Original Research Article

Determination of an expedient method for screening quorum quenching (QQ) rhizobacteria with *Choromobacterium violaceum* (CV) RU9 as bioindicator

Abstract

Quorum quenching (QQ) is the process of hindering the auto-inducer molecules that acts as quorum sensing (QS) signals. Quorum sensing signals, the Acyl homoserine lactones play a major role in induction of virulence in *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), the most acclaimed causative agent of soft rot disease in several vegetable crops. The disease sounds so alarming because it is also an infamous post-harvest affliction. Disruption of the QS signals will halt the production of QS induced secretion of the plant cell wall degrading virulence enzymes. Thus, in the present study it was attempted to screen rhizobacterial isolates for their ability to produce degradative enzymes for the disruption of the QS signal molecules. Three methods, soft agar diffusion assay, soft agar overlay assay and disc diffusion assay were trailed out with a basic modification to choose the most befitting method. For the assay we used *Choromobacterium violaceum* (CV) strain RU9 as a bioindicator rather than the mutant strain CV026. Of the three methods of screening, the soft agar overlay assay was chosen as the pre-eminent method for screening which further required standardization of cell load of CV cells for the overlay. 1% to 5% CV RU9 cells were overlaid and their violacein units were determined simultaneously. A concentration of 3% CV RU9 cells had 812 violacein units for overlay was determined to be the appropriate concentration. Soft agar overlay assay with 3% CV RU9 cells was used to determine the quorum quenching ability of the rhizobacterial strains isolated from various crops.

Keywords: *Chromobacterium violaceum*, CV 026, Overlay assay, Violacein units.

1. Introduction

Quorum sensing (QS) is the mechanism of intercellular communication in bacteria where, detection of the autoinducer (AI) signals in response to cell population density will regulate the expression of certain physiological response in many bacteria [1, 2]. Any process that interferes with the quoration, thus the physiological character expressed by them is called quorum quenching. In recent times quorum quenching is explored as the solution to check infections caused by bacterial strains resistant to antibiotics [3, 4]. Several native microflora harbor the ability to quorum quench. Scientists reported numerous rhizobacteria which were isolated and investigated for their quorum quenching based biocontrol activity. Bacterial isolate *Bacillus* sp. 240B1 obtained from soil samples was close to the earliest report of soil bacteria harboring AiiA an enzyme which attenuates the virulence of *Erwinia carotovora* by inactivating quorum sensing signal AHL molecules [5, 6]. Subsequently several investigators isolated quorum quenching microorganisms from rhizosphere of various crops [7-9]. Most studies explored the quorum quenching based biocontrol ability against Pcc [10, 11]. Four hundred bacterial isolates obtained from potato rhizosphere were screened for their ability to degrade autoinducer signal molecules secreted by soft rot pathogen P*ectobacterium carotovorum* subsp *carotovorum*. Six isolates that presented best AHL degrading activity were characterized and identified as bacteria belonging to genera *Ochrobactrum, Rhodococcus, Pseudomonas, Bacillus* and *Delftia*. Genera *Ochrobactrum* and *Delftia* were reported for the first time. These isolates had the ability to degrade natural AHL's produced by *Pcc* [8]. Chan et al., [7], discovered QQ strains that could degrade N-acyl homoserine lactones by investigating the ginger (*Zingiber officinale*) rhizosphere in the aerable lands of Malaysian rainforest. Quorum sensing inhibitor isolates were isolated from potato rhizosphere and studied for the biocontrol of *Pectobacterium aterosepticum* [9]. Garge and

Nerukar, [10] isolated AHL degrading *Bacillus* sp from the rhizosphere of Carrot, Beetroot, Maize, Fenugreek, Potato, Pigeon pea, Raddish, Pearl millet and Eggplant then studied for the biocontrol of Pcc. Out of the 97 isolates screened, 20 AHL degrading *Bacillus* sp. were obtained out of which 3 isolates displays considerable enfeeblement of Pcc soft rot. Enzymatic degradation of QS signals was the prime method analyzed in the above studies. With the referenced background it was decided to isolate rhizobacteria and screen them for their enzymatic degradation of the AHL autoinducer molecules.

Screening of rhizobacterial isolates required a meticulous technique for the identification of potent isolates. The screening methods for quorum quenching are bioindicator based. Bacteria whose property of pigmentation is driven by quorum sensing were commonly used as bioindicators. *Chromobacterium violaceum* (CV) ATCC 12472 whose regulation of pigmentation is by N-hexanoyl homoserine lactone (C6-HSL) was used as a bioindicator which was overlaid on the fully grown test bacterial culture [12]. Violacein negative, mini-Tn5 mutant *C. violaceum* (CV 026), whose pigmentation potential can be restored by extraneous addition of AHL's was seeded along with N-hexanoyl-L-homoserine lactone and the QQ antagonist supernatant were co-cultured to study quorum quenching. Sterile filter paper discs impregnated with QQ test isolates placed on a semi solid agar seeded with CV026 cells was used as a screening method to scrutinize the phytoextract of *Scutellaria baicalensis* Georgi [13]. The disc diffusion assay was further extended to study the QQ potential of several bacterial strains against soft rot caused by *Dickeya dadantii* [14].

The preliminary investigations of the present study required determination of a method best suited for our laboratory conditions, test bacteria and bioindicator strain used for assay. Methods of screening harnessed in the earlier reports were trialed out and one method that best

suited our needs and availability along with the concentration of the CV RU9 cells to be used in the assay to produce sufficient pigmentation were determined. The standardization of method and concentration was important because the non-mutant CV RU9 strain was not reported earlier as a bioindicator used infrequently in QQ screening studies.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used to confirm the screening were, the rhizospheric isolates obtained from the rhizosphere of various agricultural crops and the bacterial cultures were grown on Nutrient agar (HiMedia) media and nutrient broth at 28°C in a bioincubator (Thermocon, India). The quorum quenching activity of the test isolates were examined using the bio-indicator strain *Chromobacterium violaceum* RU9 accession number 8071 purchased from The Microbial Type Culture Collection and Gene Bank (MTCC), housed at the Institute of Microbial Technology (IMTECH), Chandigarh.

2.2. Selection of screening method for detection of quorum quenching activity

The quorum quenching activity of the bacterial isolates was determined by the employment of the bio-indicator strain, *Chromobacterium violaceum* (CV) strain RU9. Three methods of detection, soft agar diffusion assay, disc diffusion assay and soft agar overlay assay were trialed out to identify the method best adopted to advance with the screening. The methodologies adopted are as follows.

2.2.1. Preparation of culture

For the diffusion assay and soft agar diffusion assay, the overnight grown test cultures were centrifuged (Eppendorf *5430 R*, Germany) at 6000rpm for 5 minutes; supernatant was discarded form the harvested cells. About 1.5ml of phosphate buffer solution (pH 6.8, PBS, Sigma Co.) was added and the cells were lysed by sonication. The samples were sonicated

(Athena Ultrasonic Processor, ATP-120) using a single probe sonicator for 10 seconds at high intensity. After sonication, the supernatant was collected by centrifugation and used for further assay.

For the overlay assay, the bacterial isolates to be tested were inoculated by picking a single isolated colony from pure culture plates in 10ml nutrient broth and incubated overnight at 35 °C with 120rpm rotations. The cells were harvested by centrifugation at 6000rpm for 8 minutes at room temperature. The cell pellets were washed with phosphate buffer solution (PBS) (pH 7) and dispersed in 1ml phosphate buffer. The prepared cultures were further used for screening.

2.2.2. Soft agar diffusion assay

The soft agar diffusion is a monolayer assay where the basal layer is Luria Bertani (LB, HiMedia) agar containing 0.8% agar mixed with 0.5% *Chromobacter violaceum* RU9 cells. The plates were left undisturbed till the agar solidified. A well of 6mm diameter was bore at the centre of the plate with a cork borer and was filled with the culture supernatant obtained by centrifugation at 12,000 rpm for 5 minutes at 4°C after sonication. The plates were incubated at 30°C for 48hours.

2.2.3. Disc diffusion assay

Disc diffusion assay is a two layered assay both layers differing in composition and concentration of agar. The basal layer is a Luria bertani agar layer with 2% agar and the second layer is a semisolid layer with 0.5% agar mixed with 0.5% CV RU9 cells. About 2 ml of the test bacterial isolate was centrifuged at 12000rpm for 8 minutes and the supernatant was collected. The collected supernatant was filtered in a millipore 0.2 µm disc filter. Filter paper discs of 6 mm were taken and soaked in the collected supernatant for 1minute and was placed on to the surface of the dual agar layer plates. The plates were incubated at room temperature 28°C for 24

hours. Anti-quorum sensing was detected by observing the formation of clear zone around the filter paper.

2.2.4. Soft agar overlay assay

The softagar overlay assay was performed according to the procedure as described by Mclean *et al*., 2004 with minor modifications. This is also a double agar layer assay but has a temporal separation in the order of convening both layers. The basal layer is a Luria bertani agar layer with 2% agar. The LB agar plates were spot inoculated with the test bacteria cultures. The plates were incubated at 30°C till the cultures grow. After attainment of growth of the cultures, the plates were overlaid with LB media containing 0.5% agar seeded with 0.5% CV RU9 cells. The plates were incubated at 30°C and observed for the formation of zone of de-pigmentation.

2.3. Determination of percent *Chromobacter violaceum* RU9 cells for seeding

The percentage of inoculums of the bioindicator *Chromobacter violaceum* RU9 for overlay was determined as 0.5% CV cells used in prior studies did not give sufficient pigmentation. The experiment involved diluting the CV cells to different concentrations, extracting the violacein pigment, measuring the optical density and simultaneously plating the diluents as overlay on pre-inoculated test plates. The CV RU9 culture was inoculated in LB broth and incubated to attain a population of 10^9 cells. The broth was diluted in LB broth to different concentrations from 1% to 5%. The tubes containing the diluents were vortexed to have uniform dispersion of the cells. About 1 ml of each culture was pipetted out and the cells were lysed by the addition of equal volume of 10% sodium dodecyl sulfate (≥99.0%, Sigma-aldrich). The contents were incubated at room temperature for 5 minutes after through mixing by vortexing for 5 secs. After incubation, about 4 ml of water saturated butanol was added to the cell lysate vortexed for 5 secs and centrifuged at 13000 rpm for 5 minutes. The upper phase containing the violacein extract was collected and the absorbance was measured at 585nm. The absorbance of

the bioassay culture was measured at 600nm. The ratio of the absorbance of the butanol extract versus the bioassay culture density was calculated and multiplied by 1000: (A nm/O.D. nm) X 1000. The values obtained 585/ 660 are expressed as violacein units. Simultaneously, the percent diluents were overlaid onto the test cultures pre-inoculated on full strength agar plates, incubated at 30°C and observed for the development of pigmentation. The plates were observed and optimum concentration was fixed.

2.4. Screening of rhizobacterial isolates for quorum quenching ability

Ninety-six rhizobacterial isolates obtained were screened for quorum quenching activity by soft agar overlay assay. The test rhizobacterial strains were inoculated in NA broth and incubated overnight at 30°C. Then the cells were harvested, washed and diluted with phosphate buffer. The diluted inoculants were spot inoculated at the centre of the LB agar (full strength, 2%) plates and incubated for 24 hours. Once the bacterial growth was observed, the plates were over laid with LB media containing 0.5% agar seeded with 3% CV RU9 cells. The plates were incubated at 30°C and observed for the zone of de-pigmentation around the colony. The colony and zone diameter formed around the QQ positive colonies were measured.

3. Results

The quorum quenching ability of the test bacteria were due to the competitive inhibition by various AHL molecules. The method for screening the quorum quenching isolates were determined and optimized for our lab conditions. *Chromobacter violaceum* CV026, a commonly used bioindicator in quorum quenching studies is a mini-*Tn*5 *cviI* mutant of *Chromobacterium* strain ATCC 31532, which produce an easily detectable purple colour pigment violacein when supplemented with an inducing concentration of medium chain-length homoserine lactones HSLs. Instead, we used the non-mutant CV RU9 strain for our study. The strain is a non-mutant parent strain hence there was no need for the addition of exogenous AHL's. The bioindicator of

the study CV RU9 produced only minimal amounts of violacein when grown in nutrient broth. When in LB broth the culture produced a dark purple color, which represented the induction of violacein synthesis. Thus, it was decided to use LB broth for the entire study. Comparison of the three methods trialed drew us towards the following conclusions. There was no promising result obtained from the soft agar diffusion assay and disc diffusion assay. In the disc diffusion assay, there was diffusion of supernatant but there was no prominent de-pigmentation of the reporter strain around the test cultures. In the soft agar diffusion assay, the diffusion was so less and the clearance zone formed was not so evident due to the less concentration of agar used. In soft agar overlay assay, the zone formation was so prominent. The test cultures were inoculated and incubated, as the cultures grow the QS inhibitor molecules were discharged into to base media by dispersion, which when overlaid with CV RU9 formed a zone of clearance if the test culture quorum quenches. The soft agar overlay assay was chosen as designate method for screening of the rhizobacterial isolates (Fig 1).

The determination of soft agar overlay assay as the prime screening method then steered us to decide the percentage of inoculum of CV needed for the overlay. Conventionally, most prior studies used 0.5% CV cells for overlay with the addition of external AHL. But as we employed the CV RU9 as bioindicator without endogenous AHL addition, 0.5% CV cells were inadequate to produce sufficient purple color intensity in the entire plate. Hence, attempts were made to affix the optimum percent CV RU9 cells to be mixed with LB broth for the overlay. The violacein pigment was extracted determining the violacein units as simultaneous overlay was done. The results showed increase in pigmentation in the plates as the percent cells increased but there was a sudden spike in the violacein production when 3% CV RU9 cells were used (Fig 2). The violacein units also recorded the same. At 1% the violacein units was 375 and at 5% it was 906, which was the maximum. At 3% there is a prompt hike in violacein pigmentation having

812 units which then continues to increase steadily till 5%, recording 875 units at 4%. Optimizing the CV RU9 percentage, 3% of CV cells were fixed as ideal concentration for overlay. Employing soft agar overlay assay with 3% CV cells, the ninety-six rhizobacterial isolates obtained from six crops rhizospheres were screened for their ability to quorum quench [15]. This biosensor based detection of quorum quenching activity is the most common way to screen microbes quantitatively and qualitatively. The outcomes of the experiments conducted elaborated as follows. Twenty-four isolates were positive for AHLase production. Table 1 shows the cultures positive for anti-quorum sensing activity. Scoring based on the zone diameter were given [14], zone diameters above 2.45mm were given the highest scoring (+++). The zones with diameter less than 1.23mm (+) were given with the least scoring and the intermediate scoring (++) was given when the zone diameter lied between 1.23mm and 2.45mm. Twelve cultures had high scoring while rest of the cultures showed intermediate and less scores.

4. Discussion

The phenomenon of quorum quenching is displayed in laboratory by the employment of a bioindicator bacterial strains whose property of pigmentation is induced by quorum sensing. Harrison and Soby, [16] in their recent publication states that since 1997, there were about 176 publications indexed in Pubmed that used CV026 as a biosensor. The CV026 is a mutant of the parent strain *C. violaceum* ATCC 31532 which lacks the ability to quorum sense for violacein pigment production in the absence of an external acyl homoserine lactone. Earlier studies tested *Chromobacterium violaceum* (CV) and *Pseudomonas aureofaciens* as bioindicators in which the pigment production is regulated by homoserine lactone (HSL) QS. Lack of pigmentation in the cultures indicated QQ. Maclean *et al*. [12] examined both bioindicator bacteria and compared their pigmentation potential and noticed that *Chromobacterium violaceum* CV produced darker pigmentation which provided a better and readily observable visualization than *Pseudomonas*

aureofaciens. The QS inhibition by bacteria can be determined by formation of halo zone around the QQ culture [12]. *Agrobacterium tumefaciens*, was also used for screening quorum quenching microorganisms. Zhang *et al*, [17], used mutant *Agrobacterium tumefaciens* strain NT1 for screening QQ *Actinobacter* sp. Most *in vitro* screening of bacterial isolates for QS inhibition studies employs CV026 as bioindicator. In the present study the strain CV RU9, a non-mutant strain which produces purple color without addition of exogenous AHL was used as a bioindicator. Maclean and his colleges, [12] also used a non-mutant CV strain ATCC 12472 as bioindicator which also does not demand external AHL supply. The method of screening varied with time advancements. Earlier studies used cultures directly for screening [18] but recent studies used the culture supernatant for assays [19, 20]. The efforts made to determine the suitable method for screening yielded softagar overlay assay as the screening method for our study. The cell load of CV RU9 cells to be used in the overlay was determined. An overlay with a series of concentrations of CV RU9 showed that 3% would be highly suitable for our study. The 3% bioindicator cells reported high pigmentation and violacein units. At 4% and 5% also there was increase in pigmentation and violacein units but we fixed 3% because the violacein units value initiated the unforeseen ascend at this concentration. Also the assay is so sensitive that the excess concentrations of the bioindicator strain may generate cognate signals so as to block any QS inhibition [21, 22]. Thus 3% was fixed as final concentration of CV RU9 cells for the overlay.

5. Conclusion

Rhizobacteria isolated from crop rhizospheres were subjected to screening to determine the potential of the isolates to quorum quench. To enunciate the screening, it was determined to utilize the non-mutant CV RU9 bacterial strain as this eliminates the need for the external addition of synthetic AHL's making the screening inexpensive, precise and simple. After

systematic scientific exertions, it was concluded that soft agar overlay assay would be used as a method for screening with 3% concentration of the bioindicator, CV RU9 cells used for the overlay. The determined modification in the existing method would be convenient, compliant and economical.

COMPETING INTERESTS DISCLAIMER:

The authors declare there is no conflict of interest.

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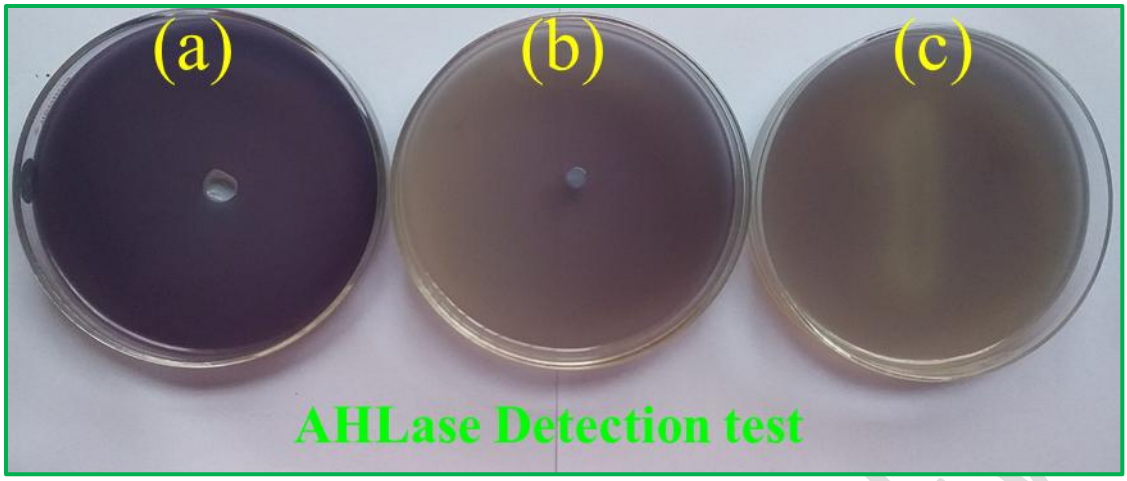

Fig.1. Comparison of the screening methods employed for testing quorum quenching **a)** Soft agar diffusion assay **b)** Disc diffusion assay **c)** Soft agar overlay technique.

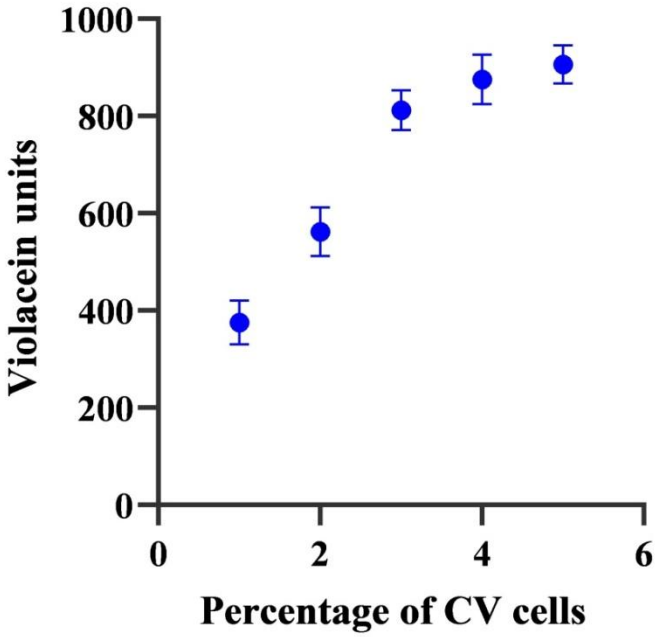

Fig.2. Violacein units as influenced by *Chromobacterium violaceium* RU9 strain.

Table 1. AHLase positive quorum quenching isolates.

Sl. No.	Isolate Code	AHLase Activity
1	**BRR05**	+++
2	**BRR06**	++
3	**BRR09**	+
4	**BMR17**	+++
5	**BMR22**	+++
6	**BMR29**	+
7	**BMR32**	+++
8	**RAR37**	++
9	**RAR38**	++
10	**RAR39**	++
11	**BBR46**	++
12	**BBR51**	+++
13	**BBR57**	+++
14	**BBR58**	+++
15	**BBR60**	+++
16	**BBR61**	++
17	**BBR62**	+++
18	**BAR70**	+++
19	**BAR75**	++
20	**BAR77**	++
21	**BAR78**	+++
22	**BAR79**	+++
23	**BFR83**	+++
24	**BFR86**	+++

Scoring based on the zone diameters in mm, + = <1.225mm, ++ = <2.45mm, +++ = >2.45mm