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Detection of Quorum Sensing Molecules and Biofilm Formation in *Ralstonia solanacearum*

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Abstract Many bacteria use small diffusible signaling molecules to communicate each other termed as quorum sensing (QS). Most Gram-negative bacteria use acyl homoserine lactone (AHL) as QS signal molecules. Using these signaling molecules, bacteria are able to express specific genes in response to population density. This work aimed to detect the production of QS signal molecules and biofilm formation in *Ralstonia solanacearum* isolated from various diseased tomato plants with symptoms of bacterial wilt. A total of 30 *R. solanacearum* strains were investigated for the production of QS signal molecules using *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NT1 (pZLR4) biosensor systems. All 30 bacterial isolates from various bacterial wilt-affected tomato plants produced AHL molecules that induced the biosensor. The microtiter plate assay demonstrated that of the 30 bacterial isolates, 60 % formed biofilm, among which four isolates exhibited a higher degree of biofilm formation. The biofilm-inducing factor was purified from these four culture supernatants. The structure of the responsible molecule was solved using nuclear magnetic resonance and mass spectroscopy and was determined to be 2-hydroxy-4-((methylamino)(phenyl)methyl) cyclopentanone (HMCP), which was confirmed by chemical synthesis and NMR. The Confocal laser scanning microscopic analysis showed well-developed biofilm architecture of bacteria when treated with HMCP. The knowledge we obtained from this study will be useful for further researcher on the role of HMCP molecule in biofilm formation.

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Introduction

Ralstonia solanacearum is a Gram-negative soil-borne β-proteobacterium that is the causal agent of bacterial wilt and one of the most devastating bacterial plant diseases in the world [14]. The strong impact of this pathogen results from its worldwide geographical distribution and its wide host range. *R. solanacearum* is pathogenic to more than 200 plant species belonging to over 50 different botanical families [23]. After entering plant roots, the pathogen invades the xylem vessels and rapidly spreads to aerial parts of the plant throughout the vascular system. Typical wilt symptoms result from altering water fluxes in the plant due to excessive production of extracellular polysaccharides within the vascular system. This pathogen relies on a complex regulation network that coordinates the expression of its various pathogenicity determinants according to the environmental stimuli. A key player in this regulatory system is hypersensitive reaction and pathogenicity (HrpG), which directly or indirectly controls the transcriptional induction of more than 350 genes in the presence of plant cells, including those directing the synthesis of the Type III secretion system (T3SS) and effector substrates that are essential for pathogenesis [26].

Acyl homoserine lactone (AHL)-mediated quorum sensing is widely prevalent in Gram-negative bacteria. It is composed of an AHL synthase (LuxI-type family protein)

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and an AHL reporter (LuxR-type family transcription regulator). LuxI is responsible for the synthesis of the AHL signaling molecule, and LuxR is the receptor and the transcriptional activator of Lux-controlled genes. In many Gram-negative bacteria, homologous LuxI/LuxR systems have been identified, each capable of producing specific AHLs. The AHLs are composed of a conserved homoserine lactone moiety and a fatty acid side chain that can vary in length (4–18 carbon atoms) [8].

The growth of *R. solanacearum* in plant tissues, particularly during disease development, probably leads to the formation of colonies that can be linked to biofilms [20]. Molecules such as AHL, oligopeptides, and amino acids (like glutamate, aspartate, and fatty methyl esters) may impact biofilm physiology [17, 19]. The QS mechanism enables bacteria to make collective decisions with respect to the expression of a specific set of genes involved in biofilm formation and virulence. QS plays a key role in orchestrating the expression of several secondary metabolites, siderophores, exoproteases, and exotoxins and participates in the development of biofilms [2]. Biofilms have a characteristic structure consisting of microcolonies enclosed in a hydrated matrix of microbially produced proteins, nucleic acids, and polysaccharides. In this complex biofilm network, the cells act less as individual entities and more as a collective living system, often with channels to deliver water and nutrients to the cells at the inner portion of the biofilm [3]. Microbial societies that form biofilms present with their own defense and communication systems. Very little is known about the factors required for biofilm formation in *R. solanacearum* [1]. There are reports suggesting that an autoinducer play an important role in biofilm formation [4, 9].

The aim of this study was to detect the production of AHLs among *R. solanacearum* isolated from various diseased tomato plants with symptoms of bacterial wilt. The isolates were tested for biofilm formation using a well-established reporter assay. Several QS detection systems have been described in the literature, the majority of which make use of reporter strains that have a different affinity for detecting different molecules [6]. These strains have been genetically modified by placing reporter genes under the control of a QS-inducing promoter. As many biosensors detect a narrow range of AHLs, each responding to different structural features, it is essential to use several biosensors when testing bacteria for AHL production. Some of the strains can detect a narrow range of AHLs, such as *C. violaceum* CV026, which detects short (C4–C8) mostly unsubstituted AHLs [19]. Other strains can detect a broader AHL range, such as *A. tumefaciens* NT1 (pZLR4), which can detect short to long AHLs (C4–C18), including most of the 3-oxo and 3-hydroxylated AHL derivatives [10]. Although it cannot provide the exact AHL structure, using a combination of different sensor strains is a simple method for the initial characterization of AHL molecules, and determining the activation pattern of different biosensors can reflect different AHL production profiles in different strains. In this study, the isolates were screened for AHL signal molecules using the biosensor strains *C. violaceum* CV026 and *A. tumefaciens* NT1 (pZLR4) and subjected to TLC assay. The isolates were tested for biofilm formation using a microtiter plate method. The differences in the formation of biofilm among the *R. solanacearum* isolates were studied using cell-free extracts, which were purified using HPLC. Structure elucidation of the compound responsible for these differences was achieved using NMR and MS techniques.

Materials and Methods

Bacterial Strains, Media, and Culture Conditions

Two different AHL monitor strains were used to screen AHL-producing isolates of *R. solanacearum*. CviR of *C. violaceum* CV026 strain regulates the production of a purple pigment when induced by AHLs [18]. *A. tumefaciens* NT1 (pZLR4) carries a lacZ fusion to TraI and produces a blue color in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in response to AHLs [10]. All strains were grown in or on Luria broth (LB) (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl), solidified with 1.2 % agar when required and supplemented with the appropriate antibiotics; 100 μg/ml kanamycin for *C. violaceum* CV026 and 30 μg/ml gentamicin for *A. tumefaciens* NT1 (pZLR4) when necessary.

The *R. solanacearum* strains were isolated from various tomato plants with symptoms of bacterial wilt. The most frequently and commonly used method for the detection and identification of *R. solanacearum* is isolation on 2,3,5-triphenyltetrazolium chloride (TZC) agar (Kelman 1954) [15]. Plant material was chopped into sterile water using a disinfected scalpel, and pieces were allowed to remain in the water for 30 min so that bacteria had time to migrate from the plant material. After 30 min, two loops full of the suspension were streaked onto TZC agar. After 48 h of incubation, colonies with morphology similar to *R. solanacearum* were purified and identified by polymerase chain reaction (PCR) using the previously published primers: 759(5′-GTCGCCGTCAACTCACTT TCC-3′) and 760(5′G TCGCCGTCAGCAATGCGGAATCG-3′) [16]. Genomic DNA was extracted using a DNA isolation kit (Bangalore Genei, Bangalore, India) according to the manufacturer's instructions. The final PCR amplification reaction mixture contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.05 mM of each dNTP, 25 pmol of primers, 1 μl of genomic template

DNA, and 0.5 U of Taq DNA polymerase. The PCR amplifications were performed using a MultiGene thermocycler (Labnet International, Inc. USA) with the following PCR temperature cycling parameters: denaturation at 94 °C for 3 min, annealing at 53 °C for 1 min, and extension at 72 °C for 1.5 min, followed by 30 cycles of 94 °C for 15 s, 60 °C for 15 s, 72 °C for 15 s, and a final extension of 72 °C for 5 min. PCR products were separated in 1.5 % agarose gels, stained with ethidium bromide and visualized under UV (302 nm) irradiation. The isolated and confirmed strains were maintained in sterile distilled water at −20 °C.

Screening of *R. solanacearum* for AHL Production

The AHL production of *R. solanacearum* was screened according to the method of Pinto et al. [22] using the biosensor strains *C. violaceum* CV026 and *A. tumefaciens* NT1 (pZLR4). These biosensors do not produce AHLs but respond to exogenous AHLs. The strain to be tested for induction, *C. violaceum* CV026 was streaked onto a LB agar plate in parallel to the monitored strain. Testing for AHL production against *A. tumefaciens* NT1 (pZLR4) was performed in a similar assay supplementing the agar with 70 µg/ml X-gal. Plates were incubated for 24 h at 25 °C, with the exception of the *A. tumefaciens* NT1 (pZLR4) streaked plates, which were incubated up to 3 days. The negative controls were the monitor strains themselves.

Preparation of Cell-Free Culture Fluids of *R. solanacearum* Strains

Extracts of culture supernatants were prepared as described by Shaw et al. [25] with minor modifications. Extracts were prepared from 500 ml cultures after growth in LB medium for 20 h at 30 °C with agitation at 100 rpm. Bacteria were removed by centrifugation at 10,000 rpm for 10 min, and the supernatants were extracted with an equivalent volume of ethyl acetate-containing glacial acetic acid at 0.1 ml/l. The mixture was shaken vigorously for 30 s using a separatory funnel, and the phases were allowed to separate. The shaking was repeated three times before the ethyl acetate-containing fraction was removed, and another 10 ml fraction was added. The whole extraction process was repeated three times. The combined ethyl acetate fractions were filtered, dried via rotary evaporation at 40–45 °C, reconstituted in 400 µl of acidified ethyl acetate and stored at −20 °C.

Thin-Layer Chromatography (TLC)

Extract samples dissolved in ethyl acetate in volumes of 10 µl were spotted onto 20 × 20 cm C18 reversed-phase TLC aluminum sheets (Merck, Germany), and chromatograms were developed using methanol/water (60:40, v/v) until the front reached the top, as described by Shaw et al. [25]. The TLC plate was allowed to dry while an agar cover layer was prepared of *C. violaceum* CV026. Thirty ml of an overnight culture of *C. violaceum* CV026 was used to inoculate 150 ml of LB medium containing 0.8 % agar; culture was mixed and spread immediately over the surface of the developed plates. After the agar was solidified, the coated plates were incubated at 25 °C overnight in a closed plastic container. Standards used on TLC included synthetic *N*-octanoyl homoserine lactone (OHL).

Biofilm Formation Assay Using the Microtiter Plate Method

The basic concept of this method was adopted from Anbazhagan et al. [2] with some modifications. Approximately 200 µl of LB was added into each well of a sterile 96-well polystyrene plate (Nunc, USA) followed by the addition of 2 µl of bacterial culture that was grown at 30 °C overnight with shaking at 100 rpm LB broth without bacterial culture was used as a control. The plate was covered and incubated without shaking at 30 °C for 18 h. At this time, 1 µl from each well was transferred into triplicate wells containing 100 µl of fresh LB and plates were incubated without shaking at 30 °C for 24 h. The supernatant was discarded carefully followed by staining each well with 150 µl of 1 % crystal violet (Qualigens, India) for 30 min at room temperature. The stain was removed, and the wells were washed twice with sterile deionized water. To the wells was added 175 µl of dimethyl sulfoxide (DMSO) to solubilize the crystal violet. The plate was then read spectrophotometrically at an absorbance wavelength of 570 nm. Three wells per experiment were used to investigate each strain in three independent experiments.

Statistical Analysis

The biofilms formed by the 30 isolates were statistically analyzed by one-way analysis of variance (ANOVA) with post hoc multiple comparisons. The P value was less than 0.05. All statistical tests were performed using the SPSS software.

Purification of HMCP from Cell-Free Extract

The extracts of the isolates which exhibited a higher degree of biofilm formation were fractionated by HPLC. The concentrated extracts of culture supernatants from isolated *R. solanacearum* strains were applied to a C8 reverse-phase analytic HPLC column (Waters, USA) and eluted with a

linear gradient of acetonitrile in water (5–100 %) at a flow rate of 1 ml/min over a 12 min period monitored at 190 and 211 nm. The eluent was collected as 0.2 ml fractions. The fractions which influenced the biofilm formation were pooled, dried, and dissolved in an appropriate solvent for NMR and mass spectrometry analysis.

Nuclear Magnetic Resonance and Mass Spectrometry

Extracts of the isolates which exhibited a higher degree of biofilm formation were subjected to NMR and mass spectrometry. Proton NMR spectra were obtained at room temperature on a Bruker Avance 400 MHz spectrometer (Bruker cop. Germany) with chemical shift values given in ppm relative to trimethylsilane (TMS) as an internal standard. Approximately five mg of the sample was dissolved in deuterated chloroform ($CDCl_3$) and was added to a clean NMR tube [28].

Mass spectral studies were carried out on a Q-TOF micro mass spectrometer (Waters Inc., USA) in ESI–MS mode. One mg of the compound was dissolved in HPLC-grade acetonitrile. The target analyte was uniquely identified by its parent-to-product ion mass transitions. Data acquisition and analysis were accomplished using the EMPOWER software package (version 3.0) [5].

Chemical Synthesis of HMCP

To a cold (−78 °C) solution of N-methyl-1-phenylmethylamine (30 mg, 0.25 mM) in freshly distilled dry tetrahydrofuran (THF) (25 ml) was added *n*-butyllithium via syringe (1.12 ml, 1.1 M in hexanes). The mixture was stirred at this temperature for 30 min and then allowed to come to room temperature. To this was added a solution of 2-hydroxy-4-methylcyclo pentanone (27 mg, 0.23 mM) in THF drop-wise, followed by continued stirring for an additional 12 h. The reaction mixture was filtered through a pad of Celite. The solvent was evaporated under reduced pressure to obtain a pale yellow solid. The compound was extracted with dichloromethane and filtered through Celite. The desired compound was obtained from the filtrate and was purified by column chromatography using ethyl acetate/petroleum ether (1:1) to yield a colorless solid.

Effect of HMCP on Biofilm Formation

The effect of HMCP on biofilm formation was tested as described under the section titled "Biofilm formation assay using the microtiter plate method". Ten isolates that were considered to be non-biofilm formers were selected. A set of analyses was carried out by adding (0.2 μg/ml) HMCP to a bacterial culture and comparing it to a bacterial culture lacking HMCP. An isolate that did not produce HMCP was used as a control. Each isolate was investigated with three wells per experiment, over three independent experiments.

Confocal Laser Scanning Microscopic (CLSM) Analysis

Overnight cultures of the *R. solanacearum* which were considered to be non-biofilm formers were added into 1 ml fresh LB medium containing cover glass of 1 cm^2 along with and without HMCP (0.2 μg/ml). After 16 h of incubation, the cover glasses were rinsed thrice with distilled water and biofilm formed on the cover glasses was stained with 20 μl of 1 % acridine orange. The excess stain was washed out, and the stained cover glasses were visualized with CLSM (Zeiss, LSM 710, Germany).

Results

Confirmation of *R. solanacearum* Isolates by PCR

The bacterial colonies that grew on TZC with morphology similar to *R. solanacearum* along with the reference culture were subjected to DNA extraction, and PCR was carried out using species-specific primers 759 and 760, which produced a single 281 bp fragment (Fig. 1) typical of *R. solanacearum*, as reported by Opina et al. [21]. PCR products were purified and sequenced. The sequencing data obtained were aligned with database deposits in NCBI GenBank that displayed maximum homology. The sequences are deposited in GenBank with the following accession numbers, AB910593-AB910597, AB911030-AB911039, AB911324-AB911333, and AB914680-AB914684.

Screening for AHL Production Among the Isolates

When the *R. solanacearum* isolates were cross-streaked with the biosensors strains, all of the 30 isolates induced the biosensor strains, indicating positive results (Fig. 2). *C. violaceum* CV026 produced violacein, indicating a positive response, which demonstrated that those isolates produced AHLs that most likely had either short or medium acyl chains. The isolates also induced *A. tumefaciens* NT1 (pZLR4), which produced a blue pigment in response to AHLs. *A. tumefaciens* NT1 (pZLR4) detects short to long AHLs (C4–C18), including most of the 3-oxo and 3-hydroxylated AHL derivatives. This demonstrated that all of the 30 *R. solanacearum* isolates produced AHL molecules that induced the biosensor strain.

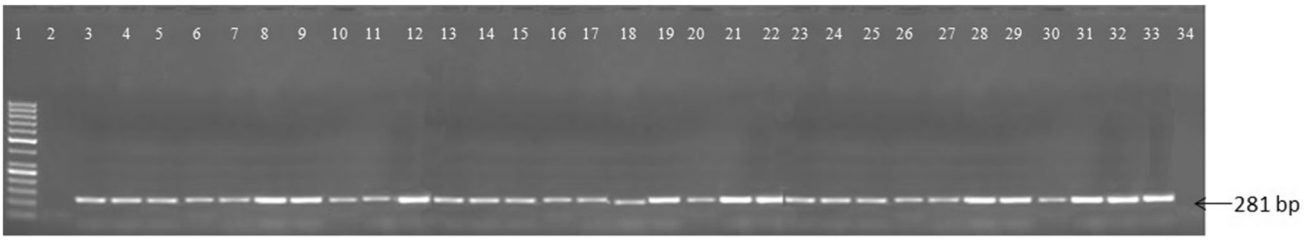

Fig. 1 PCR-based confirmation of *R. solanacearum* isolates. The amplified 281 bp PCR product is visible on the ethidium bromide-stained agarose gel. *Lane 1* DNA marker, *lane 2* negative control 1 (reaction mixture without template DNA), *lane 34* negative control 2 (reaction mixture with known *E. coli* DNA), *lane 3* positive control (an identified *R. solanacearum* strain from Central Potato Research Institute, Shimla, India), *lanes 4–33* contain amplicons from isolates

Thin-Layer Chromatography

To further confirm the agar assay results, TLCs were performed with AHL extracts from isolated *R. solanacearum* strains followed by confirmation with *C. violaceum* CV026 (Fig. 3). The TLC results indicated the presence of at least one type of AHL in the extracts of *R. solanacearum* isolates, confirming the results of the agar assay. *R. solanacearum* produced a compound which activated *C. violaceum* CV026 and migrated on the TLC plate similar to OHL (standard) with R_f value of 0.43, which are similar to R_f value found by Shaw et al. [25].

Biofilm Formation Assay Using the Microtiter Plate Method

The quantification of biofilm produced by the 30 isolates of *R. solanacearum* revealed that 18 (60 %) isolates significantly formed biofilm, among which four isolates (S2, S4, S15, and S22) exhibited higher relative amounts of biofilm formation (P value <0.05). Figure 4 displays the graphical representation of the biofilm formation among all of the 30 isolates. Isolates without a change in the optical density compared to the control were considered to be non-biofilm formers. The assay was performed in triplicate, and data are the means of these experiments.

Identification of HMCP by NMR and Mass Spectrometry

NMR spectrometric analysis revealed the presence of 2-hydroxy-4-((methylamino)(phenyl)methyl)cyclo pentanone (HMCP) in the tested four isolates (S2, S4, S15, and S22) which exhibited higher degree of biofilm formation. The proton NMR spectrum of HMCP (Fig. 5a) was recorded using a 400-MHz spectrometer and $CDCl_3$ as a solvent. The comprehensive data for HMCP are given as follows (Fig. 5b): The multiplet at δ 7.5–7.8 ppm (5H, *m*, Ar–H) was due to aromatic protons. The doublet of doublets at δ 3.31 to 3.50 (2H, *dd*, CH_2) was assigned to methyl protons. The signal at δ 2.9 ppm was due to the methyl group attached to the nitrogen atom. The signal at δ 2.75 ppm (1H, *dd*, CH) was assigned to the C-8 proton of the cyclopentanone ring. The signals in the aliphatic region δ 1.6 and δ 1.9 ppm (2H, *dd*, CH_2) were attributed to the cyclopentanone ring-bearing methyl protons.

Details of the acquisition of the mass spectra using the ESI–MS technique have already been mentioned. Very briefly, the presence of the HMCP molecule was further supported by positive mode ESI mass spectrometry, which documented a base peak at *m*/*z* 265.5 corresponding to the $[M + 2Na]^+$ ions and having a molecular formula of $C_{13}H_{17}NO_2$ (Fig. 5d). In addition, the spectrum exhibited peaks that were assignable to various fragments arising from the thermal cleavage of HMCP. To unambiguously confirm the chemical structure of HMCP, we synthesized HMCP. The synthetic product was analyzed by proton NMR and gave a spectrum identical to the purified natural HMCP (Fig. 5c).

Effect of HMCP on Biofilm Formation

HMCP resulted in a significant growth pattern in biofilm formation by bacteria. The biofilm formation rate of bacteria responded well when HMCP was added to the bacterial culture; there was increase in biofilm formation (Fig. 6) compared to the control. The CLSM analysis showed well-developed biofilm architecture of bacteria when treated with HMCP (0.2 μg/ml), with that of control (Fig. 7). This indicates that HMCP plays a very prominent role in biofilm formation by bacteria.

Discussion

QS is a regulatory mechanism which enables bacteria to make collective decisions with respect to the expression of a specific set of genes. In our study, all isolates were screened for quorum sensing signal molecule production, and these isolates effectively induced *C. violaceum* CV026

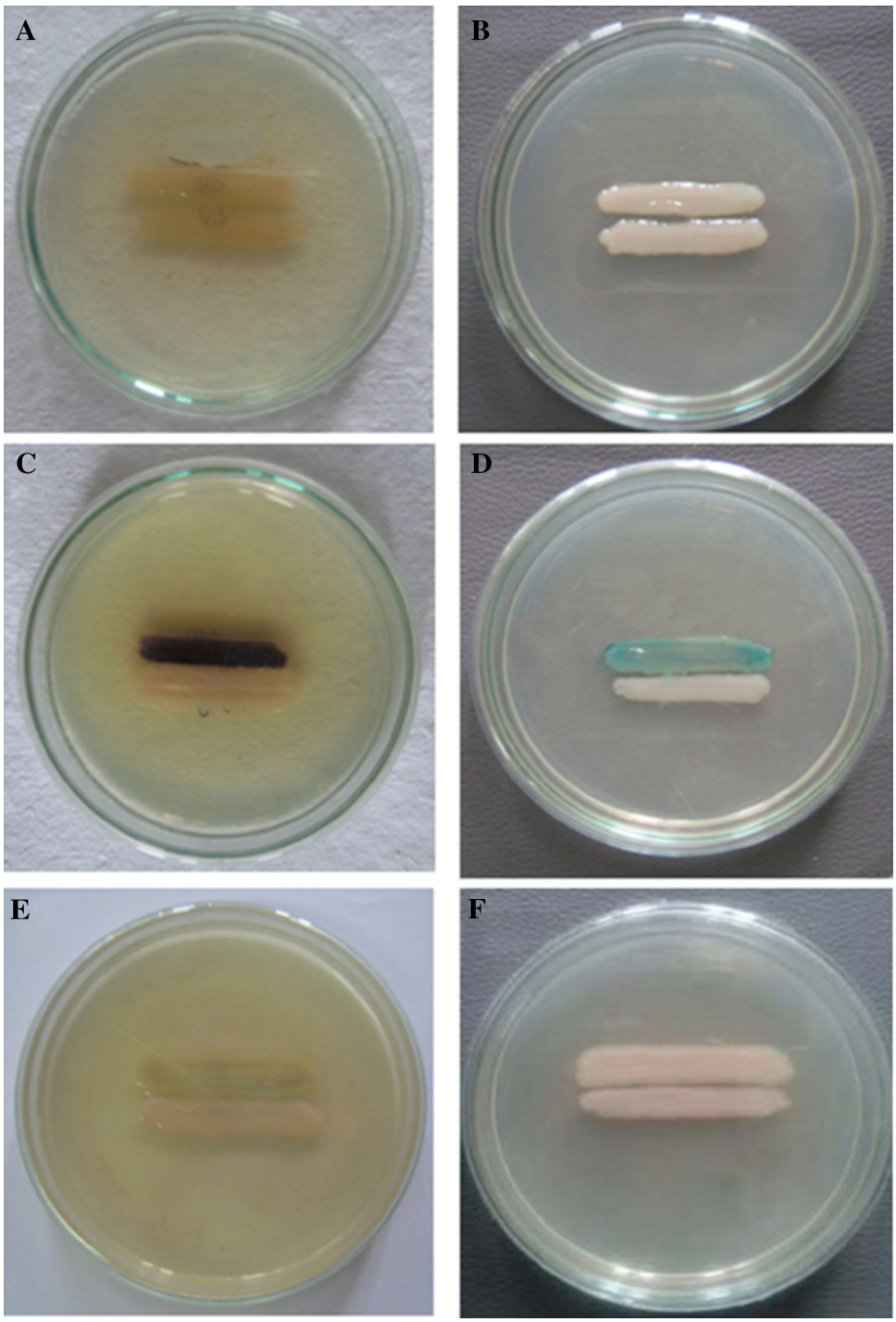

Fig. 2 Agar plate assays for screening the production of AHLs in *R. solanacearum* isolates using *C. violaceum* CV026 and *A. tumefaciens* NT1 (pZLR4) as a monitoring system. Monitor strains were streaked parallel to the tested bacteria or by themselves, as described in "Materials and Methods" section. **a** and **b** response to *C. violaceum* CV026 and *A. tumefaciens* NT1 (pZLR4), respectively, parallel to themselves relative to the negative controls; **c** and **d** response to positive result; **e** and **f** response to negative result

and *A. tumefaciens* NT1 (pZLR4) biosensor monitoring systems, which indicated that all of the isolates produced one or more AHL molecules. The method used for screening pure cultures of *R. solanacearum* for AHL production using the *C. violaceum* CV026 and *A. tumefaciens* NT1 (pZLR4) monitor systems is rapid and does not require sterile supernatants or AHL extracts from cultures. The *R. solanacearum* isolates that were positive in the induction assay of *C. violaceum* CV026 may produce AHL compounds with unsubstituted side chains from C4 to C8 in length, since this monitor strain responds to these type of AHLs [18] and *A. tumefaciens* NT1 (pZLR4) displays the broadest sensitivity to AHLs at the lowest concentrations [10]. 3-OH PAME is the only endogenous fatty acid isolated from *R. solanacearum*, other than the acyl homoserine lactones, which has been shown to be an auto-regulator

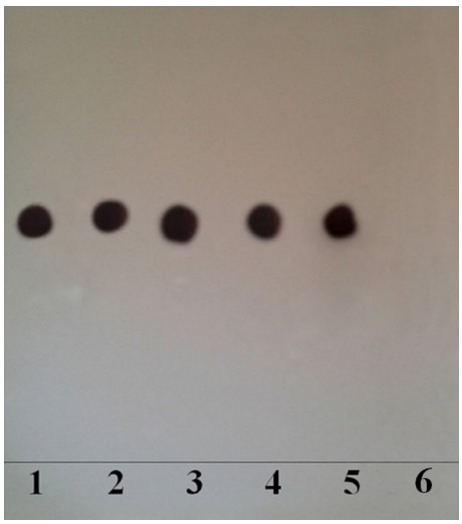

Fig. 3 A thin-layer chromatogram of the AHLs present in cell-free supernatants of *R. solanacearum* isolated from bacterial wilt-diseased plants. Samples were chromatographed on C18 reversed-phase thin-layer plates developed with methanol/water (60:40, v/v), and the spots were visualized with the *C. violaceum* CV026 reporter strain. *Lanes 1–4*, samples from culture extracts of *R. solanacearum*; *lane 5* *N*-octanoyl homoserine lactone standard; lane 6, extracts from LB medium used as a negative control

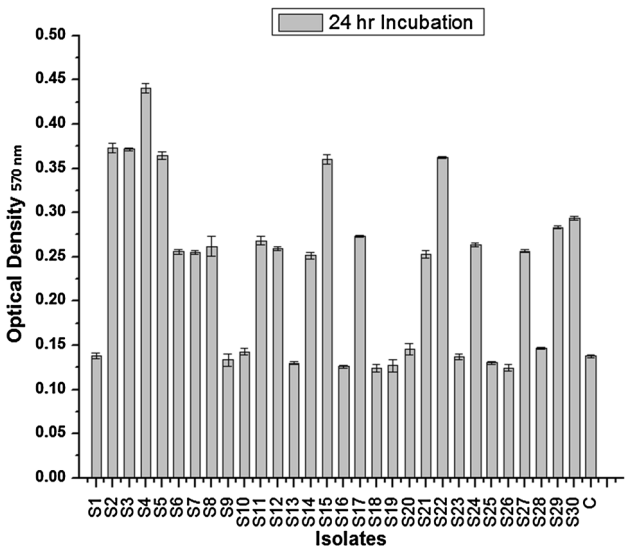

Fig. 4 Biofilm formation among the thirty isolates of *R. solanacearum* in microtiter plates. Strain numbers are designated in the *x-axis*. C is the control (without bacterial culture). Approximately 60 % of the isolates form significant biofilm. Data are pooled from triplicate experiments. The results are expressed as the mean, and the *error bars* indicate the standard deviation. The *P* value was less than 0.05 ($P < 0.05$)

and may be the first example of a new family of compounds that can mediate long distance intercellular communication [11].

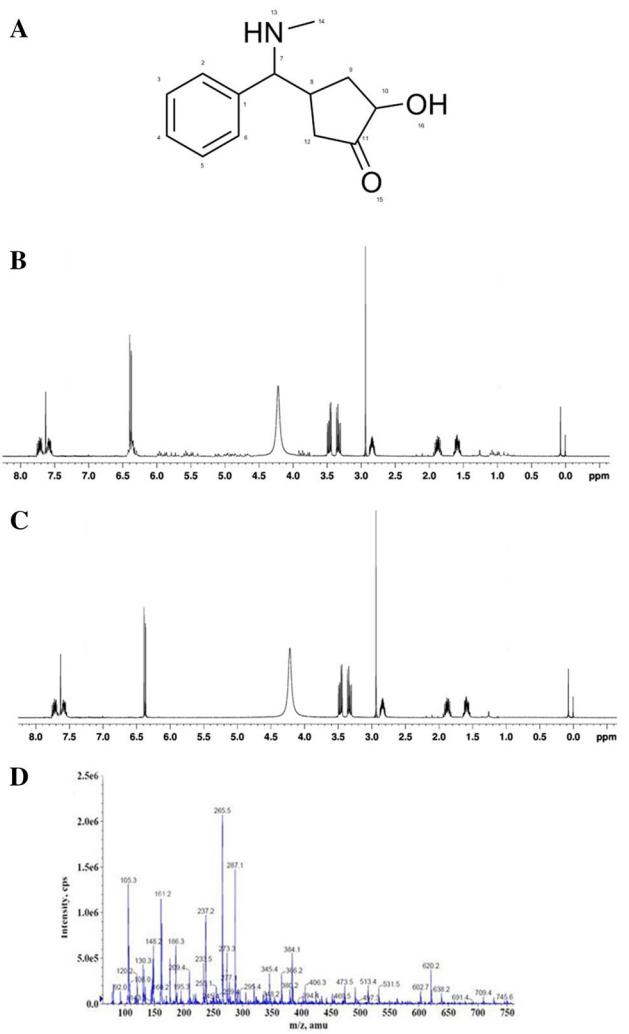

Fig. 5 NMR and mass spectra of HMCP. **a** Structure of HMCP; **b** 1H NMR spectrum of HMCP extracted from *R. solanacearum* growth medium; **c** 1H NMR spectrum of synthesized HMCP; **d** Mass spectrum of HMCP

The TLC assay developed with monitor strains has previously been used for the characterization of AHL compounds [13]. Separation by TLC coupled with biodetection gives a direct, visual catalog of the AHL signal molecules, detectable by the biosensor, present in supernatants of bacterial cultures [25]. In this study, we used the *C. violaceum* CV026 sensor system in developing TLC plates and identified AHL products from *R. solanacearum* isolated from wilt-affected tomato plants in an approach similar to that used by Cha et al. [6]. TLC profiling of several *R. solanacearum* isolates indicated that the preliminary identification of AHL products based on agar plate screening correlated well with TLC profiles. From TLC assay, it was confirmed that *R. solanacearum* isolates produced OHL. Detection of AHLs by TLC using the

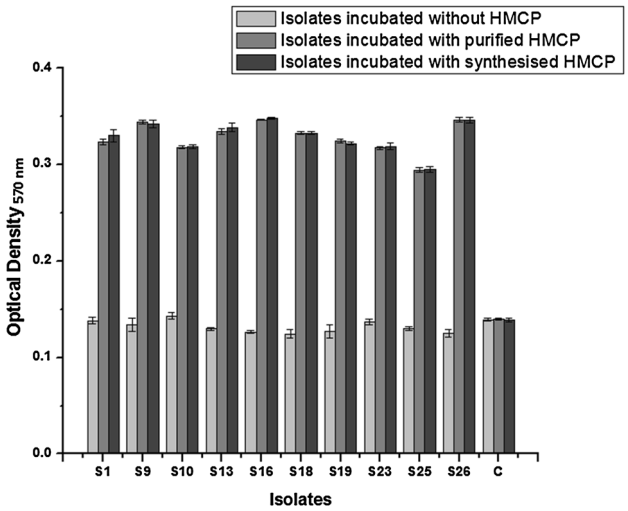

Fig. 6 Effect of HMCP on biofilm formation in microtiter plates. Strain numbers are designated in the *x-axis*. The isolates S1, S9, S10, S13, S16, S18, S19, S23, S25, and S26 were tested for biofilm formation upon incubation with and without HMCP. There was an increase in biofilm formation in the isolates incubated with HMCP when compared to those without HMCP, with reference to the control C (isolate that did not produce HMCP), demonstrating that HMCP plays a major role in biofilm formation. Data are pooled from triplicate experiments. The results are expressed as the mean, and the *error bars* indicate the standard deviation. The *P* value was less than 0.05 ($P < 0.05$)

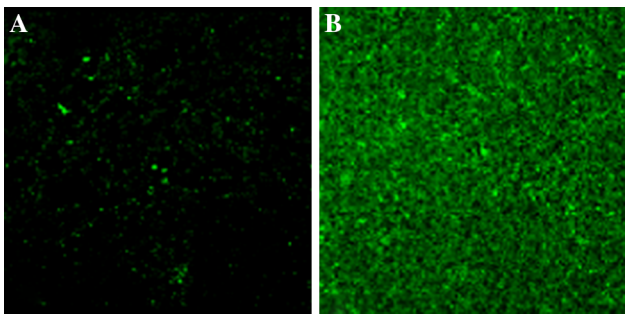

Fig. 7 Confocal laser scanning microscopy images of test bacterial biofilm grown in the **a** absence and **b** presence of HMCP (0.2 μg/ml)

biosensor strain has been reported by several scientists [12, 24, 25].

As a mechanism of communication to coordinate behavior, QS has been found to have an influence on biofilm formation [27]. Biofilm formation is now considered the dominant mode of growth for bacteria in natural habitats, clinical environments, and diseases [7]. It was observed that approximately 60 % of the *R. solanacearum* isolates formed a significant amount of biofilm, out of which four isolates (S2, S4, S15, and S22) exhibited a higher degree of biofilm formation. The biofilm-inducing factor was purified from four culture supernatants (S2, S4, S15, and S22). The structure of the responsible molecule was solved using NMR and MS, and determined to be HMCP, with a mass of 265.5 amu. This assignment was confirmed by chemical synthesis and NMR. The HMCP was subjected to bioassays, and we could found that it was not able to activate either of the biosensor strains. From this, we came to know that HMCP cannot be considered as AHL molecule.

In this study, it was demonstrated that, out of the eighteen biofilm-forming isolates, only four isolates produced HMCP. This may be because biofilm formation is a multifactorial event, and QS signals are only one of such factors. Our finding supports HMCP involvement in bacterial cell–cell signaling stems in which it induces biofilm formation. HMCP was observed in the cell extracts of the S2, S4, S15, and S22 isolates. The ten isolates that were considered to be non-biofilm formers displayed a significant increase in biofilm formation after the addition of HMCP to the culture media. The results of CLSM analysis also showed that the biofilm was well developed with the addition of HMCP. We isolated HMCP from *R. solanacearum* from wilt-infected tomato plants and characterize its structure and mass using NMR and mass spectrometry. It has also been demonstrated that HMCP plays a major role along with other AHL molecules in biofilm formation in *R. solanacearum*. The identification of HMCP will greatly facilitate future studies to unravel the contribution of HMCP dependent signaling to bacterial infection.

To conclude, we have used simple methods for the detection of QS molecules using the *C. violaceum* CV026 and *A. tumefaciens* NT1 (pZLR4) monitor systems. Based on our studies, we found that the signaling molecule in *R. solanacearum* isolates to be OHL. We also found that the HMCP plays a major role in biofilm formation. Further studies are required to elucidate the role of HMCP in development of disease in host. Still, new molecules and their effects on microbial virulence continue to be discovered.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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