has played a significant role in causing extensive losses in nurseries as well as in orchards (Gottwald and Irey, 2007). In Florida, nearly 2,57,000 orchard trees and 3,000,000 nursery plants were destroyed at a cost of over \$ 6 million during the year 1915-33 and again during 1984-86

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(Schoulties et al., 1987). The most recent outbreak of citrus canker appeared in Florida in 1995 and after ten years of eradication effort, the USDA declared that the eradication was no longer possible. The disease continues to increase its geographic range inspite of heightened regulations imposed by many countries. The disease caused major economic losses in citrus growing countries like USA (Gottwald and Irey, 2007), Argentina (Canteros, 2004) and Brazil (Leite and Mohan, 1990). The origin of citrus canker is not clear but is thought to have originated from south-east Asia or India and then widely distributed around the world (Civerolo 1984; Verniere et al., 1998). In India, the citrus canker was first reported from Punjab (Luthra and Sattar, 1942). Several workers have reported the incidence of canker on the acid lime and other varieties of citrus (Das, 2003). 7.50% incidence of citrus canker was reported in three districts i.e. Kalurkot, Daryakhan and Bhakkar of Punjab province of Pakistan (Sahi et al., 2007). In Bangladesh, eight nurseries were surveyed during January, 2012 to December, 2013 which revealed the highest incidence (72.22 %) and severity (27.55%) of citrus canker during July at Khagrachari and lowest incidence (28.22%) and severity (5.55 %) was recorded in Dhakka during January (Rashid et al., 2014). In Peninsular Malaysia, incidence and severity of citrus canker on leaves was 36.50 and 15.20%, respectively whereas, it was 18.70 and 7.50% on fruits respectively (Derso et al., 2007). According to Jain (1959), different varieties of the same citrus species were infected to almost same extent in Himachal Pradesh. In subtropical zone of Himachal Pradesh, incidence of citrus canker was recorded to range between 28–39% during 2015-16 (Anonymous, 2016). Keeping in view the importance of the disease the present studies were conducted to record the prevalence of disease in subtropical zone of Himachal Pradesh on different species of citrus.

2. Materials and Methods

2.1. Disease survey and collection of diseased samples

To record the prevalence of the disease in terms of disease incidence and severity (% disease index), a survey was conducted during 2018 in different areas of three districts (Kangra, Hamirpur and Sirmour) of Himachal Pradesh. Disease severity of citrus canker was recorded by the using the disease rating scale given by Horsfall and Heuberger (1942) and further calculated by the formulae given by Johnston and Booth (1983).

Disease severity / % disease index = $\frac{\text{Sum of individual rating}}{\text{Total number of ratings} \times \text{Maximum rating}} \times 100$

Disease incidence was calculated by the formulae:

Disease incidence (%) = $\frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$

During the survey, the diseased plants of K. lime (*C. aurantifolia*), Kinnow (*C. reticulata*), Mosambi (*C. sinensis*) and Jatti Khatti (*C. jambhiri*) showing typical canker symptoms were selected. Diseased plant parts (leaves and twigs) were

collected, packed in bags and brought to laboratory. These samples were subjected to isolation and purification and maintained for further studies of the pathogen

2.2 Isolation, designation and maintenance of isolates

The pathogen was isolated by using the method given by Janse (2005). After isolation and purification of the isolates they were maintained inside the refrigerator at 4 °C for further studies. Nine isolates of different species of citrus from different locations were isolated and maintained and were designated as isolate 1, 2, 3, 4, 5, 6, 7, 8 and 9. These isolates were sub – cultured at regular intervals.

2.3. Identification of the pathogen

The identity of different isolates was confirmed by the cultural, biochemical and pathogenicity traits of the pathogen.

2.3.1. Cultural characters

Erlenmeyer flasks of 150 ml capacity were filled with 50 ml of nutrient sodium chloride broth and autoclaved at 121°C and 15 p.s.i pressure for 20 minutes. One loopful of bacterial colonies of each isolate was inoculated in each flask and incubated at 28 ± 2 °C for 48 h. One ml bacterial suspension from each flask was serially diluted up to a dilution factor of 10⁻⁷ and was pour plated in nutrient sodium chloride agar medium. These plates were incubated at 28 ± 2°C in the BOD incubator for 48 – 72 h. Various colony characters viz., colour, shape, size and elevation of the colonies in each isolate were recorded and compared with the typical colony characters of *Xanthomonas* spp.

2.3.2. Biochemical tests

Different biochemical tests viz., esculin hydrolysis, protein digestion and Gram's staining were performed to confirm the identity of all isolates as per the methodology given by Schaad and Stall (1988) and Bergey's Manual of Determinative Bacteriology (1923). Development of black colour and a clearing solution were observed in the esculin hydrolysis and protein digestion tests, respectively while, production of reddish pink colour of cell walls under microscope was observed in Gram's staining procedure in all isolates under study.

2.3.3. Pathogenicity test

Pathogenicity test of each isolate was conducted on Kagzi lime host using detached leaf assay method given by Verniere et al. (1998).

3. Results and Discussion

3.1. Disease survey and collection of diseased samples Disease was found to be prevalent on different *Citrus* species in all the areas surveyed in three districts of the state. It is clear from the Table 1 that disease incidence ranged between 40 to 100 % whereas, disease severity ranged between 2.78 to 50.55 % on various species of citrus. As far as the prevalence of the disease in different areas of the state was concerned,



Table 1: Occurrence and magnitude of citrus canker at various locations in Himachal Pradesh

District	Area	Citrus spp.	Disease incidence (%)	Disease severity (%)
Hamirpur	Dera Parol	<i>C. sinensis</i> (Mosambi)	50.00	3.34
	Dhanwan	<i>C. aurantifolia</i> (K. lime)	100.00	22.22
	Neri	<i>C. aurantifolia</i> (K. lime)	88.45	15.06
	Putriyal	<i>C. sinensis</i> (Mosambi)	40.00	4.44
	Bara	<i>C. sinensis</i> (Santra)	40.00	2.78
	Lambloo	<i>C. jambhiri</i> (Jatti khatti)	100.00	19.44
	Mean		69.74	11.21
Sirmour	Dhaulakuan	<i>C. jambhiri</i> (Jatti khatti)	100.00	44.83
	Kolar	<i>C. sinensis</i> (Mosambi)	100.00	15.08
	Behrabala	<i>C. reticulata</i> (Kinnow)	100.00	19.04
	Surajpur	<i>C. limon</i> (Lemon)	42.80	2.78
	Mean		85.70	20.43
Kangra	Jachh	<i>C. jambhiri</i> (Jatti khatti)	100.00	50.55
	Basabzain	<i>C. reticulata</i> (Kinnow)	83.33	17.78
	Panjarda	<i>C. sinensis</i> (Mosambi)	80.00	18.88
	Mean		87.77	29.07

the mean disease incidence (87.77%) and severity (29.07%) was found to be maximum in district Kangra whereas, mean disease incidence (69.74%) and severity (11.21%) was recorded to be minimum in district Hamirpur.

In district Hamirpur, the maximum disease incidence (100.00%) was recorded on K. lime (*Citrus aurantifolia*) and Jatti khatti (*C. jambhiri*) at Dhanwan and Lambloo, respectively whereas, minimum disease incidence (40.00%) was recorded on Santra (*C. sinensis*) and Mosambi (*C. sinensis*) at Bara and Putriyal, respectively. As far as the disease severity was concerned, it was recorded to be maximum (22.22%) on K. lime (*C. aurantifolia*) at Dhanwan whereas, minimum disease severity (2.78%) was recorded on Santra (*C. sinensis*) at Bara.

In district Sirmour, disease incidence was recorded to be 100% at Dhaulakuan, Kolar and Behrabala on Jatti khatti (*C. aurantifolia*), Mosambi (*C. sinensis*) and Kinnow (*C. reticulata*), respectively while, lowest disease incidence (42.80%) was recorded on Lemon (*C. limon*) at Surajpur. As far as the disease severity was concerned, it was recorded to be maximum (44.83%) on Jatti khatti (*C. jambhiri*) at Dhaulakuan whereas, minimum disease severity (2.78%) was recorded on Lemon (*C. limon*) at Surajpur.

In district Kangra, maximum disease incidence (100.00%) and severity (50.55%) was recorded on Jatti khatti (*C. jambhiri*) at Jachh whereas, minimum disease incidence (80.00%) and disease severity (17.78%) were recorded on Mosambi (*C. sinensis*) at Panjarda and on Kinnow (*C. reticulata*) at Basabzain, respectively.

The occurrence of disease on various species of *Citrus* in surveyed areas is in accordance with Rashid et al. (2014), Singh and Thind (2014) and Ference et al. (2018) who reported

the susceptibility of different host species towards the citrus canker pathogen.

From surveyed areas in which disease severity was found to be more than 15.00%, the diseased samples from canker infected citrus plants were collected and brought to laboratory for isolation. In all, nine pathogen isolates were collected from four different species of citrus at nine locations as mentioned in Table 2, Figure 1.

Table 2: Details of various isolates collected from different areas

Isolate name	Citrus spp.	Area of collection	District
Isolate-1	<i>Citrus reticulata</i> (Kinnow)	Behrabala	Sirmour
Isolate-2	<i>C. sinensis</i> (Mosambi)	Kolar	Sirmour
Isolate-3	<i>C. jambhiri</i> (Jatti khatti)	Dhaulakuan	Sirmour
Isolate-4	<i>C. reticulata</i> (Kinnow)	Basabzain	Kangra
Isolate-5	<i>C. sinensis</i> (Mosambi)	Panjarda	Kangra
Isolate-6	<i>C. jambhiri</i> (Jatti khatti)	Jachh	Kangra
Isolate-7	<i>C. aurantifolia</i> (K. lime)	Neri	Hamirpur
Isolate-8	<i>C. aurantifolia</i> (K. lime)	Dhanwan	Hamirpur
Isolate-9	<i>C. jambhiri</i> (Jatti khatti)	Lambloo	Hamirpur

3.2. Isolation and Identification of the pathogen

The pathogen was isolated on nutrient sodium chloride agar medium and different isolates were identified on the basis of cultural, pathogenic and biochemical tests.



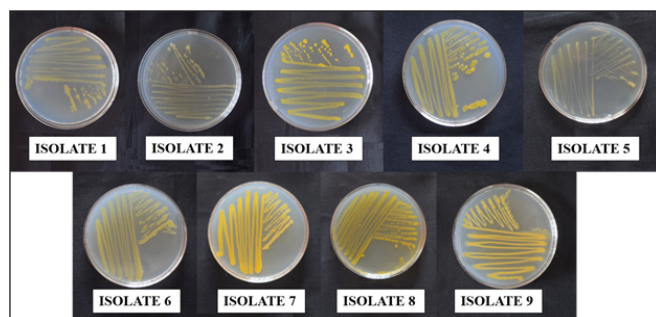


Figure 1: Pure cultures of *Xanthomonas axonopodis* pv. *citri* isolates

3.2.1. Cultural characters

Yellow coloured colonies were obtained by streaking the bacterial suspension on nutrient sodium chloride agar plates. Single colonies from these plates were picked and further streaked on new nutrient sodium chloride agar plates to get the purified isolates. The cultural and morphological characters of the pathogen isolates were also recorded and have been presented in Table 3.

It is clear from the table that isolates 1, 8 and 9 were pale in colour, isolate 5 was light yellow in colour and rest of the isolates were yellow in colour. Colony diameter of the isolates ranged from 1.45 mm in isolate 7 to 3.15 mm in

Table 3: Cultural characteristics of various isolates of *Xanthomonas axonopodis* pv. *citri* collected from different locations

Characters	Isolate								
	1	2	3	4	5	6	7	8	9
Colour	Pale	Yellow	Yellow	Yellow	Light yellow	Yellow	Yellow	Pale	Pale
Colony diameter (mm)	3.15	2.75	2.10	2.70	2.50	1.60	1.45	1.80	2.60
Elevation	Flat	Elevated	Slight elevation	Flat	Flat	Flat	Flat	Flat	Flat
Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular

isolate 1. As far as the elevation of colonies was concerned, colonies of isolate 2 were elevated and slight elevation was also observed in colonies of isolate 3 while, all other isolates formed flat colonies. Shape of colonies in all the isolates was found to be circular.

3.2.2. Biochemical tests

The identity of the pathogen was also confirmed on the basis of different biochemical tests viz., protein digestion, esculin hydrolysis and Gram's staining. All the isolates tested positive both for esculin hydrolysis indicated by blackening of esculin medium after 2 days of pathogen inoculation and protein digestion indicated by the appearance of clear solution within 10 days of inoculation (Figure 2). All the pathogen isolates stained pink in Gram's staining thus giving a negative reaction, indicating the pathogen isolates to be Gram negative.

3.2.3. Pathogenicity test

The results of pathogenicity tests have been depicted in terms

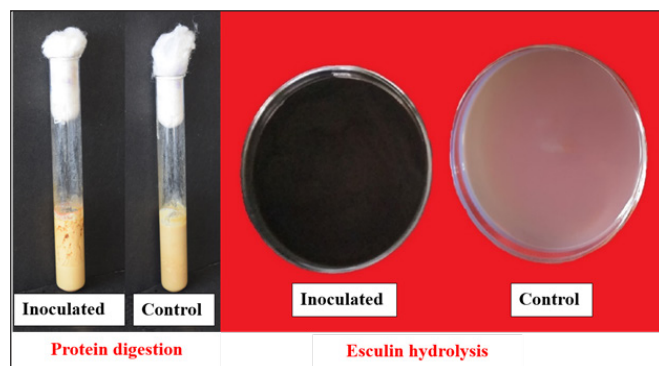


Figure 2: Identification of *Xanthomonas axonopodis* pv. *citri* isolates by biochemical tests

of incubation period and type of symptom developed in Table 4, Figure 3. It is clear from the table that incubation period varied from 3.00 days in isolate 4 to 5.00 days in isolate 6 and time taken for complete symptom development varied from 14.00 days in isolates 4, 7 and 9 to 16.00 days in isolates 1, 3, 6 and 8. On the upper surface of the leaves, yellow halo around the lesion appeared in case of isolates 1, 2 and 4, while crater like lesions appeared in rest of the isolates.

Table 4: Incubation period and type of symptoms developed in pathogenicity tests of different isolates of *Xanthomonas axonopodis* pv. *citri*

Isolate	Incubation period (days)	Symptoms on upper leaf surface
1	4.00 (16.00)	Yellow halo around the lesion
2	3.33 (15.00)	Yellow halo around the lesion
3	4.67 (16.00)	Crater like lesion
4	3.00 (14.00)	Yellow halo around the lesion
5	4.00 (15.00)	Crater like lesion
6	5.00 (16.00)	Crater like lesion
7	4.00 (14.00)	Crater like lesion
8	4.33 (16.00)	Crater like lesion
9	4.00 (14.00)	Crater like lesion

Figures in parentheses represents time (days) taken for complete symptom development

Based on the morphological, cultural and biochemical observations, the pathogen isolates were identified as *X. axonopodis* pv. *citri*. Colonies of different isolates on nutrient

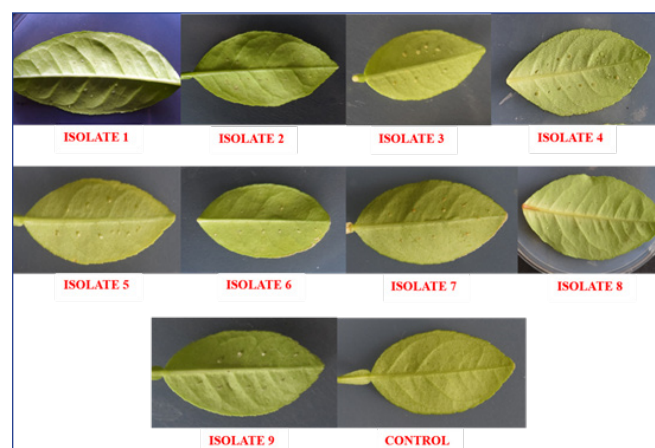


Figure 3: Pathogenicity of different isolates of *Xanthomonas axonopodis* pv. *citri* proved on detached leaves of *K. lime* by pin prick method

sodium chloride agar medium were yellow or different shades of yellow which is supported by the findings of Das (2003) who reported that colonies of *Xanthomonas* spp. on culture media are yellow as a result of xanthomonadin pigment production. The findings of cultural characters of various isolates were supported by the work of Singh and Thind (2014) who reported the variation in colour and shape of colonies of *X. axonopodis* pv. *citri* on various media. Dhakal et al. (2009) and Schaad and Stall (1988) have reported the pathogen to be Gram negative and esculin hydrolysis as well as protein digestion positive which support present findings. As far as pathogenicity of pathogen isolates was concerned, the results similar to our findings have been reported by Khalid et al. (2010) who reported that the bacterium produced symptoms on the host within 2 – 3 weeks and type of symptoms ranged from pustules to necrotic lesions consisting of erumpent corky tissue.

4. Conclusion

In Himachal Pradesh, citrus canker was prevalent on *Citrus aurantifolia*, *C. sinensis*, *C. reticulata*, *C. jambhiri* and *C. limon*. The associated pathogen was identified as *X. axonopodis* pv. *citri* on the basis of biochemical tests. Nine pathogen isolates collected, produced light yellow, pale and yellow colonies, with or without elevation, colony diameter between 1.45 to 3.15 mm on nutrient sodium chloride agar. Symptoms appeared in 3.00 to 5.00 days and developed completely in 14.00 to 16.00 days during pathogenicity tests.

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