The Bioscan 11(4): 2803-2809, 2016 (Supplement on Plant Pathology)
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.com

STUDIES ON ATTENUATING THE VIRULENCE OF *RALSTONIA SOLANACEARUM*, PATHOGEN CAUSING TOMATO WILT USING RHIZOSPHERE BACTERIA ISOLATED FROM THE INFECTED TOMATO PLANTS BY AHL BASED QUORUM SENSING SYSTEM

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KEYWORDS
Tomato
Virulence
Quorum sensing
AHL
Rhizosphere

Received on :
21.10.2016

Accepted on :
03.12.2016

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ABSTRACT
The bacterium *Ralstonia solanacearum* causes bacterial wilt on more than 200 plant species, including important crops such as potato, tomato, eggplant, pepper, tobacco and banana. Bacterial wilt caused by *R. solanacearum* is the most devastating disease on tomato. Many factors contribute to the virulence of this pathogen. AHL quorum sensing molecules are fascinating group of molecular targets for genetic and chemical manipulation. AHL based quorum sensing system has been identified in a number of plants associated bacteria and have been shown to control the virulence factor. In the current study virulence of *R. solanacearum* was tried to attenuate using rhizosphere bacteria isolated from rhizosphere soil of the infected tomato plants. The bacterial isolates were characterized at molecular level using 16S rRNA gene sequencing, and it was found that the bacterial isolates were belonging to Genus *Rhizobium* and *Brevindimonas*. The identified bacteria showed positive reaction when tested for the release of AHL molecule using Chromobacterium *violaceum*, as a biosensor indicator organism and *in-vitro* studies of these bacteria on tomato wilt were found to attenuate the virulence of *R. solanacearum* by AHL based quorum sensing system.

INTRODUCTION

Ralstonia solanacearum, previously named *Pseudomonas solanacearum* and *Burkholderia solanacearum*, is a soil-borne gram negative bacterium that causes bacterial wilt disease on more than 200 plant species from 50 botanical families, including important crops such as potato, tomato, eggplant, pepper, tobacco and banana (Hayward, 1991). *R. solanacearum* is considered as species complex-a heterogeneous group of related but genetically distinct strains (Fegan and Prior, 2005). This bacterium infects plants through root wounds or at sites of secondary root emergence, then colonizes the xylem vessels and spreads rapidly to aerial parts of plant through vascular system. In xylem vessels, the bacterial population can multiply rapidly and reach very high levels (>1010 cells/cm of stem in tomato) (Araud-Razou *et al.,* 1995; Vasse *et al.,* 1995). Typical disease symptoms include browning of the xylem, chlorosis, stunting, wilting, and the infected plants usually die rapidly. Bacterial wilt is considered one of the most destructive bacterial plant diseases because of its extreme aggressiveness, world-wide geographic distribution, and unusually broad host range (Prior *et al.,* 1998). In fact, *R. solanacearum* was ranked 2nd in a list of the top 10 most scientifically/economically important plant pathogenic bacteria pathogens in 2012 (Mansfield *et al.,* 2012). *R. solanacearum* has been widely accepted as a model organism for the study of bacterial virulence and pathogenicity in plants (Hayward, 1991). To date, many factors have been found to contribute to the virulence of *R. solanacearum*. The virulence factors of *R. solanacearum* are controlled by a complex regulatory signal transduction pathway that responds to both environmental signals and quorum sensing molecule (Flavier *et al.,* 1997; Genin *et al.,* 2005).

The process, whereby an increase in the concentration of signal molecule(s) in the extracellular milieu reflects cell population density is called 'quorum sensing' (QS) (Williams *et al.,* 2007; Williams, 2007). In many Gram-negative bacteria, QS depends on the actions of N-acyl homoserine lactone (AHL) signal molecules (Williams *et al.,* 2007; Williams, 2007). *Quorum sensing* in prokaryotic biology refers to the ability of a bacterium to sense information from other cells in the population when they reach a critical concentration (i.e. a *Quorum*) and communicate with them. The "language" used for this intercellular communication is based on small, self-generated signal molecules called as autoinducers (Antariksh *et al.,* 2011). *Quorum sensing* is thought to afford pathogenic bacteria a mechanism to minimize host immune responses by delaying the production of tissue-damaging virulence factors until sufficient bacteria have amassed and are prepared to overwhelm host defense mechanisms and establish infection (Antariksh *et al.,* 2011).

AHL-mediated *Quorum sensing* systems are studied in a large number of Gram negative bacterial species belonging to the **á, â** and ã subclasses of proteobacteria, including bacteria in the genera *Agrobacterium*, *Aeromonas*, *Burkholderia*, *Chromobacterium*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Hafnia*, *Nitrosomonas*, *Obesumbacterium*, *Pantoea*, *Pseudomonas*, *Rahnella*, *Ralstonia*, *Rhodobacter*, *Rhizobium*, *Serratia*, *Vibrio*, *Xenorhabdus*, and *Yersinia*. The vast majority of gram negative *Quorum sensing* systems that have been studied thus far utilize N- AHLs as signaling molecules (Kempner and Hanson, 1968; De-Kievit *et al.,* 2000). As reported by McClean *et al.* (1997) and Florence and Allan, (2002), *Chromobacterium violaceum*, a Gram-negative bacterium commonly found in soil and water, produces the characteristic purple pigment violacein., is used as the biosensor indicator organism to detect the AHL signals released by other Gram negative bacteria.

The development of treatments based on QS interference is largely driven by the need of alternative or complementary approaches to phytochemicals and antibiotics In this view, the current study aimed at attenuating the virulence of *R. solanacearum* using rhizosphere bacteria isolated from rhizosphere soil of infected tomato plants by AHL based quorum sensing system using *C. violaceum* as a biosensor indicator organism.

MATERIALS AND METHODS

Collection of the rhizosphere samples

Rhizosphere samples were collected in sterile condition from the tomato growing regions of Dharmapuri, Tamilnadu State during August 2014. and tomato goring regions of Kolar, Karnataka State during January 2015. Randomly collected soil samples in this areas were brought to the laboratories in sterile conditions and further processed for isolating the bacteria.

Isolation of *R. solanacearum* and rhiosphere bacteria from tomato rhizosphere soil

TTC media (Hugh and Leifson, 1953) and 523 media (Kelman, 1954) were used for isolation and maintenance of *R. solanacearum*. TTC media (one liter) contained Peptone (10 g), Casein Hydrolysate (1.0 g), Glucose (5.0 g) and Agar (15 g), while 523 media (one liter) was made with $MgSO_4.7H_2O$ (0.3 g), K_2HPO_4 (0.2 g), Yeast Extract (4.0 g), Casein Hydrolysate (8.0 g), Sucrose (10 g) and Agar (15 g). In TTC media 5 ml of 1% 2, 3, 5- triphenyltetrazolium chloride was added to the sterilized medium before pouring into the plates. Similarly Nutrient Agar (Hi-Media) was used for the isolation and maintenance of non-pathogenic rhizophere bacterial isolates. Ten grams of rhizosphere soil were taken into a 250 mL of conical flask and 90 mL of sterile distilled water was added to it. The flask was shaken for 10 min on a rotary shaker. One milliliter of suspension was added to 10ml vial and shaken for 2 min. Serial dilution technique was performed up to 10^{-7} dilution. An aliquot (0.1 ml) of this suspension was spread on the plates of TTC agar medium as well as Nutrient agar medium. Plates were incubated for 3 days at 30ºC to observe the colonies of bacteria. Bacterial colonies were streaked to other sterile TTC plates (for *R. solanacearum*) and NA plates (for rhizosphere bacteria) and the plates were incubated at 30ºC for 3 days. Typical bacterial colonies were observed over the streak. Well isolated single colony was picked up and re-streaked to fresh TTC and NA agar plate and incubated similarly (Kushwaha *et al.,* 2013; Aabid-Hussain *et al.,* 2014).

Biochemical tests for confirmation of *R. solanacearum*

Isolates of *R. solanacearum* were studied according to specific biochemical tests *i.*e., gram staining (Schaad, 1980), potassium hydroxide test (Suslow *et al.,* 1982), catalase oxidase test (Schaad, 1980), kovacs oxidase test, levan production from sucrose (Schaad, 1980), lipase activity (Sierra, 1957), production of fluorescent pigment (King *et al.,* 1954) and Oxidation of glucose (Hayward, 1964).

Detection of AHL signals using indicator organisms

AHL signals was detected using the indicator organism *Chromobacterium violaceum*, a Gram-negative bacterium commonly found in soil and water, produces the characteristic purple pigment violacein, is used as the biosensor indicator organism to detect he AHL signals released by other Gram negative bacteria. The biosensor organism capable of detecting a range of AHLs were used to determine whether QSI is due to the production of interfering AHLs competing with the C6-HSL regulation of *C. violaceum* pigment production (Florence and Allan, 2002). *C. violaceum* were procured from IMMTECH, India and the pure cultures of bacteria was maintained in the laboratory on nutrient agar plates for further *Invitro* study of quorum quenching of *R. solanacearum*

In-vitro study of quorum quenching of *R. solanacearum*.

Microtitre plate assay method was followed with modification (Shahbaz *et al.,* 2015). 100 μL of sterile nutrient broth was added to 96 well plate, followed by the addition of 20μl of pure nutrient broth culture of *C. violaceum* to all the 96 wells and then 20μl pure broth cultures of different rhizosphere bacterial isolates was added to 90 wells. Pure broth cultures of *C. violaceum* and *R. solanacearum* maintained in their respective medium was added in two well lines as control for colour detection. The plates in replica were incubated at 30ºC and observed for the colour changes at different time intervals of 24h, 48h and 76h respectively.

Molecular characterization of isolated rhizosphere bacteria involved in quorum sensing

Bacterial DNA isolation

Bacterial genomic DNA was isolated as per the standard protocol (Hoffman and Winston, 1987), single colony from each of the bacterial cultures was innoculated on nutrient broth and grown for 48 h at 30ºC. Cells were harvested from 5 ml of the culture. To this 100 μL of lysozyme was added and incubated at RT for 30 min followed by the addition of 700 μl of cell lysis buffer (Guanidium isothiocyanate, SDS, Tris-EDTA). The contents were mixed by inverting the vial for 5 min with gentle mixing till the suspension looked almost transparent. 700 μl of isopropanol was layered on top of the solution. The two layers were mixed gently till white strands of DNA were seen. The DNA extracted from the aqueous layer was ethanol precipitated. The DNA pellet was dried and dissolved in 100 μl of 1X TE buffer. The quality of DNA was checked by running on agarose gel (0.8%) stained with ethidium bromide (0.5 μg/ μl). A single intense band with slight smearing was noted. The extracted genomic DNA of the bacterial isolates was used as template DNA for amplification of the 16S rRNA gene.

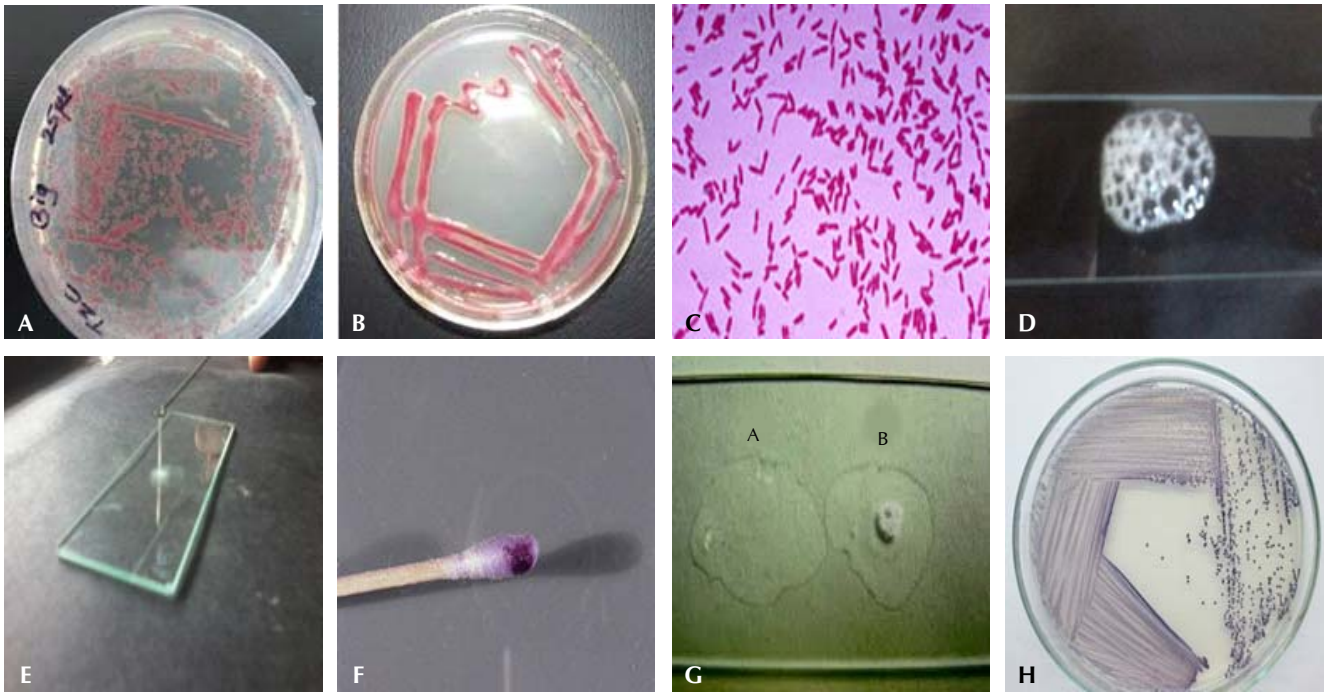

Plate 1: A-H:A- *R. solanacearum* colonies grown on TTC media, B-Pure streaked colonies of *R. solanacearum*, C-Gram staining of *R. solanacearum* showing Gram negative rods, D- *R. solanacearum* showing positive for catalase test, E-Slime threads of *R. solanacearum* showing positive for potassium hydroxide test, F- *R. solanacearum* showing positive for Kovac's oxidase test, G-Production of bubbles by *R. solanacearum* showing positive catalase oxidase Test, H-Pure culture of *C. violaceum* on NA plate.

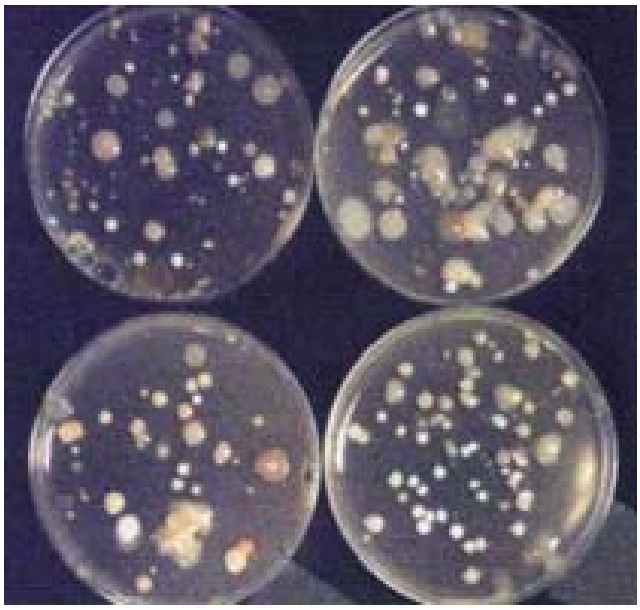

Plate 2: Bacterial colonies isolated from the Tomato rhizosphere soil

Oligonucleotide primers

16S rRNA gene primers were procured from Aristogene Biosciences (P) Ltd, Bangalore. The oligonucleotides were reconstituted to 100 ng/μl stocks in sterile TE buffer. The primers were used at working concentration 100 ng/μl in sterile filtered distilled water. The sequence of the primers was as follows:

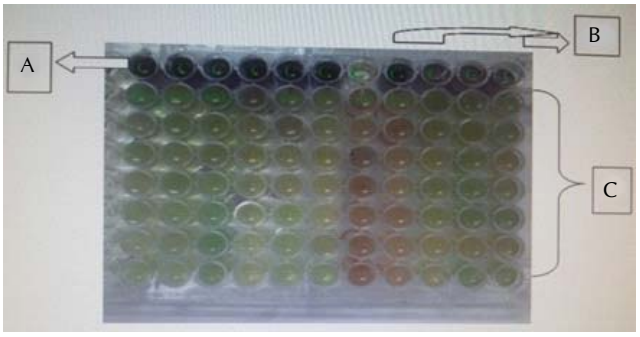

Plate 3: Detection of AHL signals in 96 well plate by using *C. violaceum* as signal detector-A- *R. solanacearum*: + *C. violaceum*, B- *R. solanacearum*: + TL1 and TL2 rhizosphere bacterial samples, C- *C. violaceum* + other non-reacting rhizophere bacterial samples

Forward primer - 5'-ACTCCTACGGGA GGCAG CAG-3'

Reverse primer - 5'-ATTACCG CGGCTGCTGG-3'

Amplification of 16S rRNA gene by PCR

Optimum annealing temperature was determined by employing gradient PCR. Amplification reaction was performed in 0.5 ml tubes. Individual reaction (50 μl) contained 100 ng of the extracted DNA, 1X PCR assay buffer (250 mM Tris-HCl, 10 mM KCl, 1.5 mM $MgCl_2$), 100 mM dNTP's, 100 ng/μL each of forward and reverse primers, 1 unit of *Taq* DNA polymerase (Sigma, USA). Reactions were thermally cycled in a thermal cycler system. PCR performed with forward and reverse primers involved an initial denaturation for 2 mins at 94°C, followed by 30 cycles of 94°C denaturation for 1 min,

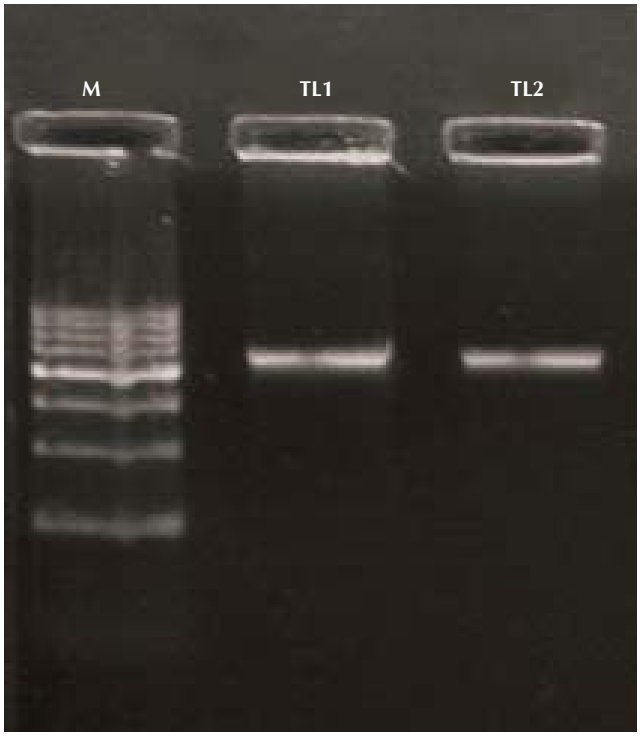

Figure 1: Agarose gel electrophoresis of genomic DNA from two bacterial isolates form Rhizosphere soil. M- Standard DNA Marker, TL1 – TL2 – Genomic DNA of the bacterial isolates

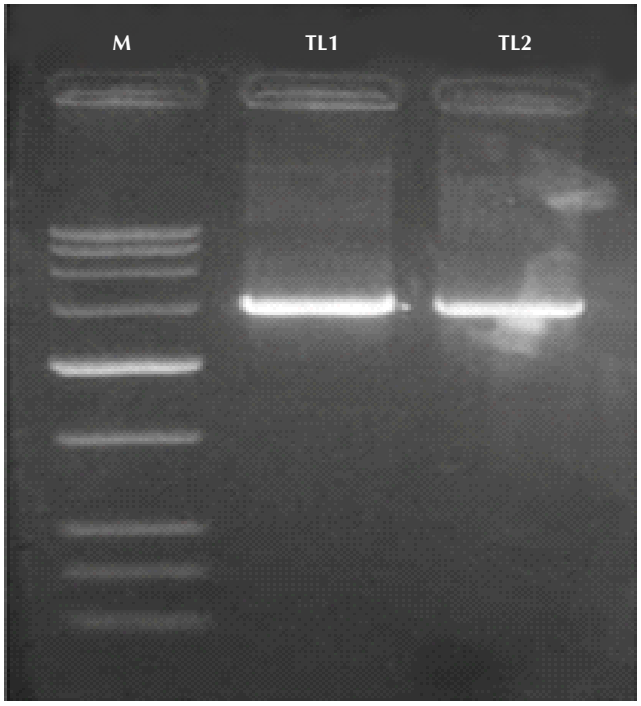

Figure 2: Agarose gel electrophoresis of 16S rRNA PCR amplicon from two bacterial isolates form Rhizosphere soil. M- 0.1-3 kb Low range marker: sizes- 100bp, 200bp, 300bp, 600bp, 1kb, 1.5kb, 2 kb, 2.5kb, 3 kb)., TL1 – TL2 – 16S rRNA PCR amplicon of the bacterial isolates

58ºC for annealing for 30 s and extension at 72 ºC for 1 min 30 s. Finally the reactions were healed at 72ºC for 5 min. Specific and optimum amplification of the gene was seen at 58 ºC of annealing temperature. Subsequently the gene was amplified at 58ºC and the amplified PCR product (1.5 Kb) was purified from low melting agarose gel, stained with ethidium bromide as per the standard protocols (Sambrook *et al.,* 2001) for further sequencing.

Sequence analysis

Sequencing of the 16S rRNA gene of all the bacterial isolates was done at sequencing facility of Aristogene Biosciences (P) Ltd, Bangalore, India from both the directions. The sequence obtained was subjected to BLAST search and bacterial species were determined. The percentages of sequence matching were also analysed and the sequences were submitted to NCBI-Gen Bank to obtain accession numbers.

Study on effect of quorum quenching bacteria on tomato wilt

Quorum quenching effect of rhizosphere bacterial isolates on *R. solanacearum* causing tomato wilt along with biosensor indicator bacteria *C. violaceum* was done with Pot experiment (Kushwaha *et al.,* 2013). Soil sample was collected from the plots where tomato crop was grown for the past several years. The soil was sieved and mixed with thoroughly washed and dried sand in a proportion of 3:1 (soil: sand). The soil and sand mixture was then autoclaved for 15 mins at 121ºC. Seeds of tomato were surface sterilized with 0.5% (w/v) $HgCl_2$ at room temperature and thoroughly washed with sterilized distilled water (Siriskandarajah *et al.,* 1993). Pure cultures of *R. solanacearum*, *C. violaceum* and rhizosphere bacterial isolates (TL1 and TL2) were inoculated into 50mL TTC broth and nutrient broth respectively and incubated for 24 h at 30ºC and required number of sterilized tomato seeds were made to infect with *R. solanacearum* by adding it to 10mL of TTC broth containing the bacterial culture. After 24 h of incubation, 5 seeds were sown to each of the 4 plastic pots and control seeds were incubated into sterilized distilled water. All pots were watered daily and allowed for the emergence of the tomato seedlings for 1 week. After 1 week 10 mL of pure broth cultures *C. violaceum* and rhizosphere bacterial isolates (TL1 and TL2) was added into the rhizosphere zone of the infected tomato seedlings. The naming of the pots and inoculation of the bacterial cultures was done as under:

Pot A

Control-normal uninfected tomato seedlings.

Pot B

R. solanacearum infected tomato seedling inoculated with pure culture of TL1 isolate.

Pot C

R. solanacearum infected tomato seedling inoculated with pure culture of TL2 isolate.

Pot D

R. solanacearum infected tomato seedling inoculated with pure culture of *C. violaceum*.

Pot E

R. solanacearum infected tomato seedling only.

The pots were kept in sunlight in natural conditions and watered regularly for 2 weeks. After 2 weeks the morphological observations were made.

RESULTS

Isolation of *R. solanacerum* and other rhizosphere bacteria from the tomato rhizosphere soil

R. solanacerum was isolated from rhizosphere soil and was grown on TTC media. Red color colonies of the bacteria were seen on TTC agar plates (Plate – 1-A). They were sub cultured and pure cultures of bacteria were maintained on TTC agar plates as shown in Plate-1-B. The bacteria were Gram negative rods (Plate-1-C). On the basis of biochemical test performed, *R. solanacearum* were positive for catalase test, potassium hydroxide test, kovacs oxidase test, catalase oxidase test (Plate-1-D-G). Differential positive test were observed for levan production from sucrose, lipase activity, production of fluorescent pigment and Oxidation of glucose (plate not shown). Similarly the other rhizosphere bacteria from tomato rhizosphere soil were isolated by serial dilution on nutrient agar plates (Plate-2). Selected isolated colonies were sub cultured on nutrient agar plates for further molecular characterization and other *in vitro* studies.

Detection of AHL signals using indicator organisms

AHL signals was detected using the indicator organism *C. violaceum,* which is a facultative anaerobic microorganism acting as a signal indicator. The biosensor organism is capable of detecting a range of AHLs were used to determine whether QSI is due to the production of interfering AHLs competing with C6-HSL regulation of *C. violaceum* pigment production. *C. violaceum* was procured from IMMRECH, India As per the growth conditions of the bacteria *C. violaceum*, was grown on nutrient agar. Dark pigmented colonies of the bacteria were seen on nutrient agar plates and the pure cultures (Plate-1-H) of these bacteria were maintained in the laboratory for further studies.

In-vitro study of quorum quenching of *R. solanacearum*

Rhizosphere bacterial isolates were tested for quorum sensing activity of *R. solanacearum* in 96 well plates. *C. violaceum* was used as indicator organism against *R. solanacearum* for the color change. The plate was incubated at 35ºC and observed for the colour changes at different time intervals of 24 h, 48 h, and 76 h respectively. After 24 h the plate was observed for the color reactions. As shown in Plate-3, A-Line was positive for the color change and this line in the plate was considered as the control, wherein the wells contained *C. violaceum* and *R. solanacearum.* Similarly the B-Line showed

Table 1: Partial 16S rRNA sequence and identified bacterial species of the bacterial isolates from tomato rhizosphere soil involved in quorum quenching

Isolate	Partial 16S rRNA gene sequence	Size (bp)	Identified Bacteria By BLASTn
TL1	ACCCATGCAGTCGAACGAACCTTCGGAGTTAGTGGCGGACGGGTGAGTAACAC GTGGGAACGTGCCTTTAGGTTCGGAATAACTCAGGGAACTTGTGCTAATACCGAATGTGCCC TTCGGGGGAAAGATTTATCGCCTTTAGAGCGGCCGCGTCTGATTAGCTTGTTGGTGGGG TAATGGCCCACCAAGGCTACGATCAGTAGCTGGTCTGAGAGGATGACCAGCCACATT GGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAAT GGGCGAAAGCCTGACGCAGCCATGCCGCGTGTATGATGAAGGTCTTAGGATTGTAAAAT ACTTTCACCGGTGAAGATAATGACTGTAGCCGGAGAAGAAGCCCCGGCTAACTTCGTGCCA GCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATTACTGGGCGTAAAGGGA GCGTAGGCGGACATTTAAGTCAGAGGTGAAATCCCGGAGCTAACTTCGGAACTGCCTTTGATA CTGGGTGTCTTGAGTGTGAGAGAGGTATGTGGAACTCCGAGTGTAGAGGTGAAATTCGTAGAT ATTCGGAAGAACACCAGTGGCGAAGGCGACATATGGCTCATTACTGACGCTGAGGCTCGAAAG CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGATTGCTAGTTGTC GGGAAGCTTGCTTCTCGGTGACGCAGCTAACGCATTAAGCAATCCGCCTGGGGGAGTACGGT CGCAAGATTAAAACTCAAAGGATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT TAATCGAAGCAACGCGCAGAACTTACCACCTTTTGACATGCCTGGACCGCCAGAGAGATC TGGCTTTCCCTTCGGGGACTAGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTG AGATGTTGGGGTAGTCCCG CACGAGCGCA	992	*Brevendimonas Sp.*
TL2	GGCGTCGATGATGTAGCTGTTGGGGCAGTTCACTGCTTCGGTGGCGCAGCTAACGCATTAAAGCATGAC CGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT GGAGCATGTGGTTTAATTCGAAGCAACGCGCAAAACCTTACCACCCCTTGACATGCCTGGAACG CCCAAGAAATTTGGTCTTGCTCTCCGGATAAACGGAACACAGGTGCTGCATGGCTGTCGTCA GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTATTTG CTACCATATGGTTGTCCACTCAAAGGGCAGTGGACGTGATACACGAAGAGGGGGGTGAGGACGACTC CTCGTCCTCCTGGCCCTTAGGGGTTGGGCTCCCCTAGTGCTACGGGGACGGAGGGAGGGGAACCCGAGAAG GCGAGCCAATCCCAAACTGTCAATCGCATCTCGGATCGCATTGTGCTCTTCAACTCCAGTGCGTGAGAAT CGATTCTCATCTAATATCGGACCGTCATGGCGCGCTGAATACCTGCCCTTGCATTGTAGCACCGCAC GTCTGCCAGTGGGTTTGGTTTTACCCGAAGGCGCTGTGCGCACGGGGGGGGGATTACCACGACCACGATTC GGACTGGGTTGGAGGTGAAACGTAAGGAAAGATGAAGTTCTTTCCGTTGGGTGGGGGTACGTTCCCACG TTTTACTACGCTCTGATTCCCCCCCCAGGGGCGCTACAATATTAAATCTTAAACACTCTCCCCCCCTTTTT CTTTGACCAAACATAAAAAAAATAAAAAAATCTGGGTTTACATTTTGAAGGGTTAAGGGTGAGCGTGCC CCCCTGGAATCTCACCTCACAATTTATAAAAGCACCGAGGGGGGGGGGGTTTTCCCCCAATTTTTT TAAATAACCGCGCGCCCCCCCTCTTAAATAAAACGGCGGGGGGGGGGGGAAAAAAAAAAGG CGGGGGTGTTTTTTTTTTGGGGGGGGAGAATATTTTTTTGGGGGGGAGGAGAAAATTATTT TTTTTTTCAGGGTATTTGTTTTCTTCTTCCCCCCGGGGGGGGGTTGTTATCTCCCCCCCCCCACGGTA GGATAAAAATAAAACTCCTTCTCTCCCCCACTAAAAAAAAGAGAGGGGGGGCGGGCGCC CCCTCAACAGAAATAGATAATATCGTTTTTCTCTCAACTGCAAAGAAAGGAGGTGATGGTGG TCCGCCTGCGGGTGGAGAAAAATTTCTATCTCTTCCACGCCATGGATTTATTTGATAAAGAAGC AGGGGCGGGCGCACCCCGCCGGTGATGAGTATGGGTAGAGTTTAAAA	1366	*Rhizobium* sp

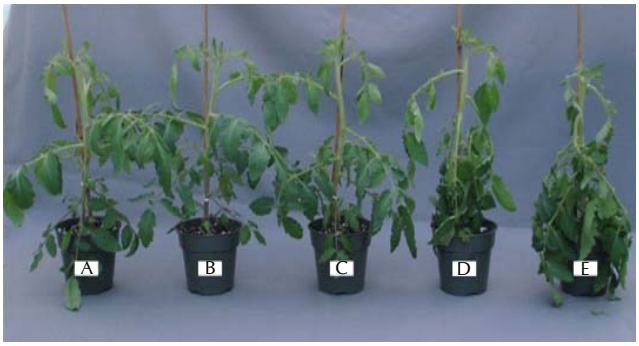

Figure 3: *In vitro* study on effect of quorum quenching bacteria on tomato wilts

positive reaction for the color change, wherein the wells contained *C. violaceum* and two different rhizosphere bacterial isolates TL1 and TL2. The well in the C-Line contained *C. violaceum* and other rhizosphere bacterial isolates showed non-reactive for the color change. The positive rhizosphere bacterial isolates TL1 and TL2, which was able to release AHL signals were identified using 16S rRNA gene sequencing.

Molecular characterization of rhizosphere bacteria interfering in quorum sensing

The bacterial isolates isolated from rhizosphere soil interfering in QS of *R. solanacearum* were characterized using 16S rRNA gene based molecular technique by isolating the genomic DNA of the bacteria as shown in agarose gel electrophoresis (Figure 1). The isolated genomic DNA was used as the template for amplification of 16S rRNA gene using PCR and run on agarose gel electrophoresis (Figure 2). The amplicon was marching to 1500 bp marker DNA in the DNA ladder. The PCR product was purified and sequenced. The sequence was BLAST analyzed and found the bacterial isolated TL1 was *Brevendimonas sp.* and TL2 was *Rhizobium sp.* The sequence of *Rhizobium sp.* was about 1366 bp. Similarly, the sequence of *Brevendimonas sp.* was about 992 bp as shown in Table 1.

Study on effect of quorum quenching bacteria on tomato wilt

10mL of pure broth cultures *C. violaceum* and rhizosphere bacterial isolates (TL1 and TL2) was added into the rhizosphere zone of the infected tomato seedlings 4 pits with control pot The naming of the pots (Figure 3) and inoculation of the bacterial cultures was done as under:

Pot A: Control-normal uninfected tomato seedlings.

Pot B: *R. solanacearum* infected tomato seedling inoculated with culture of *Brevindimonas sp.*.

Pot C: *R. solanacearum* infected tomato seedling inoculated with pure culture of *Rhizobium sp.*.

Pot D: *R. solanacearum* infected tomato seedling inoculated with pure culture of *C. violaceum*.

Pot E: *R. solanacearum* infected tomato seedling only.

The pots were kept in sunlight in natural conditions and watered regularly for 2 weeks. After 2 weeks morphological observations were made. From the observations (Figure 3) it was found that no wilt in control (Pot-A), plants in Pot-B, Pot-C showed no wilt. But, bacterial wilt was seen in tomato plants potted in Pot-D and Pot-E respectively. Absence of bacterial wilt in Pot-B and C is due to the inoculation of rhizophere bacteria involving in quorum sensing. Two rhizosphere bacteria *Brevindimonas sp.* and *Rhizobium sp.* were found to be effective quorum quenchers against *R. solanacearum* responsible for the wilt disease of tomato.

DISCUSSIONS

In the current study, we tried attenuating the virulence of *R. solanacearum* using rhizosphere bacteria isolated from rhizosphere soil of infected tomato plants by AHL based quorum sensing system using *C. violaceum* as a biosensor indicator organism. Isolation of pure bacterial cultures of the pathogen, *R. solanacearum* was confirmed by conducting specific biochemical tests and the positive results of the tests were in accordance with (Shahbaz *et al.*, 2015). Two rhizosphere bacterial isolates found positive for the release of AHL signals using *C. violaceum* as indicator organism, was identified at molecular level using 16Sr RNA gene sequencing. The use of 16S rRNA as a molecular fingerprint to identify and classify bacteria in different natural and environmental samples has been reported (Ohkuma and Kudo, 1996; Muyzer, 1999; Madhusudan *et al.*, 2011). In our studies *C. violaceum* was used as the biosensor indicator bacteria in detecting of release of the AHL signals of other bacterial species. McClean *et al.*, (1997) and Florence and Allan, (2002) also have reported about the use of this bacteria in detecting AHL signals. Rhizosphere bacterial isolates which was positive for the release of AHL signals was identified as *Brevindimonas sp* and *Rhizobium sp.* *In-vitro* studies on attenuating the virulence of *R. solanacearum* using *Brevindimonas sp* ,and *Rhizobium sp.* as well as *C. violaceum* in controlling tomato wilt with pot experiment, it was found that *Brevindimonas sp.* and *Rhizobium sp.* were able to control tomato wilt. *C. violaceum* is not effective in attenuating the virulence of *R. solanacearum* since the bacteria is mutant strain that no longer produce its cognate AHL (C6-HSL) due to inactivation of its luxI-like gene; thus it can be used as an indicator for exogenous AHLs as it produces the purple pigment violacein in response to added AHLs (McClean *et al.*, 1997). Release of AHL signals and attenuating the virulence of other pathogenic bacteria by different species of *Rhiozobium* have been reported (Florence and Allan, 2002). According to Flavier, (1997) the plant pathogen *R. solanacearum* produces 3-hydroxypalmitic acid methyl ester as a novel signaling molecule that with AHL was used to regulate the virulence. The current study also aimed towards AHL signaling system in regulating the virulence of *R. solanacearum* using rhizosphere bacteria. From the current study it can be concluded that two rhizosphere soil gram negative bacteria *Brevindimonas sp.* and *Rhizobium sp.*, were found to be effective quorum quenchers against *R. solanacearum* that were responsible for the wilt disease of tomato. Based on this research, it can be further concluded that there is need to develop eco-friendly, cost-effective, biological control system such as use of quorum quenching of quorum sensing molecules in the management of important diseases of tomato. Biological control strategies could be easily incorporated into integrated management system.

ACKNOWLEDGMENT

The authors thank the financial support from University Grants Commission, India under the Minor Research Projects.

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