



## Pathogenicity and inoculum concentration effects of *Clavibacter michiganensis* subsp. *michiganensis* on severity of bacterial canker of tomato

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### Abstract

A total of 100 tomato plant samples exhibiting typical symptoms of bacterial canker were collected from tomato-growing areas of north-western areas of Pakistan. On isolation of the causal agent of this disease, 47 out of 100 samples yielded typical colony morphology of *Clavibacter michiganensis* subsp. *michiganensis*, when grown on Nutrient agar (NA) medium. 34 isolates out of the 47 exhibited *Clavibacter michiganensis* subsp. *michiganensis* like cultural characteristics when grown on Yeast extract-Dextrose-CaCO<sub>3</sub> (YDC) medium. However, 27 isolates only out of these 34 were confirmed to be Gram positive. Pathogenicity of the 27 *Clavibacter michiganensis* subsp. *michiganensis* isolates were confirmed using cotyledon, young seedlings and old seedlings assays. Results showed that all these isolates were able to cause bacterial canker disease symptoms on tomato plants. Cotyledon assay was negative for isolates from Mansehra (MNS2); whereas, MNG1 isolate didn't produce any symptoms for all three tests. Old seedlings test was negative for four isolates mainly; MKD2, MNG1, DIK1 and NWS2. To investigate the hypothesis that appearance of symptoms depends on inoculum concentration of pathogen; two aggressive isolates of *Clavibacter michiganensis* subsp. *michiganensis* i.e., MKD1 (from Malakand) and KLM1 (from Kalam) were inoculated into tomato plants at different concentrations (i.e.  $1 \times 10^2$  -  $10^8$  cells/ml). Results reported that earlier and more severe disease symptoms of bacterial canker were produced by the more aggressive isolate (MKD1); compared to the moderately aggressive isolate (KLM1), which produced milder and delayed disease symptoms on tomato plants.

**Keywords:** Tomato, *Clavibacter michiganensis* subsp. *michiganensis*, Pathogenicity, inoculum concentration

### 1. Introduction

Tomato (*Solanum lycopersicum*) is grown worldwide for its edible fruits. Due to its high nutritive value; tomato consumption is believed to

benefit the heart and reduce cardiovascular diseases (Shidfar *et al.*, 2011). Moreover; tomato consumption has been associated with decreased risks of breast

cancer (Zhang *et al.*, 2009), head and neck cancers (Freedman *et al.*, 2008), moreover, is strongly protective against neurodegenerative diseases (Rao *et al.*, 2002; Sukanuma *et al.*, 2002).

There are about 7500 varieties of tomato which were grown for different purposes (Allen, 2008). Regarding the annual production of tomatoes worldwide; it reached 148.26 million tons (MT) in 2013. China is the largest producer (50.55 MT); accounted for about one quarter of the global output, and is followed by India (18.23 MT) and United States (12.57 MT). Pakistan produced 0.57 MT of tomatoes during 2013 (FAOSTAT. 2015). Tomato crop is grown all over the Pakistan, and about 58196 hectares areas were grown with different varieties of this crop. Regarding the average per hectare global production of tomatoes; Belgium and Netherland were on top with  $5.0 \times 10^6$  and  $4.9 \times 10^6$  kg/ ha; respectively. On the other hand, Pakistan holds 142<sup>th</sup> position with a much lower average production of  $9.8 \times 10^3$  kg/ ha.

Occurrence of different tomato diseases is one of the major reasons responsible for the lower yields of this crop. Mildews, blights, wilts and cankers are some of the most common infectious diseases. Tomato canker; also known as bacterial canker or bird's eye, is one of the most important bacterial diseases. Bacterial canker of tomato was first reported from USA in 1910, probably originated there. Ranges of disease incidence were recorded to be 10-80 % (Vasinauskienė, 2002) with about 20-30 % annual losses (A. Rafi, personal communication).

Bacterial canker disease is caused by *Clavibacter michiganensis* subsp. *michiganensis*. This pathogen is seed-borne, and is also able to survive in soil for more than two years in infected plant debris (ASTA. 2011). Typical canker symptoms develop at 23-28°C, when relative humidity is 80 % or more (Xu *et al.*, 2012). The first disease symptom in case of greenhouse grown tomatoes was the reversible wilting of leaves during hot mid-day hours. Later, wilting becomes

irreversible and the whole plant dies. Under field conditions; the edge of the leaflets of lower leaves start drying out; and then small whitish pustules were produced on leaf veins and petioles. During the advanced stages of the disease, a reddish brown discoloration of the vascular tissue can easily be recognized at the union of stems and branches; petiole and peduncle etc. (OEPP/EPPO Bulletin. 2013). The disease affects the fruits, so they may fail to develop and fall, or ripen unevenly. Moreover; fruits may sometimes develop characteristic "bird's eye" spots. Through cracks in the stem, slimy masses of bacteria ooze out to the surface during wet weather (Agrios, 2005).

Efficient management of this disease requires integrated control measures. The current study aimed to evaluate the pathogenicity of *Clavibacter michiganensis* subsp. *michiganensis* isolates on three different tomato growth stages; and to find the effect of inoculum concentration on disease severity of bacterial canker of this crop.

## 2. Materials and methods

### 2.1. Survey and sampling

In order to collect plant samples from tomato fields; a comprehensive survey was conducted in tomato-growing areas of Khyber Pakhtunkhwa (KP), from April to August, 2012. KP was divided into five agro-ecological zones based on; weather, temperature, precipitation and geographic location etc. i.e. Northern KP; consisting of Malakand, Buner, Mingora, Swat, Shangla, Dir upper, Dir lower, Chitral and Kalam, Southern KP; consisting of D. I. Khan, Bannu, Hangu, Kohat, Karak, Lakki Marwat and Tank, Eastern KP; consisting of Abbottabad, Naran, Kaghan, Mansehra, Haripur and Battagram, and Western KP; consisting of Khyber agency, Orakzai agency, Mohmand agency, North and South Waziristan, and the most important Central KP; consisting of Mardan, Nowshera, Peshawar, Swabi and Charsadda.

## 2.2. Isolation of *Clavibacter michiganensis* subsp. *michiganensis* from tomato samples

The bacterium was isolated from diseased tomato plant tissue/ fruit according to Zainal *et al.*, (2008). Each sample (5 g) was first soaked in 15 ml of phosphate buffer tris (PBT) at 4°C for 15 min., homogenized using a mortar and pestle and then filtered through muslin cloth. The filtrate (50 µl) was plated onto NA medium; plates were then incubated at 23-27°C for 2 weeks. All bacterial colonies showing morphological characteristics similar to these of *Clavibacter michiganensis* subsp. *michiganensis* as reported by Zainal *et al.*, (2008), were transferred onto YDC medium. Bacterial colonies growing on YDC which had yellow, wet, and mucoid cultural characteristics were preserved on YDC slants; before being subjected to pathogenicity assays.

## 2.3. Pathogenicity assays

Pathogenicity of the 27 *Clavibacter michiganensis* subsp. *michiganensis* isolates were confirmed by using the following methods:

### 2.3.1. Cotyledon test

This was a simple and rapid *in vivo* test to confirm pathogenicity of occasional strains which cause wilting of young tomato plants in the greenhouse. Using all suspected isolates, 10<sup>9</sup> cells/ml suspensions were prepared in sterile saline (0.85 %) using 24-48 h old culture grown on NA. Freshly germinated tomato seedlings (in pots) with expanding cotyledons were used for the test. A droplet of bacterial suspension was rubbed over the upper surface of each cotyledon; and then pots were covered with a polythene bag. To maintain high humidity, wet cotton was placed in each pot for 48 h; and 4 plants were inoculated for each isolate. Control seedlings cotyledons were rubbed with sterile dist. water. White blisters or craters on the cotyledon surface (indicating pathogenicity of isolates) were examined with a hand lens. A droplet of sterile saline rubbed over healthy cotyledons

served as negative control (OEPP/EPPO Bulletin. 2013).

### 2.3.2. Young seedlings test

Four tomato seedlings (10 days old) were inoculated with 50 µl of 10<sup>9</sup> cells/ml bacterial suspension of each isolate at the stem of cotyledons. Test plants were grown at 26± 2°C, and at > 70 % relative humidity in the greenhouse. Six days after inoculation; slight reversible wilting was detected in some seedlings up to 9 days. Control seedlings were inoculated with sterile dist. water. The bacterium was re-isolated from wilted plants by removing 1 cm stem section from above the inoculation point; and then suspending it in phosphate buffer followed by cultivation through dilution plating on NA medium (OEPP/EPPO Bulletin. 2013).

### 2.3.3. Old seedlings test

Suspected isolates were subjected to pathogenicity assay on tomato seedlings of a single variety (Rachana) in the greenhouse. According to Xu *et al.*, (2010); stems of 4 week-old tomato seedlings were punctured with needle dipped in a suspension of bacteria containing 10<sup>8</sup> cells/ml. The inoculum was prepared from a bacterial culture grown overnight on NA; and then suspended in dist. water. These seedlings were maintained at 28± 2°C; and symptoms were recorded 7–21 days after inoculation. Control seedlings were inoculated with sterile dist. water. For each isolate, five tomato seedlings were used.

## 2.4. Effect of inoculum concentration on disease severity

This assay was adopted according to Burokiene *et al.*, (2005); to find the minimum concentration of *Clavibacter michiganensis* subsp. *michiganensis* that can cause canker disease on tomato plants. For this purpose; two isolates aggressive and moderately aggressive ones i.e. MKD1 and KLM1, respectively were used. Tomato plants (three susceptible varieties; Rachana, Rio-Grand and Lyreka) were

grown for 5 weeks in the greenhouse and then inoculated as described before in old seedlings test. The bacterial inoculum was prepared and serially diluted up to 10 folds in PBS buffer. Concentrations ranging from  $10^2$  -  $10^8$  cells/ ml were used for inoculating tomato plants. Inoculated plants (three per each concentration and per each variety) were kept under greenhouse conditions. These plants were sprayed with water; and then covered with plastic bags for first 48 h after inoculation. They were observed daily for three weeks for bacterial canker disease symptoms development.

Disease severity was rated in reference to Bibi and Ahmad, (2016) disease rating scale. Different categories of the scale are: 0 = no symptoms, 1= few (1/3 of the) leaves wilted, 2= more than 1/3 and up to 2/3 of the leaves wilted, 3= more than 2/3 of the leaves wilted except for the terminal leaves on the main shoot, 4= terminal leaves of the main shoot also wilted, 5= plant completely dead. Five control plants (stabbed with sterile water-dipped needle) from each variety were also included. Disease ratings on different tomato varieties were averaged for calculating effect of inoculum concentration.

## 2.5. Statistical analysis

The different values of the scale were analyzed by using Bdiya and Dahiru, (2006) statistical equation with minor modification:

$$S = \frac{\sum n}{4N}$$

Where; S = severity,  $\sum n$  = sum of individual ratings, N = total number of plants assessed, and 4 = highest score of the severity scale.

## 3. Results

### 3.1. Survey and sampling

Surveys conducted in various locations of KP resulted in the collection of a total of 100 tomato samples showing typical symptoms of bacterial

canker such as; bird's-eye-spot on leaves/ stems or/ and fruits, wilted leaves, tunneling and splitting of stem, retarded growth, and necrosis of leaf margins.

### 3.2. Isolation of *Clavibacter michiganensis* subsp. *michiganensis* from tomato samples

Regarding colony morphology, when tomato samples were grown on NA medium, 47 out of 100 of these samples yielded colonies morphology typical to that of *Clavibacter michiganensis* subsp. *michiganensis*, i.e. yellow, wet and mucoid colonies; as reported previously by Zainal *et al.*, (2008). Culture characteristics on YDC showed that 13 isolates were not *Clavibacter michiganensis* subsp. *michiganensis*; as their growth rate were comparatively fast and their colors were whitish to off-white. The remaining 34 isolates exhibited a pale yellow to orange color and were mucous with slow growth rate; and thus suspected to be *Clavibacter michiganensis* subsp. *michiganensis* (Anwar *et al.*, 2005). For further confirmation; Gram staining and 3 % KOH solubility test were performed for all isolates; where only 27 out of the 34 isolates were confirmed as Gram positive.

### 3.3. Pathogenicity assays

The 27 Gram positive isolates were tested for their pathogenicity on tomato plants using cotyledon test, young seedlings and old seedlings tests. Results shown in Table (1) revealed that all the isolates were able to cause disease symptoms on tomato plants. However, isolates from Mingora (MNG1) and Mansehra (MNS2) did not produce the white blisters or craters on the surface of cotyledon, in cotyledon test. When the test was performed on young seedlings, all isolates produced wilting symptoms and killed the inoculated seedlings except MNG1 isolate. On the other hand for old seedlings test; isolates MKD2, MNG1, DIK1 and NWS2 did not produce canker symptoms; and inoculated seedlings appeared healthy.

**Table 1:** Pathogenicity of 27 *Clavibacter michiganensis* subsp. *michiganensis* isolates using cotyledon, young and old seedlings assays

S. no.	Isolate code	Origin of the isolate	Pathogenicity assays		
			Cotyledon test	Young seedlings test	Old seedlings test
1	MKD1	Malakand	+	+	+
2	MKD2	Malakand	+	+	-
3	BNR1	Buneir	+	+	+
4	MNG1	Mangora	-	-	-
5	SWT1	Swat	+	+	+
6	SWT3	Swat	+	+	+
7	SNG1	Shangla	+	+	+
8	DUP1	Dir upper	+	+	+
9	DLW1	Dir lower	+	+	+
10	CTL1	Chitral	+	+	+
11	KLM1	Kalam	+	+	+
12	DIK1	D.I.Khan	+	+	-
13	KHT1	Kohat	+	+	+
14	ABD1	Abottabad	+	+	+
15	ABD2	Abottabad	+	+	+
16	NRN1	Naran	+	+	+
17	MNS1	Mansehra	+	+	+
18	MNS2	Mansehra	-	+	+
19	HRP1	Hari pur	+	+	+
20	MDN1	Mardan	+	+	+
21	MDN2	Mardan	+	+	+
22	NWS1	Nowshera	+	+	+
23	NWS2	Nowshera	+	+	-
24	PSH1	Peshawar	+	+	+
25	SWB1	Swabi	+	+	+
26	CHD1	Charsadda	+	+	+
27	KAG1	Khyber agency	+	+	+

Where; (+) symptoms produced, (-) symptoms not produced

### 3.4. Effect of inoculum concentration on severity of bacterial canker disease

Current results indicated that inoculum concentration of bacterial suspension influenced timing and development of bacterial canker disease symptoms on tomato plants. Plants inoculated with the highest concentrations ( $10^8$  cells/ ml) of the two strains (MKD1 and KLM1) wilted earlier; and showed infection symptoms when data were recorded at 9<sup>th</sup> day after inoculation. Meanwhile, plants inoculated with ( $10^2$  cells/ ml and  $10^3$  cells/ ml) concentrations needed more time to develop symptoms than those inoculated with higher

concentrations ( $10^5$ – $10^8$  cells/ ml). The time needed to develop canker symptoms on inoculated tomato plants also depended on the isolate virulence. The more virulent isolate MKD1 showed symptoms earlier than the less virulent isolate KLM1 (Table 2).

## 4. Discussion

Current results of pathogenicity assays were similar to those of Ftayeh *et al.*, (2010), who reported that all of their isolates and also the reference strains induced typical symptoms of bacterial canker on mechanically inoculated young tomato plants in 10 to 15 days.

**Table 2:** Effect of *Clavibacter michiganensis* subsp. *michiganensis* inoculum concentrations on severity of bacterial canker disease of tomato plants

Isolate	Inoculum concentration	Average disease severity scale (Days after inoculation)								
		Day 9	Day 11	Day 13	Day 15	Day 17	Day 19	Day 21	Day 23	Day 25
MKD1	1×10 <sup>2</sup>	0	0	0	0	0	0	0.67	1.33	2
	1×10 <sup>3</sup>	0	0	0	0	1	1	2	2	3
	1×10 <sup>4</sup>	0	0	0	0	1	2	2	2	4
	1×10 <sup>5</sup>	0	0	0	2	3	3	4	4	5
	1×10 <sup>6</sup>	0	0	1	3	3	4	4	5	5
	1×10 <sup>7</sup>	0	1	2	2	4	4	5	5	5
	1×10 <sup>8</sup>	1.33	2	2	4	4	4.33	5	5	5
KLM1	1×10 <sup>2</sup>	0	0	0	0	0	0.33	0.33	1.33	2
	1×10 <sup>3</sup>	0	0	0	0	0	0.33	1	2	3
	1×10 <sup>4</sup>	0	0	0	0	1	2	2	3	3
	1×10 <sup>5</sup>	0	0	0.67	1	1	2	3	3	3
	1×10 <sup>6</sup>	0	0	1	2	2	2	3	3	3
	1×10 <sup>7</sup>	0	1	1.33	2	2	3	3.67	4.33	5
	1×10 <sup>8</sup>	0.33	1	2	2	3	4	4	5	5

Where; MKD1 (collected from Malakend) as the most aggressive isolate; whereas, KLM1 (collected from Kalam) as moderately aggressive isolate. Concentrations of bacterial suspensions (cells/ ml) were adjusted through 10 fold serial dilutions. Disease severities were rated according to scales of Bibi and Ahmad, (2016).

These symptoms included unilateral wilting of leaflets and canker on the stems; followed finally by wilting of the entire plants. In our study, few isolates either did not cause or caused disease symptoms after a longer period of incubation. These non-production or delayed appearance of symptoms might be attributed to plant defense mechanisms; where chemical changes thus have occurred with prolonged incubation of the pathogen inside the vascular tissue. Similarly, Behrendt *et al.*, (2002) observed wilting symptoms on tomato seedlings in some of their treatments with increased incubation periods; conversely, decreased disease severity was observed in other treatments.

Several studies suggested that the main factor that has an influence on the epidemiology of the disease is the incubation period between infection and the onset of symptoms. The duration of

incubation normally ranges from 7- 84 days and is generally affected by; temperature, plant age, and inoculum concentration of pathogen. Longer incubation periods were observed however in older plants, cool temperature, and lower inoculum concentration (Burokiene *et al.*, 2005). Currently, when *Clavibacter michiganensis* subsp. *michiganensis* isolates were inoculated into tomato seedlings; they produced varied symptoms of canker disease including wilted cotyledons, hypocotyls, and finally wilting of the entire seedlings. However in other treatments; clear cut results were not observed and the inoculated tomato seedlings did not show clear symptoms of *Clavibacter michiganensis* subsp. *michiganensis* infection. Current results were consistent with those of Anwar *et al.*, (2005). Current variations in results of pathogenicity assays might be attributed to the effect of environmental conditions; and to the nature of tomato variety used

during these assays. In accordance, Zainal *et al.*, (2008) also reported inconsistencies in their pathogenicity assays. Sen *et al.*, (2015) concluded that environmental conditions might not be optimum for disease development; tomato cultivar was not appropriate for the pathogenicity assay, or the pathogen might have exhibited different degrees of virulence against the used tomato cultivar.

In the current study, pathogen inoculum concentration has influenced the timing and development of bacterial canker disease symptoms. Plants inoculated with the highest bacterial concentrations ( $10^8$  cells/ml) got infected and wilted earlier. However, plants inoculated with lower concentration of inoculum such as;  $10^2$  cells/ml and  $10^3$  cells/ml; needed more incubation time to develop disease symptoms. Nevertheless, even at the lowest concentrations of the bacterial inoculum ( $10^2$  and  $10^3$  cells/ml); plants were infected and wilted. Our results were similar to those of Burokiene *et al.*, (2005), who found that inoculum concentration affected the timing of symptoms development. Sen *et al.*, (2015) also reported that inoculum concentration have effective role on the incubation period and then on disease development. For earlier disease symptoms production, virulence of the pathogenic isolate used must also be considered. Thus more virulent isolates produced disease symptoms earlier, in accordance with Burokiene *et al.*, (2005).

## Conclusion

Our study reported that appearance of bacterial canker disease symptoms; and disease severity depends upon virulence of the pathogen, and upon its inoculum concentrations. More virulent and concentrated inoculum of *Clavibacter michiganensis* subsp. *michiganensis* caused early and more severe canker disease symptoms on tomato plants.

## Conflict of interests

The authors declare no conflict of interests.

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