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EVALUATION OF CULTURAL AND PATHOGENIC VARIABILITY, HOST RANGE SCREENING OF *CLAVIBACTER MICHIGANENSIS* SUBSP *MICHIGANENSIS* AND ASSESSMENT OF METHODS OF INOCULATION FOR DISEASE INCEPTION

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Key words : *C. michiganensis subsp michiganensis, Solanaceous host, Pathogen spread, Inoculation methods*

Abstract– *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) causes bacterial wilt and canker diseases in tomato. Studies on cultural and pathogenic variability of *Cmm* has been carried under greenhouse and laboratory conditions using 10 isolates of *Cmm,* collected from different regions of Uttarakhand and Himachal Pradesh States of India. A susceptible tomato cultivar Arka Vikash was used to evaluate the pathogenic variability. All the isolates of *Cmm* exhibited differential reaction on host as regard to symptom expression. Isolate *Cmm* 10 was observed to be the most virulent isolate which caused complete plant death within 18th day of inoculation. During studies on cultural variability, the bacterial colonies of most of the isolates were initially small, (1-4 mm diameter), mucoid and concave with three different pigmentations viz., yellow, pale-white and orange which became glistening with the increase in age. Among the different inoculation methods used, stem inoculation method followed by the foliar spray was found to be the most effective method for quick spread of the pathogen within the plant. Out of the five host species, particularly of solaneceous family (*Capicum frutescens*, *Capsicum annum, Solanum tuberosum, Solanum nigrum*), except *Chenopodium album* when screened for host range of the pathogen, the bacterium exhibited the diseased symptoms only in bell pepper.

INTRODUCTION

Tomatoes (*Lycopersicon esculentum* Mill.) belonging to family Solanaceae (nightshade family), genus. *Lycopersicon*, subfamily *Solonoaideae* and tribe *Solanae* (Taylor, 1986), are amongst one of the most extensively cultivated and consumed 'vegetables' around the globe, for both the fresh fruit market as well as processed fruit industries **(**Heuvelink, 2005). They are referred to be one of the most accepted vegetable in the major parts of world, owing to its high nutritive value, easy adaptability in new environment and varied uses. The major tomato growing states in the India are Madhya Pradesh, Karnataka, Andhra Pradesh, Telangana, Gujarat, Odisha, West Bengal, Bihar, Maharashtra, Chhattisgarh, Tamil Nadu, Uttar Pradesh, Haryana, Himachal Pradesh, Assam, Jharkhand and Punjab, accounting for about 97% of the total production of the tomato in the country. Tomato being an important commercial crop grown in a large area of 849'000 Ha (NHB,2017), it's proneness to a number of fungal, bacterial and viral diseases leads to a significant reduction in yield. The crop is being attacked by different bacterial pathogens amongst them, one of the bacterial disease, i.e. bacterial canker and wilt of tomato caused by *Clavibacter michiginensis* subsp *michiganensis* (*Cmm*) is of utmost importance (Chang *et al.,* 1992). *Cmm* is a Gram positive bacterium, i.e. aerobic, rod shaped and nonsporulating in nature. The bacterium belongs to genus *Clavibacter* that is currently differentiated into five subsp. (ssp) on the basis of host species amongst this ssp., *C .m.* subsp. *michiganensis* infects tomato (Davis *et al.,* 1984; Strider, 1969). Bacterial canker disease is usually manifested as cankerous lesion on

stem, bird's eye spot surrounded by whitish halo in the fruit, unilateral wilting that eventually leads to complete plant death (Sen *et al.,* 2015). The insufficient understanding of intricate behavior of *Cmm* as phytopathogen, unavailability of efficient management tools and the lack of resistant genotypes are the main factors responsible for the lower rate of successful pathogen management (Chang *et al.,* 1991) thereby making it an economically devastating pathogen that inflicts considerable damage in most of the tomato-producing regions around the world (Gleason, 1993). The present investigation was conducted to assess the cultural and pathogenic variability in the *Cmm* isolates, to study the best suitable method for the pathogen spread through varied inoculation methods and host range screening for the purpose of understanding the mode of survival of the bacterium in the absence of the host.

MATERIALS AND METHODS

Isolation, purification and preservation of the bacterium

The bacterium was isolated, from infected seeds, seedlings, plant parts and fruits, purified and preserved following the method described by Janse, (2004) for subsequent studies. Infected tissues were surface sterilized by alcohol (70%) and were then placed in a test tube having sterilized water. Tissue from each infected plant part was left for 30 minutes in water and crushed gently so that the bacterium can be released out of the tissue into water. Subsequently 100µl of suspension was poured into petriplate containing Nutrient Agar Glucose Yeast medium (NGY). The seeds collected from infected fruits exhibiting the disease symptom were placed on the NGY medium and incubated in the growth chamber at 28±1ºC. The seeds were examined after 72hrs to record the recovery of the bacterium.

Purification of the bacterium was done on D_2ANX medium, that shows the characteristic feature of the bacterium by streaking freshly growing single colonies from NGY medium and incubated at 28 ± 1 ºC for five days. *Cmm* isolates were stored in NGY slants at 4ºC and in glycerol stocks at -20ºC for long term storage for further studies.

Studies on variability of *Clavibacter michiganensis* subsp *michiganensis* isolates

Cultural variability of *Cmm* isolates

For cultural variability, all the ten (10) isolates of the bacterium were grown on NGY medium at 25^0C. The medium having 7.0 pH was sterilized, cooled to be lukewarm and poured in sterilized Petri-plates. On solidification of the medium, these plates were inoculated with different isolates streaking method and incubated at 25±1°C for 72 hrs. After 72hrs of incubation, the plates were examined for bacterial growth and colony characters.

Pathogenic variability of different isolates of *Cmm*

All the ten isolates of *Cmm* were evaluated for the expression of wilt and canker symptom under glass house conditions on the tomato variety Arka Vikash. For that purpose, seeds of tomato variety "Arka Vikash" were sown in pots (25cm in dia.) filled with sterilized soil + sand (2: 1: w/w) and kept in protected conditions. One seedling was maintained per pot. The plants were inoculated with the pathogen at 5th week stage.

For the preparation of bacterial cell suspension one loop full culture of 48h old bacterial colony was inoculated into 50mL of autoclaved nutrient broth in 150 ml "Erlenmayer flask. The suspension was adjusted to an OD value of 0.06nm at 660 nm that corresponds to 10^8 cfu ml^{-1} by adding required quantity of sterilized distilled water. Pathogenicity tests were conducted on five week old tomato seedlings.

The seedlings were inoculated with sterilized hypodermic syringe in the stem region of five week old seedlings with 0.2 ml of standardized bacterial

Rating scale	Disease score	Symptoms
0	No disease	no leaves showing wilting
1	1-/10% of leaves with wilt	slight marginal wilting,
2	11-/25% of leaves with wilt	Unilateral wilting of the leaves
3	26-/49% of leaves showing wilting	sectored wilting, and canker formation, associated with chlorosis
4	50-/74% of leaves showing wilting	pronounced leaf collapse
5	whole leaf wilted	Complete plant death

suspension (1.0x 10^8 cfu/ml). Plants were kept under alternate 14h light and 10h dark periods at 32 º C temperatures and were observed periodically for the appearance of the symptoms.

Observations

Data on disease development was recorded on the basis of percent wilting and canker formation on the stem. Evaluation of disease appearance and development was determined using 0-/5 arbitrary scale (Soyulu *et al.,* 2013).

Studies on growth of bacterium in different temperature conditions

To know the effect of temperature on multiplication of the bacteria, it was incubated at different temperature range. NGY broth in 150 ml 'Erlenmeyer' flasks, each containing 50 ml of sterilized nutrient broth (pH 7.0) were autoclaved at 15 lbs. /Inc^2 for 20 minutes to avoid contamination of the media. Sterilized broth was inoculated with 48h culture using straight inoculation and then incubated at a series of temperature ranging from 10 ^oC, 20 ^oC, 30 ^oC and 40 ^oC. Each treatment was replicated thrice. The OD value at 660nm by using Spectrophotometer was observed for each replication.

Studies on growth of bacterium at different pH conditions

To know the effect of pH on multiplication of the bacteria, it was incubated at different pH range. NGY broth in 150 ml 'Erlenmeyer' flasks, each containing 50 ml of sterilized nutrient broth ranging from pH 4.0 – 8.0 were autoclaved at 15 lbs. /Inc^2 for 20 minutes to restrict the growth of the pathogen in the media. Sterilized broth was inoculated with 48h culture using straight inoculation and then 28 ± 1ºC for 72 hrs. Each treatment was replicated thrice. The OD value at 660nm by using Spectrophotometer was observed for each pH.

Screening for the host range of the bacterium

To determine the alternate host, the pathogen was syringe inoculated to different crop plant belonging to the family Solanaceae such as pepper, bellpepper (*Capsicum annum* group), brinjal (*Solanum melongena*), potato (*Solanum tuberosum*) and weed host makoi (*Solanum nigrum*), including bathuaa non Solanaceous host (*Chenopodium album*). Evaluation of disease appearance and development was determined using a 0-/5 scale (Soyulu *et al.,* 2013).

Effect of inoculation methods on incubation period of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seedlings

To find out the best inoculation method for the expression of bacterial canker symptoms in tomato plants, the plants were inoculated with different methods as described:

Inoculation methods used

Seed inoculation

Seeds (50 g) of tomato cultivar "Arka Vikash" were packed in a muslin cloth bag and placed in a 500mL flask (Borosil) containing 200 ml of the bacterial suspension (1.0x10^8 cfu/ml). The seeds were left in the bacterial cell suspension for about half an hour and the bacterial suspension was removed by placing the muslin cloth inside the vacuum suction cups and suction pressure was applied for the removal. Then seeds were kept for drying on a sterilized blotter paper sheet under sterilized conditions.

Foliar spray inoculation

Bacterial suspension (1.0x10^8 cfu/ml) was sprayed on to the leaves with the help of atomizer. Inoculated plants were covered with polythene sheet and were frequently sprayed with distilled water for 48 h to maintain high relative humidity. The plants were observed weekly for the appearance of the symptoms.

Syringe inoculation of stem

In the stem of five week old seedlings, 0.2 ml of standardized bacterial suspension (1.0x10^8 cfu/ml) was inoculated through injection with the help of a sterilized hypodermic syringe. The plants were observed periodically for the appearance of the symptoms.

Wound inoculation of stem with toothpick

The stem of five week old seedlings were inoculated through toothpick method in which the tooth picks dipped in the bacterial suspension (1.0x10^8 cfu/ml) were inserted into the stems at the collar region. The seedlings were observed periodically for the appearance of the symptoms.

Foliar spray of leaves and stem inoculation

Bacterial suspension (1.0x10^8 cfu/ml) was sprayed on the leaves with the help of atomizer and 0.1 ml of

standardized bacterial suspension ($1.0x10^8$ cfu/ml) was injected with the help of a sterilized hypodermic syringe.

Syringe inoculation of peduncles

The peduncles of the plant were injected with 0.2 ml of standardized bacterial suspension ($1.0x10^8$ cfu/ml) with the help of a sterilized hypodermic syringe. The plants were observed periodically for the appearance of the symptoms.

Statistical analysis

The data was analyzed using simple ANNOVA on Completely Randomized Design (CRD) in *invitro* studies and Randomized Block Design (RBD) in *invivo* studies. Analysis of variance (ANOVA) was performed using STPR software package versions 2 and 3, where significance level of 0.05 was used for all statistical interpretation.

RESULTS AND DISCUSSIONS

Symptomatology

The pathogen *Clavibacter michiganensis* subsp *michiganesis* (Smith) Davis showed an array of symptoms in tomato seedlings and plants. Artificial infection through inoculation of the pathogen on different plant parts exhibited marginal necrosis in the leaves, canker on stem & fruits and unilateral wilting followed by wilting of the entire plant leading to the plant death.

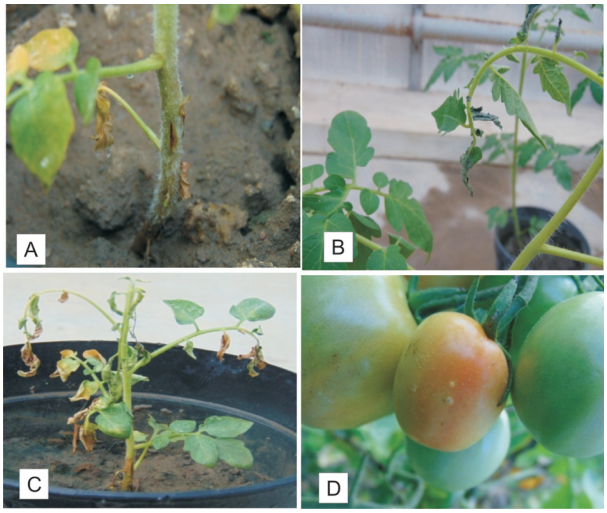

Plate 1. Symptom expression by *Cmm* on different parts of tomato plant; A, showing canker on stem of the tomato seedling; B.Unilateral wilting of the leaves of tomato seedlings; C. whole plant wilting; D canker on tomato fruits

Under natural field conditions, the disease is generally observed during the months of May-August, where high temperature is accompanied by rainfall. The rain splashes act as the resource for the spread of the bacterium. The symptoms of the disease became visible on the stem region as cankerous lesion, which increases in size chronically (A), infection on the stem often also girdles the stem and may cause premature plant death, The symptom on the leaves appears in the form of unilateral wilting in plant (B), that appeared starting from the top most part and follows downside, whole plant wilting (C), symptom on the fruit appeared in the form of bird's eye spot formation (D) (Plate 1). A wide range of symptoms are produced by the pathogen on the basis of plant stages time of infection, intercultural operations, location of production under glasshouse or field conditions and cultivar, etc (EPPO Bulletin, 2016).

Cultural variability of different isolates of *Cmm*

On NGY medium, the bacterial colonies of most of the isolates were small, 1-4 mm in diameter and developed within 72-96 h from the day of inoculation. The bacterial colonies were light yellow, orange, round and semifluidal (Plate 2).

Colonies become deeper yellow and glistening with longer period of incubation (Table 1). The

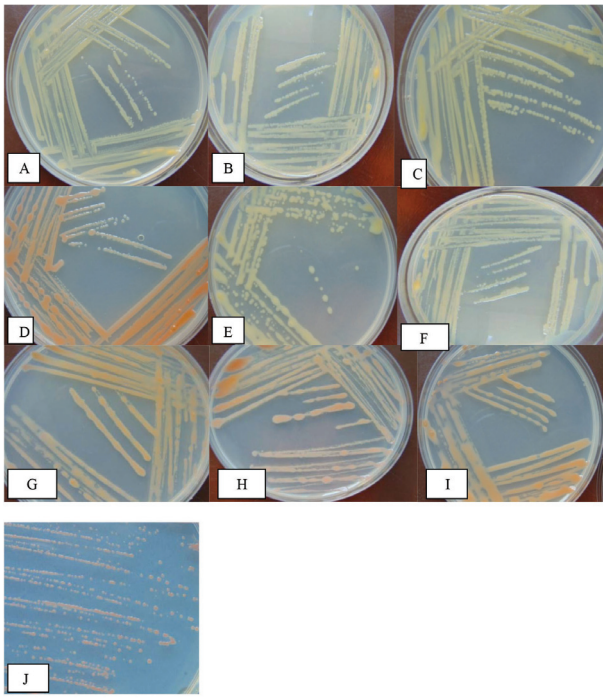

Plate 2. Cultural variability of different isolates (Cmm^1-Cmm^{10}) of the bacterium (A-I).

colony color of the bacterial isolates *Cmm*1, 3, 4, 6 and 8 was yellow and that of isolate *Cmm*2 was creamish white to yellow while it was orange in isolate *Cmm* 5, 7, 9 and 10. The shape of colonies was concave to dome shaped in case of all the isolates. Different colony characteristics including dry, sticky, mucoid and less mucoid, pink, red, yellow, orange, white or colorless colonies are also been reported (Davis and Vidaver, 2001; Kaneshiro *et al.,* 2001).

Pathogenic variability of different isolates of *Cmm*

The ten of the bacterial isolates were inoculated into the variety "Arka Vikash" by the method of syringe inoculation at the nodal point of true leaf emergence and the observation for disease rating was taken as described below:

Artificial inoculation of the tomato seedlings with different isolates revealed that the seedlings inoculated with isolates *Cmm*5, *Cmm* 6 and *Cmm* 10 showed first symptom on leaf as marginal necrosis and unilateral wilting on 6th day of inoculation (Table 2). Isolates, *Cmm*1, *Cmm* 2, *Cmm* 3, *Cmm* 8 and *Cmm* 9 showed symptoms on leaf on 12th day of inoculation, while isolates *Cmm* 4 and *Cmm* 7 showed symptoms on 18th day of inoculation. Isolate *Cmm* 10 was the most virulent which caused complete plant death within 18th day of inoculation, while isolate *Cmm* 8 was observed to be the least virulent amongst the all 10 isolates and showed significant symptoms on 5th week after inoculation. Isolate *Cmm* 10 which show the signs of the most virulent isolate was further taken up for glasshouse and open field studies. The results obtained were in confirmation with Mendez *et al.,* 2020 who also reported the presence of pathogenic variability amongst the isolated pathogenic *Cmm* strains.

Studies on growth of bacterium in different temperature conditions

For the purpose of identification of the optimum temperature for the growth of *Cmm* the six virulent bacterial isolates *Cmm* (1, 3, 5, 6, 8, and 10) were subjected to the following range of temperature.

The studies for assessing the most suitable temperature for the growth and multiplication of the six virulent isolates of *Cmm* (1, 3, 5, 6, 8, 10) on a range of temperatures viz., 10ºC, 15ºC, 20º, 25ºC, 30ºC, 35ºC and 45ºC was done, wherein, 25ºC

Table 1. Colony characteristics of different isolates of bacterial canker pathogen on nutrient agar glucose yeast medium.

S. No.	Isolates	Colony characteristics		
		Color	Shape	Size (mm)
A	*Cmm1*	Yellow	Round, mucoid	1-2
B	*Cmm2*	Creamish white to yellow	Round	1-3
C	*Cmm3*	Yellow	Circular, fluidal	1-3
D	*Cmm4*	Yellow	Circular	2-4
E	*Cmm5*	Yellow	Round, mucoid	2-3
F	*Cmm6*	Orange	Round	1-3
G	*Cmm7*	Orange	Round	1-3
H	*Cmm8*	Orange	Circular, fluidal	2-4
I	*Cmm9*	Orange	Circular	2-3
J	*Cmm10*	Orange	Round, mucoid	1-3

Table 2. Pathogenic variability of *Cmm* isolates on tomato seedlings

Isolates	Method of inoculation	Days after inoculation							
		0	6	12	18	24	30	36	42
Cmm1	Stem inoculation	0	0	1	2	3	3	3	4
Cmm2	Stem inoculation	0	0	1	2	3	4	4	4
Cmm3	Stem inoculation	0	0	1	2	3	3	4	4
Cmm4	Stem inoculation	0	0	0	1	2	3	3	3
Cmm5	Stem inoculation	0	1	2	3	3	4	4	4
Cmm6	Stem inoculation	0	1	2	3	4	4	4	4
Cmm7	Stem inoculation	0	0	0	1	2	2	3	3
Cmm8	Stem inoculation	0	0	1	1	2	2	2	2
Cmm9	Stem inoculation	0	0	1	1	2	3	3	3
Cmm10	Stem inoculation	0	3	4	5	5	5	5	5

temperature was found to be the optimum temperature for the growth and multiplication of all the isolates (Table 3**)**. Growth of *Cmm* in the similar temperature ranges has been also reported earlier (Davis and Vidaver, 2001)**,** who also confirmed 25ºC - 30 ºC as optimum temperature range for pathogen growth.

Studies on growth of bacterium at different pH conditions

To determine the optimum pH for the growth of *Cmm* the six virulent bacterial isolates *Cmm* (1, 3, 5, 6, 8, 10), were subjected to the pH range of 4-8

The studies for observing the most suitable pH conditions for the bacterium were conducted on a range of pH viz., 4,5,6,7 and 8. Amongst the varied pH conditions observed for the growth of the bacterium pH 7 was found to be the optimum temperature for bacterial growth (Table 4). The results are in agreement with the findings of the earlier studies (Singh and Bharat, 2017).

Host range screening

For the purpose of screening the virulence of the isolates on crops other than tomato, the host range screening for the pathogen was done with five solanaceous host and one non solanaceous host.

The findings indicate that out of the six different plant species, the cankerous lesion on stem, at the point of inoculation, were observed only in the bell pepper eighteen days after inoculating. The symptoms were expressed as cancerous growth on

Table 3. Growth of bacterium in different temperature conditions

Isolates	OD value (660 nm) of bacterial isolates at different temperature						
	10°C	15°C	20°C	25°C	30°C	35°C	45°C
Cmm1	0.480	0.946	1.163	1.253	1.280	0.956	0.153
Cmm2	0.327	0.528	0.892	1.182	1.205	0.813	0.236
Cmm3	0.946	1.053	1.263	1.336	1.263	1.056	0.190
Cmm4	0.209	0.492	0.813	1.201	1.225	0.926	0.343
Cmm5	0.550	0.940	1.140	1.233	1.160	0.850	0.250
Cmm6	0.950	1.146	1.263	1.350	1.256	1.153	0.273
Cmm7	0.416	0.917	1.256	1.319	1.284	0.918	0.307
Cmm8	0.450	0.950	1.070	1.176	1.130	0.970	0.260
Cmm9	0.315	0.502	0.935	1.215	1.275	1.192	0.115
Cmm10	0.946	1.160	1.271	1.353	1.256	0.940	0.160
CD at 5%	a= 0.0155;		b= 0.0168;		axb=0.0412		

a= interaction within the isolates
b= interaction within the temperature conditions
axb= interaction between isolates and temperature conditions

Table 4. Growth of bacterium in different pH conditions

Isolates	OD value (660nm) of bacterial isolates at different pH				
	4	5	6	7	8
Cmm1	0.070	0.80	1.34	1.43	1.26
Cmm2	0.031	0.094	0.62	1.18	1.05
Cmm3	0.046	0.14	0.81	1.26	0.95
Cmm4	0.061	0.11	0.72	1.25	1.13
Cmm5	0.083	0.45	1.16	1.34	1.04
Cmm6	0.070	0.36	1.28	1.35	1.16
Cmm7	0.038	0.89	1.19	1.23	1.10
Cmm8	0.040	0.25	1.24	1.32	1.15
Cmm9	0.018	0.12	0.83	1.07	0.92
Cmm10	0.073	0.45	1.36	1.47	1.24
CD at 5%	a = 0.11;		b = 0.101;		axb = 0.247

a= interaction within the isolates
b= interaction within the pH conditions
axb= interaction between isolates and pH conditions

stem whereas on fruit of bell pepper brown colored water soaked lesions were observed. However, no symptoms were produced in any other solanaceous host. In non-solanaceous host, *C.album* the bacterium on artificial inoculation in stem exhibited small restricted cancerous growth at the point of inoculation (Table 5). However the researchers also confirmed the infection of the pathogen in pepper plant, which was not confirmed in the present study, as no symptoms were observed in pepper plant (Singh and Bharat, 2017).

Effect of inoculation methods on incubation period of *Clavibacter michiganensis* subsp. *michiganensis* on tomato seedlings

Different methods of artificial inoculation when applied using most virulent isolate *Cmm10* on the 5 weeks old tomato seedlings, the symptoms were noticed in tomato seeds 5th days after germination. The symptoms appeared on 5 weeks old tomato seedlings at 11th day when inoculated together with foliar spray and syringe inoculation of stem. In 5 weeks old tomato seedlings, symptoms were observed at 8th days when the bacterium was inoculated with syringe inoculation method at the junction of first true leaf., In wound inoculation (toothpick method), the disease expression was at 10th days of bacterial inoculation. Foliar spray along with syringe inoculation of stem, syringe inoculation of stem at junction of first true leaf followed by stem inoculation with toothpick were adjudged as effective methods of artificial inoculation of the bacterium *C. michiganensis* subsp. *michiganensis* to the tomato seedlings. These results are in substantiation with the previous findings (Singh and Bharat, 2017), where they described syringe inoculation of leaves and branches, stem

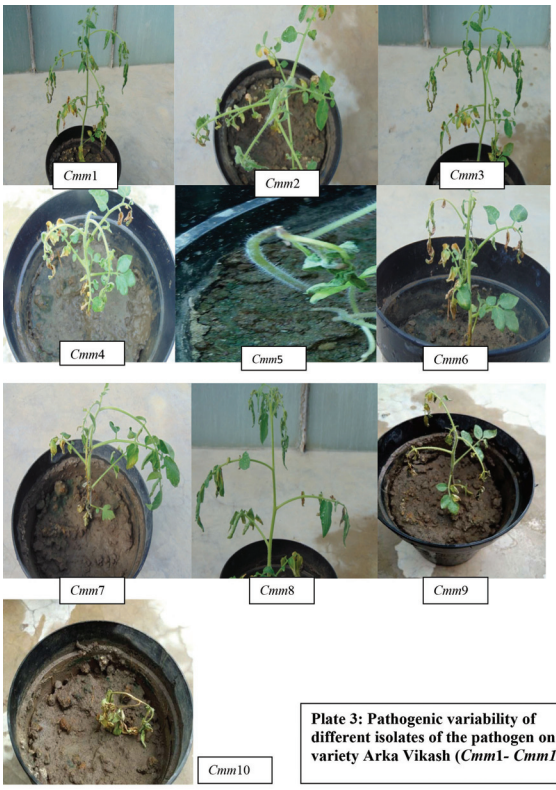

Plate 3: Pathogenic variability of different isolates of the pathogen on variety Arka Vikash (*Cmm*1- *Cmm10*)

Table 5. Plants used for studying the host range of *Cmm*

Treatment	Scientific name	Common name	Family	Variety	Days after Planting				
					6	12	18	24	30
*Cmm*10	*C. annum*	Bell pepper	Solanaceae	Indra	0	0	1	1	1
*Cmm*10	*C.annum*	Pepper	Solanaceae	P.C.1	0	0	0	0	0
*Cmm*10	*S.tuberosum*	Potato	Solanaceae	K.Jyoti	0	0	0	0	0
*Cmm*10	*S.melongena*	Brinjal	Solanaceae	P.Samrat	0	0	0	0	0
*Cmm*10	*C.album*	Bathua	Amaranthaceae	-	0	0	1	1	1
*Cmm*10	*S.nigrum*	Makoi	Solanaceae	-	0	0	0	0	0

Table 6. Effect of different inoculation methods on incubation period of *Cmm* 10 isolate *on* tomato seedlings

Types of inoculation	Disease Rating (days after inoculation)							
	0	6	12	18	24	30	36	42
Seed inoculation	0	2	3	3	3	3	3	3
Syringe inoculation of stem	0	0	1	2	2	3	4	4
Syringe inoculation of leaves	0	0	1	1	1	2	2	2
Toothpick inoculation of stem	0	0	0	1	2	2	3	4
Foliar spray of leaves and stem inoculation	0	1	2	2	3	4	4	5
Syringe inoculation of peduncles	0	0	1	1	2	2	3	3

inoculation with toothpick and leaf clipping methods as effective methods of artificial inoculation (Table 6, Plate 4).

Evaluation of disease appearance and development was determined using a 0-/5 arbitrary scale (Soyulu *et al.,* 2013).

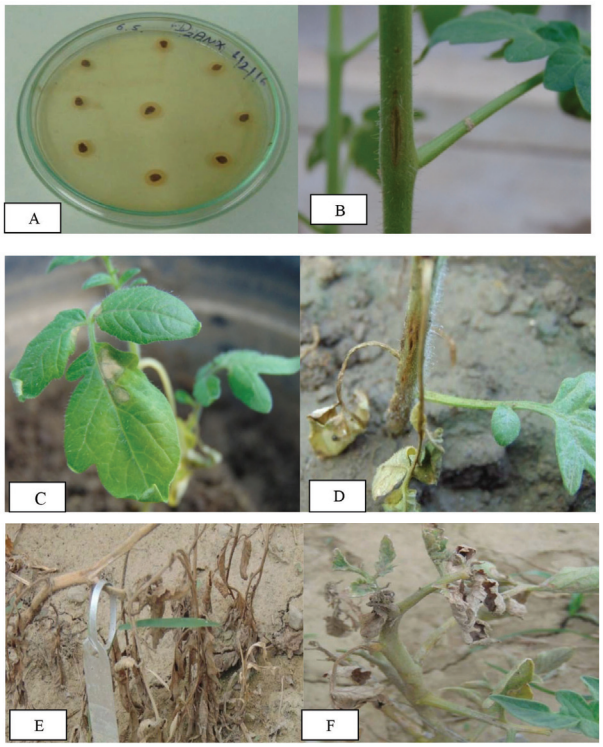

Plate 4. Symptom expression through different methods of inoculation of Cmm on variety Arka Vikash (A-F)

Perusal of the data in Table 6 showed that the symptoms were noticed in tomato seeds 5th days after germination, in the tomato seedlings at 8th days of inoculation with syringe inoculation at the junction of first true leaf, on 10th days after inoculation in clipping and stem inoculation with toothpick. Whereas, the symptoms appeared on 11th days of inoculation with foliar spray and syringe inoculation of stem.

CONCLUSION

In conclusion, this study showed the presence of pathogenic and cultural variability amongst the *Cmm* strains, exhibited by the differential infection rate and varied colony characteristics respectively. The optimum condition for *Cmm* infection through *invitro* studies were observed to be at a temperature range of 25-30ºC and pH of 7. The host range screening showed bellpepper as an important solanaceous host of the pathogen. Further research may be needed for considering the effect of other host crop on the transmission of the pathogen.

Conflicts of Interests

The authors declare that there are no conflicts of interest within them.

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