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A simple method for direct isolation of N-acyl-L-homoserine lactone mediated biofilm-forming rhizobacteria from roots

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ABSTRACT

Plant-associated bacteria produce quorum sensing (QS) signals for community (biofilm) formation and functioning in the rhizosphere. The QS-positive biofilm-forming rhizobacteria that excel benefits to the plants are now gaining increased importance for agricultural use due to their high competitiveness. However, there is no method available to distinguish these bacteria from the roots of a plant to ease the isolation. Currently, all the plant-associated bacteria have to be isolated, purified and subsequently screened for the QS activity using biosensor strains. This study describes a direct isolation method for N-acyl-homoserine lactone (AHL) type quorum sensing signal producing bacteria from the plant root. In this method, the root sample collected from the field was overlaid directly with the bacterial growth medium seeded with the biosensor reporter, *Chromobacterium violaceum* (CV026). The AHL produced by QS positive rhizobacteria residing on the surface of the root will be recognized by violacein production of CV026. The bacterial isolates recovered from rice root using this method were further confirmed for the QS activity and biofilm formation. All the QS-positive strains produced N-butyryl DL-homoserine lactone (a C4-AHL type) signal in the culture medium and had biofilm formation during *in vitro* culturing. The 16S rRNA gene sequences of these QS-positive biofilm-forming rhizobacteria revealed that these strains are phylogenetically close to *Pseudomonas siluiensis*, *Aeromonas hydrophila* and *A. caviae*. Therefore, this could be a simple, rapid and straightforward procedure for isolation and characterization of quorum-sensing rhizobacteria from plant roots.

1. Introduction

The soil bacteria attracted by root exudates, root cell lysates and mucilages, profusely colonize in the rhizosphere and on the surface of the roots. These bacteria multiply to high densities in this vicinity due to ample nutrients provided by the rhizodeposits (Venturi and Keel, 2016). Most of these plant-associated rhizobacteria use quorum sensing (QS) signals for cell aggregation, which ultimately improve the rhizosphere colonization through biofilm formation (Loh et al., 2002). The Gram-positive bacteria produce short-chain polypeptide derivative signals, while the Gram-negative bacteria produce N-acyl homoserine lactone as autoinducer or QS signal molecule for the cell to cell communication (Papenfort and Bassler, 2016). The LuxR – LuxI type regulatory system is responsible for the quorum sensing mechanism present in the rhizobacteria (Fuqua et al., 1994; Gray and Garey, 2001). The commensal, mutualistic and pathogenic bacteria use more or less same mechanisms for effective colonization in the rhizosphere (Danhorn and Fuqua, 2007; Morris and Monier, 2003).

Plant-growth promoting rhizobacteria (PGPR) with effective root colonizing ability through biofilm formation is always advantageous than those strains without biofilm (Velmourougane et al., 2017). Hence, the QS-based biofilm-formation would be an important trait for the PGPR strains to excel their maximum benefits to the crops. However, the pathogenic bacteria that are associated with plants were well characterized for their quorum-sensing which includes *Agrobacterium tumifaciens*, *Burkolderia glumae*, *Dickeya solani*, *Erwinina carotovora*, *E. stewartii*, *Pantoea stewartii, Ralstonia solanacearum*, *Pseudomonas syringae*, *Xanthomonas* spp. and *Xylella fastidiosa* [reviewed by Ansari and Ahmad (2018), Sibanda et al. (2018)] with limited knowledge on PGPR QS for *Ensifer* spp., *Rhizobium* spp., *Sinorhizobium meliloti,* various species of *Pseudomonas* (*P. aeruginosa, P. putida, P. fluorescens, P. chlororaphis*), *Azotobacter vinelandii* and *Azospirillum lipoferum* [reviewed by

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Harjai and Sabharwal, 2017, Velmourougane et al. (2017)]. The mechanisms of quorum sensing and biofilm formation by plant-associated bacteria are well known in recent days (Barriuso, 2017; Carlier et al., 2015; Danhorn and Fuqua, 2007; Primo et al., 2015).

The current microbiological procedure requires two steps to identify the QS positive biofilm-forming rhizobacteria from the plants. The first step involves the routine isolation of all the plant-associated bacteria by standard plating method, followed by purification and then the subsequent step involves the screening of isolates for QS signals through bioassay using reporter strains *viz.*, *Chromobacterium violaceum* (CV026) and *Agrobacterium tumefaciens* (NTL1) (Steindler and Venturi, 2007). This procedure is time-consuming, laborious and interfering with a high number of non-QS bacterial background. In the present work, we have simplified the procedure for direct isolation of the QS positive biofilm-forming rhizobacteria from the plant root. We have used the reporter strain-seeded agar medium for direct isolation of QS-positive rhizobacteria from the rice root.

2. Materials and methods

2.1. Bacterial culture and media

The AHL biosensor reporter strain *Chromobacterium violaceum* CV026 grown in Luria Bertani medium at 30 °C was used for the isolation of QS-positive rhizobacteria from the rice root. This strain can detect the presence of exogenous AHLs with four to eight carbon (McClean et al., 1997). The purple-colored violacein pigment production will be induced in *C. violaceum* CV026 if suitable AHL molecule is excreted or present in the medium. The violacein-positive strain *Chromobacterium violaceum* ATCC12472 routinely grown on LB medium at 30 °C was used as positive control. *Escherichia coli* DH5α used as negative control for QS assay was routinely grown on LB medium at 37 °C.

2.2. Isolation and purification of QS-positive rhizobacteria from rice root using C. violaceum as a biosensor

The CV026 was grown in LB broth at 30 °C in an incubator (Lab Companion, USA) for 18 h to reach the cell density of ≈ 10^8 cells per ml. One ml of this culture was transferred to 100-ml of LB agar at a lukewarm temperature (about 50 °C) and used as a reporter for isolation of QS bacteria from rice roots. The rice seedlings from both nursery and transplanted fields of Wetlands Farm, Tamil Nadu Agricultural University, Coimbatore were sampled by uprooting without damaging the roots, transferred to sterile bags (HiMedia, India) and brought to the laboratory. The soil adhered on the root was carefully removed by a gentle rinse with sterile water. The root portions were cut off and placed in a 100 sq. cm sterile petri dish and nearly 50-ml of CV026 seeded LB agar medium at a lukewarm temperature (about 50 °C) was poured over the root and allowed to rest without disturbance in the laminar airflow chamber until complete solidification. The petri dishes were incubated at 30 °C in an incubator (Lab Companion, USA) for 1 to 2 days and observed for violacein pigment production near the root region. The presence of purple-colored pigment production indicates the presence of QS-positive bacteria on the root surface. The non-pigmented colonies appeared close to the root that is surrounded by pigmented reporter (CV026) colonies were marked as QS positive bacteria and those colonies were carefully picked by a sterile toothpick and streaked to a new LB plate.

For authentication of QS positive isolates, freshly grown isolates and the biosensor reporter strain CV026 were streaked parallel to each other on petri plates containing LB agar and incubated at 30 °C. *Chromobacterium violaceum* ATCC12472 was used as positive control. The plates were observed after 24 h and 48 h for violacein production by CV026 due to AHL production by the QS isolate.

2.3. AHL profiling by thin-layer chromatography (TLC)

All the QS-positive isolates were further characterized for the N-acyl-L-homoserine lactone by TLC (Silica gel 60 F254, Merck, Germany) by adopting the method of Shaw et al. (1997). Two identical TLC plates were run for each isolate and one plate (reporter plate) was used for TLC-bioassay with CV026 (Shaw et al., 1997) and another plate (preparative plate) was used for identification of AHL compound by GC/MS.

2.4. Gas chromatography/mass spectrometry (GC/MS) analysis for AHL

After identifying the AHL spot in the reporter plate with CV026, the regions of the preparative plate corresponding to the compound to be analyzed were scraped off and extracted three times with 1 ml of 1:1 (vol/vol) chloroform: dichloromethane. The combined extracts were then centrifuged; filtered through 0.2 µm syringe filter (Axiva SFNY25 R) and dried at room temperature. The residue was redissolved in HPLC-grade methanol and used for GC/MS analysis. The AHL compounds extracted from the isolates were analyzed using GC/MS (PerkinElmer CLARUS SQ8C) connected to a mass selective detector with NIST library (Cataldi et al., 2004). Identification was performed using the DB-5 MS capillary standard non – polar column with dimensions 30 m × 0.25 mm ID and 0.25 µm film thicknesses, where pure helium was used as carrier gas. The GC oven temperature was increased from 75 °C (held for 2 min) to 150 °C (held for 2 min) and then to 220 °C (held for 1 min) and finally to 250 °C (held for 5.5 min). Samples of 1 µl volume were injected in a splitless mode. The conditions of mass spectrometry were ionization source set to 70 eV, emission current 500 mA, MS Source temperature at 230 °C and MSQuad at 150 °C. The mass spectrometer was run in full-scan mode (m/z 15–800). The software provided with the GC/MS was used to perform mass calculations and predictions with NIST library.

2.5. Biofilm assay

All the bacterial isolates were assessed for the formation of biofilm in 96-well titer plate (Pierce et al., 2008) and quantified the biofilm production by crystal violet staining as described by O'Toole (2011).

2.6. Phylogenetic analysis

All the QS-positive biofilm-forming bacteria isolated from rice root were identified after amplification and sequencing of nearly full-length of 16S rRNA gene sequence (Weisburg et al., 1991). The sequences obtained for the isolates were deposited in GenBank with the accession numbers of MK007297 (QSRB1); MK007298 (QSRB2); MK007299 (QSRB3); MK007300 (QSRB4) and MK007301 (QSRB5).

3. Results

3.1. Isolation and authentication of QS-positive rhizobacteria from rice root

The procedure postulated for direct isolation of AHL-producing bacteria from the rhizosphere of a crop plant was repeated three times on ~ 18 to 20-days old rice seedlings collected from the field. In all the technical replicates of the experimentation, we could isolate the AHL-producing bacteria adhered on the surface of the rice root through the appearance of purple pigmentation of CV026 as a reporter (Fig. 1A). In each rice root, three or four such positive colonies have appeared. For validation of the method, we have pooled all those colonies appeared in one technical replicate and used for further analysis. All the purified colonies were reconfirmed for their AHL production using CV026 bioassay. The result clearly indicated that all these strains produced AHL in the medium, which made purple pigmentation of CV026 (Fig. 1B). All these five isolates were designated as QSRB 1 to 5 (quorum-sensing positive rhizobacteria).

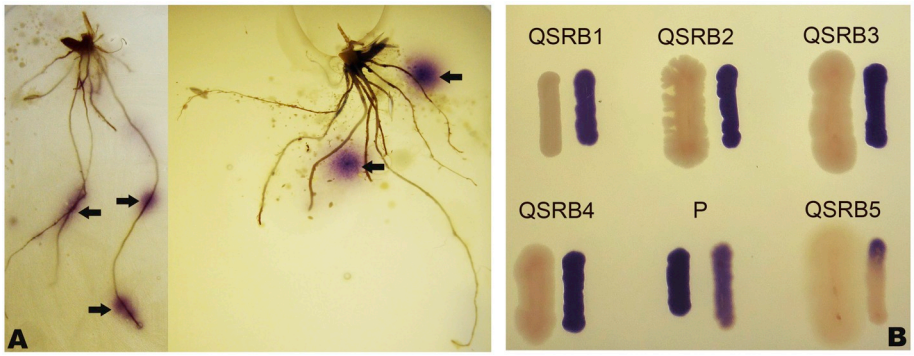

Fig. 1. (A) Direct isolation of quorum-sensing positive bacteria from the rice root using *Chromobacterium violaceum* (CV026) as reporter strain. (B) Authentication of the QS-positive isolates by parallel streaking with reporter strain, *Chromobacterium violaceum* (CV026) in LB agar. QSRB1–5: QS positive strains from rice root; P – Positive control (*Chromobacterium violaceum* (ATCC12472)).

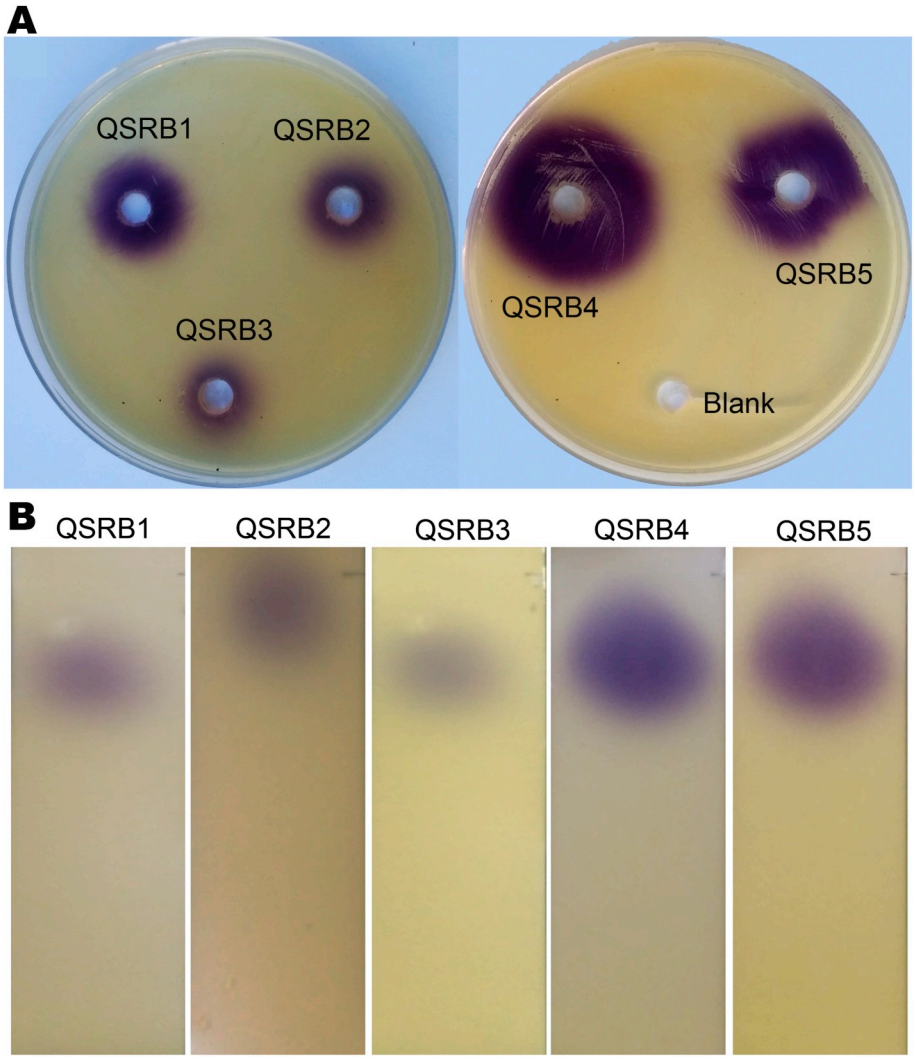

Fig. 2. Detection of N-acyl L-homoserine lactone from the supernatant extracts of QS-positive bacterial isolates using CV026 reporter strain (A) and by TLC bioassay (B). QSRB1–5: QS positive strains from rice root. A volume of 10 µl of extracted AHL from rhizobacterial isolates was spotted in the well formed on an agar plate overlaid with *C. violaceum* CV026 and incubated.

3.2. Identification of AHL produced by QS-positive isolates

The supernatant of the QSRB cultures grown in LB medium for 24 h showed the presence of AHL compounds. The concentration of AHL purified from the culture supernatants differed among the isolates. QSRB4 and QSRB5 had higher concentrations of AHL in the supernatant than other isolates, while in QSRB3 extract found the lowest level (Fig. 2A). The TLC bioassay performed for the AHL extracts detected only one AHL compound with more or less same Rf value, irrespective of the QSRB isolates (Fig. 2B). The GC/MS analysis of the TLC-separated extract identified the AHL as N-butyryl DL-homoserine lactone (retention time – about 13 min; *m/z* – 144; 95.5 to 96.0 purity) in all the five QSRB isolates (Fig. 3).

3.3. Biofilm formation by QS-positive isolates

The biofilm-forming capacity was compared between the five QSRB isolates grown under static culture for seven days and assessed by

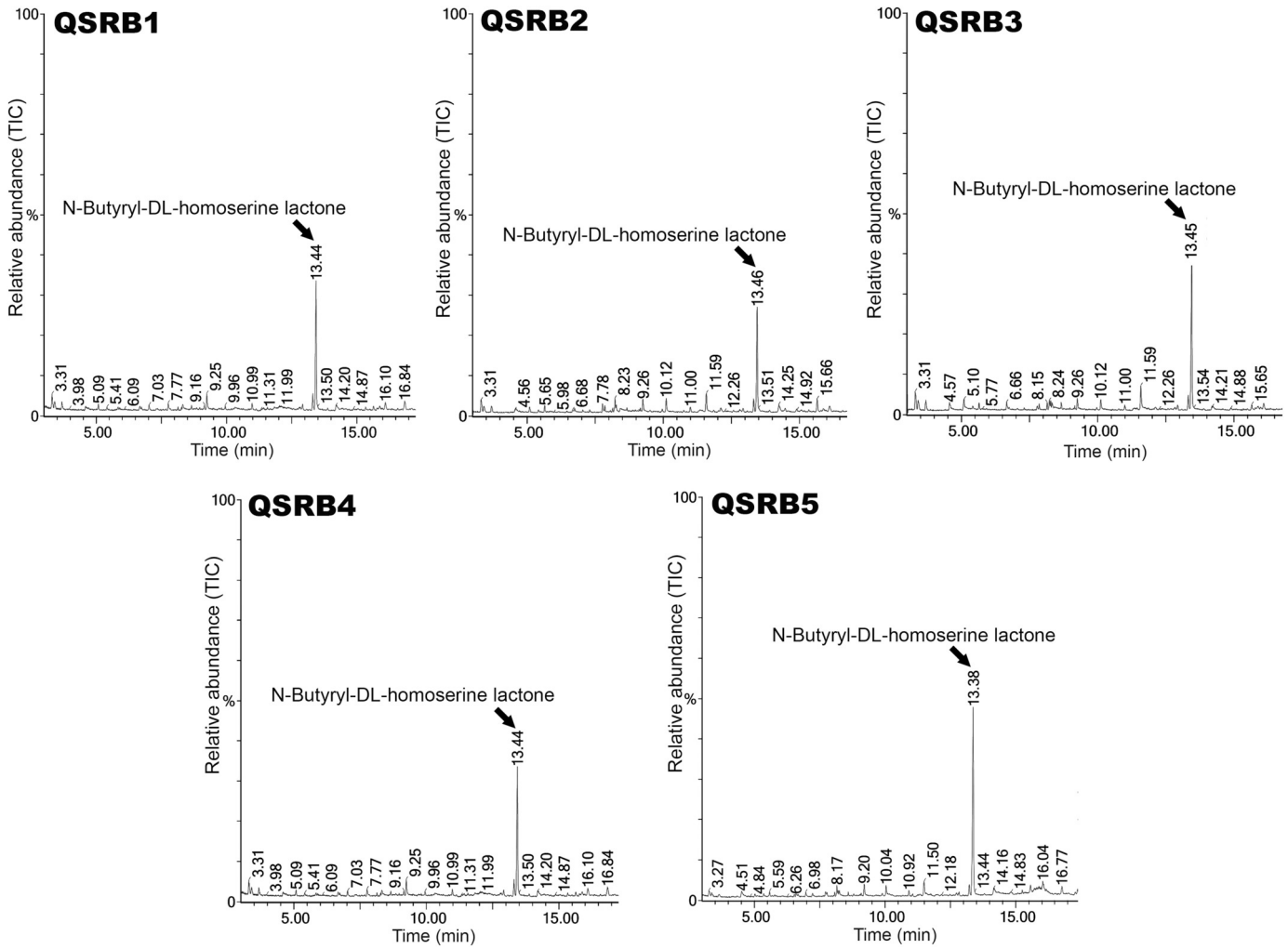

Fig. 3. GC/MS analysis of extracted and TLC-run AHL of supernatant from QS-positive bacterial strains. QSRB1–5: QS positive strains from rice root. The AHL compound was identified using NIST library provided with the instrument.

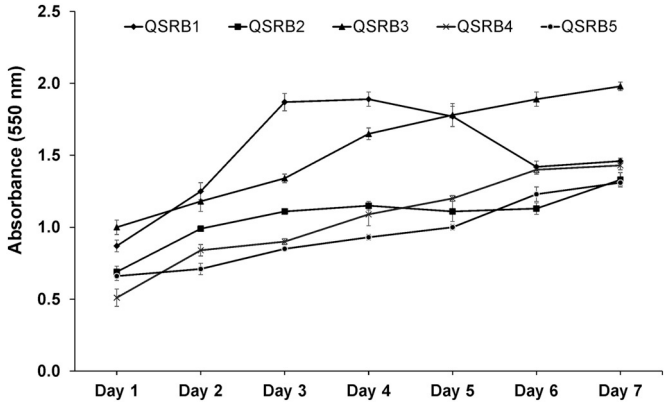

Fig. 4. Biofilm production of the QS-positive strains (QSBR1–5) as measured by crystal violet straining method in microtiter plates. Means of three replicate values plotted and error bar indicates the standard error.

crystal violet-based microtiter plate method. Among the five, QSRB3 showed the highest biofilm accumulation, reached 1.98 (OD at 550 nm) on the 7th day followed by the rest of the isolates. All the QSRB isolates except QSRB1 gradually increased the biofilm-based population intensity with the time course of incubation. The isolate QSRB1 reached the maximum biofilm formation on the 3rd day of incubation and then declined it after 5 days (Fig. 4).

3.4. Phylogenetic analysis of QS-positive isolates

The QSRB isolates were identified based on their 16S rRNA gene sequence homology with the nucleotide database using BLAST search and the results were summarized in Table 1. The QSRB1 is close to *Pseudomonas sihuiensis* with 99% sequence homology, while rest of the isolates belong to *Aeromonas* spp. The highest homology for QSRB2 and QSRB5 was found with *Aeromonas hydrophila* (98 and 99% respectively), while QSRB3 and QSRB4 with *Aeromonas caviae* (99% for both the isolates).

4. Discussion

Quorum sensing is an important event happening in the rhizosphere for microbial community formation and functioning. The rhizospheric bacteria use the QS-regulated genes for biofilm formation and for the synthesis of antimicrobial compounds against competitors (Pieterse et al., 2016). Quorum sensing is a regulated gene expression of key signaling process, increases the cell density of a bacterial strain. The adherent cells further become embedded within a slimy extracellular matrix that is composed of extracellular polymeric substances (EPS) which are structurally referred to as a biofilm. The cells within a biofilm

Table 1
Authentication of QS-positive bacterial isolates from rice rhizosphere using 16S rRNA gene sequence homology.

Strain	GenBank Accession number	16S rRNA gene sequence similarity based on BLAST on NCBI database			
		Closest species	Strain	Identity (%)	Accession Number
QSRB1	MK007297	*Pseudomonas sihuiensis*	KCTC32246	99%	LT629797.1
QSRB2	MK007298	*Aeromonas hydrophila*	HLY6	98%	KR094132.1
QSRB3	MK007299	*Aeromonas caviae*	AH07	99%	KU975029.1
QSRB4	MK007300	*Aeromonas caviae*	AH07	99%	KU975029.1
QSRB5	MK007301	*Aeromonas hydrophila*	KCTC32246	99%	LT629797.1

produce EPS components which are typically polymeric conglomeration of polysaccharides and proteins (Flemming et al., 2016; Loh et al., 2002). The biofilm-forming capability of a PGPR has an added advantage over the non-biofilm forming strains, as the former can effectively colonize and elicit its beneficial roles to the plants, while the latter has space and nutritional competition issues with native microbiome. Hence, PGPR strains capable of biofilm formation in the rhizosphere are in need of the future agriculture for effective nutrient transformation, control the soil-borne pathogens and for abiotic stress mitigation (Seneviratne et al., 2011; Velmourougane et al., 2017). In order to isolate QS-positive rhizobacteria from a plant root, no simple and straightforward procedure is available. In the present work, we have successfully standardized a simple method to isolate QS-positive rhizobacteria directly from the rice root, without intricate screening procedures. The principle behind the method involves the detection of AHL compounds produced by a rhizobacterial isolate adhered on the surface of the root directly in a bacterial growth medium using a biosensor reporter strain overlaid in it. If the AHL produced by the rhizobacteria is recognized by the biosensor strain, the QS bacterial colonies will appear as usual creamy or white colonies, which are surrounded by purple-colored (violacein-produced) CV026. This indicates the exogenous production of AHL by the bacteria appeared from the root, induced the violacein production of CV026 through QS. By careful distinction between the rhizobacterial colonies from the CV026 background, we can obtain the QS-positive strains.

Bradyrhizobium strains from groundnut (Nievas et al., 2012), *Enterobacter* from lettuce leaves (Lau et al., 2013), *Serratia glossinae* from sesame rhizosphere (Jung et al., 2017) and *Sinorhizobium meliloti* from arbuscular mycorrhizal spores (Palla et al., 2018) were isolated routinely using conventional methods and later screened for AHL production using reporter strains such as *Chromobacterium violaceum* (CV026) and *Agrobacterium tumefaciens* (NTL1). Compared to these, the method standardized by us could be easier and reliable to spot the QS-positive bacteria adhered on the surface of the root. In the present work, we further characterized these five isolates obtained from rice root for AHL identity and biofilm formation, in order to validate the methodology. We could find that all the five isolates produced a single AHL molecule in the culture supernatant, which was identified as N-butyryl L-homoserine lactone. It is a C4-HSL type QS molecule, commonly produced by several plant-associated bacteria such as *Pseudomonas aeruginosa* (Parsek and Greenberg, 2000; Singh et al., 2000), *Aeromonas veronii* (Zhao et al., 2018), *Aeromonas hydrophila* (Yang et al., 2018), *Aeromonas caviae* (Lim et al., 2014) and *Enterobacter asburiae* (Lau et al., 2013). In the present study, we got *Pseudomonas sihuiensis, Aeromonas hydrophila,* and *A. caviae* as QS-positive bacteria from rice root through the new method. This result confirms that the method standardized for isolation of QS-positive bacteria from root surface could detect the bacterial isolates without any false background and also reveals that more than one species of bacteria present in the root can be isolated. We have used *Chromobacterium violaceum* (CV026) as reporter strain for this method which can detect the AHL with the chain length of C4 to C8 only (McClean et al., 1997). For detection of long-chain AHL producing rhizobacteria, *Agrobacterium tumefaciens* (strain NTL1) could be used instead of CV026 (Farrand et al., 2002). We also assume that this method can detect the quorum-quenching bacteria residing in the plant roots, which produce inhibitors for quorum sensing.

We conclude that by overlaying the biosensor strain with the plant root in a growth medium, the quorum-sensing positive rhizobacteria could be isolated directly. This method is rapid, simple and highly reproducible, which can be modified by different biosensor strains according to the target of the length of AHLs or for isolation of quorum quenching bacteria.

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Conflict of interest

The authors declare that they have no competing interests.

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