Modulation of quorum sensing controlled behaviour of bacteria by growing seedling, seed and seedling extracts of leguminous plants

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Received: 28 September 2008 / Accepted: 31 December 2008

Abstract Effect of growing seedling, seeds and seedlings extracts from seven leguminous plants (*Pisum sativum*, *Vigna radiata*, *Vigna mungo*, *Cajanus cajan*, *Lentil culinaris*, *Cicer arietinum* and *Trigonella foenum graecum*) were screened for their ability to influence quorum sensing controlled pigment production in *Chromobacterium violaceum* indicator strains (CV12472 and CVO26). Germinating seedling and seedling extracts of only *P. sativum* (pea) showed inhibition of violacein production. Interestingly, the *T. foenum graecum* (fenugreek) seed extracts enhances the pigment production. Quorum sensing regulated swarming motility in *Pseudomonas aeruginosa* PAO1 was reduced by pea seedling extract while enhanced by the fenugreek seed extracts. These findings suggest that plant metabolites of some legumes interact actively with bacterial quorum sensing and could modulate its associated functions.

Keywords Legume · Seedling and seed extracts · Quorum sensing modulation · *Chromobacterium violaceum* · Swarming motility

Introduction

Many Gram negative bacteria regulate expression of gene sets in response to population density. This regulatory mechanism, called autoinduction or quorum sensing (QS), is based on production of a small, diffusible signal molecule called, autoinducer, by the bacteria. In Gram negative bacteria, both plant and human associated pathogens, most common type of QS signal molecules are N- acyl homoserine lactones (AHLs) [1]. The quorum sensing - regulated bacterial functions have been characterized in *P. aeruginosa*, *C. violaceum*, *Burkholderia cepacia*, *Vibrio spp.*, *Erwinia carotovora*, *Pantoea stewartii*, *Ralstonia solanacearum* and *Xanthomonas campestris* by several workers [2]. Various functions such as biosynthesis of antibiotics, production of virulence factors, exopolysaccharide biosynthesis, bacterial swarming, plasmid conjugal transfer, as well as transition into stationary phase have been found under QS regulation in one or other Gram negative bacteria [3]. The AHLs produced by cells of one species can induce population density dependent responses in cells of other species in natural rhizospheric environment [4]. It is known that plants have evolved strategies to interfere with bacterial AHL signaling system, prevent initiation of pathogens attack. Such interference could include the production of signal mimics, signal blockers, signal degrading enzymes or production of compounds that block the activity of AHL producing enzymes [5]. The first example of AHL mimic compounds produced was halogenated furanone from marine red algae *Delisea pulchra* [6]. The furanones are structurally related to AHLs and inhibit the induction of AHL stimulated actions by binding the AHL receptor proteins [6]. The report that exudates of pea seedlings contain several activities that

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mimic AHL signals and stimulate or inhibit AHL regulated actions [5] has indicated the possible role of plant secreted metabolites on bacterial quorum sensing and plant–microbe interactions. In the recent years, QS inhibition and modulation of AHL activity by the extracts of medicinal and food plants have been documented [7, 8].

We hypothesized that like pea plant, QS interfering compounds might also be present in seed and seedling of other leguminous plants. Thus the objective of study was to detect the presence of QS modulatory activity in growing seedlings, seed and seedling extracts of seven leguminous plants using QS biosensor bacterial strains.

Materials and methods

Bacterial strains and growth conditions

C. violaceum CV12472 is a wild type bioreporter strain and produces purple pigment, violacein, under QS control. It produces and responds to the cognate autoinducer molecules i.e. C_4 and C_6 AHLs. *C. violaceum* (CVO26) is a mini Tn_5 mutant indicator strain derived from wild type *C. violaceum* (CV31532) strain and is unable to synthesize its own AHL but retains the ability to respond exogenous C_4 and C_6 AHL [9]. *C. violaceum* (CV31532) is a non pigmented, C_6 AHL over producing strain and hence being exploited to obtain signal molecules to be used in bioassay using CVO26 strain. *P. aeruginosa* PAO1 is a pathogenic strain and various behaviors of this strain including swarming motility are QS controlled. Unless other wise stated, all strains were grown in or on LB broth (1% peptone, 0.5% yeast extract, 0.5% NaCl) solidified with 1.5% agar when required and supplemented with antibiotics (kanamycin 20 µg/ml for CVO26). Growth temperature was 28°C and 37°C for *C. violaceum* and *P. aeruginosa* respectively.

Preparation of plant samples

The procedure for seed surface sterilization, seedling growth and bioassay for seedling were adopted as described by Teplitski et al [5] with little modifications wherever needed. Seven day old aseptically grown seedlings (100–150) were macerated in sterile distill water (5 ml) aseptically and extracted in methanol (200 ml) and ethanol (200 ml) separately, filtered and sterilized by membrane filter (0.2 µm). Similarly, surface sterilized seeds (500g) of test legumes were imbibed in sterile distill water (100 ml) for 6–8 h and ground aseptically and extracted with methanol (500ml) for 5 days with intermittent stirring. After the end of extraction, the filtrate was separated with Whatman filter paper No. 1 and then centrifuged at 10,000 rpm. Clear fil-

trates from above preparations were concentrated on rotary evaporator at 40°C. The plant material was also sequentially extracted in ethanol (500 ml) and processed as above. The dried extract was reconstituted in DMSO and different amount of the extract was tested in the bioassay.

Isolation of natural AHL from *C. violaceum* CV31532

The standard method, as described by Shaw et al [10] was adopted for isolation of AHL by growing *C. violaceum* CV31532 in 4L Luria broth on shaking incubator at 28°C for 18 h. The culture filtrate obtained by centrifugation was extracted with acidified ethyl acetate (0.1% v/v acetic acid) in the ratio 7:3 (supernatant: acidified ethyl acetate, v/v) and, finally concentrated and dried by rotary evaporator at 40°C and reconstituted in acetonitrile. The amount of partially purified AHL needed for production of violacein by CVO26 was first standardized by agar well diffusion plate assay.

Assay for inhibition of violacein production by growing seedlings

0.1 ml of freshly grown and appropriately diluted ($\sim 2.5 \times 10^6$ cfu/ml) CV12472 and CVO26 were mixed with 5 ml warm LB containing 0.5% agar, separately. For CVO26 strain, standardized amount (500 µl) of crude preparation of C_6 AHL was added in the soft agar suspension of test bacteria. Seven day old aseptically grown seedlings were placed on thin layer of soft agar containing the bacteria and incubated for overnight at 28°C [5]. Inhibition of pigment production near the seedling indicated quorum sensing inhibition.

Assay for QS modulation by seedling and seed extracts

Agar well diffusion assay [11] was performed with *C. violaceum* CV12472 and CVO26 for determination of pigment inhibition or enhancement activity of seed and seedling extracts. LB agar plates were spread with 0.1 ml of appropriately diluted ($\sim 2.5 \times 10^6$ cfu/ml) freshly grown cultures and wells of 8 mm diameter were made and sealed at the bottom by soft agar. Varying amounts of non inhibitory concentrations (20 µl to 100 µl) of the extracts were loaded in the agar well. Solvent and Luria broth was used as control. Plates were incubated at 28°C for 18–24 h to check the zone diameter of pigment inhibition/ enhancement around the well.

Swarming assay with *P. aeruginosa* PAO1

Effect of non inhibitory concentrations of pea seedling and fenugreek seed extracts on swarming of *Pseudomonas aeruginosa* was determined by the method as described by

Vattem et al [7]. Extent of swarming was determined by measuring the diameter of swarm.

Results and discussion

Quorum sensing is now known to regulate a number of bacterial functions both in Gram negative and Gram positive bacteria. In Gram negative bacteria most common signal molecules produced are acyl homoserine lactones. Interference with this cell to cell signaling process has been reported by certain natural and synthetic compounds. Development of bioassay based on QS indicator strains has made the easy detection of such activity. *C. violaceum* CV12472 and its mutant CVO26 are efficient strains which produce and or respond to C4 and C6 AHLs. Production of violacein (a violet pigment) is under QS. Inhibition or enhancement of this activity was detected by above assay. Similarly *P. aeruginosa* PAO1 is well studied QS indicator strain where a number of bacterial behaviors including swarming motility (a virulence factor) are under QS control. We have screened the QS modulatory activity of common legumes such

P. sativum, *V. radiata*, *V. mungo*, *C. cajan*, *L. culinaris*, *C. arietinum* and *T. foenum graecum* using *C. violaceum* strains. Growing seedling of Pea showed inhibition of pigment production in CV12472 and CVO26 strains while seedlings of other six legumes could not influence the pigment production (Table 1, Fig. 1). Assay with CVO26 strain

Fig. 1 Inhibition of violacein production by 7 day old germinating pea seedling on Luria agar plate

Table 1 Effect of seedling, seed and seedling extracts on quorum sensing controlled behavior of bacteria

Plant material	Effect on violacein production (diameter of zone of inhibition/enhancement in mm)		Effect on swarming motility (% reduction/increase in swarm diameter)*	Effect on the growth of <i>Chromobacterium violaceum</i> CV31532
	<i>Chromobacterium violaceum</i> CV12472	<i>Chromobacterium violaceum</i> CVO26	<i>Pseudomonas aeruginosa</i> PAO1	
<i>P. sativum</i>				
Germinating seedling	+	+	NT	-
Seedling extract	+ (25)	+ (23)	(37.93%) (18mm)	-
Seed extract (methanolic)	-	-	-	-
Seed extract (ethanolic)	-	-	-	-
<i>T. foenum graecum</i>				
Germinating seedling	-	-	-	-
Seedling extract	-	-	-	-
Seed extract (methanolic)	+ (15)	+ (14)	(34.48%) (39mm)	-
Seed extract (ethanolic)	+ (13)	+ (13)	-	-
<i>Other legumes</i>				
<i>V. radiata</i> , <i>V. mungo</i> , <i>C. cajan</i> , <i>L. culinaris</i> , <i>C. arietinum</i>				
Germinating seedling	-	-	-	-
Seedling extract	-	-	-	-
Seed extract (methanolic)	-	-	-	-
Seed extract (ethanolic)	-	-	-	-

+ = active, - = No effect, = enhancement, = inhibition, NT= not tested

*Diameter of swarm in control was 29 mm

required standardized amount of natural AHL extracted from CV31532 strain (Fig. 2).

Seedling extract of pea at non inhibitory concentration (100 μ l) showed zone of pigment inhibition of 25 mm and

Fig. 2 Induction of violacein formation by *Chromobacterium violaceum* CVO26 in presence of natural C₆ AHL extracted from

23 mm in CV12472 and CVO26 strains, respectively. Other plants could not influence the pigment production. On the other hand, seed extracts of *T. foenum graecum* showed zone of pigment enhancement (13 to 15 mm) at 100 μ l against both test strains. The tested concentration of seed extracts were non inhibitory to the growth of all test strains. Seed extracts of other legumes were inactive (Table 1, Fig. 4). This is the first report on fenugreek seed extracts showing QS modulation activity. On the other hand methanolic extracts of pea seedling and fenugreek seed extracts demonstrated increase and decrease in swarming motility of *Pseudomonas aeruginosa* PAO1 respectively. Pea seedling extract at 40 μ l significantly reduced the swarm by 37.93%. In contrast, fenugreek seed extract resulted in the enhancement of swarm by 34.48% over control (Table 1, Fig. 5). On the basis of these observations, it seems that anti-QS compound in pea is produced during seedling growth only and is not present in the seeds. These outcomes are in agreement with the reports of Teplitski et al [5]. Further, inhibition of swarming motility in *P. aeruginosa* PAO1 confirmed

Fig. 3 Inhibition of violacein production by methanolic extract of pea seedling

Fig. 4 Enhancement of violacein production by methanolic extract of *Trigonella* seed

Fig. 5 Swarming assay with *Pseudomonas aeruginosa* PAO1 (a) Control (b) In the presence of methanolic extract (40 μ l) of pea seedling (c) In the presence of methanolic extract (40 μ l) of *Trigonella* seed

the interference of QS controlled behavior by pea seedling extracts. Similar observation was also made by Teplitski et al [5] against *Serratia liquefaciens*. Although, the active compounds responsible for above activity has not yet been characterized. It seems that the active compound is specific to pea and not common to other tested legumes as the seedling of other six plants were inactive.

The QS enhancement activity in *C. violaceum* and *P. aeruginosa* by *Trigonella* seed extracts indicated that plants might produce compounds that are directly or indirectly altering the bacterial AHL based signaling system and such compounds could prove to be important in determining the outcome of interactions between higher plants and bacteria. Few workers have also reported the interference on QS regulated virulence and modulation by extracts from dietary and medicinal plants [6, 8, 12]. It is expected that compounds produced by plants may have regulatory role in defining the nature of plant-bacteria interactions [2]. These findings have highlighted the possible QS regulatory role of seed and seedling exudates in the rhizosphere and require further in-depth study using advanced methods of exploring QS interference [13]. The plant seedlings and root exudates of other leguminous and non leguminous plants should be studied in search of such activities. Further isolation and identification of bioactive compounds from plants is needed to assess their mode of action and possible role in plant-microbe interactions.

Acknowledgement We are grateful to Professor Robert JC McLean, Department of Biology, Texas State University, USA for his kind cooperation and providing all biosensor bacterial strains used in this study. We are also thankful to University Grant Commission, New Delhi for providing financial assistance in the form of Research Project No. FN 33-208/2007(SR).

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