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Evaluation of Germplasm and Biocontrol Agents against Bacterial Canker (*Clavibacter michiganensis* Subsp. *Michiganensis*) of Tomato in Himachal Pradesh

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ABSTRACT

Bacterial canker (Clavibacter michiganensis subsp. michiganensis) of tomato (Solanum lycopersicum) is a newly emerging bacterial disease in mid hills of Himachal Pradesh, India. The disease is causing huge losses to the tomato production in this region. Under the present investigations, the bacterial canker pathogen was isolated in pure culture. For inculcation of bacteria in young tomato plants for pathogenicity testing and germplasm screening, methods like syringe inoculation of leaves and branches, leaf clipping and stem inoculation with toothpick were adjudged effective. Out of 18 tomato lines/ cultivars tested against the pathogen, none was found resistant to the disease after artificial inoculation in pot culture. However, seven lines viz., EC – 521086, EC – 521074, EC – 251649, EC – 25265, EC – 521054, EC – 35322 and EC – 2791 exhibited moderately susceptible reaction and rest of the lines/ cultivars were either susceptible or highly susceptible. Among five bacterial biocontrol agents tested against the bacterial pathogen in vitro, Pseudomonas flourescens strain PfS-1 was found effective in inhibiting the growth of the pathogen. This biocontrol agent when inoculated through seed, improved germination and delayed the expression of disease symptoms in pot culture.

Key words: *Bacterial canker of tomato, disease management, biological control, germplasm screening*

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is the most important and widely grown vegetables in the world. In India, tomato is grown in states like Bihar, Karnataka, Andhra Pradesh, Maharashtra, Madhya Pradesh, Tamil Nadu, Orissa and Himachal Pradesh. Himachal Pradesh is known for off-season production of tomato in the country with an area of 10,000 ha and annual production of 3,88,000 metric tonnes[13]. In this hilly state major tomato growing areas are located in the mid hills of district Solan, Shimla, Kullu and Sirmour.

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In the mid hills tomato, popularly called "Lal Sona", is the major economic crop grown under open and protected conditions. Due to intensive cultivation of tomato crop various diseases caused by fungi, bacteria, viruses, nematodes and abiotic factors appear during the cropping season and cause yield losses. Among bacterial diseases, Bacterial wilt (*Ralstonia solanacearum*) and Bacterial spot (*Xanthomonas campestris* pv. *vesicatoria*) were of common occurrence[8]. During last two to three years Bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* (Smith)[18]. It has also been observed in tomato growing localities in Solan and Sirmour districts[17]. It is now a major disease of tomato under open field as well as under greenhouse conditions and is very difficult to control[16]. The disease can cause yield losses of up to 70 per cent[18]. The bacterial canker pathogen was previously reported to occur in Karnataka state with an incidence up to 48 per cent[19]. Bacterial canker is one of the most difficult tomato diseases to control. Once it has been established in the crop, it can be extremely contagious. Detection of infected plants can be very difficult and there are no effective means of chemical treatment. Therefore, other measures like use of resistant cultivars, biocontrol agents etc. have been suggested[10].

The present investigations were thus conducted to screen the available tomato germplasm or lines to find out any tomato line/cultivar resistant to the disease. Biological control agents were also tested for their use in the disease management programme.

MATERIALS AND METHODS

The present investigations were carried out in The Department of Plant Pathology, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (HP) during 2012-13. The materials and methods used during the studies are explained here under:

Isolation of Bacterial canker pathogen

Small bits of the diseased tissues taken from affected leaves and fruits (Fig. 1A & B) along the margin of lesions were cut with the help of sterilized scalpel. The bits were sterilized by dipping in 1 per cent mercuric chloride solution (4% Clorax) for few seconds, washed thrice in sterile water and placed in sterile water drops on a flamed glass slide under aseptic conditions. In order to obtain bacterial ooze, incision was given in the centre of each bit with a sterile blade. Another set of these slides were also examined under the microscope for the presence of bacterial cells. A loopful of suspension was streaked on sterilized nutrient agar plates under aseptic conditions. These Petri plates were incubated at 25 ± 2^0C for 48 h and observed for colony formation of the pathogen. Circular, creamy, and creamy to yellow colony of the bacterium were picked from the Petri plate and Gram staining was performed. The colonies which were Gram +ve were selected (Fig. 2A & B). The identification of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) was done on the basis of morphological, physiological and biochemical characters as suggested in the Laboratory Guide for Identification of Plant Pathogenic Bacteria[15].

Effect of Different Inoculation Methods on Incubation Period of Bacterial canker

To find out best artificial inoculation method for further studies on bacterial canker related to germplasm screening, different inoculation methods were performed on young (25-30 days old) tomato seedlings of cv. "ArkaVikas". Bacterial cell suspension was prepared by suspending one loopful of 48 h old bacterial colonies raised on Petri plates in 50 ml of sterilized nutrient broth. The suspension was adjusted to 0.26 OD value (3.0 x 10^8cfu/ml) by adding required quantity of sterilized water. Tomato seeds were dipped in bacterial suspension for 15 minutes and dried in shade before sowing. Leaves, branches and stem of tomato plants were injected with the help of sterilized hypodermic syringe filled with bacterial suspension (Fig 3). In spray inoculation the seedlings were sprayed with

bacterial suspension through hand atomizer. In stem inoculation method tomato plants were inoculated with the help of toothpick. For leaf clipping method tomato leaves intact with the seedlings were cut with scissor and dipped in bacterial suspension for a while. Inoculated plants were covered with polythene bags for 48 h to maintain high relative humidity by frequently spraying distilled water and were observed periodically for the appearance of the symptoms. Some tomato plants and seeds were left without any treatment (uninoculated control) for comparison.

Disease Management studies

Germplasm screening

In all, 18 tomato lines/cultivars procured from the Department of Vegetable Science, UHF, Nauni were screened against the bacterial canker pathogen after artificial inoculation conditions in pots. The seedlings of different germplasm lines were raised in pots containing sterilized soil for 25-30 days. The plants were inoculated with the bacterial culture following wound inoculation of stem with toothpick as discussed earlier. The appearance of disease on different germplasm lines were recorded at a regular interval and the evaluation was done as per the scale used by Foster and Chandi[6] with some modifications.

Evaluation of Biological Control Agents

***In vitro* evaluation**

Five bacterial antagonists viz., *Pseudomonas flourescens* strain PfS-1, *Pseudomonas flourescens* strain PfS-2, *Pseudomonas flourescens* strain PfS-3, *Bacilus subtilis* strain BsS-1, *Pseudomonas aurigenosa* strain PaS-1 procured from the Department of Plant Pathology, Dr. Y S Parmar University of Horticulture and Forestry, Nauni (H.P.) were screened under *in vitro* conditions against *C. michiganensis* subsp. *michiganensis* by using well diffusion method[20]. Nutrient agar was poured in the Petri plates under aseptic conditions. After solidification of the medium, one loop of bacterial pathogen culture was spread in each of the Petri plates (90 mm dia.). A well of 4 mm diameter was cut with a cork borer in to center of Petri plate and 0.5 ml of bacterial suspension of test bacterial biocontrol agent was pipetted in the well. Plates were incubated at 27^0C. The diameter of inhibition zone was measured after 72 h of inoculation and the per cent inhibition was calculated using the formula suggested by Vincent[21].

Evaluation biocontrol agents through seed inoculation method under pot culture

Seeds of a susceptible variety ArkaVikas were first inoculated with the pathogen suspension in sterilized water. The inoculated seeds were dried for a while and then treated with the suspension of different biocontrol agents following the same method, separately[16]. The seeds after different treatments were sown in pots containing sterilized soil under greenhouse conditions in completely randomized design. The plants were observed for the expression of disease symptoms periodically.

Statistical Analysis

The data obtained from laboratory as well as greenhouse experiments were subjected to appropriate statistical analysis wherever necessary using standard procedure as described by Gomez and Gomez[7].

RESULTS AND DISCUSSION

Effect of different inoculation methods on incubation period of Bacterial canker

For various studies on disease development and germplasm screening artificial inoculation of tomato seedlings is needed. Various methods of inoculation of bacterial pathogens have been described and used by different workers. Seven such methods of inoculation were also used to find out most efficient inoculation method under the present study. On the basis of symptom expression and incubation period four methods viz., syringe inoculation of leaves, syringe inoculation of branches, leaf clipping and stem inoculation with toothpick were observed to be good inculcation methods for bacterial canker of

tomato out of seven different inoculation methods used (Table 1). Syringe inoculation of stem has been advocated as a method for bacterial inoculation by many workers[4]. While top pruning or leaf clipping methods were suggested as new inoculation methods for bacterial canker of tomato for various pathological studies[11].

Germplasm screening

Use of resistant cultivars is considered as the ultimate solution for management of a plant disease. Hence, under present study an attempt was made to find out any tomato line/cultivar resistant to bacterial canker. Out of 18 tomato lines/cultivars screened none of them showed resistant or moderately resistant reaction after artificial inoculation of bacterial canker pathogen (Table 2). However, seven lines viz., EC – 521086, EC – 521074, EC – 251649, EC – 25265, EC – 521054, EC – 35322 and EC – 2791 exhibited moderately susceptible reaction. All other tested germplasm exhibited susceptible to highly susceptible reaction. So in the present investigation no resistant cultivar/ line of tomato could be screened out. In other countries some resistant tomato lines have been found against the bacterium. However, most of the commercial cultivars were observed susceptible against the disease[12,14].

Evaluation of biocontrol agents against *Clavibacter michiganensis* subsp. *michiganensis*

***In vitro* evaluation**

Biocontrol agents also provide an alternate and eco-friendly method for the management of bacterial diseases. Under *in vitro* conditions the biocontrol agents *Pseudomonas fluorescens* PfS-1 provided 17.33 per cent growth inhibition of the bacterium and was found better than other biocontrol agents (Table 3). Colin *et al*[5]., and Amkraz *et al*[1]., demonstrated that *Pseudomonas fluorescens* strains exhibit a various degree of antagonism towards *Clavibacter michiganensis* subsp. *michiganensis in vitro*. Other authors have also reported that species of *Pseudomonas* and *Bacillus* strains isolated from the rhizosphere of forest plants and soil have an ability to inhibit the growth of *Clavibacter michiganensis* subsp. *Michiganensis*[9]. The majority of the fluorescent *Psuedomonads* provided growth inhibition of this bacterial pathogen under *in vitro* conditions[3]. These workers have suggested that antibiotic compounds and siderophores produced by fluorescent *Psuedomonads*, constituted an important factor in the suppression of bacterial canker pathogen.

Evaluation of biocontrol agents in pots through seed inoculation method

These biocontrol agents when used as seed inoculation before sowing provided better results. The biocontrol agent *Psuedomonas fluorescens* strain PfS-1 and *Bacillus subtilis* strain BsS-1provided better seed germination and delayed in the development of disease symptoms when applied alone and in combination with pathogen suspension than that of the pathogen inoculation alone (Table 4). Bakker *et al*[2]., has observed reduction in the infection of bacterial canker of tomato after seed treatment with fluorescent *Psuedomonads* strain HF-142. Biological seed treatment with antagonistic *Psuedomonas fluorescens* has also been known to improve seed quality under laboratory condition and reduced the severity of bacterial canker of tomato in the field condition[19]. This treatment increased the population of introduced antagonistic bacteria on the seed and soil. Hence, the competition for substrate and space of the introduced bacteria was improved. As competition for nutrients probably occurs in most interactions between bacteria and pathogens on the root, and is partly responsible for the observed biocontrol by fluorescent *Psuedomonads*.

Table 1: Effect of different inoculation methods on incubation period of *Clavibacter michiganensis* subsp. *michiganensis* on tomato seedlings

Inoculation method	Symptom expression after days of inoculation														
	0	6	9	12	15	18	21	24	27	30	33	36	39	42	45
Seed inoculation	0	0	0	0	0	0	1	1	1	1	2	2	3	3	4
Foliar spray	0	0	1	1	1	2	2	2	3	3	3	4	4	4	4
Syringe inoculation of leaves	0	1	1	1	2	2	2	3	3	3	4	4	4	4	4
Syringe inoculation of branches	0	1	1	1	1	2	2	2	3	3	3	4	4	4	4
Syringe inoculation of stem	0	0	1	1	2	2	2	3	3	3	3	3	4	4	4
Clipping	0	1	1	2	2	2	2	3	3	3	3	4	4	4	4
Stem inoculation with toothpick	0	1	1	2	2	2	3	3	3	3	4	4	4	5	5

Where: 0 – no symptoms, 1 – up to 1/3 of the leaves showed marginal necrosis and yellowing, 2 – marginal necrosis and up to 2/3 of the leaves was yellow and wilted, 3 – more than 2/3 of leaves and leaflets shriveled and wilted but the terminal leaves on the main shoot not wilted, 4 – terminal leaves of the main shoot and most leaves wilted, 5 – totally plant death.

Table 2: Reaction of tomato germplasm to bacterial canker of tomato (*Clavibacter michiganensis* subsp *michiganensis*)

Sr. No.	Tomato lines	Disease severity (%)	Reaction
1	EC – 521 052	39.51 (38.93)	S
2	EC – 521086	22.22 (28.11)	MS
3	EC – 521074	24.92 (29.92)	MS
4	EC – 521046	31.36 (34.04)	S
5	EC – 126902	40.64 (39.59)	S
6	EC – 251649	20.99 (27.23)	MS
7	EC – 251646	32.44 (34.70)	S
8	EC – 521041	28.36 (32.15)	S
9	EC – 25265	44.28 (41.70)	MS
10	EC – 521054	30.54 (33.53)	MS
11	EC – 35322	23.82 (29.20)	MS
12	EC – 2791	17.21 (24.49)	MS
13	EC – 2798	49.62 (44.76)	S
14	EC – 6486	27.68 (31.72)	S
15	EC – 8591	42.17 (40.48)	S
16	EC – 520075	54.20 (47.39)	HS
17	Arkavikas	61.92 (50.88)	HS
18	Naveen	58.18 (49.69)	HS
	C.D.(0.05)	1.89	

MR = Moderately resistant (1.1-10.0 %)
MS = Moderately susceptible (10.1-25.0 %)
S = Susceptible (25.1-50.0 %)
HS = Highly susceptible (> 50 %)
Figures in the parentheses are arc sine transformed values

Table 3: *In vitro* evaluation of different biocontrol agents against *Clavibacter michiganensis* subsp. *michiganensis*

Biocontrol agent	Per cent growth inhibition
Pseudomonas fluorescens strain PfS-1	17.33 (24.59)
Pseudomonas fluorescens strain PfS-2	14.40 (22.27)
Pseudomonas fluorescens strainPfS-3	16.36 (23.83)
Bacilus subtilis strain BsS-1	13.75 (21.75)
Pseudomonas aurigenosa strain PaS-1	12.26 (20.49)
C.D. (0.05)	**1.13**

Figures in the parentheses are arc sine transformed values

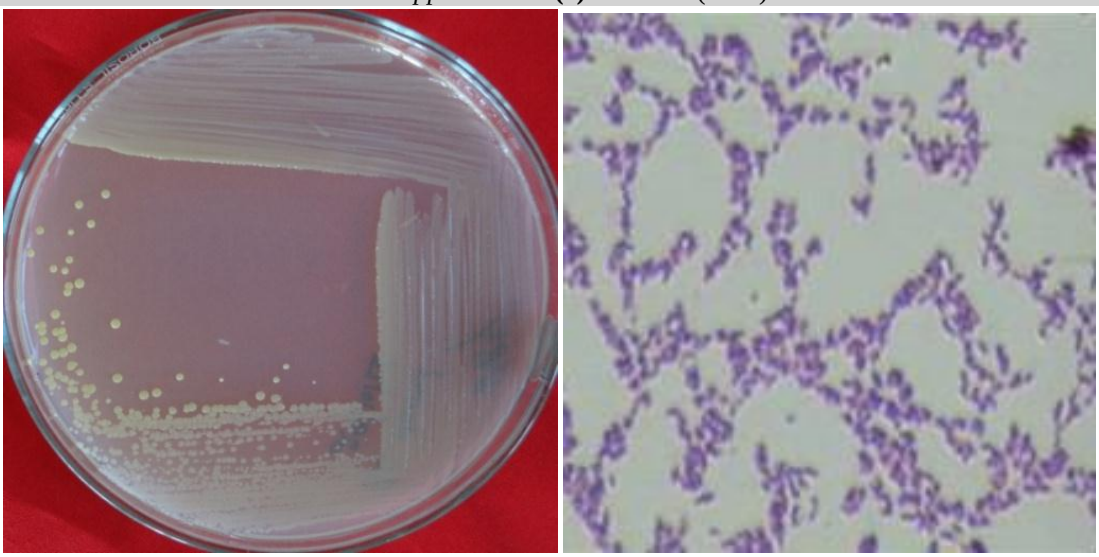
Table 4: Evaluation of biocontrol agents in pots through seed treatment against bacterial canker of tomato

Seed Treatment	Seed Germination (%)	Symptom appeared Days After Seed Sowing											
		21	24	27	30	33	36	39	42	45	48	51	54
Cmm	46.55 (43.00)	0	1	1	1	2	2	2	3	4	4	4	5
Cmm+ PfS-1	57.04 (49.03)	0	0	0	1	1	1	2	2	3	3	4	4
Cmm+ PfS- 2	53.38 (46.92)	0	0	1	1	2	2	2	3	3	4	4	4
Cmm+ BsS- 1	50.00 (44.98)	0	1	1	2	2	2	3	3	3	4	4	4
PfS-1	60.33 (50.94)	0	0	0	0	0	0	1	1	1	2	2	2
PfS-2	54.54 (47.59)	0	0	0	0	1	1	2	2	3	3	3	3
BsS- 1	57.04 (49.03)	0	0	0	0	1	1	2	2	2	3	3	3
Un- inoculated	48.33 (44.03)	0	0	0	1	1	2	2	2	3	3	3	4
C.D.(0.05)	3.37 (1.95)												

Where, 0 – no symptoms, 1 – up to 1/3 of the leaves showed marginal necrosis and yellowing, 2 – marginal necrosis and up to 2/3 of the leaves was yellow and wilted, 3 – more than 2/3 of leaves and leaflets shriveled and wilted but the terminal leaves on the main shoot not wilted, 4 – terminal leaves of the main shoot and most leaves wilted, 5 – totally plant death.

A. **B.**

Fig. 1: Different types of symptoms of bacterial canker of tomato: A: Marginal necrosis of leaf, B. Bird's eye spots on young fruit



A. **B.**

Fig. 2: A) Colonies of *Clavibacter michiganensis* subsp. *michiganensis* on Nutrient agar medium and B) bacterial cells showing Gram +ve reaction

A. **B.**

Fig. 3: Syringe inoculation method of artificial inoculation: A: inoculation of tomato leaf and B: expression of disease symptom after artificial inoculation

CONCLUSIONS

Among the 18 tomato lines/cultivars screened none of the tomato germplasm lines were found resistant to the bacterial canker pathogen. Biocontrol agents like *Pseudomonas fluorescens* have the potential to be used as seed treatment against the disease.

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