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ORIGINAL PAPER

Arachis hypogaea L. produces mimic and inhibitory quorum sensing like molecules

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Abstract A wide variety of plant–associated soil bacteria (rhizobacteria) communicate with each other by quorum sensing (QS). Plants are able to detect and produce mimics and inhibitor molecules of the QS bacterial communicative process. *Arachis hypogaea* L. (peanut) establishes a nitrogen-fixing symbiosis with rhizobia belonging to the genus *Bradyrhizobium*. These bacteria use a QS mechanism dependent on the synthesis of *N*-acyl homoserine lactones (AHLs). Given the relevance that plant–rhizobacteria interactions have at the ecological level, this work addresses the involvement of peanut in taking part in the QS mechanism. By using biosensor bacterial strains capable of detecting AHLs, a series of standard and original bioassays were performed in order to determine both (i) the production of QS-like molecules in vegetal materials and (ii) the expression of the QS mechanism throughout plant–bacteria interaction. Mimic QS-like molecules (mQS) linked to AHLs with long acyl chains (lac-AHL), and inhibitor QS-like molecules (iQS) linked to AHLs with short acyl chains (sac-AHL) were detected in seed and root exudates. The results revealed that synthesis of specific signaling molecules by the plant (such as mQS and iQS) probably modulates the function and composition of the bacterial community established in its rhizosphere. Novel bioassays of QS detection during peanut–*Bradyrhizobium* interaction showed an intense production of QS signals in the contact zone between root and bacteria. It is demonstrated that root exudates stimulate the root colonization and synthesis of lac-AHL by *Bradyrhizobium* strains in the plant rhizosphere, which leads to the early stages of the development of beneficial plant–bacteria interactions.

Keywords *Bradyrhizobium* · Peanut · Quorum sensing · Rhizosphere colonization

Introduction

Bacteria are capable of communicating among themselves to coordinate group responses in order to adapt their physiology to environmental factors. A common bacterial mechanism of communication takes place when the population reaches a growth threshold at which bacteria detect and respond to their own chemical signals (autoinducers) through regulating gene expression. This communicative regulated process is defined as quorum sensing (QS) (Papenfort and Bassler 2016). *N*-acyl homoserine lactones (AHLs or acyl-HSL) are the most common autoinducers in Gram-negative bacteria (Hawver et al. 2016) and genes regulated by QS are often involved in

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coordinating interactive processes among bacteria and between bacteria and eukaryotic organisms (Sanchez-Contreras et al. 2007).

It has been widely shown that soil microorganisms interact with both the roots of plants and the soil constituents in the root–soil interface, a microniche defined as the rhizosphere (Tkacz et al. 2015; Hinsinger et al. 2009). This set of interactions contributes to plant growth by increasing the availability of nutrients and inhibiting the development of pathogenic microorganisms (Mendes et al. 2013). In turn, the composition and activity of the bacterial community are strongly influenced by the type of vegetation present in the soil (Thomson et al. 2010; Semmartin et al. 2010.). The optimal functioning of this complex microecosystem depends on different signaling processes between its constituent members. Particularly, the mechanism of interkingdom communication by QS seems to be one of the key processes governing the behavior of bacterial ecophysiology in the rhizosphere of superior plants, and therefore determines the development of different interaction processes (Vacheron et al. 2013).

Coevolution between plants and bacteria has determined the need for participation of the eukaryotic organism in the bacterial dialogue. Plants are capable of both detecting bacterial AHLs to adapt their physiology to bacterial populations established in their environment (Mathesius et al. 2003; Zarkani et al. 2013), and producing molecules that affect QS responses in bacteria (Corral-Lugo et al. 2016; Perez-Montaño et al. 2013). Several studies have demonstrated that bacterial AHLs are recognized by plants affecting essential phenotypes through regulating plant gene expression (von Rad et al. 2008; Schikora et al. 2011; Schenk et al. 2012). On the other hand, plants interfere with bacterial communication by producing root exudates containing signal molecules that act as agonists or antagonists of QS mainly linked to AHLs (Gao et al. 2003; Teplitski et al. 2004; Degrassi et al. 2007). Thus, plants are capable of synthesizing autoinducer molecules with similar structure to AHLs that mimic the activity of bacterial signal molecules enhancing QS regulated phenotypes (mQS); or synthesizing molecules that inhibit bacterial communication of QS by repressing the expression of processes involved in this mechanism (iQS) (Dong et al. 2001; Roche et al. 2004). In this regard, the signaling crisscross between organisms may be an adaptive strategy to allow bacteria and eukaryotes to monitor their surroundings and adjust their behavior in order to achieve more successful interactive processes, such as beneficial associations, pathogenesis, and inhibition of pathogenesis, among others. This back and forth circuit of interkingdom signaling appears to be essential for plant associated rhizobacteria as well as for the plant host (Venturi and Keel 2016).

Rhizobia-legume symbiosis is the result of a complex interaction between eukaryotic and prokaryotic organisms. The regulation of the symbiotic process includes the relatively simple model of two signals exchange (plant flavonoids, bacterial Nod factors) and a more complex system of signals (Jones et al. 2007). It comprises several compounds from both plant and bacteria to direct the course of root infection (Cooper 2007). AHLs produced by rhizobia that communicate by QS could be included in the list of symbiotic signals given the importance that they acquire in various phenomena related to the development of the symbiotic program. The processes regulated by QS in rhizobia include production of surface polysaccharides, growth inhibition, adaptation to stationary phase, efficiency of nodulation, symbiosome development and biological nitrogen fixation (BNF), all important aspects for establishing a successful symbiosis (Sanchez-Contreras et al. 2007).

Arachis hypogaea L. (peanut) constitutes an important legume crop with high relevance in different agroecological areas worldwide. Peanut establishes a nitrogen-fixing symbiosis with genetically diverse rhizobia grouped as *Bradyrhizobium* sp. (Urtz and Elkan 1996; Bogino et al. 2010; Nievas et al. 2012a). Studies addressing QS processes in *Bradyrhizobium* sp. showed that peanut-nodulating strains were characterised as producers of AHLs with long acyl chain, associated with regulation of processes linked to bacterial survival such as biofilm formation, motility, and autoaggregation (Nievas et al. 2012b; Bogino et al. 2015). In spite of the importance of such processes in driving rhizosphere and root colonization, knowledge is fragmented since the role of the plant in bacterial communication in the rhizosphere is still unknown.

Given the agricultural importance of the peanut legume, the lack of knowledge on the communication processes between peanut and rhizobacteria and the relevance that such mechanisms acquire in the rhizosphere, the aim of this study was to determine the role of peanut in the mechanism of bacterial QS through

(i) detection of mimic or inhibitory QS-like signals and (ii) evaluation of QS activity throughout *Bradyrhizobium* sp.–peanut interaction. It is proposed that the development of this work will provide the first evidence related to bacteria–peanut dialogue through QS in the rhizosphere of this legume.

Materials and methods

Bacterial strains and culture conditions

The bacterial biosensor strains *Chromobacterium violaceum* CV026 (McClean et al. 1997) and *Agrobacterium tumefaciens* NTL4 (pZLR4) (Cha et al. 1998) were employed for the different assays, and grown on LB medium (Sambrook et al. 1982) or AB medium (Chilton and Barbano 1974) respectively. The *Bradyrhizobium* sp. peanut symbiont strains P8A (Bogino et al. 2006), 62B and 20AG (Bogino et al. 2010) were routinely grown on TY medium (Beringer 1974) at 28 °C with rotary shaking at 150 rpm.

Plant material

A runner Virginia-type peanut cultivar (Manigran) from 'Criadero El Carmen', Córdoba, Argentina, was employed in this work.

Preparation of *A. hypogaea* seed and root exudates and extracts

Peanut seeds were surface sterilized according to the conditions described by Bogino et al. (2006). To ensure seed sterility, sterilization controls were made by plating aliquots of the water coming from the last step of seed washing on nutrient agar. Seed exudates were obtained by soaking 20 surface-sterilized peanut seeds at 100 rpm during 4 h, in 100 ml of sterile phosphate buffer pH 7.0. Subsequently, seeds were removed and the remaining crude exudate was prepared for lyophilization. For the root exudates preparation, sterile seeds were placed in Petri dishes containing agarized water 0.8%, incubated at 28 °C in darkness during 5–7 days, until obtaining peanut seedlings. Then each seedling was placed into a glass tube (diameter: 30 mm, length: 20 cm) containing a metallic support for the seedling and 25 ml of complete Hoagland solution (Löbler and Hirsch 1993). Peanut seedlings in the glass tubes were grown in a greenhouse under controlled hydroponics conditions (16/8 h light/dark, 28/24 °C). After 21 days, plants were removed and the Hoagland solution containing the crude root exudate was obtained and prepared for lyophilization. Both seed and root lyophilized exudates were concentrated tenfold in sterile deionized water. Crude and concentrated exudates were tested to discard microbial contamination by plating them on rich medium culture (nutrient agar). Non contaminated materials were further sterilized by filtration (filter MC-PES-02S, pore size 0.22 μm).

For the extracts preparation, sterile crude exudates were treated with three equal volumes of ethyl acetate, and the extracts were dried and resuspended in 500 μl ethyl acetate (Shaw et al. 2009). Extracts were sterilized by filtration as described above.

Detection of QS-like signals from peanut

Plate bioassays

Qualitative production of mimic or inhibitory QS-like signals (mQS or iQS) was carried out by using bacterial biosensors in both concentrated exudates and extracts from seed or root peanut. In order to determine mQS molecules direct assays were performed with *C. violaceum* CV026 for the detection of AHLs with short acyl chains (sac-AHLs) (McClean et al. 1997) and with *A. tumefaciens* NTL4 (pZLR4) for the detection of AHLs with long acyl chains (lac-AHLs) (Farrand et al. 2002). Initial inoculum of each biosensor strain was 1×10^7 ufc ml^{-1}. Aliquots of 40 μl of concentrated exudates or extracts were deposited into wells made on the surface of the LB or AB mediums. To determine iQS molecules in peanut materials, inverse assays were carried out. Inhibition of production of sac-AHLs was performed by adding C_6-HSL 100 μM (*N*-hexanoyl-DL-homoserine lactone) to the biosensor *C. violaceum* and identifying the presence of clear circles of no induction of violacein synthesis (Keshavan et al. 2005). Similarly, the detection of lac-AHLs was carried out by adding 3-O-C_{12}-AHL 100 μM (*N*-3-oxododecanoyl-L-homoserine lactone) to the biosensor *A. tumefaciens*, and identifying the presence of clear circles of inhibition of X-Gal (5-bromo-4-chloro-3-

indolyl β-D-galactopyranoside) hydrolysis (no induction of β-galactosidase enzyme) (Farrand et al. 2002).

In vivo bioassays

Sterile peanut seedlings, obtained as described above, were transferred to sterile glass tubes (diameter: 30 mm, length: 20 cm) containing 25 ml of semi-solid agarized complete Hoagland solution 0.8%. Semi-solid medium for growth of peanut plants was additionally supplemented with different bacterial strains at 10^7 cel ml^{-1} (biosensor strain *C. violaceum* CV026 or *A. tumefaciens* NTL4 pZLR4; peanut symbiont strains P8A, 62B or 20AG). X-Gal 40 μg ml^{-1} and/or AHLs 100 μM (C_6-HSL or 3-O-C_{10}-HSL) were also added when necessary. Peanut plantlets in the glass tubes were grown during different times in a growth chamber under controlled conditions as described above. Experimental controls were performed to ensure the functionality of our experimental procedures. Bioassays were carried out as follows (controls with asterisk),

(i) To determine mimics AHLs in vivo:

Tube A*: plant growing in Hoagland medium. Plants were grown for 28 days.
Tube B*: plant growing in Hoagland medium with C_6-HSL, *C. violaceum* CV026. Plants were grown for 7 days
Tube C: plant growing in Hoagland medium, *C. violaceum* CV026. Plants were grown for 28 days.
Tube D*: plant growing in Hoagland medium with X-Gal and 3-O-C_{10}-HSL, *A. tumefaciens* NTL4 (pZLR4). Plants were grown for 7 days.
Tube E: plant growing in Hoagland medium, *A. tumefaciens* NTL4 (pZLR4). Plants were grown for 28 days

(ii) To determine sac-AHLs in vivo:

Tube A*: Hoagland medium, *C. violaceum* CV026, peanut symbiont strain. Tubes were incubated for 21 days.
Tube B: plant growing in Hoagland medium, *C. violaceum* CV026. Plants were grown for 21 days.
Tube C*: plant growing in Hoagland medium with C_6-HSL, *C. violaceum* CV026. Plants were grown for 7 days.
Tube D: plant growing in Hoagland medium, *C. violaceum* CV026, peanut symbiont strain. Plants were grown for 21 days.

(iii) To determine lac-AHLs in vivo:

Tube A*: Hoagland medium with X-Gal, *A. tumefaciens* NTL4 (pZLR4), peanut symbiont strain. Tubes were incubated for 21 days.
Tube B*: plant growing in Hoagland medium with X-Gal. Plants were grown for 21 days.
Tube C*: plant growing in Hoagland medium with X-Gal, *Bradyrhizobium* P8A peanut symbiont strain. Plants were grown for 21 days.
Tube D*: plant growing in Hoagland medium with X-Gal, *A. tumefaciens* NTL4 (pZLR4). Plants were grown for 21 days.
Tube E*: plant growing in Hoagland medium with X-Gal and 3-O-C_{10}-HSL, *A. tumefaciens* NTL4 (pZLR4). Plants were grown for 7 days.
Tubes F, G, H: plant growing in Hoagland medium with X-Gal, *A. tumefaciens* NTL4 (pZLR4), peanut symbiont strain. Plants were grown for 7 days.

For each analysis, a set of 4 tubes were assayed and experiments were performed in triplicate.

Results and discussion

Production of QS-like molecules by peanut

The nitrogen fixing symbiosis between rhizobia and leguminous plants is dependent on the chemical dialogue of these partners in the rhizospheric micro-niche (Gage 2004; Jones et al. 2007). In this regard, many signaling molecules, from both plant and bacteria, are involved to direct the course of the root colonization and infection (Cooper). AHLs molecules

produced by rhizobia that communicate through QS could be included in the list of symbiotic signals gi2007ven the importance that they acquire in various phenomena linked to the development of the interaction program of these bacteria (Sanchez-Contreras et al. 2007). In addition, plants are able to synthesize molecules that interfere, either positively or negatively, in the mechanism of bacterial QS, leading to an increase in the complexity of the interactive processes in the rhizosphere (Venturi and Keel 2016).

Rhizobia-legume symbiosis in general, and *Bradyrhizobium* sp.–peanut in particular, acquire special importance at the agroecological level. The production of lac-AHL by native *Bradyrhizobium* sp. strains isolated from peanut has been previously reported (Nievas et al. 2012b). The production of QS-like molecules in materials from the peanut plant was determined here in order to study the possible role of the legume on the bacterial QS mechanism. Plate bioassays were carried out on concentrated crude exudates and on concentrated organic extracts of seeds and roots of peanut, to detect mQS and iQS molecules related to sac-AHLs or lac-AHLs (Fig. 1; Table 1). Results showed that seeds and roots of peanut produce an exudate characterised by the presence of mQS type lac-AHL-like molecules (Fig. 1b). The absence of mQS related to sac-AHL-like molecules was also determined in such materials (Fig. 1a). The mQS-like molecules were detected in both exudate and extract materials from roots, whereas these signals were only detected in the organic extract of the seed exudate and not in the concentrated crude seed exudates, probably due to the low concentration of mQS-like molecules in the latter. The plate bioassays carried out to detect iQS-like molecules showed the presence of QS inhibition for sac-AHLs in both seed and root organic extracts (Fig. 1c). No inhibition of QS mechanisms of lac-AHLs was detected in any of the tested materials (Fig. 1d; Table 1). These results suggest that the detected iQS-like molecules probably correspond to lipophilic compounds secreted at a very low concentration. What remains as an unknown effect is whether the negative impact of iQS-like molecules from peanut on sac-AHLs is on their stability, availability or regulatory effects (Huang et al. 2016).

In order to further validate the obtained results, an in vivo assay was developed to assess mQS-like molecules during the growth of the peanut plant (Fig. 2). The realization of this in vivo assay helped to evaluate a more real situation, since QS metabolites were determined in the rhizosphere of the plant during a longer time of growth (28 days), thus allowing the peanut plant to express its entire potential for root exudation. The results obtained demonstrate and confirm that peanut plant produced a root exudate containing only mimics of lac-AHL-like molecules (Fig. 2e). In spite of the growing plant and the longer time of growth, no mimics of sac-AHL-like molecules were detected in the rhizosphere of peanut plants (Fig. 2c). Results obtained from control tubes confirm that the experimental procedure carried out in the present work was efficient and reliable. In this sense no detection of active molecules was observed during plant growth in the absence of biosensor strains (Fig. 2a) whereas the biodetection of synthetic externally added AHL by the biosensor strains was clearly positive (Fig. 2b, d). Remarkably, detection of QS signals was observed from the beginning of the assay (24–48 h) at the medium-air interface, probably due to the aerobic requirements of the biosensors strains. For *C. violaceum* CV026 the detection of C_6-HSL was partly observed in the whole medium, which turned slightly more colored compared to other tubes (Figs. 2b, 3c). For *A. tumefaciens* NTL4 (pZLR4) the detection of 3-O-C_{10}-HSL was clearly noted at the contact zone with the growing root, indicating rhizosphere colonization and signal sensing at this microenvironment (Figs. 2d, 4e).

An in vivo assay for the detection of iQS-like molecules during the growth of the peanut plant was not possible to develop since the addition of AHL to the medium turned it immediately colorful due to violacein production or X-Gal hydrolysis at the interface medium-air (Fig. 2b, d). In this sense, the determination of QS inhibition is prevented because of the time lag between the early detection of the signal by the biosensor and the possibly more delayed synthesis of iQS molecules by the growing plant.

The overall result of mQS and iQS in vegetal materials from peanut, revealed the presence of mQS-like molecules related to lac-AHLs and iQS-like molecules related to sac-AHLs. This evidence suggests that the peanut plant could be able to modulate the physiology and probably the composition of the bacterial community established around its root system. It was previously determined that peanut nodulating strains were only positive for QS mechanisms related to lac-AHLs (Nievas et al. 2012b). Moreover, a survey of production of AHLs by non-symbiotic

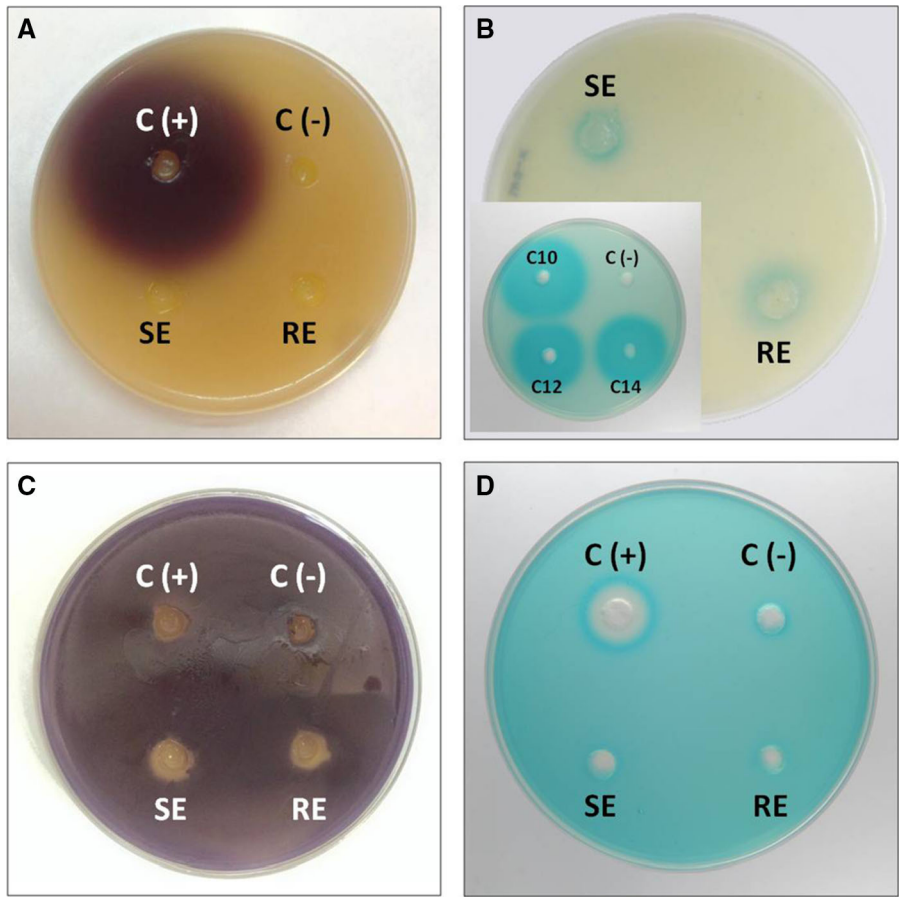

Fig. 1 Detection of QS-like molecules in vegetal materials from *Arachis hypogaea* L. *SE* Seed extract, *RE* root extract. Control negative (*C*−) ethyl acetate. **a** Direct bioassay to detect mimics AHLs with short acyl chain, C_6-HSL was employed as positive control (*C*+). **b** Direct bioassay to detect mimics AHLs with long acyl chain. 3-O-C_{10}-HSL (C10), 3-O-C_{12}-HSL (C12) and 3-O-C_{14}-HSL (C14) were employed as positive controls. **c** Indirect bioassay to detect activity inhibition of AHLs with short acyl chain, 3-O-C_{10}-HSL was employed as positive control (*C*+). **d** Indirect bioassay to detect activity inhibition of AHLs with long acyl chain, supernatant of *Bacillus subtilis* GB03 culture in stationary growth phase was employed as positive control (*C*+)

Table 1 QS-like activities of different vegetal materials from peanut

Vegetal material	mQS activity		iQS activity	
	sac-HSL	lac-HSL	sac-HSL	lac-HSL
Seed exudate	−	−	−	−
Seed extract	−	+	+	−
Root exudate	−	+	−	−
Root extract	−	+	+	−

mQS mimics QS, *iQS* inhibition of QS, *HSL* homoserine lactone, *sac* short acyl chain, *lac* long acyl chain

bacteria isolated from rhizospheric soil of peanut, showed the production of lac-AHLs by near 45% of rhizobacterial strains, and the absence of detection of sac-AHLs by 100% of the rhizobacterial strains tested (Nievas et al. unpublished data). In short, by considering the previous reports in bacteria and the results in the plant showed here, it could be speculated that the peanut plant selects members with QS mechanisms linked to lac-AHLs (e.g., their symbionts *Bradyrhizobium* sp.) and disrupts the QS mechanisms of bacteria that communicate through sac-AHLs (e.g., plant

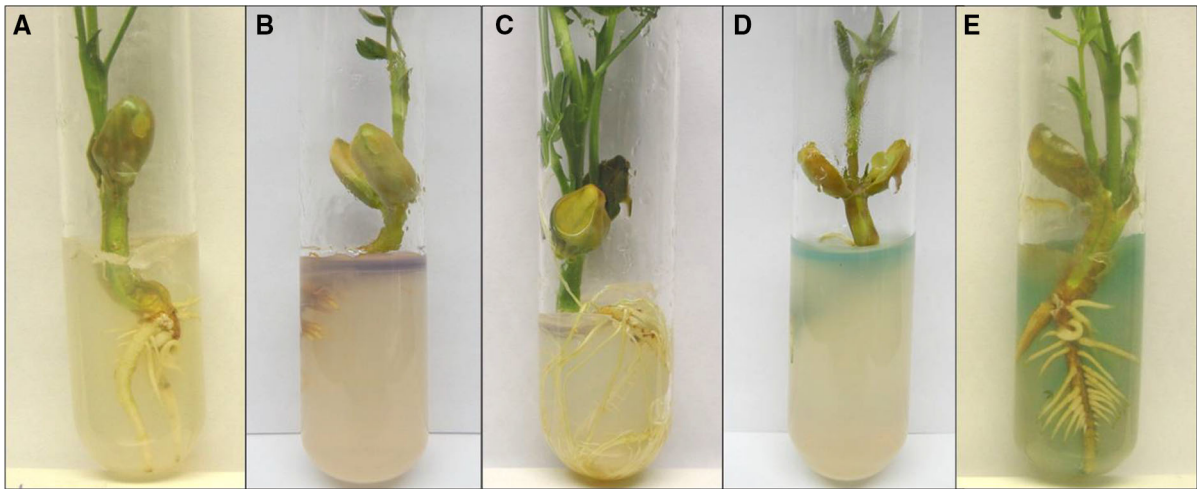

Fig. 2 Determination of mimics AHLs from peanut plants growing in vivo. **a** Peanut plant grown in Hoagland medium. **b** Peanut plant grown in Hoagland medium supplemented with C_6-HSL and *C. violaceum* CV026. **c** Peanut plant grown in Hoagland medium supplemented with *C. violaceum* CV026. **d** Peanut plant grown in Hoagland medium supplemented with C_{10}-HSL, X-Gal, and *A. tumefaciens* NTL4 (pZLR4). **e** Peanut plant grown in Hoagland medium supplemented with X-Gal and *A. tumefaciens* NTL4 (pZLR4)

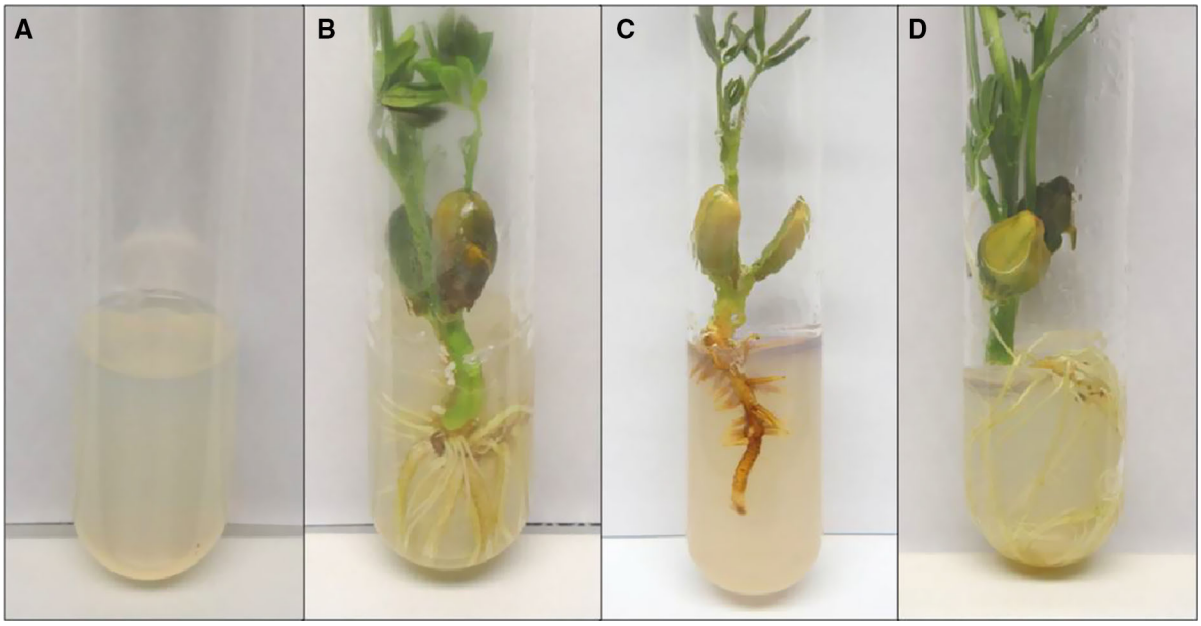

Fig. 3 In vivo detection of QS mechanism dependent on AHLs with short acyl chain. **a** *C. violaceum* CV026 and peanut symbiont strain P8A. **b** Peanut plant grown in Hoagland medium supplemented with *C. violaceum* CV026. **c** Peanut plant grown in Hoagland medium supplemented with C_6–HSL and *C. violaceum* CV026. **d** Peanut plant grown in Hoagland medium supplemented with *C. violaceum* CV026 and peanut symbiont strain P8A

pathogenic bacteria). Although the precise mechanisms are unknown, the general concept is that sac-AHLs promote plant growth by modulating the hormonal balance in the plant, whereas lac-AHLs have been related to both, contributing to root development as well as inducing resistance against microbial pathogens (Gonzalez and Venturi 2013; Schikora et al. 2016). According to the results

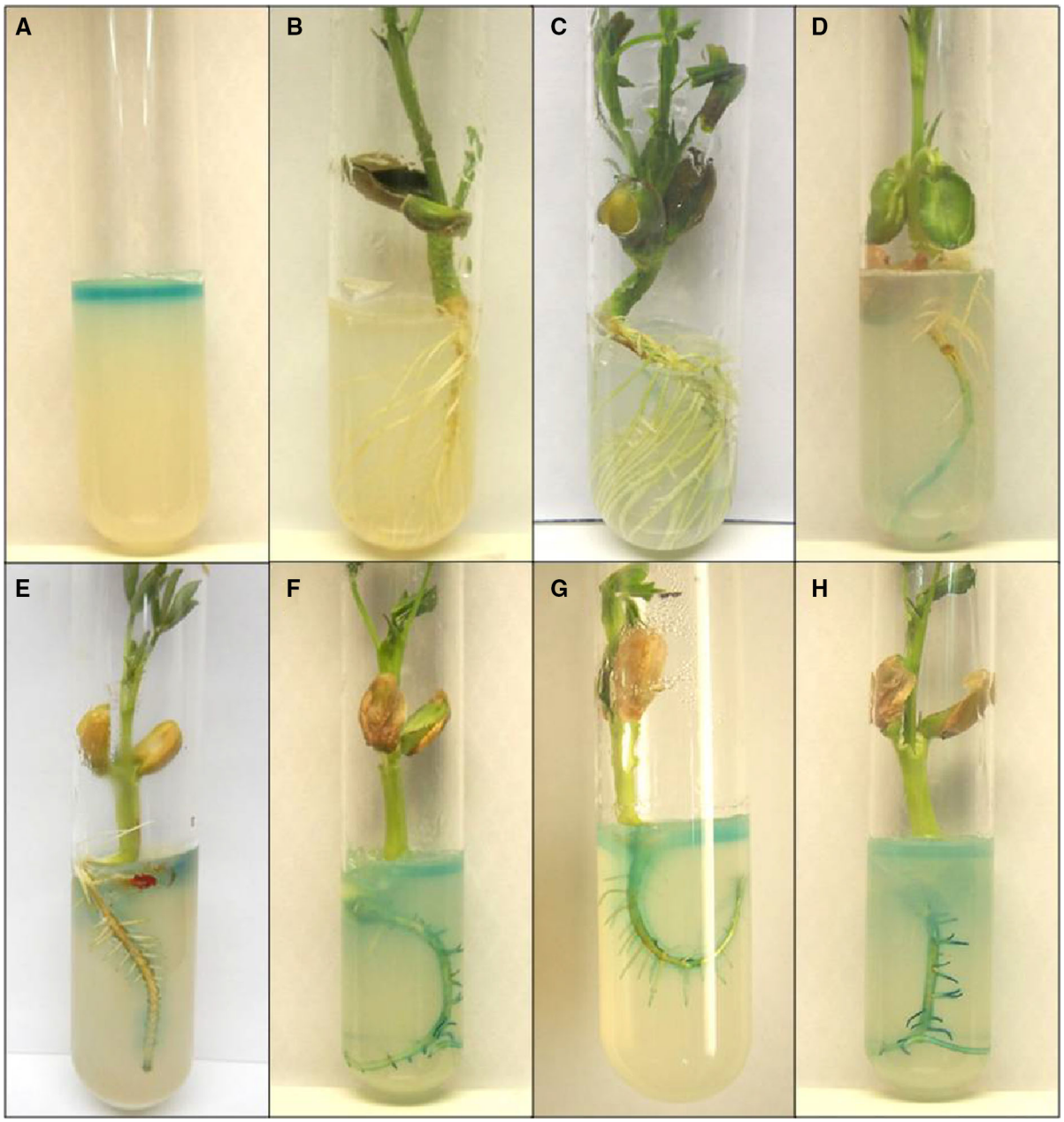

Fig. 4 In vivo detection of QS mechanism dependent on AHLs with long acyl chain. **a** Hoagland medium supplemented with X-Gal, *A. tumefaciens* NTL4 (pZLR4) and peanut symbiont strain P8A. **b** Peanut plant grown in Hoagland medium supplemented with X-Gal. **c** Peanut plant grown in Hoagland medium supplemented with X-Gal and peanut symbiont strain P8A. **d** Peanut plant grown in Hoagland medium supplemented with X-Gal and *A. tumefaciens* NTL4 (pZLR4). **e** Peanut plant grown in Hoagland medium supplemented with C_{10}–HSL, X-Gal, and *A. tumefaciens* NTL4 (pZLR4). **f**, **g**, **h** Peanut plants grown in Hoagland medium supplemented with X-Gal, *A. tumefaciens* NTL4 (pZLR4) and a peanut symbiont strain (62B for **f**, P8A for **g** and 20AG for **h**)

obtained here, the legume peanut would direct the chemical dialogue to select specific bacterial members on its root surroundings (i.e., lac-AHL against sac-AHL producers) in order to modulate the root anatomy (probably to improve symbiosis) and to avoid pathogenesis. However, the molecular mechanism of plant

QS activity remains unknown and more efforts are required to chemically identify these QS-like molecules in order to determine their mechanism of action.

Detection of QS activity throughout *Bradyrhizobium* sp.–peanut interaction

Although the results shown above are quite conclusive with respect to QS-like molecules produced by peanut, another work scheme was carried out by developing in vivo experiences in order to decipher the QS activity that takes place at the level of rhizospheric interactions between bacteria and plant. This design helped to determine a clearer evidence of communicative phenomena among bacteria–bacteria and between bacteria–plant in situ, in the rhizosphere, and to demonstrate the behavior of the partners throughout the interaction. The set of assays showed the production of QS molecules when rhizobacteria, in this case the rhizobia and peanut plants, are in contact.

Figure 3 shows the result obtained for the detection of sac-AHLs. For this purpose, control tubes without plant were processed in order to verify the behavior of the *Bradyrhizobium* sp. P8A strain in contact with the biosensor strain *C. violaceum* CV026 in the Hoagland medium. In agreement with previous results (Nievas et al. 2012b) the peanut nodulating P8A strain was incapable of synthesizing sac-AHLs, and the Hoagland medium remained colorless due to the lack of induction in the violacein synthesis after 21 days of incubation (Fig. 3a). Another set of tubes containing the plant and the *C. violaceum* CV026 biosensor were included in the experiment to test the behavior of peanut plant growing with the biosensor in absence of the symbiont strain. As above, no mQS-like signals were detected after 21 days of incubation (Fig. 3b), indicating the inability of peanut plant to produce mimic sac-AHLs in vivo. To further validate the assay, an experimental control with C_6-HSL was carried out. Strong development of pigmentation at the interface medium-air as well as slightly coloration of the entire tube confirmed the ability of *C. violaceum* CV026 to sense QS molecules (Fig. 3c). Finally, the complete biological system, constituted by the symbiotic partners (rhizobia and plant) and the biosensor strain (*C. violaceum*), showed the non-induction in the violacein synthesis after 21 days of incubation (Fig. 3d), revealing no involvement of sac-AHLs during the interaction between *Bradyrhizobium* sp. and peanut plants. It also demonstrated that the interaction between these partners did not promote the production of QS-like molecules linked to sac-AHLs, i.e., neither the plant synthesized mQS by being in contact with their nodulating strain, nor the rhizobia produced these kinds of QS-like molecules by being in contact with their plant host.

Although the results shown in Fig. 3 were interesting, they were predictable and confirmed previous assumptions. According to this, the determination of lac-AHLs in vivo by following an experimental design similar to that presented above, acquired a special relevance since both rhizobia and plant are capable of synthesizing these kinds of QS-like molecules. To achieve this, the detection of these molecules was monitored with the biosensor strain *A. tumefaciens* NTL4 (pZLR4) in the Hoagland medium supplemented with X-Gal. A series of experimental controls (Fig. 4a–e) were performed in order to check the characteristic behavior of each studied member and to validate the reliability of the system. In this sense, the *Bradyrhizobium* sp. P8A strain in contact with the biosensor strain in absence of the plant was capable of producing lac-AHLs (Fig. 4a). This result is in agreement with previous results (Nievas et al. 2012b). However, the novelty is that the current results show that the synthesis of lac-AHLs by the *Bradyrhizobium* sp. P8A strain is only reached at the medium-air interface and not in the entire medium, which demonstrates that the quorum required for the production of the QS signal is probably dependent on bacterial motility up to the air interface which is linked to the aerobic behavior of these bacteria. Moreover, the production of QS molecules was detected after 5 days of incubation and remained detectable until the end of the assay (21 days). The controls with plants included four series of tubes. The first plant control was carried out by growing the peanuts in Hoagland medium with X-Gal and without the biosensor bacteria. This assay showed the absence of degradation of the reactant itself or by the plant after 21 days of incubation (Fig. 4b). Another plant control was developed by growing the peanuts in Hoagland medium with X-Gal and the peanut symbiont strain P8A. No hydrolysis of X-Gal was detected, indicating that P8A strain alone is unable to degrade the reactant (Fig. 4c). By growing the peanuts in Hoagland medium with X-Gal and the *A. tumefaciens* biosensor

another plant control was developed. The result showed a slight production of mQS-like lac-AHLs by the plant (Fig. 4d), manifested by the development of a light blue color on the roots, which confirmed previous results. The synthesis of such molecules was detected after 15 or 18 days of incubation. At the end of the assay (21 days), the degradation of X-Gal was weakly detected around the roots, indicating that mQS-like molecules are exudated by the roots at a very low concentration and, as shown in Fig. 2e, more time is required for the diffusion and detection in the entire medium. Another control tube showed the inability of the *A. tumefaciens* biosensor to degrade X-Gal (not shown). This clear effect is shown in Fig. 4d in spite of the presence of the plant. Finally, this series of bioassays were further validated by processing an experimental control with synthetic 3-O-C_{10}-HSL, peanuts and the *A. tumefaciens* biosensor (Fig. 4e). As previously shown in Fig. 2e, X-Gal hydrolysis was detected at the medium-air interface and around the roots, demonstrating the ability of *A. tumefaciens* to effectively sense QS signal under these experimental conditions.

Figure 4f–h show the result obtained in terms of QS linked to lac-AHLs when symbiotic partners (rhizobia and plant) and the biosensor *A. tumefaciens* were in contact. In these cases, a faster and clearer effect was detected after 7 days of incubation. The rhizobial population, of the genus *Bradyrhizobium*, symbiotic with peanut, was clearly driven to activate its QS mechanism in two subpopulations, one at the medium-air interface and another in association with peanut roots. As shown in the in vivo assays, the production of lac-AHLs by different native peanut strains, i.e., 62B (Fig. 4f), P8A (Fig. 4g) and 20AG (Fig. 4h), was detected by the biosensor strain and expressed as the development of zones of color at the air interface and around the peanut roots. By comparing these results with those obtained with the control tube of *A. tumefaciens* exposed to synthetic AHL (Fig. 4e), we can conclude that peanut symbiotic strains probably produced a high amount of different lac-AHLs, which were detected even with more intensity than the synthetic AHL. The results shown here highlight a double effect of the plant on bacterial populations: (i) root exudates drive to bacterial colonization of the rhizosphere, allowing bacteria to establish a population associated with the peanut root and subsequently to express different biological effects (i.e., development of symbiosis), and (ii) the process of bacterial communication by QS was intensified at the rhizosphere, indicating that the peanut plant promotes the development of such communication mechanisms on its root, probably favoring or stimulating the subsequent expression of beneficial bacterial behavior. According to the results shown here, probably the rich nutritive root exudates containing agonist QS signals for peanut symbiotic bacteria and antagonist QS signals for other non-symbiotic or even pathogenic bacteria, can guide the course of initial crucial phenomena of root colonization by selected bacterial populations, leading to subsequent development of beneficial interactions (Hartmann et al. 2014). In agreement, an increase in the nodulation in roots of *Medicago truncatula* produced by 3-oxo-C14-AHL of *Sinorhizobium meliloti* has previously been reported (Veliz-Vallejos et al. 2014).

Interestingly, results achieved by applying this novel methodology (Fig. 4e–h) demonstrated that the bacterial population in the peanut rhizosphere was a mixed consortium of two different genera of rhizobacteria, i.e., *Bradyrhizobium* and *Agrobacterium*, which effectively interacted and colonized the root system of the legume plant. We suggest application of this approach as a useful tool to study rhizosphere colonization of bacteria that communicate through QS mechanisms.

Results presented in this work are the first evidence of QS activity produced by the legume peanut. Further studies are needed to determine the chemical nature of the active compounds as well as their influence on the behavior of bacterial QS in *Bradyrhizobium* sp.

Conclusions

This is the first report of the detection of QS-like activity in materials from peanut. Such activity is related to mQS lac-AHLs-like molecules and to iQS sac-AHLs-like molecules, highlighting the importance of the legume in the selection of specific bacterial populations. This work also shows an original methodology to detect QS signals during the plant growth. It allowed the successful assessment of peanut rhizosphere colonization by *Bradyrhizobium* sp., showing the process of early interkingdom communication and interaction between bacteria and peanut.

The study of biological systems related to efficient sustainable agricultural practices is becoming crucial

to increase crops yields and to provide high quality food. Increasing knowledge of plant–rhizobacteria interactions by studying different aspects of its establishment can be key for the understanding of improving crops growth. Knowledge of QS at the interkingdom level and its handling could be a useful tool for applying strategies aimed at reducing pathogenic interactions and promoting beneficial ones.

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