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In vitro lytic enzyme activity of antagonistic rhizobacteria against tomato bacterial canker

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Abstract
Bacterial canker is the most contagious and destructive disease of tomato caused by *Clavibacter michiganensis* ssp. *michiganensis* (Cmm) can drastically reduce tomato yield and quality, thus causing substantial economic losses both in greenhouses and in open-field production. The pathogen is seed borne, persists in plant debris in soil and on contaminated greenhouse structures and infected seeds. It infects host plants via roots or wounds and invade the xylem vessels, followed by a systemic infection of the host which results in leaf lesions, wilting, fruit lesions and ultimately yield loss of marketable fruits. Biological control through the use of beneficial microorganisms or by the combination of multiple antagonists colonizing on the rhizosphere, surface and inner tissues of healthy plants has emerged as a promising alternative to chemical pesticides as a more rational and safer crop management over disease control. A total of 550 rhizobacterial isolates from different horticultural, vegetable crops and medicinal plants grown under mid hills and high hills of Himachal Pradesh were screened for antagonistic activity against *Clavibacter michiganensis.* Out of total, only 40 bacterial isolates showed antagonistic activity by depicting inhibition zone in the range of 3.20 to 12 mm. Twenty isolates were screened for production of different lytic enzymes viz. amylase, cellulase, lipase, protease and chitinase. A lot of variation was observed in amylase activity ranging from 0.12 to 2.19 E.I. (Enzyme index). Maximum E.I (2.19) was recorded for isolate KU_2 S1 whereas the isolate $RO_{4(5)}$ showed minimum E.I. (0.12).

Keywords: Tomato, clavibacter michiganensis, bacterial canker, rhizobacteria, lytic enzymes

Introduction
The rhizosphere is the narrow zone of soil specifically influenced by the root system and is hot spot of microbial abundance and the activity is due to presence of root exudates and rhizodeposits (Samalla *et al.*, 2006) [17]. This zone is rich in nutrients when compared with the bulk soil due to the accumulation of a variety of plant exudates, such as amino acids and sugars, providing a rich source of energy and nutrients for bacteria (Gray and Smith, 2005) [10] and the bacteria colonizing this habitat are called rhizobacteria. Beneficial bacteria are referred to as PGPR, constitutes only 1-2% of the total population (Antoun and Kloepper, 2001) [3] and affect plant growth in two different ways, direct and indirect. The direct promotion of plant growth by PGPR entails either providing the plant with a compound that is synthesized by the bacterium, for example phytohormones, or facilitating the uptake of certain nutrients from the environment (Bhattacharya and Jha, 2012) [4]. The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms. This can happen by producing antagonistic substances or by inducing resistance against pathogens (Sivasakthi *et al.*, 2014) [19]. This can happen by producing antagonistic substances or by inducing resistance against pathogens (Sivasakthi *et al.*, 2014) [19].
Tomato (*Lycopersicon esculentum Mill*) is one of the most widely grown vegetables in the world. Cultivated tomato is a diploid, self-pollinating perennial herb and its popularity among consumers has made it an important source of vitamins A and C in diets. Bacterial canker is the most contagious and destructive disease of tomato caused by *Clavibacter michiganensis* ssp. *michiganensis* (Cmm) can drastically reduce tomato yield and quality, thus causing substantial economic losses both in greenhouses and in open-field production. The pathogen is seed borne, persists in plant debris in soil and on contaminated greenhouse structures and infected seeds (Agrawal *et al.*, 2012) [1]. It infects host plants via roots or wounds and invade the xylem vessels, followed by a systemic infection of the host which results in leaf lesions, wilting, fruit lesions and ultimately yield loss of marketable fruits (Girish & Umesha, 2005) [9]. Biological control through the use of beneficial microorganisms or by the combination of multiple antagonists colonizing on the rhizosphere, surface and inner tissues of healthy plants

has emerged as a promising alternative to chemical pesticides as a more rational and safer crop management over disease control. PGPR exhibit several mechanisms of biological disease control, most of which involve competition and production of metabolites like antibiotics, cell wall degrading enzymes, siderophores, and HCN and inducing the systemic resistance (Induced Systemic Resistance).

Therefore, in the present study, our objective was to evaluate the potential of beneficial microbes as a bioinoculant to control bacterial canker of tomato *in-vitro*.

Materials and Methods

Agar Diffusion Test

In vitro antagonism studies between rhizobacterial isolates and the pathogenic strains of *Clavibacter michiganensis* were carried out on nutrient agar plates using agar diffusion method (Mitchell and Carter, 2000) [13].

Four hundred microlitres of *C. michiganensis* suspension containing 10^8 cfu/ml was spread on nutrient agar plates and four wells of 8mm diameter punched into the agar. In these wells 100µl suspension of each test antagonist (10^8 cfu/ml) was added and the plates incubated at 28˚C for 48h. Inhibition of *C. michiganensis* growth was assessed by measuring the diameter of inhibition zone (mm) after incubation for 48h at 28˚C.

Cell wall degrading enzyme production

Chitinase assay was performed as per the protocol given by Robert and Selitrennikoff, 1988 [16]. Protease activity assay was performed as per the protocol given by Fleming *et al*., 1975 [7]. Amylase activity was measured by method given by Shaw *et al*., 1995 [18]. Cellulase activity was measured by the method given by Ghose, 1987 [8]. Lipase activity was measured by the method given by Kumar *et al*., 2012 [11].

Results and Discussion

In vitro screening of bacterial isolates for antagonistic activity against *Clavibacter michiganensis* by using agar diffusion test

Bacterial canker is a very contagious and destructive disease of greenhouse as well as field grown tomatoes. But, the biological control of canker, caused by *Clavibacter michiganensis* ssp. *michiganensis* has not been worked out. Consequently, this study was undertaken with the aim of identifying a potential biocontrol agent of the pathogen. A total of 550 rhizobacterial isolates from different horticultural, vegetable crops and medicinal plants grown under mid hills and high hills of Himachal Pradesh were screened for antagonistic activity against *Clavibacter michiganensis.* These crops were from different agroclimatic conditions with respect to altitude and soil pH. Out of total, only 40 bacterial isolates showed antagonistic activity by depicting inhibition zone in the range of 3.20 to 12 mm.

Cell wall degrading enzyme production

Twenty isolates were screened for production of different lytic enzymes viz. amylase, cellulase, lipase, protease and chitinase (Plate 1). Out of twenty isolates, eleven (55%) exhibited amylase activity. A lot of variation was observed in amylase activity ranging from 0.12 to 2.19 E.I. (Enzyme index). Maximum E.I (2.19) was recorded for isolate KU_2 S1 whereas the isolate $RO_{4(5)}$ showed minimum E.I. (0.12).

Seven (35%) out of twenty isolates exhibited cellulase activity. Variation in cellulase activity was observed in the range of 0.40 to 1.50 E.I. Maximum cellulase activity was recorded for isolate $KU_{3(3)}$ (1.50 E.I.) and isolate $KU_{1(5)}$ (0.40 E.I.) was recorded with minimum enzyme acttvity. Thirteen (65%) out of twenty isolates exhibited lipase activity. Lipase activity was observed within the range of 1.01 to 1.73 E.I. Maximum lipase activity was recorded for isolate S 1 (1.73 E.I.) and minimum lipase activity for isolate $RO_{5(1)}$ (1.01 E.I.) was recorded. Fifeen (75%) out of twenty isolates exhibited protease activity. Protease activity was observed within the range of 0.21 to 1.87 E.I. Maximum enzyme activity was recorded for isolate $RO_{5(6)}$ (1.87 E.I.) and minimum enzyme activity was recorded for isolate $Ra_{2(2)}$ (0.21 E.I.). Nineteen (95%) out of twenty isolates exhibited chitinase activity. Variation in chitinase activity was observed in the range of 0.43 to 1.52 E.I. Isolate which exhibited maximum chitinase activity was $KU_{3(1)}$ (1.52 E.I.) and isolate that exhibited minimum chitinase activity was $RO_{2(7)}$ (0.43).

Table 1: Cell wall degrading enzyme production

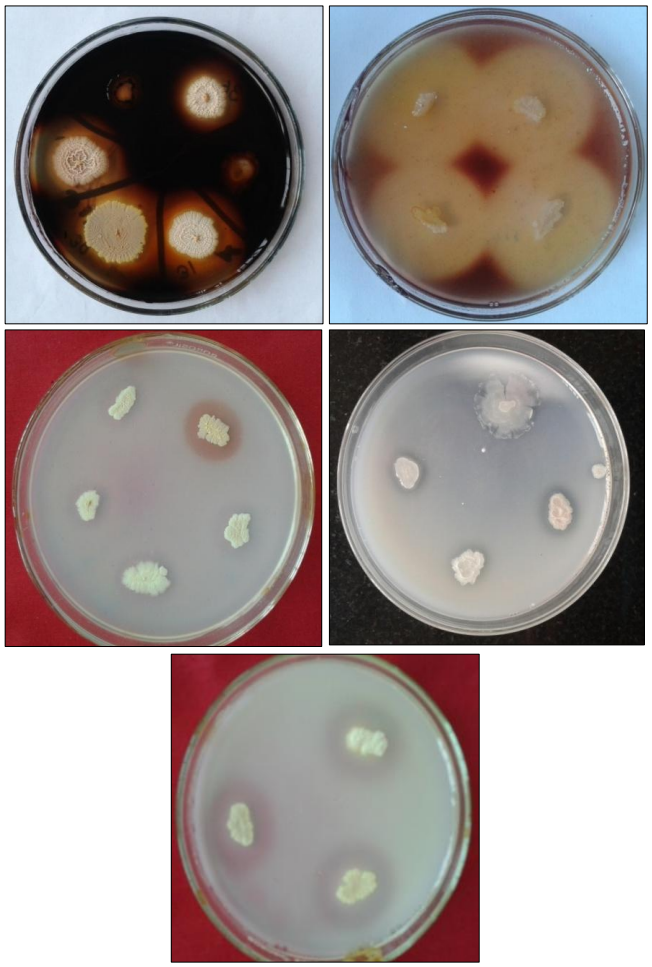
Isolate	Amylase	Cellulase	Lipase	Protease	Chitinase
	E.I.***	E.I.	E.I.	E.I.	E.I.
KU_2 S 1	2.19	1.47	1.68	1.80	1.24
NA(2)	1.80	-	1.68	1.72	1.13
NA(5)	-	1.30	1.50	1.72	1.25
S 1	1.80	-	1.73	1.62	1.13
$KU_{3(1)}$	-	1.30	1.50	1.72	1.52
$R_2S_{(1)}$	-	-	1.53	1.76	1.42
$RO_{5(6)}$	1.66	-	1.63	1.87	1.45
Ra $_{34(5)}$	1.33	-	-	1.41	1.50
NA(6)	1.51	-	1.42	1.10	0.56
Ra $_{1(3)}$	1.31	-	1.12	1.33	1.04
Ra $_{31(5)}$	-	1.10	-	-	1.50
$KU_{3(3)}$	-	1.50	1.51	1.69	1.22
KU_3	-	-	1.60	1.76	0.72
Na-12 S 1	0.78	1.20	-	-	0.90
Ra $_{2(2)}$	-	-	1.23	0.21	1.22
$RO_{4(5)}$	0.12	-	-	-	0.80
$RO_{2(7)}$	0.70	-	-	0.43	0.43
Na 8	-	-	-	0.80	1.45
$KU_{1(5)}$	1.12	0.40		-	-
$RO_{5(1)}$	-	-	1.01	-	1.20

E.I.*** = $\frac{A}{B}$

Where, A = Diameter of halo zone, B = Colony diameter

Lytic enzymes have been studied as potential antibacterial agents against bacterial plant pathogens because the enzymes play a key role in the mechanism of parasitic entry in to host cells (Dahiya *et al*., 2006 and Nguyen *et al*., 2008) [6, 15]. Production of different lytic enzymes viz. amylase, cellulase, lipase, protease and chitinase was also tested for these antagonists. Overall, 55% of the isolates were able to produce amylase, 32.5% were cellulase producers, 55% were lipase producers, 60% protease producers and 97.5% were chitinase producers. Chakraborty *et al.* (2013) [5] also screened three bacterial antagonists for PGP characteristics (P-solubilization, IAA production, siderophore production, protease and chitinase production). It has been reported that several mechanisms are responsible for suppression of pathogen involving the lytic enzymes that play a key role in biocontrol potential against different plant pathogens (bacterial, fungal and viral). The proposed mechanism to provide a protective effect on the roots through antagonism towards the

phytopathogenic bacteria is by producing metabolites such as siderophores, lytic enzymes like amylase, protease, cellulase, lipase and chitinase; plant hormones like auxins and IAA (Amaresan *et al*., 2011; Neeraja *et al.,* 2010; Maksimov *et al.*, 2011) [2, 14, 12].



a) Amylase
b) Protease
c) Lipase
d) Chitinase
e) Cellulase

Plate 1: Screening of antagonistic bacterial antagonists from different horticultural crops for lytic enzymes production

Summary and conclusion

The present study was aimed to explore the diversity of bacterial communities colonizing the rhizosphere soil and roots of different horticultural and vegetable crops and medicinal plants grown in natural conditions for evaluating their biocontrol potential against *Clavibacter michiganensis*, causing bacterial canker of tomato. In conclusion, high diversity of antagonistic bacteria in the rhizosphere of different horticultural crops viz. strawberry, apple and apricot was observed. *In vitro* evaluation of antagonistic bacterial isolates revealed that it reduced the disease index of bacterial canker of tomato and production of different lytic enzymes. Further, more studies are required to harness the potential of antagonistic isolates as bioinoculants in agriculture.

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