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RESEARCH ARTICLE

Biocontrol activities of rhizobacteria associated with apple, apricot and kiwi rhizosphere against bacterial canker caused by *Clavibacter michiganensis*

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Abstract
Bacterial canker, a destructive disease caused by *Clavibacter michiganensis* subsp. *michiganensis* causes significant economic losses to tomato production worldwide. Biological control has been proposed as an alternative to current chemical protectants, however, a little headway has so far been made in developing biocontrol methods against this destructive bacterial pathogen. To narrow this knowledge gap, we investigated the antagonistic capacity of different rhizobacterial isolates from three horticultural crops viz., apple, apricot and strawberry against *C. michiganensis* under in vitro conditions. The potential antagonistic strains showing in vitro inhibition against *C. michiganensis* were further screened for multifarious plant growth promoting and biocontrol activities (P-solubilization, IAA production, siderophore production, lytic enzyme activity, and ability to fix atmospheric nitrogen). Increase in concentration of cell free supernatant from 0.25 to 1.00% (v/v) revealed significant increase in antagonistic activity against *C. michiganensis* with most prominent increase being observed in isolate S_1 with 9.90 mm zone of inhibition at 0.25% concentration, which increased to 15.40 mm with increase in concentration to 1.0%. Five most efficient antagonists identified under in vitro conditions were tested for biocontrol potential against *C. michiganensis* under net house conditions. Isolate S_1 showed maximum reduction in disease incidence (70.00%) with minimum disease severity recorded (28.55%), besides significant increase in various plant growth parameters. The isolate was identified as *Bacillus amyloliquefaciens* by 16S rDNA sequencing. Therefore, strain S_1 holds considerable biocontrol as well as growth promoting potential and could be used as a potential biocontrol agent against bacterial canker of tomato.

Keywords Bacterial canker · *Bacillus amyloliquefaciens* · Biocontrol · *Clavibacter michiganensis* · Tomato

Introduction

Bacterial canker is the most contagious and destructive disease of tomato caused by *Clavibacter michiganensis* ssp. *michiganensis* (Cmm). It can drastically reduce tomato yield and quality, causing significant crop losses both in greenhouses and in open-field production. This bacterial pathogen is seed borne which persists in soil in infected seeds and dead plant debris. The pathogen poses drastic impact across the world and at 30–80% of systemic seedlings infection and up to 46% of yield losses may occur under favourable conditions (Agrawal et al. 2012). *Clavibacter*, a gram positive actinomycete enters the plant through natural openings and wounds and enters the xylem vessels, there it lead to characteristic disease symptoms such as light brown discoloration of the vascular bundles, wilting of leaves with one-sided asymmetric, necrotic lesions, necrotic lesions on stems and petioles. If pathogen attacks at seed or seedlings stage, a systemic disease infection will lead to death of plant, and if infection occurs at later stage of plant development, the plant will survive and generate fruits that can have bird's eye spots (Calis et al. 2012). It requires relatively warm temperature and high relative humidity for infection and development. It is known to spread through contaminated seed to the new areas. Bacterial canker has emerged as a major disease

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of tomato in open as well as protected cultivation conditions in the southern parts of India caused huge losses to the growers (Sarala and Shetty 2005; Umesha 2006). In Himachal Pradesh the disease was reported first time by Singh et al. (2015). The average temperature during most of the time period remains mild in this region. Therefore, tomato is grown during summer months unlike other parts of the country. During summers the congenial conditions for the development of bacterial canker do prevail in the month of June when the temperature is high and pre-monsoon showers provide humidity.

Conventional management strategies include use of soil amendments, plant debris removal, disinfection in green houses, use of certified seeds and crop rotation, but none of them have been proven sufficiently effective in controlling this destructive plant pathogen (Marcic et al. 2012). Antibacterial antibiotics and copper containing compounds have been used but their use is not encouraged as they lead to the selection of resistant bacterial population and have limited efficacy under conditions favourable for pathogen growth (Soylu et al. 2003). The decreasing efficacy of the chemical pesticides and the risks associated with them has highlighted the need for an alternative safe and more effective control measures (Haggag 2010; Deshwal and Kumar 2013). Microbial control agents provide an effective route to eco-friendly plant protection. The induction of plant resistance by using microbial bioagents may be a useful method for the reduction of severity of this disease (Romero et al. 2003; Girish and Umesha 2005). In this context, the use of plant growth promoting rhizobacteria (PGPR) as biocontrol agents is an effective alternative to the agro-chemicals (Villacieros et al. 2003). PGPR are known to rapidly colonize the rhizosphere and suppress deleterious microorganisms as well as soilborne pathogens at the root surface (Rangajaran et al. 2003). These organisms can also be beneficial to the plant by stimulating growth (Bloemberg and Lugtenberg 2001).

Bacterial canker caused by *C. michiganensis* is very prevalent and destructive disease of tomato in tropical, subtropical and temperate tomato growing regions in India. Previous studies indicated the possibilities to control bacterial pathogens of tomato under in vitro and in vivo conditions, using antagonistic strains of PGPR (Kurabachew and Wydra 2013; Alyie et al. 2008). Unfortunately very little information is available about induced resistance caused by PGPR against *C. michiganensis* in tomato. Therefore, the objectives of the present study were to evaluate the antagonistic potential of rhizobacterial strains from three different fruit crops against *C. michiganensis* and to evaluate the efficacy of selected antagonistic PGPR to reduce bacterial canker of tomato under net house conditions.

Materials and methods

Bacterial strains and test pathogen

A total of 150 bacterial strains previously recovered from rhizosphere of 3 horticultural crops viz., apple, apricot and strawberry were used in the present study. The strains were initially maintained in glycerol stock (nutrient broth amended with 30% glycerol) and preserved at − 20 °C. The strains were revived and streaked onto nutrient agar plates, incubated at 35 °C for 24–48 h and used further.

The bacterial pathogen, *C. michiganensis* causing bacterial canker of tomato was obtained from the culture stock of Department of Plant Pathology, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, India. The pathogen was maintained at 25 ± 20 °C for 48 h and observed for colony formation of the pathogen (Singh and Bharat 2017).

Antimicrobial activity of bacterial isolates against *C. michiganensis*

In vitro antimicrobial activity of bacterial strains against *C. michiganensis* was determined using agar diffusion method (Mitchell and Carter 2000). Four-hundred microlitres of *C. michiganensis* suspension containing 10^8 cfu/ml was spreaded on nutrient agar (NA) plates and four wells of 8 mm diameter were punched into the agar. In the wells, 100 µl suspension of each bacterial strain (10^8 cfu/ml) was added and the plates were incubated at 28 °C for 48 h. Inhibition of *C. michiganensis* growth was assessed by measuring the diameter of inhibition zone (mm) after incubation of 48 h at 28 °C.

The bacterial strains showing antagonistic activity against *C. michiganensis* were used further.

Characterization of antagonists as PGP bacteria

The selected antagonistic strains were in vitro characterized for additional PGP and biocontrol activities. The strains were screened for production of IAA as per the method of Gordon and Paleg (1957). The strains were grown in 25 ml Luria–Bertani (LB) broth (amended with 5 mM L-tryptophan, 0.065% sodium dodecyl sulphate and 1% glycerol) and incubated at 37 °C for 72 h at shake conditions. 3 ml of culture supernatant was mixed with 2 ml of Salkowski's reagent (2 ml 0.5 M $FeCl_3$ + 98 ml 35% $HClO_4$) and absorbance was measured spectrophotometrically at 535 nm for the development of pink colour. Solubilization of tricalcium phosphate in PVK agar was determined by the method of Pikovskaya (1948). The P-solubilization efficiency (%SE) was calculated using the equation:

$$\%\mathrm{SE} = \frac{Z - C}{C} \times 100,$$

where Z is the diameter of the colony + halo zone and C is the diameter of the colony.

The antagonistic strains were tested for their ability to grow on nitrogen free medium (Baldani and Döbereiner 1980).

Siderophore production was evaluated using chrome azurol-S (CAS) assay method and zone of clearance obtained on CAS medium was recorded (Schwyn and Neilands 1987). The selected bacterial antagonists were also evaluated for their ability produce cell wall degrading enzymes; the chitinase activity was detected on minimal agar plates amended with 0.3% colloidal chitin and diameter of halo zones was measured (Robert and Selintrennikoff 1988). Cellulase enzyme activity was detected by the method of Ghose (1987). Protease activity was detected by inoculating bacterial strains on skim milk agar plates (Fleming et al. 1975). Lipase activity was detected by using the method of Kumar et al. (2012). Enzyme activities were recorded in terms of enzyme index (EI), which was calculated as:

$$\mathrm{EI} = \frac{A}{B},$$

where A is the diameter of the colony + halo zone and B is the diameter of the colony.

The bacterial isolates showing significantly higher inhibition against *C. michiganensis* in well diffusion assay and possess other PGP activities were selected further for in vitro evaluation of antibacterial activity at different concentrations of cell free supernatant.

In vitro inhibition of *C. michiganensis* at different concentrations of cell free supernatant

Four-hundred microlitres of *C. michiganensis* suspension containing 10^8 cfu/ml was spreaded on nutrient agar plates and four wells of 8 mm diameter were punched onto the agar plates. Supernatant of 48 h old bacterial antagonist culture was added in the wells at different concentrations i.e. 0.25%, 0.50%, 0.75% and 1.00% (v/v). The plates were incubated at 28 °C for 48 h. Antagonism against *C. michiganensis* was assessed by measuring the diameter of inhibition zone (mm).

Evaluation of antagonistic activity against *C. michiganensis* under net house conditions

Growth of plants

Potting mixture consisted of soil, sand and farmyard manure (2:1:1), was sterilized by 3 repetitive autoclave cycles of 1 h each. 1.5 kg of potting mixture was filled in 12 cm diameter pots. Tomato seeds cv. Solan Vajr were surface sterilised with 0.1% $HgCl_2$ for 1 min, washed thoroughly with sterilised water and then planted in the pots containing sterilized potting mixture (50 seeds pot^{-1}). The plants were maintained in net house at 30 ± 3 °C, 70–100% relative humidity and seedlings were watered with sterilized water when necessary.

Bacterial pathogen and antagonists' inoculum preparation

The pathogen inoculum was prepared by culturing *C. michiganensis* in nutrient broth and subsequently incubated at 28 °C for 48 h on a rotary shaker at 120 rpm. The 1 OD culture with population size approximately, 10^8 cfu/ml was used for the pathogen challenge at 4–5 true leaved tomato seedlings.

The bioprotective effect of five most efficient bacterial antagonists screened under in vitro conditions was studied on tomato plants artificially challenged with *C. michiganensis* under net house conditions. For this, the antagonists' inoculum was prepared by culturing the five best antagonistic PGPRs in nutrient broth for 48 h at 35 ± 2 °C (10^8 cfu/ml) on a rotary shaker at 120 rpm. The liquid cultures were used for seedling dip and drenching of tomato seedlings at 4–5 leaves stage.

Biocontrol experiment

1.5 kg of the sterilized potting mixture was mixed with 100 ml inoculum of *C. michiganensis* (10^8 cfu/ml) and filled in 12 cm diameter pots. One week after incorporation of pathogen into the potting mixture and 1 day before transplanting the seedlings, antagonists (10^8 cfu/ml) were incorporated into the mixture at a rate of 75 ml pot^{-1}. Four weeks old tomato seedlings raised in the separate pots were root dipped in liquid culture of antagonistic strains (10^8 cfu/ml) for 60 min and transplanted into pathogen-antagonist mixture soil (Lemessa and Zeller 2007). Treatments were replicated four times with five plants per pot. Positive control was maintained which was only inoculated with the pathogen and negative control was also maintained without any inoculation of pathogen or antagonist PGPR. Seedlings treated with bacterial strains but not challenged with pathogen were kept to study the effect of bacterial antagonists on plant growth promotion. Temperature of net house varied between 25 and 35 °C and relative humidity between 70 and 100% during the course of the experiment.

Disease ratings were recorded 3 weeks after pathogen challenge using a 0–7 scale (0 = No symptoms; 1 = Trace; one leaf symptomatic; 2 = Slight, two leaves symptomatic; 3 = Moderate, > 2 leaves symptomatic; 4 = Severe, half plant affected; 5 = Very severe, more than half plant affected; 6 = Stunted with extensive collapse; 7 = Dead plant) given by Shenge et al. (2010). Disease severity was calculated as per the formula:

Disease severity index

$$= \frac{\sum (rating \times no.\ of\ plants\ rated)}{Total\ no.\ of\ plants\ observed \times highest\ rating} \times 100.$$

Disease incidence was calculated as:

$$\text{Disease incidence} = \frac{\text{No. of diseased plants}}{Total\ no.\ of\ plants\ observed} \times 100.$$

Biocontrol efficacy was calculated using the formula:

$$\text{Biocontrol efficacy} = \frac{\text{Disease incidence of control} - \text{Disease incidence of plants treated with antagonists}}{Disease\ incidence\ of\ control} \times 100.$$

The statistical analyses were done using Duncan's multiple range test at 5% significance level.

Rhizospheric colonization by *C. michiganensis* and inoculated bacterial antagonists

The rhizospheric soil from each treatment inoculated with bacterial antagonists was collected after the trial and their colonization was studied by cfu count. The reisolated bacterial colonies were characterized on the basis of morphological and biochemical characters and the strains showing same morphological and metabolic profile with that of the inoculated strains were counted. Soil adhering to roots was taken and appropriate dilutions were plated onto nutrient agar medium and incubated at 35 ± 2 °C and observed for number of cfu after 24–48 h.

Phylogenetic identification of selected bacterial antagonist

The antagonist showing most promising biocontrol results under net house conditions with significant PGP and biocontrol activities was identified to species level by using 16S rDNA technique. A universal primer set (forward 5′GCAAGT CGAGCGGACAGATGGGAGC3′ and reverse 5′AACTCT CGTGGTGTGACGGGCGGTG3′) was used to amplify 16S rDNA of selected bacterial antagonist (Mehta et al. 2013). The genomic DNA was isolated by method of Sambrook et al. (1989). A 20 µl PCR reaction was prepared containing 50 ng of template DNA, 20 pmol of each primer, 0.2 mM dNTPs and 1 U Taq polymerase in 1 × PCR buffer. Reactions were cycled 35 times as 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min 30 s followed by final extension at 72 °C for 10 min. Amplified PCR products were separated on 1.2% (w/v) agarose gel. A band of 1400 bp was excised from the gel and purified using gel extraction kit (RBCs Real Genomics, New Taipei City, Taiwan). The purified DNA was sequenced from Xcleris lab, Ahmedabad, India using same forward and reverse primers. Based on 16S rRNA gene sequences, phylogenetically related bacteria were aligned using BLAST search (Altschul et al. 1997). Multiple alignment with sequences of related taxa was implemented using CLUSTAL W (Thompson et al. 1994). A neighbour-joining phylogenetic tree was constructed with 20 other 16S rRNA gene sequences of genus *Bacillus* retrieved from GenBank using MEGA 6 software. The sequence was submitted to NCBI GenBank database and accession number was assigned.

Statistical analysis

Experimental data were analysed using standard analysis of variance (ANOVA) followed by Duncan's multiple comparison tests ($p < 0.05$). Standard errors were calculated for all mean values. Arcsine transformation was applied to all data expressed as percentages, before analysis.

Results and discussion

Screening of bacterial isolates for antagonistic activity against *C. michiganensis*

The present study was aimed to screen the rhizobacterial isolates previously isolated from rhizosphere of three horticultural fruit crops viz., strawberry, apple and apricot for their biocontrol potential against *C. michiganensis*, causing bacterial canker of tomato. The induction of plant resistance by using microbial bioagents may be useful method for the reduction of severity of this disease (Girish and Umesha 2005). Under in vitro conditions the screening of rhizobacteria with the putative antagonistic activity is the first step towards selection of candidate PGPR for development of biological control of plant diseases. A total of 150 rhizobacterial isolates isolated from rhizosphere of three horticultural fruit crops were screened for antagonistic activity against *C. michiganensis* on NA plates using well diffusion method. Out of total, only 40 bacterial isolates showed antagonistic activity against *C. michiganensis*. The sources and relevant characteristics of the 40 antagonistic strains are listed in Table 1.

The zone of inhibition ranged between 3.20 and 12 mm in size and out of total 40, five antagonists were rated excellent, five were rated very well and rest of the thirty antagonists were rated good on the basis of degree of antagonism. This study showed that strawberry, apple and apricot rhizospheres

Table 1 Screening of rhizobacterial isolates for antagonism against *Clavibacter michiganensis*

Isolate	Origin[a]	Host plant	Site of isolation	Zone of inhibition[b] (mm)	Degree of antagonism
KU_2S_1	RS	Strawberry	Kufri, Shimla	10.33 ± 0.10	+++
$NA_{(2)}$	RS	Strawberry	Nauni, Solan	4.50 ± 0.30	+
$NA_{(5)}$	RS	Strawberry	Nauni, Solan	4.90 ± 0.30	+
S_1	RS	Strawberry	Rohru, Shimla	12.00 ± 0.20	+++
$KU_{3(1)}$	RS	Strawberry	Kufri, Shimla	8.67 ± 0.13	++
$R_2S_{(1)}$	RS	Strawberry	Rohru, Shimla	10.00 ± 0.40	+++
$RO_{5(6)}$	ER	Strawberry	Rohru, Shimla	8.66 ± 0.34	++
$RA_{34(5)}$	RS	Strawberry	Rajgarh, Sirmaur	3.90 ± 0.50	+
$NA_{(6)}$	RS	Strawberry	Nauni, Solan	4.30 ± 0.70	+
$RA_{1(3)}$	ER	Strawberry	Rajgarh, Sirmaur	5.00 ± 0.50	+
$RA_{3(5)}$	RS	Strawberry	Rajgarh, Sirmaur	4.50 ± 0.40	+
$KU_{3(3)}$	ER	Strawberry	Kufri, Shimla	8.43 ± 0.30	++
KU_3	RS	Strawberry	Kufri, Shimla	9.34 ± 0.66	++
$NA_{12}S_1$	RS	Strawberry	Nauni, Solan	3.20 ± 0.80	+
$RA_{2(2)}$	ER	Strawberry	Rajgarh, Sirmaur	4.60 ± 0.40	+
$RO_{4(5)}$	RS	Strawberry	Rohru, Shimla	4.70 ± 0.30	+
$RO_{2(7)}$	RS	Strawberry	Rohru, Shimla	3.80 ± 0.20	+
NA_8	RS	Strawberry	Nauni, Sirmaur	3.20 ± 0.60	+
$KU_{1(5)}$	RS	Strawberry	Kufri, Shimla	3.91 ± 0.59	+
$RO_{5(1)}$	ER	Strawberry	Rohru, Shimla	4.80 ± 0.20	+
$RG_{1(3)}$	RS	Apple	Matiana, Shimla	10.66 ± 0.34	+++
$G_{2(6)}$	RS	Apple	Matiana, Shimla	4.33 ± 0.50	+
$RG_{2(1)}$	RS	Apple	Matiana, Shimla	4.30 ± 0.30	+
$R_{5(2)}$	ER	Apple	Matiana, Shimla	4.72 ± 0.80	+
$R_{3(4)}$	RS	Apple	Matiana, Shimla	4.99 ± 0.60	+
CH_8A	RS	Apple	Chamba	9.33 ± 0.50	++
CK_9A	RS	Apple	Chamba	4.30 ± 0.70	+
KPO_8A	RS	Apple	Recong Peo Kinnaur	3.90 ± 0.50	+
ES_3A	RS	Apple	Chopal, Shimla	3.56 ± 0.44	+
CH_3B	RS	Apple	Chamba	4.80 ± 0.20	+
$AP_{3(1)}$	RS	Apricot	Recong Peo, Kinnaur	4.10 ± 0.80	+
$AP_{2(1)}$	RS	Apricot	Recong Peo, Kinnaur	3.60 ± 0.50	+
$AT_{1(1)}$	RS	Apricot	Tabo, Kinnaur	3.89 ± 0.70	+
$AG_{1(7)}$	RS	Apricot	Gaura, Sirmaur	10.33 ± 0.30	+++
$AG_{5(1)}$	ER	Apricot	Gaura, Sirmaur	4.90 ± 0.50	+
$AK_{1(4)}$	RS	Apricot	Kandaghat, Solan	4.98 ± 0.72	+
$AN_{5(2)}$	ER	Apricot	Nauni, Solan	4.60 ± 0.40	+
$AN_{1(1)}$	RS	Apricot	Nauni, Solan	4.50 ± 0.50	+
$AN_{2(2)}$	RS	Apricot	Nauni, Solan	4.83 ± 0.33	+
$AS_{3(2)}$	RS	Apricot	Subathu, Solan	4.65 ± 0.55	+
lsd[c]	NA	NA	NA	0.79	NA

+Good zone size ranging from 0 to 5 mm

++Very well zone size ranging from > 5 to < 10 mm

+++Excellent zone size from 10 to 15 mm

[a]Origin: *RS* rhizospheric, *ER* endorhizospheric

[b]Zone of inhibition zone size-well size

[c]*lsd* least significant difference at $p \leq 0.05$

Table 2 Screening of bacterial antagonists for multifarious PGP traits

Isolate	Growth promoting			Biocontrol activities				
	Indole-3-acetic acid (μg ml^{-1})	Phosphate solubilization (%SE)	Growth on nitrogen free medium	Siderophore production (zone size in mm)	Cellulase (EI)	Lipase (EI)	Protease (EI)	Chitinase (EI)
KU_2S_1	24.00 ± 2.00	76.59 ± 5.00	+	3.23 ± 0.47	1.47 ± 0.53	1.68 ± 0.10	1.80 ± 0.20	1.24 ± 0.10
$NA_{(2)}$	5.00 ± 1.00	ND	ND	ND	ND	1.68 ± 0.12	1.72 ± 0.28	1.13 ± 0.20
$NA_{(5)}$	7.00 ± 1.50	33.33 ± 7.00	ND	ND	1.30 ± 0.30	1.50 ± 0.40	1.72 ± 0.28	1.25 ± 0.25
S_1	27.00 ± 2.00	94.16 ± 3.50	+	10.00 ± 0.40	ND	1.73 ± 0.10	1.62 ± 0.20	1.13 ± 0.17
$KU_{3(1)}$	35.00 ± 1.00	113.25 ± 3.25	+	3.33 ± 0.33	1.30 ± 0.20	1.50 ± 0.30	1.72 ± 0.10	1.54 ± 0.30
$R_2S_{(1)}$	23.00 ± 2.00	33.33 ± 2.00	+	2.56 ± 0.44	ND	1.53 ± 0.20	1.76 ± 0.14	1.42 ± 0.10
$RO_{5(6)}$	20.00 ± 1.50	30.00 ± 1.50	ND	2.99 ± 0.51	ND	1.63 ± 0.17	1.87 ± 0.13	1.45 ± 0.20
$Ra_{34(5)}$	3.00 ± 1.00	ND	+	ND	ND	ND	1.41 ± 0.10	1.50 ± 0.40
$NA_{(6)}$	7.00 ± 3.00	33.00 ± 2.00	ND	ND	ND	1.42 ± 0.08	1.10 ± 0.40	0.56 ± 0.40
$RA_{1(3)}$	5.00 ± 2.00	11.33 ± 3.20	ND	ND	ND	1.12 ± 0.10	1.33 ± 0.33	1.04 ± 0.10
$RA_{31(5)}$	6.00 ± 0.50	30.00 ± 3.00	+	ND	1.10 ± 0.50	ND	ND	1.50 ± 0.30
$KU_{3(3)}$	22.00 ± 4.00	87.36 ± 1.64	+	5.00 ± 0.50	1.50 ± 0.40	1.51 ± 0.20	1.69 ± 0.30	1.22 ± 0.22
KU_3	24.00 ± 1.00	44.32 ± 3.00	+	8.70 ± 0.30	ND	1.60 ± 0.40	1.76 ± 0.14	0.72 ± 0.28
$NA_{12}S_1$	4.00 ± 0.50	ND	ND	ND	1.20 ± 0.30	ND	ND	0.90 ± 0.10
$RA_{2(2)}$	9.00 ± 3.00	21.74 ± 2.60	+	ND	ND	1.23 ± 0.23	0.21 ± 0.10	1.22 ± 0.22
$RO_{4(5)}$	2.00 ± 0.50	25.00 ± 2.00	ND	3.56 ± 0.44	ND	ND	ND	0.80 ± 0.30
$RO_{2(7)}$	5.50 ± 2.00	32.00 ± 4.90	ND	ND	ND	ND	0.43 ± 0.10	0.43 ± 0.10
NA8	6.20 ± 0.80	ND	ND	ND	ND	ND	0.80 ± 0.20	1.45 ± 0.30
$KU_{1(5)}$	8.00 ± 1.00	ND	+	ND	0.40 ± 0.10	ND	ND	ND
$RO_{5(1)}$	9.00 ± 2.00	23.60 ± 2.50	ND	ND	ND	1.01 ± 0.40	ND	1.20 ± 0.20
$RG_{1(3)}$	70.00 ± 5.00	62.14 ± 3.00	+	7.00 ± 0.40	ND	1.52 ± 0.30	1.81 ± 0.19	1.26 ± 0.20
$G_{2(6)}$	3.00 ± 0.50	20.00 ± 3.00	ND	3.90 ± 0.60	ND	ND	1.21 ± 0.11	0.80 ± 0.20
$RG_{2(1)}$	1.50 ± 0.50	18.20 ± 1.00	ND	6.10 ± 0.60	ND	1.12 ± 0.12	ND	0.99 ± 0.20
$R_{5(2)}$	2.00 ± 0.50	ND	+	4.78 ± 0.22	0.23 ± 0.10	ND	0.89 ± 0.11	1.34 ± 0.20
$R_{3(4)}$	4.00 ± 1.00	38.80 ± 4.20	ND	5.20 ± 0.70	0.45 ± 0.10	ND	ND	1.35 ± 0.15
CH_8A	17.00 ± 2.00	64.52 ± 3.48	+	11.00 ± 0.30	1.10 ± 0.20	1.46 ± 0.30	1.73 ± 0.20	1.24 ± 0.20
CK_9A	5.50 ± 1.50	30.10 ± 1.90	ND	ND	ND	1.11 ± 0.20	ND	1.55 ± 0.10
KPO_8A	6.40 ± 1.00	17.10 ± 2.90	+	ND	0.78 ± 0.22	ND	ND	1.22 ± 0.22
ES_3A	5.00 ± 0.50	19.78 ± 5.00	ND	ND	ND	ND	1.33 ± 0.33	1.06 ± 0.20
CH_3B	2.00 ± 0.50	26.30 ± 0.70	ND	ND	ND	0.78 ± 0.22	ND	1.13 ± 0.13
$AP_{3(1)}$	10.00 ± 2.00	53.10 ± 1.00	ND	2.11 ± 0.39	ND	0.98 ± 0.20	1.21 ± 0.10	1.24 ± 0.02
$AP_{2(1)}$	11.00 ± 2.00	ND	+	2.33 ± 0.37	ND	ND	ND	1.22 ± 0.22
$AT_{1(1)}$	5.00 ± 2.00	30.56 ± 3.94	+	ND	1.32 ± 0.20	ND	1.15 ± 0.15	1.25 ± 0.25
$AG_{1(7)}$	40.00 ± 3.00	64.52 ± 2.00	+	2.58 ± 0.42	ND	ND	1.52 ± 0.20	1.50 ± 0.40
$AG_{5(1)}$	6.50 ± 1.00	28.6 ± 1.40	ND	ND	ND	ND	ND	1.30 ± 0.30
$AK_{1(4)}$	7.00 ± 1.60	ND	ND	1.91 ± 0.59	ND	0.77 ± 0.23	ND	1.26 ± 0.20
$AN_{5(2)}$	3.00 ± 1.00	41.12 ± 3.00	+	ND	0.67 ± 0.10	0.86 ± 0.34	ND	1.19 ± 0.19
$AN_{1(1)}$	4.00 ± 0.50	25.60 ± 4.10	ND	1.56 ± 0.44	ND	ND	1.30 ± 0.30	1.15 ± 0.30
$AN_{2(2)}$	6.00 ± 1.00	38.23 ± 1.77	+	ND	ND	ND	ND	1.33 ± 0.40
$AS_{3(2)}$	4.50 ± 1.50	24.08 ± 2.00	+	ND	ND	1.14 ± 0.10	1.17 ± 0.10	1.45 ± 0.20
[a]lsd	2.96	5.17		0.76	0.48	0.39	0.34	0.38

[a]*lsd* least significant difference at $p \leq 0.05$

support a diverse population of bacteria antagonistic to *C. michiganensis*. Gautam et al. (2019) also screened 150 bacterial strains from different horticultural crops grown in mid hills and high hills of Himachal Pradesh against *C. michiganensis*, out of which 40 rhizobacterial isolates showed in vitro antagonistic activity.

Functional PGP traits of antagonistic bacterial isolates

In the present study the 40 antagonistic isolates were further screened for PGP and biocontrol traits (Table 2). Plant growth promotion by PGPR is a complex phenomenon, which is achieved by their one or more plant growth promoting activities (Aslantas et al. 2007). The present study revealed that the antagonistic bacterial isolates from rhizosphere of these horticultural crops possessed one or more plant growth promoting and biocontrol activities. The in vitro screening of rhizobacteria from different crops rhizosphere for multifarious PGP and biocontrol traits and their use as biostimulants and bioprotectants under net house conditions has also been reported earlier (Sharma et al. 2015). The plant hormone IAA is considered the most important native auxin (Ali et al. 2010). It acts as an important signal molecule that regulates organogenesis, tropic responses, cell expansion, division, differentiation and in gene regulation of plants as well (Ryu and Patten 2008). In the present investigation, all the antagonistic isolates had the ability to produce IAA in the range of 1.50–70.00 µg ml^{-1}. Strain $RG_{1(3)}$ showed highest concentration of IAA (70 µg ml^{-1}) which was significantly higher than all the other strains.

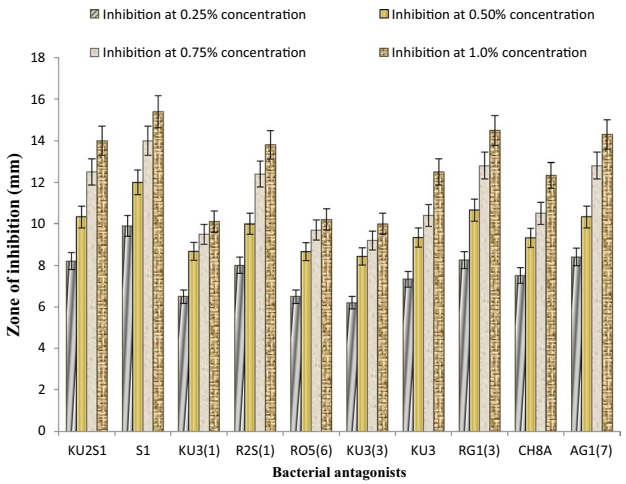
Similar to our work the antagonistic *Bacillus* strains producing IAA in the range of 15.00–95.00 µg ml^{-1} stimulated tomato growth under net house conditions (Sharma et al. 2015).

P-solubilization is considered as one of the most important attribute of the PGPR. The screening protocol used for the identification of PSB isolates in the present study relies on halozone formation on PVK agar plates containing tri-calcium phosphate as the insoluble P-source. Out of 40 antagonistic strains, 32 strains showed P-solubilization on PVK agar and 20 strains showed growth on nitrogen free Jensen's medium. Highest P-solubilization (113.25%SE) was exhibited by the strain $KU_{3(1)}$ which was statistically at par with strain S_1 (94.16%SE). It is well established fact that improved phosphorus nutrition influences overall plant growth and root development (Patel et al. 2008; Joseph and Jisha 2009) as evident in the present study where P-solubilizing bacterial isolates resulted in significant growth promotion of tomato plants.

The production of siderophore and cell wall lysing enzymes such as cellulase, lipase, protease, and chitinase is considered important for biocontrol (Hameeda et al. 2006; Suresh et al. 2010; Ahemed and Khan 2011; Wahyudi et al. 2011). In the present study, out of 40, 19 isolates produced siderophore with zone size ranging from 1.56 to 11 mm. Maximum zone was produced by strain CH_8A (11.00 mm) which was statistically at par with strain S_1 (10.00 mm). The occurrence of siderophore producing antagonistic isolates is of direct significance to plants as it helps in iron sequestering near the roots, especially in iron deficient conditions (Ramos Solano et al. 2010). 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 into host cells (Dahiya et al. 2006; Nguyen et al. 2008). In the present study all the 40 antagonists were positive for at least one of the lytic enzyme activities. Significantly the highest cellulase activity was exhibited by strain $KU_{3(3)}$ (1.50 EI) which was statistically at par with isolate KU_2S_1 (1.47 EI). Highest lipase activity was observed in strain S_1 (1.73 EI) and highest protease activity by strain $RO_{5(6)}$ (1.87 EI) which was statistically at par with isolates $RG_{1(3)}$ (1.81 EI) and KU_2S_1 (1.80 EI). The highest chitinase activity was exhibited by isolate CK_9A (1.55 EI) which was statistically at par with isolates R_8 (1.8 EI) and $KU_{3(1)}$ (1.54 EI). 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, chitinase and production of plant hormones like auxins (Amaresan et al. 2012; Neeraja et al. 2010; Maksimov et al. 2011; Chakraborty et al. 2013).

Antibacterial activity of selected bacterial antagonists against *C. michiganensis* at different concentrations of cell free supernatants

The antibacterial activities of ten most efficient antagonists with zone of inhibition > 5 mm and possessing significant PGP traits were further evaluated at four different concentrations of cell free supernatants (Fig. 1). The selected



lsd ($p \leq 0.05$) for inhibition zone at, 0.25% concentration of culture filterate = 0.34;at 0.50% concentration = 2.87; at 0.75% concentration = 0.57; at 1% concentration (CD) = 0.53

Fig. 1 Antibacterial activity of bacterial antagonists against *C. michiganensis* at different concentrations of cell free supernatant

ten bacterial antagonists showed variation in antibacterial activity with zone of inhibition, ranging from 6.20 to 15.40 mm. The zone of inhibition increased with increase in concentration of supernatant from 0.25 to 1% (v/v). The maximum antibacterial activity at 0.25% concentration of cell free supernatant was recorded for antagonist S_1 with 9.90 mm zone of inhibition, which increased to 15.40 mm with increase in concentration to 1%. Following S_1, isolate $AG_{1(7)}$, showed 8.40 mm zone of inhibition and isolate $RG_{1(3)}$ showed 8.30 mm zone of inhibition at 0.25% concentration, which increased up to 14.30 mm and 14.50 mm respectively at 1% concentration of cell free supernatant.

Ahmed Sheikh (2010) also studied the effect of different concentrations of bacterial cell free supernatants against the growth of pathogenic *Staphylococcus aureus* and methicillin resistant *S. aureus* (MRSA). The growth of *S. aureus* and MRSA was decreased with increasing the concentration of the bacterial filtrate and consequently the percentage of inhibition increased. Similarly, Yoshida et al. (2000) developed the antimicrobial spectrum of antagonistic *Bacillus amyloliquefaciens* RC2 against several phytopathogenic bacteria using different concentrations of culture filtrate. The filtrate strongly inhibited the growth of *Agrobacterium tumefaciens*, *C. michiganensis* subsp. *michiganensis*, *Erwinia carotovora* subsp. *carotovora*, and *Xanthomonas campestris* pv. *campestris* even at four fold dilution of culture filtrate. Suppression of bacterial growth by the cell free extract of antagonistic isolates and formation of inhibition zone were presumably due to the metabolites being released from bacteria into the culture medium (Lanteigne et al. 2012).

In planta evaluation of biocontrol potential against *C. michiganensis*

The biocontrol potential of five most efficient bacterial antagonists (KU_2S_1, S_1, $R_2S_{(1)}$, $RG_{1(3)}$ and $AG_{1(7)}$) under in vitro conditions was evaluated on tomato plants inoculated with *C. michiganensis* under net house conditions. The canker symptoms in control plants appeared after 2 weeks since the seedlings were transplanted and the disease severity index and disease incidence were assessed after 3 weeks since transplantation. Seedling dip and drenching of soil with liquid based culture of antagonistic strains significantly decreased the severity of bacterial canker over positive control (uninoculated but pathogen challenged) (Table 3).

Disease severity index in the positive control was 82.80% whereas it ranged from 28.55 to 76.62% in antagonists treated plant. The isolate S_1 was found to be most promising biocontrol treatment that reduced the disease incidence by 70.00% with minimum 28.55% disease severity index which was significantly lower than control and all the other antagonists. Similar studies were carried out by Girish and Umesha (2005), showing that three PGPR strains effectively reduced the canker incidence; however the biocontrol efficacy of the most efficient strain *B. amyloliquefaciens* IN937a (56%) was significantly lower than the biocontrol efficacy recorded in our strain S_1 (70%). Strain $RG_{1(3)}$ also showed significant protective effect against *C. michiganensis* and resulted in 50% decrease in bacterial canker incidence. The tested strains inhibiting bacterial pathogen under net house conditions also exhibited in vitro antibacterial metabolite production, siderophore production and lytic enzymes production. These activities might have contributed to the bioprotection against *C. michiganensis*. Boudyach et al. (2001) also reported the significant reduction in the infection when applied with three strains (HF22, HF142 and HF183) as compared to untreated control. It has been suggested that the ability of antagonistic bacteria to induce disease resistance might be due to some metabolites or certain compounds (Miyazawa et al. 1998). Development of induced systemic resistance (ISR) in plants is also associated with the coordinate expression of a complex set of PR proteins, so-called 'SAR genes' (Conrath et al. 2001). Furthermore, breakdown products of pathogen and/or plant cell wall components released by the activities of various hydrolytic enzymes have been shown to act as elicitors of plant defense responses (Van Loon 1997). Hence, it could be assumed that biocontrol potential of the present isolate

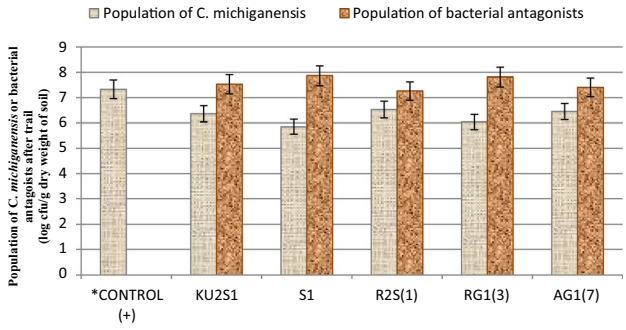
Table 3 Biocontrol efficacy of selected bacterial antagonists towards *Clavibacter michiganensis* under net house conditions

Treatment	Disease severity index (%)	Disease incidence (%)	Biocontrol efficacy (%)
CONTROL[a] (+)	82.80 ± 3.00 (65.57)	98.00 ± 2.00 (90.00)	–
KU_2S_1	56.47 ± 2.50 (48.75)	65.00 ± 4.00 (54.22)	35.00 ± 2.00 (35.78)
S_1	28.55 ± 2.45 (32.17)	30.00 ± 2.50 (32.90)	70.00 ± 3.20 (57.10)
$R_2S_{(1)}$	76.62 ± 1.38 (61.81)	85.00 ± 2.00 (70.08)	15.00 ± 3.00 (19.92)
$RG_{1(3)}$	43.75 ± 3.00 (41.38)	50.00 ± 2.60 (45.00)	50.00 ± 2.40 (45.00)
$AG_{1(7)}$	65.75 ± 3.95 (54.26)	70.00 ± 1.40 (57.10)	30.00 ± 3.10 (32.90)
lsd[b]	5.07	4.50	4.56

Figure in parenthesis () are arc sign transformed values

[a]Positive control, inoculated with *Clavibacter michiganensis*

[b]*lsd* least significant difference at $p \leq 0.05$



lsd at $p \leq 0.05$ For Population of *Clavibacter*= 0.22; For Population of bacterial antagonist = 0.23

Fig. 2 Rhizospheric population of *C. michiganensis* and inoculated bacterial antagonists after trial

S_1 might be due to ISR mediated by cumulative effect of certain genes, enzymes and metabolites accumulated by the test strain. Singh et al. (2017) tested five bacterial biocontrol agents against the *C. michiganensis* under in vitro conditions, *Pseudomonas fluorescens* strain PfS-1 was found effective in inhibiting the growth of the pathogen. This biocontrol agent when inoculated through seed which improved germination and delayed the expression of disease symptoms in pot culture.

Rhizosphere colonization by the *C. michiganensis* and inoculated bacterial antagonists

It has been well established that rhizosphere colonization capacity of an antagonistic bacterium is a key factor affecting its suppression effect against soil-borne pathogens (Dhingra et al. 2003; Andreote et al. 2009). Soil-borne pathogens and beneficial microorganisms interact and compete with each other for available resources in the rhizosphere (Raaijmakers et al. 2009). In present work, the data obtained after population density study of antagonists and pathogen revealed that application of antagonistic strains significantly reduce the pathogen population in soil (Fig. 2), thus protecting plant roots from pathogen attack.

The population of *C. michiganensis* in the rhizosphere remained high (7.33 log cfu/g dry soil weight) towards the end of the experiment in control whereas the population reduced after treatment with bacterial antagonists. The results about the population of antagonistic bacterial strains revealed the highest population of strain S_1 (7.86 log cfu/g dry soil weight) which was statistically at par with the population of strain $RG_{1(3)}$ (7.81 log cfu/g dry soil weight). The smallest population of antagonistic isolate was recorded for strain $R_2S_{(1)}$ (7.26 log cfu/g dry soil weight). Our work is in agreement with study of Ding et al. (2013), who reported that strain BIO23-treated plants showed reduction in population of *R. solanacearum* from 1.1×10^7 to 1.3×10^6 cfu/g of soil.

Effect of bacterial antagonists on growth promotion of tomato

Tomato seedlings treated with antagonistic strains but not challenged with the pathogen were used to study the growth promotion effect of inoculated strains. In present study, the antagonistic strains consistently increased different plant attributes such as shoot and root length, dry weight over uninoculated control (Table 4). Highest shoot length, root length, shoot dry weight and root dry weight was recorded in plants treated with strain S_1 which were significantly higher than the control and all the other treatments. Studies have also highlighted the increase in growth and biomass of crop plants in response to PGPR inoculation (Walia et al. 2013; Mehta et al. 2010; Kumar et al. 2015). The enhanced plant growth by treatment with bacterial antagonists could be attributed to their production of growth stimulating substances such as IAA, P-solubilization and nitrogen fixation. Moreover, the production of siderophores by the test isolates might give them competitive advantage to make Fe^{3+} unavailable to the pathogen. Romero et al. (2003) studied

Table 4 Effect of liquid formulation of selected bacterial isolates on tomato seedlings and their growth promotion under net house conditions

Treatment	Shoot length (cm)	Root length (cm)	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})
CONTROL[a] (−)	26.87 ± 1.00	2.33 ± 0.10	8.20 ± 0.40	1.53 ± 0.03
KU_2S_1	33.73 ± 1.27	3.17 ± 0.08	14.57 ± 0.10	2.46 ± 0.46
S_1	49.17 ± 2.83	7.43 ± 0.43	15.1 ± 0.90	3.23 ± 0.10
$R_2S_{(1)}$	28.37 ± 2.00	3.03 ± 0.50	9.67 ± 0.10	2.06 ± 0.14
$RG_{1(3)}$	33.50 ± 1.50	3.32 ± 0.40	14.30 ± 0.30	2.46 ± 0.06
$AG_{1(7)}$	31.90 ± 1.10	3.13 ± 0.30	13.37 ± 0.13	2.40 ± 0.40
lsd[b]	3.12	0.61	0.76	0.46

[a]Negative control, without pathogen and antagonists' inoculation

[b]*lsd* least significant difference at $p \leq 0.05$

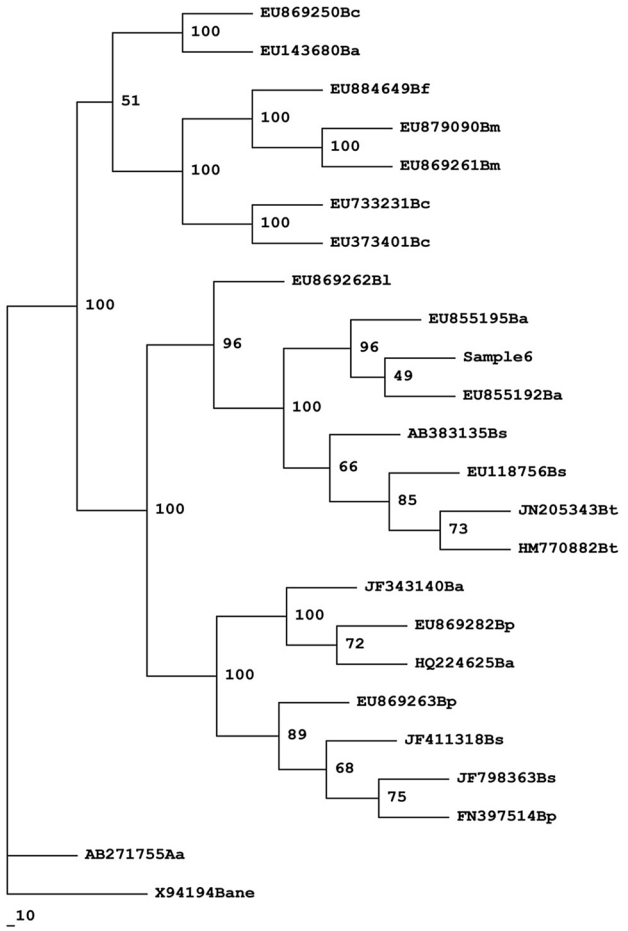


Fig. 3 Neighbour—joining tree based on 16S rDNA gene sequence showing the phylogenetic relationship of isolate S1 with the analysed sequence

the biocontrol potential of *Azospirillum brasilense* Sp7 against *C. michiganensis* and its growth promotional effect on tomato. Their study revealed that *Azospirillum* treated plants showed 20–30% control in disease incidence and significant increase in plant height from 7.2 cm in control plants to 9.5 cm in treated plants.

Phylogenetic analysis of selected isolate by 16S rDNA sequence analysis

Among the antagonistic strains tested, strain S_1 was identified as most promising biocontrol treatment against bacterial canker and growth promotion of tomato. Therefore strain S_1 was selected for identification to species level by 16S rRNA gene sequencing.

The 16S rDNA fragment was amplified using selected primers and a fragment of 1400 bp was obtained and sequenced. The sequence so obtained showed 99% homology with *B. amyloliquefaciens* (EU855192). Phylogenetic analysis (Fig. 3) also revealed the identity of strain S_1 as *B. amyloliquefaciens*, as the isolate was closely clustered with *B. amyloliquefaciens* (EU855192) with high boot strap value. The sequence of strain S_1 was deposited in NCBI Genbank under accession number KM658175. Strains of *B. amyloliquefaciens* are considered particularly promising as microbial biocontrol agents with the properties of controlling different classes of bacterial and fungal plant pathogens (Yin et al. 2011). Some strains of *B. amyloliquefaciens* and *B. subtilis* are known to produce a wide range of antibiotic substances such as surfactin, fengycin, iturin A and TasA (Arguelles-Arias et al. 2009; Chen et al. 2009; Raaijmakers et al. 2010; Vitullo et al. 2012) and plant growth-promoting metabolites such as IAA (Idris et al. 2007; Yuan et al. 2013). The antagonistic activity and plant growth-promoting effect of strain S_1 suggest that our bacteria, as found in other research with other bacterial strains, could produce such biologically active compounds.

In conclusion, *B. amyloliquefaciens* strain S_1 isolated from the apple rhizosphere can effectively control bacterial canker caused by *C. michiganensis* under greenhouse conditions, thus indicating that this strain could serve as a potential alternative to chemical pesticides for biocontrol of *C. michiganensis*. However the evaluation of biocontrol potential under field conditions and more insight into mechanisms of suppression of *C. michiganensis* by *B. amyloliquefaciens* S_1 need to be further clarified in future experiments.

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