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RESEARCH PAPER

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Quorum sensing communication: *Bradyrhizobium-Azospirillum* interaction via N-acyl-homoserine lactones in the promotion of soybean symbiosis

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Quorum-sensing (QS) mechanisms are important in intra- and inter-specific communication among bacteria. We investigated QS mechanisms in *Bradyrhizobium japonicum* strain CPAC 15 and *Azospirillum brasilense* strains Ab-V5 and Ab-V6, used in commercial co-inoculants for the soybean crop in Brazil. A transconjugant of CPAC 15-QS with partial inactivation of N-acyl-homoserine lactones (AHLs) was obtained and several parameters were evaluated; *in vitro,* CPAC 15 and the transconjugant differed in growth, but not in biofilm formation, and no differences were observed in the symbiotic performance in vivo. The genome of CPAC 15 carries functional *luxI* and *luxR* genes and low amounts of three AHL molecules were detected: 3-OH-C12-AHL, 3-OH-C14-AHL, and 3-oxo-C14-AHL. Multiple copies of *luxR*-like genes, but not of *luxI* are present in the genomes of Ab-V5 and Ab-V6, and differences in gene expression were observed when the strains were co-cultured with *B. japonicum*; we may infer that the *luxR*-genes of *A. brasilense* may perceive the AHL molecules of *B. japonicum*. Soybean symbiotic performance was improved especially by co-inoculation with Ab-V6, which, contrarily to Ab-V5, did not respond to the AHLs of CPAC 15. We concluded that *A. brasilense* Ab-V5, but not Ab-V6, responded to the QS signals of CPAC 15, and that the synergistic interaction may be credited, at least partially, to the QS interaction. In addition, we confirmed inter- and intra-species QS communication between *B. japonicum* and *A. brasilense* and, for *Azospirillum,* at the strain level, impacting several steps of the symbiosis, from cell growth to plant nodulation and growth.

KEYWORDS
Azospirillum brasilense, *Bradyrhizobium japonicum*, biofilm, co-inoculation, LuxI

1 | INTRODUCTION

Quorum sensing (QS) in bacteria can be described as an intra- and/or inter-specific communication mechanism, involving chemical mediators (autoinducers) capable of inducing responses dependent on the population density. Several bacteria use autoinducers such as N-acyl-homoserine lactones (AHLs) to control gene expression [1]. The signals are produced by members of the LuxI protein family and are detected by transcription factors of the LuxR family; the *luxI* and *luxR* genes are often adjacent to each other [2]. AHL autoinducers pass freely through the cell membrane and reach high intracellular concentrations with increasing population density until establishing a critical level that triggers a specific response [3].

The number of LuxI and LuxR proteins may vary with the bacterium. In a QS circuit, a LuxI protein has a corresponding LuxR that binds to its AHL, but often more LuxR proteins are present in the genome. Also, it is possible to find bacteria that do not possess a complete QS circuit, presenting *luxI* or *luxR* homologous genes in their genomes that can induce or respond to exogenous AHLs, respectively [4].

Important phenotypes are controlled by QS in bacteria, such as biofilm formation, production of toxins, synthesis of exopolysaccharides (EPS), virulence factors, and motility, that are essential properties for the establishment of symbiotic and pathogenic relationships with eukaryotic hosts [5]. In the N_2-fixing symbiosis of soybean with *Bradyrhizobium* spp., nodulation involves participation of QS mechanisms, and the transcription of the nodulation (*nod*) genes is controlled by population density [6]. In addition, QS mechanisms have been linked to other important rhizobium-legume symbiosis features, including nodulation efficiency, symbiosome development, EPS production, and N_2 fixation [3].

The synthesis of AHLs by *Bradyrhizobium* strains has been reported [7,8]. The first *Bradyrhizobium* genome sequenced (strain USDA 110) revealed the gene pair *bjaI* (homologue of *lux1*) and *bjaR* (homologue of *luxR*) [9]; a decade later, it was demonstrated that USDA 110 can synthesize an isovaleryl-homoserine lactone (IV-AHL) [10]. The delay in detecting the molecule is explained by its very low concentration, in the order of 5 nM, whereas in most bacteria with QS mediated by AHL, the concentration of the autoinducers is in the order of 0.1–10 μM [10].

The genomic analysis of *B. japonicum* CPAC 15, an important strain used in inoculants for soybean crops in Brazil [11] revealed genes homologous to *luxI* and *luxR* [12], suggesting that the strain could produce and respond to AHLs. High nucleotide identity (>94%) was found between the *luxI* and *luxR* genes of CPAC 15 and *bjaI* and *bjaR* of USDA 110 [12]; however, the detection and characterization of the AHL produced by CPAC 15, as well as the phenotypes regulated by the QS mechanism, remained to be studied.

Azospirillum encompasses plant-growth-promoting bacteria (PGPB) used in agriculture, the main effects of which result from the stimulation of root formation, attributed to the intense production and secretion of indole-3-acetic acid, benefiting many plant species [13]. The QS mechanism mediated by AHL seems unusual in *Azospirillum*, as Vial et al. [14] demonstrated that only four out of 40 *Azospirillum* strains tested produced AHL; the four strains belonged to the species *A. lipoferum*. For the strains of *Azospirillum brasilense* Ab-V5 and Ab-V6, selected in Brazil for inoculation of grasses [15,16], no LuxI has been found either [17,18].

Multi-strain inoculants, including PGPB and N_2-fixing rhizobia, have received increasing attention in agriculture. One recent example are the benefits of co-inoculation of soybean with *Bradyrhizobium* spp. and *A. brasilense* in Brazil, including precocity and increased nodulation, higher N_2-fixation rates and grain yield, in addition to other benefits such as improved tolerance of water stress [15,19–21]. The benefits of co-inoculation have been attributed to root promotion by *Azospirillum*, increasing the sites for nodulation by *Bradyrhizobium*; however, the possibility of involvement of more complex mechanisms such as QS deserves investigation.

The hypothesis of our study is that QS mechanisms (via AHL) participate in intra- and inter-specific communication in *B. japonicum* and *A. brasilense*, impacting processes as bacterial growth, biofilm, soybean nodulation, and growth. To investigate this hypothesis we used molecular and physiological approaches.

2 | MATERIALS AND METHODS

2.1 | *Bradyrhizobium japonicum* and *Azospirillum brasilense* strains and growth conditions

Bradyrhizobium japonicum strain CPAC 15 (=SEMIA 5079) was grown in modified-YM medium [22] and *Azospirillum brasilense* strains Ab-V5 and Ab-V6 were grown in DYGS medium [17], both at 28 °C, in the dark (full growth reached at three days for *Azospirillum* and seven days for *Bradyrhizobium*). The three strains are used in commercial inoculants in Brazil: CPAC 15 for soybean (*Glycine max* (L.) Merr.) and Ab-V5 and Ab-V6 for inoculation of maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), brachiarias (*Urochloa* spp.) and co-inoculation of soybean, and common bean (*Phaseolus vulgaris*). All strains used in this study are deposited at the Diazotrophic and Plant Growth Promoting Bacteria Culture of Embrapa Soja (WFCC Collection #1213, WDCC Collection #1054), located in Londrina, Paraná State, Brazil; and at the Microbiology Department of the University of Seville, Seville, Spain.

2.2 | Obtaining a transconjugant strain of *B. japonicum*

For the conjugation, the transfer plasmid pME6863 of *Escherichia coli* [23] was used. The plasmid contains the *aiiA* gene from *Bacillus* sp. A24, responsible for the synthesis of an acyl-homoserine lactonase, capable of inactivating AHLs. The plasmid was transferred to *B. japonicum* strain CPAC 15, as described before [24].

To confirm the presence of *aiiA* in the transconjugant of CPAC 15, the DNA was extracted (PureLink ™ Quik Plasmid Miniprep kit – Invitrogen™). The *aiiA* gene was amplified by PCR using the primers aiiA-F (5 ′GCAGGTCGTTGTTGGA 3′) and aiiA-R (5 ′CAGGGAACACTTTACATCCC 3′), designed based on the sequence of *Bacillus* sp. A24, retrieved from the GenBank database (AF397400) [23]. The amplification conditions (DNA Engine® Thermal Cycler, Research INC, USA) consisted of an initial denaturation step of 95 °C for 2 min; followed by 45 cycles of 95 °C for 45 s, 59 °C for 3 s, 72 °C for 1 min and 30 s; and a final extension cycle of 72 °C for 7 min.

The transconjugant strain obtained was denominated as CPAC 15-QS and was maintained in modified-YMA:glycerol (60:40) supplied with antibiotic (40 μg ml^{-1} tetracycline) at −80 °C; the presence of the plasmid was confirmed by both the resistance to tetracycline (40 μg ml^{-1}), and the amplification with primers *aiiA* gene. Before and after each experiment we verified and confirmed the plasmid stability.

2.3 | Structural determination of AHLs of *B. japonicum* CPAC 15 by LC/MS

B. japonicum strain CPAC 15 (wild-type) was grown in modified-YM medium for 7 days at 28 °C, in the dark, under agitation. The culture was centrifuged, the supernatant was extracted with dichloromethane and the organic phase was evaporated. The extract was dissolved in 1 ml of methanol: water (1:1) containing 0.1% (v/v) of formic acid, filtered (0.2 μm) and 20 μL were injected in an UHPLC-QExactive system equipped with Tracer Hypersyl ODS column (250 × 4.6 mm, particle size 5 μm) (Teknokroma, Spain).

Elutions were performed at room temperature with flow rate of 400 μl min^{-1} using a gradient of water and methanol, both acidified with 0.1% of formic acid [25]. HPLC analyzes were performed on a Perkin-Elmer Series 200 System (Waltham, USA). The determination of the chemical structure of the AHL molecules produced by *B. japonicum* strain CPAC 15 was performed by liquid chromatography and mass spectrometry *in tandem* [26]. Multiple-Reaction ion Monitoring (MRM) is a method *in tandem* of MS/MS in which specific ions selected are transmitted through a first analyzer (quadrupole, Q1) and specific fragments selected resulting by collision-induced dissociation (CID) in Q2 are introduced into a second analyzer (Q3). In addition to the chemical identification method mentioned above, the detected AHL structures were verified by comparison of the mass spectrum of the standards according to the potentiation of ion production (EPI) method.

All spectrometric analyzes were performed on a mass spectrometer 2000 QTRAP hybrid triple-quadrupole-linear trap (Applied Biosystems, USA) equipped with a turbo ion source used in electrospray mode of positive ions. The spectrometric conditions were optimized by infusion of standard solutions dissolved in methanol (100 μg ml^{-1}) at a flow of 10–100 μl min^{-1}: C4-AHL, C6-AHL, C8-AHL, C10-AHL, C12-AHL, C14-AHL, 3-oxo-C6-AHL, 3-oxo-C8-AHL, 3-oxo-C10-AHL, 3-oxo-C12-AHL, 3-oxo-C14-AHL, 3-OH-C6-AHL, 3-OH-C12-AHL, and 3-OH-C14-AHL.

2.4 | Growth curves for *B. japonicum* and *A. brasilense*

B. japonicum CPAC 15 and *A. brasilense* Ab-V5 and Ab-V6 were individually pre-cultivated in 10 ml of modified-YM and DYGS media, respectively, and evaluated by optical density (OD) in a spectrophotometer at 600 nm; growth was standardized in OD_{600} 0.6. One ml of each bacterial culture (or, in the case of co-culture of *Bradyrhizobium* and *Azospirillum*, 800:200 μl, respectively, from each culture, due to differences in growth rate) were transferred to glass vials containing 150 ml of buffered liquid medium consisting of 2 g of yeast extract, 1.5 g of glutamic acid, 8 g of mannitol, and 0.25 g of KH_2PO_4 per liter, pH adjusted to 6.8. The vials were incubated under the same conditions as described above, but for 50 days. Eight treatments were included, each with three replicates, as follows: 1-CPAC 15; 2-CPAC 15-QS; 3-CPAC 15 + Ab-V5; 4-CPAC 15-QS + Ab-V5; 5-CPAC 15 + Ab-V6; 6-CPAC 15-QS + Ab-V6; 7-Ab-V5; and 8-Ab-V6.

The evaluation of bacterial growth was performed by analyzing the OD_{600} and by counting colonies after serial dilutions and plating on Petri plates containing modified modified-YMA or DYGS media. Bacterial growth was verified in 15 different times (T, in hours), each with three biological replicates, from T 0 to T 1200 h (50 days).

2.5 | Bioprospection of QS genes in the genome of *A. brasilense* strains Ab-V5 and Ab-V6

QS genes were searched in the genomes of *A. brasilense* strains Ab-V5 (Accession # POQV01000000) and Ab-V6 (Accession # POTD01000000), available at the NCBI database [18]. The nucleotide sequences of the *lux* genes identified were aligned and the similarity of nucleotides between them was analyzed using the MEGA 7 Program (Molecular Evolutionary Genetics Analysis) [27].

2.6 | Expression of *luxI* and/or *luxR* genes of *B. japonicum* and *A. brasilense* by RT-qPCR

B. japonicum CPAC 15 wild-type and the transconjugant CPAC 15-QS were pre-cultivated in 10 ml of modified-YM media for 72 h, and OD_{600} verified. The strains were transferred in triplicate for vials containing 30 ml of culture medium, under agitation of 120 rpm and 28 °C. The treatments studied were: 1-CPAC 15; 2-CPAC 15-QS; 3-CPAC 15 + Ab-V5; 4-CPAC 15-QS + Ab-V5; 5-CPAC 15 + Ab-V6; 6-CPAC 15-QS + Ab-V6. Total RNA was extracted and purified from the cultures after seven days, using Trizol® (Life Technologies), as described before [28]. Total RNA concentration was evaluated in a NanoDrop™ ND-1000 spectrophotometer (Thermo Scientific™) and the integrity of the material verified by electrophoresis in agarose gel (1%). For cDNA synthesis, the enzyme Reverse Transcriptase (Superscript III™, Invitrogen™) was used following the manufacturer's protocol.

For the analysis of the differential expression analysis by RT-qPCR, the *luxI* and *luxR* genes of *B. japonicum* CPAC 15 [12] and eight *luxR*-like genes of *A. brasilense* Ab-V5 and Ab-V6 [18] were selected. The primers were designed using the Primer3plus online tool and are shown in Supporting Information, Table S1. The 16S ribosomal gene was used as a normalizer of the relative expression of target genes.

The RT-qPCR reactions were performed in the Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Foster, CA, USA) with three replicates for each of the three biological replicates. The Platinum® SYBR Green® Master Mix kit (Applied Biosystems) was used, following the manufacturer's protocol. The cycles were as follows: 50 °C for 2 min, 95 °C for 10 min, 45 cycles at 95 °C for 2 min, 60 °C for 30 s and a final extension cycle of 72 °C for 30 s. The Rest 2009 software was used to evaluate the results by statistical analysis ($p \leq 0.05$). CPAC 15-QS was used as control to compare the gene expression (value 1).

2.7 | Analysis of biofilm formation

The evaluation of biofilm was performed on polystyrene microplates with U-bottom (Deltalab S.L.), as described before [28], with slight modifications. Eight treatments were studied, the same as in the growth curves study, and tests were performed with three biological replicates. The biofilm analysis was based on the addition of: (a) flavonoid genistein (Sigma-Aldrich®) (50 µl 10 ml^{-1}); (b) Nod factor (lipochitin-oligosaccharides) (LCO) extracted from *B. diazoefficiens* strain USDA 110, as described before [29] (10 µl 10 ml^{-1}); (c) exudates of soybean seeds, extracted as described before [30] (100 µl 10 ml^{-1}); (d) 3-oxo-C6-AHL autoinducing molecule (50 µl 10 ml^{-1}); and (e) control (without addition of any substrate). First, the strains were single inoculated in the presence and in the absence of the mentioned molecules, in 10 ml of modified-YM medium and grown for 24–48 h at 28 °C. When the strains were co-cultured, the *Bradyrhizobium* + *Azospirillum* ratio was of 70:30 µl, respectively, due to differences in the growth rate. After this period, the OD_{600} was evaluated and the bacterial cultures were diluted to OD_{600} of 0.3. Next, 100 µl of the dilutions were added to each well of the microplate. The plate was covered with a polystyrene cap and carefully inverted and introduced into a moist chamber. The plates were incubated at 28 °C for 14 days. After this, the OD_{600} was evaluated and the culture medium was carefully removed. The plate was dried at 60 °C for 1 h. After this, three washes were performed by immersing the microplate in 0.9% NaCl, and the plate was placed again to dry at 60 °C for 1 h. Following, 100 µl of 0.1% of crystal violet per well was added, and the plate was maintained with the dye for 20 min. The plate was then washed three times with distilled water by submerging the plate. After drying for a further 1 h at 60 °C, 100 µl of 96% ethanol was added to each well of the plate, and the plate was allowed to stir gently until all the violet crystal was dissolved. Finally, to OD was analyzed at 570 nm.

2.8 | Greenhouse and field experiments

The experiment included the eight treatments described in the growth curve experiment, in addition to a ninth treatment consisting of a non-inoculated control. The treatments were arranged in a completely randomized experimental design with six replicates. The experiment was performed under sterile conditions, in Leonard jars receiving N-free nutrient solution [31]. Before sowing, soybean seeds of cultivar BRS 1010 were surface disinfested with alcohol and sodium hypochlorite. The cell concentrations used as inoculant followed the Brazilian recommendation for the soybean co-inoculation, of 1.2×10^6 cells $seed^{-1}$ of *Bradyrhizobium* and 1.2×10^5 cells $seed^{-1}$ of *Azospirillum* (10-fold less than bradyrhizobia) [19], and the inoculants were added at these concentrations to the seeds.

Four seeds were sown per pot, and 5 days after the emergence plants were thinned to two plants per pot. After 35 days of emergence, the chlorophyll content was measured using a portable meter SPAD-502 (Konica Minolta Sensing Inc., Osaka, Japan), in the first recently expanded trifoliated leaf of each plant; the chlorophyll concentration in leaves was estimated according to the calibration curve, as described before [32]. Plants were harvest, roots and shoots were separated in the laboratory, carefully washed and oven-dried at 65 °C for approximately 72 h. Nodules were detached from dried roots and counted. The following parameters were determined: shoot dry weight, root dry weight, number of nodules, and nodules dry weight. The nitrogen content of shoots was obtained by the green salicylate method [33].

The performance of the wild-type CPAC 15 strain was also evaluated in a field experiment performed at the experimental station of Embrapa Soybean in Londrina-PR, Brazil (23°11′S, 51°11′W, 620 m altitude, Köpen-Geiger climate type Cfa). Soil sampling was performed before sowing for evaluation of physical and chemical properties, as described before [34] and the results are shown in Supporting Information, Table S2. Populations of soybean rhizobia [34] and of diazotrophic bacteria [19] in the soil were also determined in the same soil samples and the results are shown in Supporting Information, Table S2.

Soybean cultivar BRS 360RR was used with the following treatments: 1- Non-inoculated control; 2- non-inoculated control receiving 200 kg of N ha^{-1}; 3- inoculated with *B. japonicum* CPAC 15; 4- co-inoculated with *B. japonicum* CPAC 15 + *A. brasilense* Ab-V5; 5- co-inoculated with *B. japonicum* CPAC 15 + *A. brasilense* Ab-V6.

Inoculant concentrations applied to the seeds were the same as of the greenhouse experiment. The experiment was performed in a complete randomized block design, with six replicates, and each plot measured 6 m long × 4 m wide (24 m^2); the plots were separated by 0.5 m-wide rows and 1.5 m-wide terraces to avoid cross contamination from surface flushes containing bacteria or fertilizers that may occur in consequence of heavy rainfall.

At sowing, all plots received 300 kg ha^{-1} of N-P-K (0-28-20) fertilizer, applied in-furrow. No N-fertilizer was applied, except where specified (in treatment 2), that received 200 kg N ha^{-1} as urea, split in two broadcast applications of 100 kg N ha^{-1}, at sowing and as side dressing at 35 days after seedling emergence. Row spacing was 50 cm, with approximately 18 plants m^{-1}, and resulted in a final population of approximately 300,000 plants ha^{-1}.

All plants received leaf sprays of Mo (20 g ha^{-1}) and Co (2 g ha^{-1}) at 30 days after emergence, as recommended for the soybean crop in Brazil. Pests and weed control were as described before [34]. At 35 days, plants were harvested to evaluate the same parameters as in the greenhouse experiment, except for the chlorophyll content. At the physiological maturity, grains were harvested from a central area of 7 m^2 (2 × 3.5 m) of each plot; seeds were collected, cleaned, weighed, and seed moisture was determined and adjusted to 13% to estimate grain yield.

2.9 | Statistical analysis

The data for bacterial growth, biofilm, greenhouse, and field experiments were first submitted to tests of normality and homogeneity of variances. When these conditions were confirmed, the data were submitted to analysis of variance (ANOVA), and when a statistically significant value ($p \leq 0.05$) was confirmed, a multiple comparison of means was performed by the Tukey's test at 5% of probability level.

3 | RESULTS

3.1 | Transconjugant strain of *B. japonicum* CPAC 15 for QS

One transconjugant strain of *B. japonicum* (CPAC 15) containing the plasmid pME6863 was obtained (CPAC 15-QS) and confirmed by tetracycline-resistance selection and amplification of the *aiiA* gene of *Bacillus* sp. A24. Our assumption is that in the transconjugant CPAC 15-QS the AHL-mediated mechanism would be inactivated by the activity of the lactonase of *Bacillus* sp. A24 [23], allowing the comparison between the wild-type CPAC 15 and the transconjugant carrying QS.

3.2 | Detecting and characterizing the AHLs of *B. japonicum* CPAC 15

The QS signals found for the strain CPAC 15 were: 3-OH-C12-AHL, 3-OH-C14-AHL, and 3-oxo-C14-AHL (Supporting Information, Figs. S1 and S2). However, only traces of these molecules were obtained and they soon disappeared; therefore, quantification was not possible. In the CPAC 15-QS strain, the only molecule detected was 3-OH-C14-AHL, with considerably lower signal than in the wild-type CPAC 15; the molecule also disappeared very soon. Although in extremely low concentration, as 3-OH-C14-AHL was detected in CPAC 15-QS, we will say that the plasmid containing the *aiiA* gene resulted in the partial inactivation of AHLs.

3.3 | Analysis of bacterial growth *in vitro*

The initial OD_{600} for all treatments was zero, reaching up to 2.3 for the *B. japonicum* strains and, due to cell aggregation by *A. brasilense* in both single- and co-cultured treatments, great variability was detected in the OD, reaching a maximum value of 1.5.

Bacterial growth was verified for a long period, to check if cell density an viability could be affected by QS mechanisms. In general, no differences in growth between the wild and the transconjugant *B. japonicum* strains were observed up to the late stationary phase (Fig. 1A). It is worth mentioning that the presence of the plasmid was checked throughout the growth of the transconjugant. Despite some punctual differences, in general strains Ab-V5 and Ab-V6 of *A. brasilense* also showed similar growth (Fig. 1B).

When *B. japonicum* was co-cultured with *Azospirillum*, CFUs ranged from about 5×10^7 to 5×10^9 UFC ml^{-1} (Figs. 1C and 1E). In the presence of Ab-V5, statistical differences between the growth of CPAC 15 and CPAC 15-QS were observed at 7, 10, 21, and 36 days (T 168, 240, 504, and 864 h, respectively), and CPAC 15 always

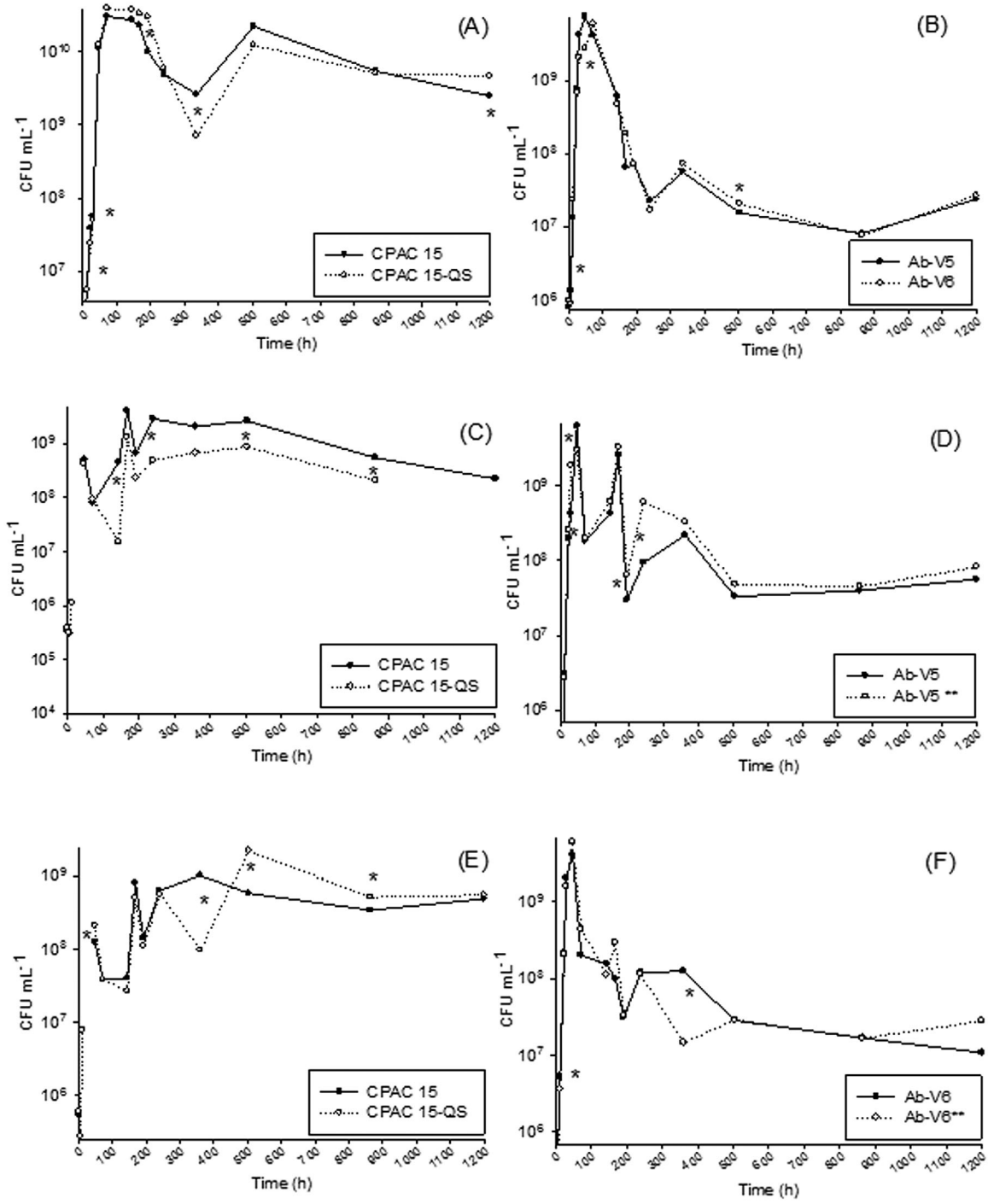

FIGURE 1 Growth curves obtained by the colony forming units per ml (CFU ml^{-1}) of single cultures of (A) *Bradyrhizobium japonicum* strains CPAC 15 and CPAC 15-QS and (B) *Azospirillum brasilense* strains Ab-V5 and Ab-V6. Growth curves of *B. japonicum* CPAC 15 and CPAC 15-QS when co-cultured with (C) Ab-V5 and (E) Ab-V6. Growth curve of (D) Ab-V5 and (F) Ab-V6 when co-cultured with CPAC 15 and CPAC 15-QS. *Means statistical difference ($p < 0.05$) (ANOVA), $N = 3$. **Cultured in the presence of strain CPAC 15-QS

showed higher OD than the CPAC 15-QS strain (Fig. 1C). In regard to the CFU of *A. brasilense* Ab-V5, when co-cultured with *Bradyrhizobium* (Fig. 1D), at T 30 h OD was higher with the wild-type CPAC 15, but at T 48, 192, and 240 h it was higher with CPAC 15-QS (Fig. 1D). In the co-cultures of *B. japonicum* and Ab-V6, in most evaluations (T 48, 336, 504, and 864 h) counts were higher for the transconjugant strain CPAC 15-QS (Fig. 1E). In regard to the growth of *A. brasilense* strain Ab-V6, statistical differences were observed only at T 0 and 336 h, where the OD was higher in the co-culture with the wild-type CPAC 15 (Fig. 1F).

3.4 | Prospection of *lux* genes in the genomes of *Azospirillum brasilense* strains Ab-V5 and Ab-V6

When searching in the genomes of *A. brasilense* strains Ab-V5 (Accession # POQV01000000) and Ab-V6 (Accession # POTD01000000), we found 14 putative *luxR*-like genes (LuxR homologues) in Ab-V5, and 15 putative *luxR*-like genes in Ab-V6. The analysis has shown that, in both strains, that the *luxR*-like genes seem to be duplicated throughout the genome, as some copies were identical, resulting in a total of eight different *luxR*-like genes, and primers were designed based on their sequences (Supporting Information, Table S1). Although apparently duplicated, we should consider that each allele may specify a different function, or be expressed in different conditions. No *luxI*-like genes were found. An analysis of nucleotide similarity of these putative genes has shown a maximum value of 58.5% between *luxR* genes of *B. japonicum* and *A. brasilense*; therefore, in general nucleotide identity was low. In relation to the comparisons of *luxR*-like genes of *A. brasilense* strains Ab-V5 and Ab-V6, nucleotide identities ranged from 61.6 to 100%.

3.5 | Expression of *luxI* and *luxR* genes in *B. japonicum* and *A. brasilense*

To improve our understanding of the roles of the Lux-family of genes in intra- and inter-specific communication between *B. japonicum* and *A. brasilense*, we evaluated the relative expressions of *luxI* and *luxR* of *B. japonicum* CPAC 15 in comparison to the transconjugant CPAC 15-QS and when co-cultured with the *A. brasilense* strains Ab-V5 and Ab-V6. The efficiency test indicated that three (*luxR* Azo1; *luxR* Azo4; *luxR* Azo8) out of the eight primers of the LuxR family of *A. brasilense* strains Ab-V5 and Ab-V6 (Supporting Information, Table S1) had low-efficiency values (below 0.8), and, therefore, were discarded.

The results obtained in the RT-qPCR analysis are shown in Fig. 2, and, although the expressions of both *luxI* and *luxR* were higher in the wild-type than in the transconjugant CPAC 15-QS, statistical difference was observed only for the *luxI* gene. In the co-culture evaluations, we observed increases or decreases in the relative expression of *lux* genes, depending on the gene and on the strain. When CPAC 15 and CPAC 15-QS were co-culture with *A. brasilense* Ab-V5, the relative expression of the *luxI* and *luxR* genes of CPAC 15 was significantly higher than in the CPAC 15-QS (1.8- and 5-fold, respectively) (Figs. 3A and 3B). When the Lux family of Ab-V5 was evaluated, significantly increased expression in co-cultures of *B. japonicum* and Ab-V5 were observed only for the *luxR* Azo3 and Azo6 genes (6- and 8-fold higher, respectively), and higher with CPAC 15 than with the transconjugant CPAC 15-QS (Figs. 3D and 3F). In the co-culture of *B. japonicum* with *A. brasilense* Ab-V6, although the relative expressions of both *luxI* and *luxR* were increased in the wild-type CPAC 15 in comparison to the CPAC 15-QS, statistical significance was obtained only for the *luxR* gene (8.8-fold) (Fig. 4B). However, contrary to the observations with Ab-V5, the co-culture with Ab-V6 with CPAC 15 resulted in decreased expression of two *luxR*-like genes, Azo 3 and Azo 5 (0.15- and 0.41-fold, respectively) in the wild-type CPAC 15 in comparison to the CPAC 15-QS (Figs. 4D and 4E).

3.6 | Biofilm formation *in vitro* in *Bradyrhizobium japonicum* and *Azospirillum brasilense*

In relation to evaluation of the biofilm, the values of OD 570 nm ranged from 1.7 to 2.6, and, in most cases, the biofilm values were higher in co-cultures than in single cultivation (Fig. 5). No statistical difference was detected between the CPAC 15 and the CPAC 15-QS strains when singly inoculated in any of the treatments, but strain Ab-V5 synthesized more biofilm than the Ab-V6 in the control treatment, with LCO and with soybean-seed exudates. In the control treatment, the highest value for the biofilm was observed in the co-culture of CPAC 15 + Ab-V5, but in none of the other treatments did the co-culture with Ab-V5 increase the OD in comparison with the single CPAC 15 or CPAC 15-QS; on the contrary, a decrease was observed for both *A. brasilense* strains in the presence of LCO. In the co-culture with Ab-V6, statistically significant increases were observed for the CPAC 15, but not for CPAC 15-QS, in the presence of genistein, seed exudate, or 3-oxo-C6-AHL (Fig. 5). Both *A. brasilense* strains produced lower biofilm values than the *B. japonicum* CPAC 15 and CPAC 15-QS strains in all conditions assayed (Fig. 5).

3.7 | Greenhouse experiment

After 35 days of growth under greenhouse conditions, the chlorophyll content showed highest value with the co-inoculation of CPAC 15-QS + Ab-V6, which did not differ statistically from the wild-type strain co-inoculated with the same strain of *Azospirillum* (Fig. 6A). Shoot dry-weight results indicated the superior performance of co-inoculation of CPAC 15-QS with either Ab-V5 or Ab-V6 and of CPAC 15 with Ab-V6 (Fig. 6B). No differences were observed in N content of plants inoculated with *B. japonicum* or co-inoculated and, as expected; N concentration ($mg\ g^{-1}$) in shoots of plants singly inoculated with *Azospirillum* was very low (Fig. 6C). On the contrary, total N content in shoots ($mg\ N\ pl^{-1}$) highlighted the superior symbiotic performance

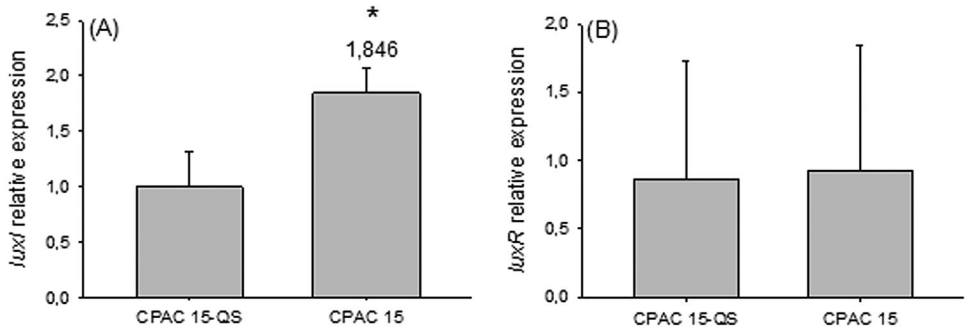

FIGURE 2 RT-qPCR analysis of the relative expression of the *luxI* and *luxR* genes of the wild-type *B. japonicum* CPAC 15 and the CPAC 15-QS transconjugant. (A) *luxI* gene of the wild-type over the CPAC 15-QS; (B) *luxR* gene idem to A; data were normalized in relation to the endogenous control (16S rRNA). Error bars are shown and asterisks indicate significant expression at $\alpha = 5\%$ determined by the REST2009 software; $N = 9$

of CPAC 15-QS + Ab-V6, but did not show statistical difference from CPAC 15 + Ab-V6 and CPAC 15-QS + Ab-V5 (Fig. 6D).

The results obtained for root growth (Fig. 7A) were similar to those for shoot growth (Fig. 6B). In relation to nodulation, nodule number (Fig. 7B) and nodule dry weight (Fig. 7C) followed the same trend as root dry weight (Fig. 7A), with higher values with the wild-type CPAC 15 or with the transconjugant CPAC 15-QS co-inoculated with Ab-V6 and the transconjugant with Ab-V5. Therefore, in relation to nodulation, for nodule number the inoculation of *B. japonicum* with Ab-V6 was better, regardless of QS, and AHL molecules had a negative effect only with Ab-V5.

3.8 | Field experiment

It was not possible to include the transconjugant strain CPAC 15-QS in the field experiment, due to restrictions in introducing genetically-modified microorganisms to the soil, but it was important to conduct a field experiment to confirm the efficacy of single inoculation with CPAC 15 and of the co-inoculation with the two strains of *A. brasilense*. The experiment was performed in a traditional area of soybean cropping that has received inoculant containing *Bradyrhizobium* for several years and thus had a high, well established population of compatible rhizobia (Supporting Information, Table S2). No differences between the treatments were observed in nodule number, indicating that the naturalized population was capable of nodulating the soybean; however, both single and co-inoculation increased nodule dry weight (Table 1). As expected, N-fertilizer reduced both nodule number and dry weight. No differences between the treatments were observed in shoot dry weight at 35 days after sowing, but total N in shoots increased in response to inoculation/co-inoculation and application of N-fertilizer. The highest grain yield was achieved when soybean was co-inoculated with *B. japonicum* and *A. brasilense* Ab-V6, but with no statistical difference from the co-inoculation with Ab-V5. All inoculated treatments and the control receiving N-fertilizer accumulated similar amounts of N in grains, and higher than the non-inoculated control (Table 1).

4 | DISCUSSION

A transconjugant of *B. japonicum* CPAC 15 was obtained in which the AHL-mediated mechanism was almost entirely inactivated by the activity of the lactonase [23]. It was then possible to investigate differences between the wild-type and the transconjugant CPAC 15-QS related to the bacterium-bacterium (intra- and inter-specific) and plant-bacterium interactions, which could be attributed to QS mechanisms.

Synthesis and activity of AHL molecules are related to genes known as *luxI*- and *luxR*-types. *B. diazoefficiens* USDA 110 carries one *luxI* and three *luxR* genes, and the complete genome of this strain highlighted the main pair of genes *bjaI* (homologue to *lux1*) and *bjaR* (homologue to *luxR*) [2,9], in addition to other putative *luxR* genes [35], while *B. japonicum* USDA 6 [35] and CPAC 15 [12] have lower numbers of *luxR* copies. Other *Bradyrhizobium* strains investigated by Case et al. [2] had three *luxI* and four *luxR* (strain BTAi1), or only one pair of *luxI-luxR* (strain ORS278). In the genome of *B. japonicum* CPAC 15, the description of genes homologous to *bjaI* and *bjaR* [12] suggests that the strain might produce AHL molecules such as by strain USDA 110, identified as an isovaleryl-homoserine lactone (IV-AHL) [10]. In our study, we have determined that CPAC 15 synthesizes three different types of molecules, but we were unable to identify IV-AHL. Interestingly, the 3-OH-C14-AHL has been reported as an inducer of survival in *Rhizobium leguminosarum* at the stationary phase, as well as of nodulation genes in the rhizosphere [36]. Only traces of the AHL molecules were detected in CPAC 15, and they soon disappeared, consistent with reports for strain USDA 110 by [8], as well as for the

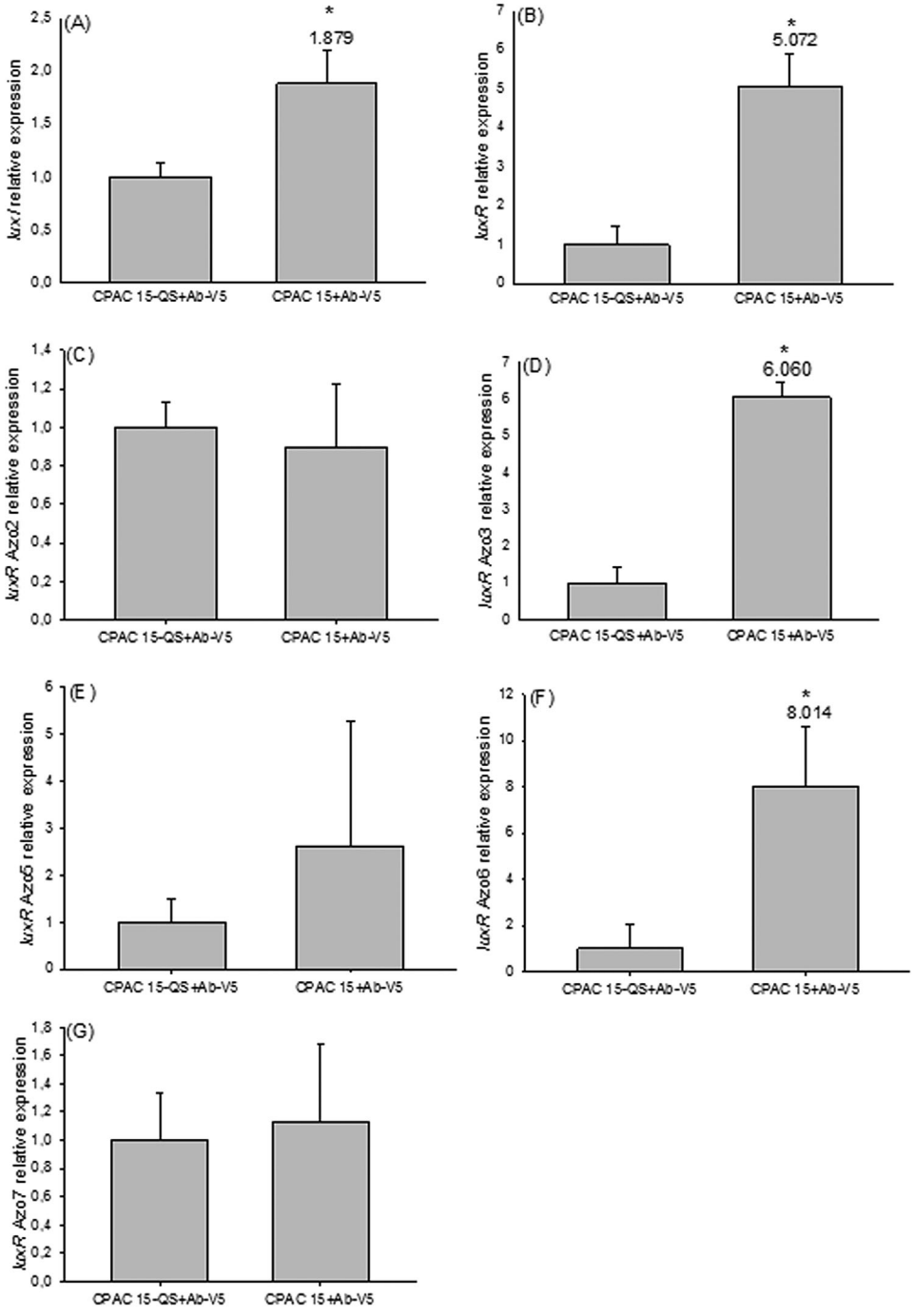

FIGURE 3 RT-qPCR analysis of the relative expression of the *luxI* and *luxR* genes in the co-culture between *B. japonicum* and *A. brasilense* strain Ab-V5. (A) *luxI* gene of the wild-type *B. japonicum* CPAC 15 over the CPAC 15-QS transconjugant in the presence of *A. brasilense* Ab-V5; (B) *luxR* gene idem to A; (C) *luxR* Azo2 gene of the *A. brasilense* strain Ab-V5 in the presence of CPAC 15 over the CPAC 15-QS; (D) *luxR* Azo3 gene idem to C; (E) *luxR* Azo5 gene idem to C; (F) *luxR* Azo6 gene idem to C; (G) *luxR* Azo7 gene idem to C. Data were normalized in relation to the endogenous control (16S rRNA). Error bars are shown and asterisks indicate significant expression at $\alpha = 5\%$ determined by the REST2009 software; $N = 9$

cinnamyl-AHL produced in picomolar concentrations by *Bradyrhizobium* ORS278 [37]. Despite the low concentrations, it has been shown that these molecules can induce gene transcription in *Bradyrhizobium*, by the activation of the LuxR receptors [10,37].

In relation to the PGPB *A. brasilense* strains Ab-V5 and Ab-V6, we have shown that their genomes encompass 14 and 15 putative *lux* genes, respectively, representing eight different putative *luxR*-type transcriptional regulatory genes, duplicated throughout the genomes; no *luxI* gene was detected in any of the

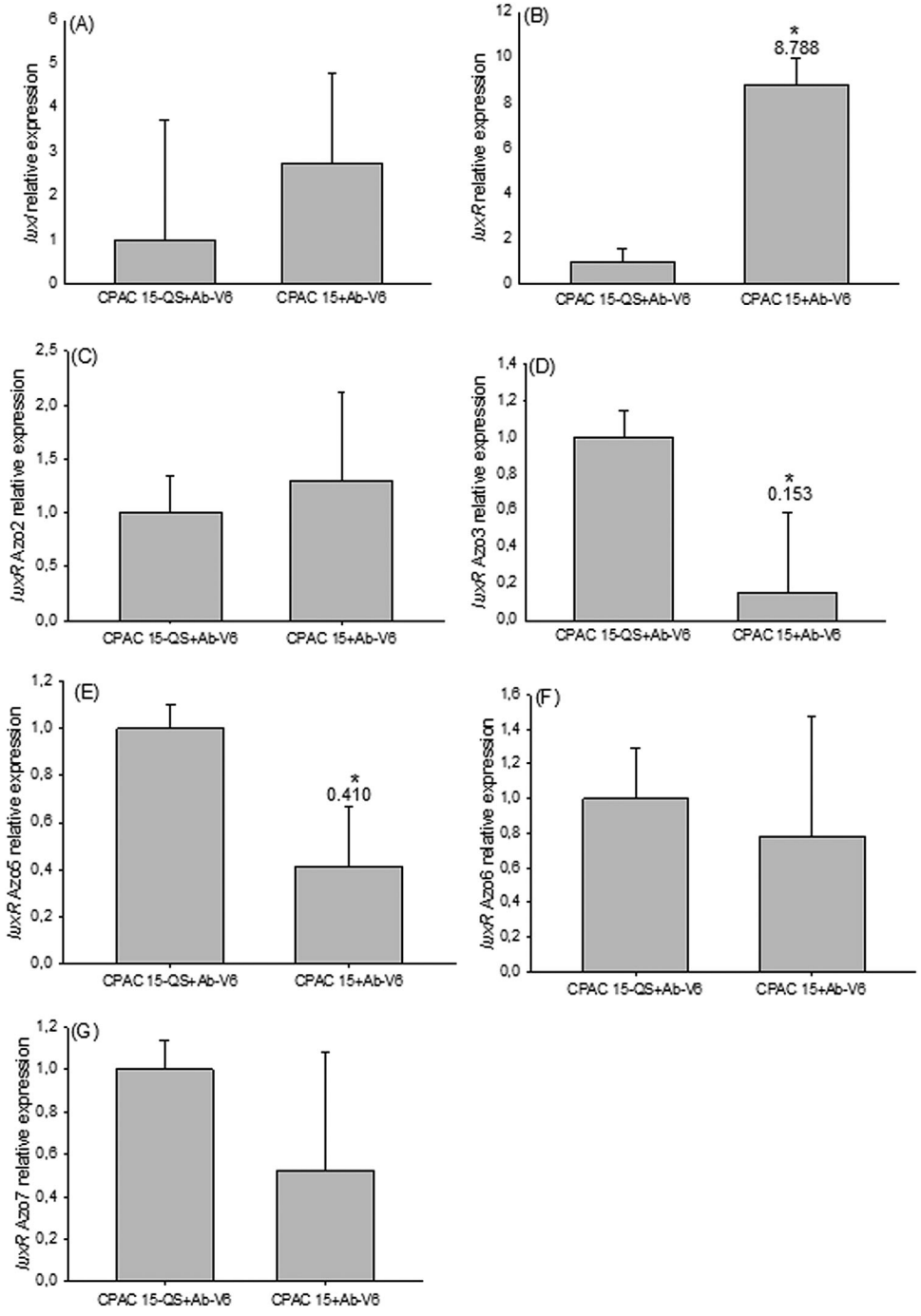

FIGURE 4 RT-qPCR analysis of the relative expression of the *luxI* and *luxR* genes in the co-cultured between *B. japonicum* and *A. brasilense* strain Ab-V6. (A) *luxI* gene of the wild-type *B. japonicum* CPAC 15 over the CPAC 15-QS transconjugant in the presence of *A. brasilense* Ab-V6; (B) *luxR* gene idem to A; (C) *luxR* Azo2 gene of the *A. brasilense* strain Ab-V6 in the presence of the wild-type *B. japonicum* CPAC 15 over the CPAC 15-QS transconjugate strain; (D) *luxR* Azo3 gene idem to C; (E) *luxR* Azo5 gene idem to C; (F) *luxR* Azo6 gene idem to C; (G) *luxR* Azo7 gene idem to C. Data were normalized in relation to the endogenous control (16S rRNA). Error bars are shown and asterisks indicate significant expression at $\alpha = 5\%$ determined by the REST2009 software; $N = 9$

strains, confirming our previous report [17]. This observation is in agreement with the reports that *luxI* genes are rare in *Azospirillum*, although they have been detected in *A. lipoferum* [14], but not in *A. brasilense* [14]. Therefore, our results indicate that Ab-V5 and Ab-V6 would not be able to produce AHL molecules; however, as they have several putative copies of *luxR*, they could respond to AHLs produced by other bacteria in the environment, or in co-culture, or in co-inoculation.

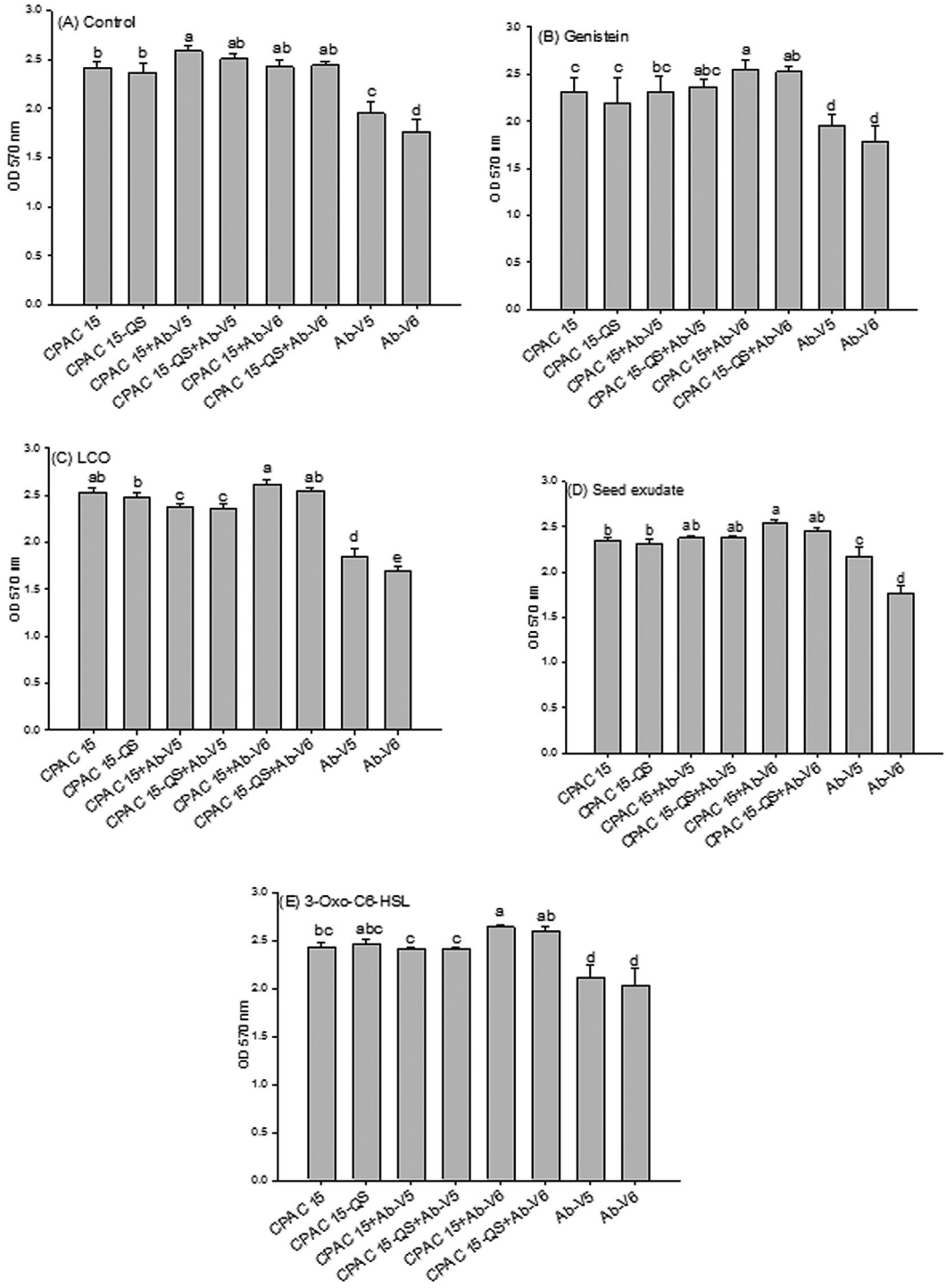

FIGURE 5 Analysis of bacterial biofilm formation *in vitro*, in modified-YM medium adapted for co-culture of *B. japonicum* and *A. brasilense*. Evaluation of biofilm synthesis by strains CPAC 15 and CPAC 15-QS and *A. brasilense* strains Ab-V5 and Ab-V6 when grown alone or in co-culture. (A) Control; (B) genistein; (C) lipoquitine oligosaccharides (LCO); (D) exudate of the soybean seeds; (E) 3-oxo-C6-AHL. Error bars are shown and means followed by the same letter do not differ by Tukey's test at $p < 0.05$ probability

In our study, in the transconjugant of *B. japonicum* there was a decreased expression of *luxI* and *luxR*. In co-culture with both strains of *A. brasilense*, the expression of *luxI* and *luxR* genes was higher in CPAC 15 than in CPAC 15-QS, with an emphasis on the co-culture with Ab-V5. Nevertheless, particularly interesting were the results of *luxR* genes of *A. brasilense*, as in Ab-V5 the expression of two genes was significantly increased (Azo3 and Azo6) in co-culture with *B. japonicum*, whereas, in Ab-V6, two genes were repressed (Azo3 and Azo5). Altogether, the results indicate an intricate

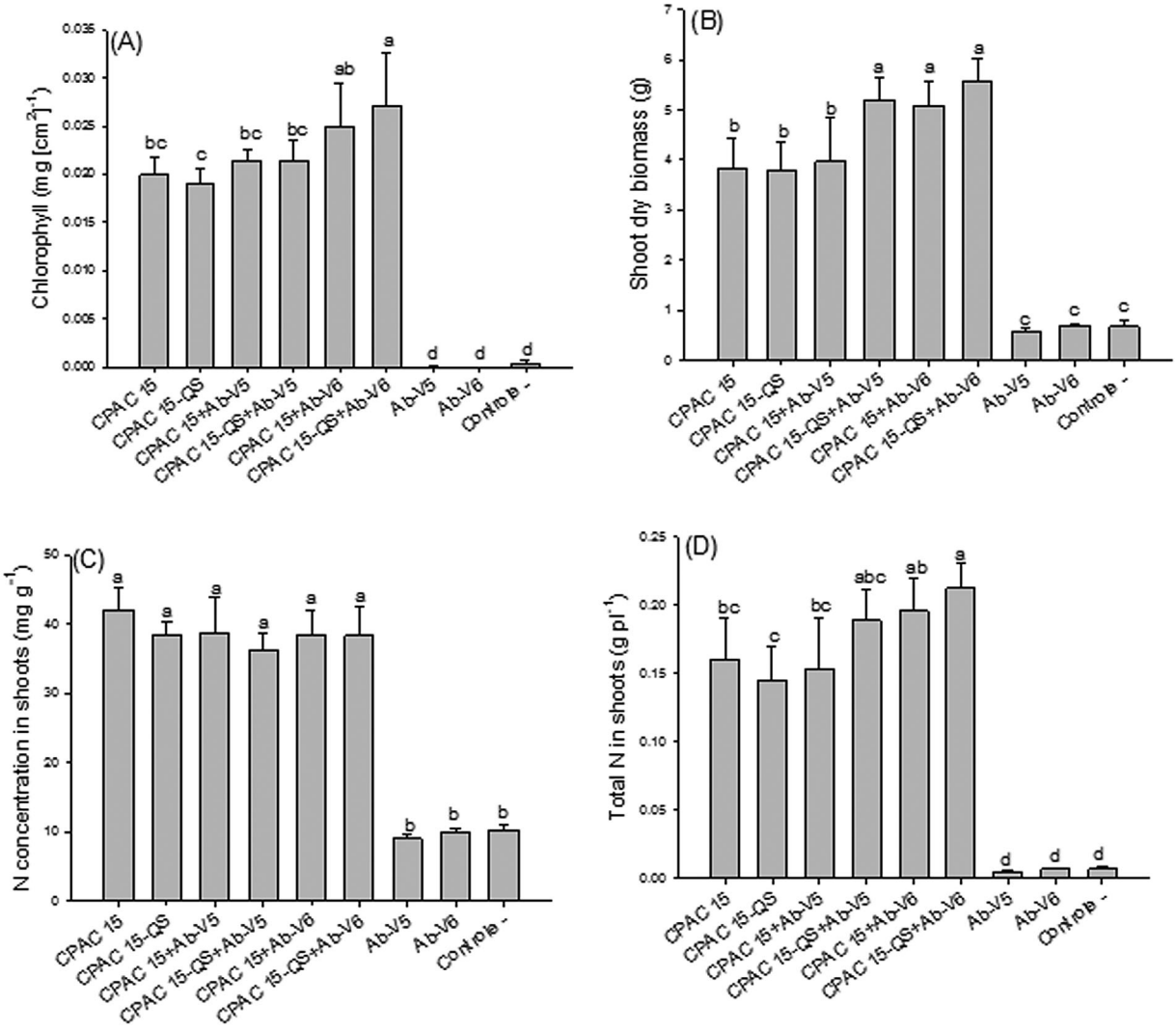

FIGURE 6 Evaluation of soybean growth when single inoculated or co-inoculated with *Bradyrhizobium japonicum* strains CPAC 15 and CPAC 15-QS and *Azospirillum brasilense* strains Ab-V5 and Ab-V6. (A) Chlorophyll content; (B) shoot dry biomass; (C) N content in shoots; (D) total N accumulated in shoots. Soybean plants of cultivar BRS 1010 grown under greenhouse conditions and evaluated at 35 days after emergence. Error bars are shown and means followed by the same letter do not differ by the Tukeýs test at 5% probability; $N = 6$

mechanism of production and recognition of AHL molecules acting not only intra- and inter-species, but which can go deeper at the very specific level of strain. In the rhizosphere, this cross-talk must occur between microorganisms possessing inducers and/or receptors, homologous genes of the Lux family [1–3,8].

In relation to growth, it is well known that AHL autoinducers may reach high intracellular concentrations with increasing population density until establishing a critical level that will lead to a specific response [3]. In our study, in general no differences in growth were observed in the comparison of CPAC 15 and CPAC 15-QS. However, the transconjugant showed higher growth at the final stationary phase. As CPAC 15-QS contains the *aiiA* gene, we may suppose that the absence of autoinducers resulted in higher growth after reaching high cell density, attributing a probable role of AHL molecules in controlling growth in *B. japonicum* CPAC 15. Similar responses have been reported in *B. diazoefficiens* USDA 110 via bradyoxetin, which acts as a siderophore in iron-poor media and at high cell densities [1]. Benefits in terms of controlling growth would rely on a preparation to enter the stationary phase, anticipating limiting or stressing conditions [38]. In our study, when in co-cultures, microbial growth responses varied with the species and also at the strain level. For example, Ab-V5 increased growth in later stages when co-cultured with CPAC 15-QS, indicating that it might be a response to the absence of AHL molecules from *B. japonicum*, while with Ab-V6 cell density was higher with CPAC 15. In addition, growth of *B. japonicum* wild-type and transconjugant also varied with the *Azospirillum* strain.

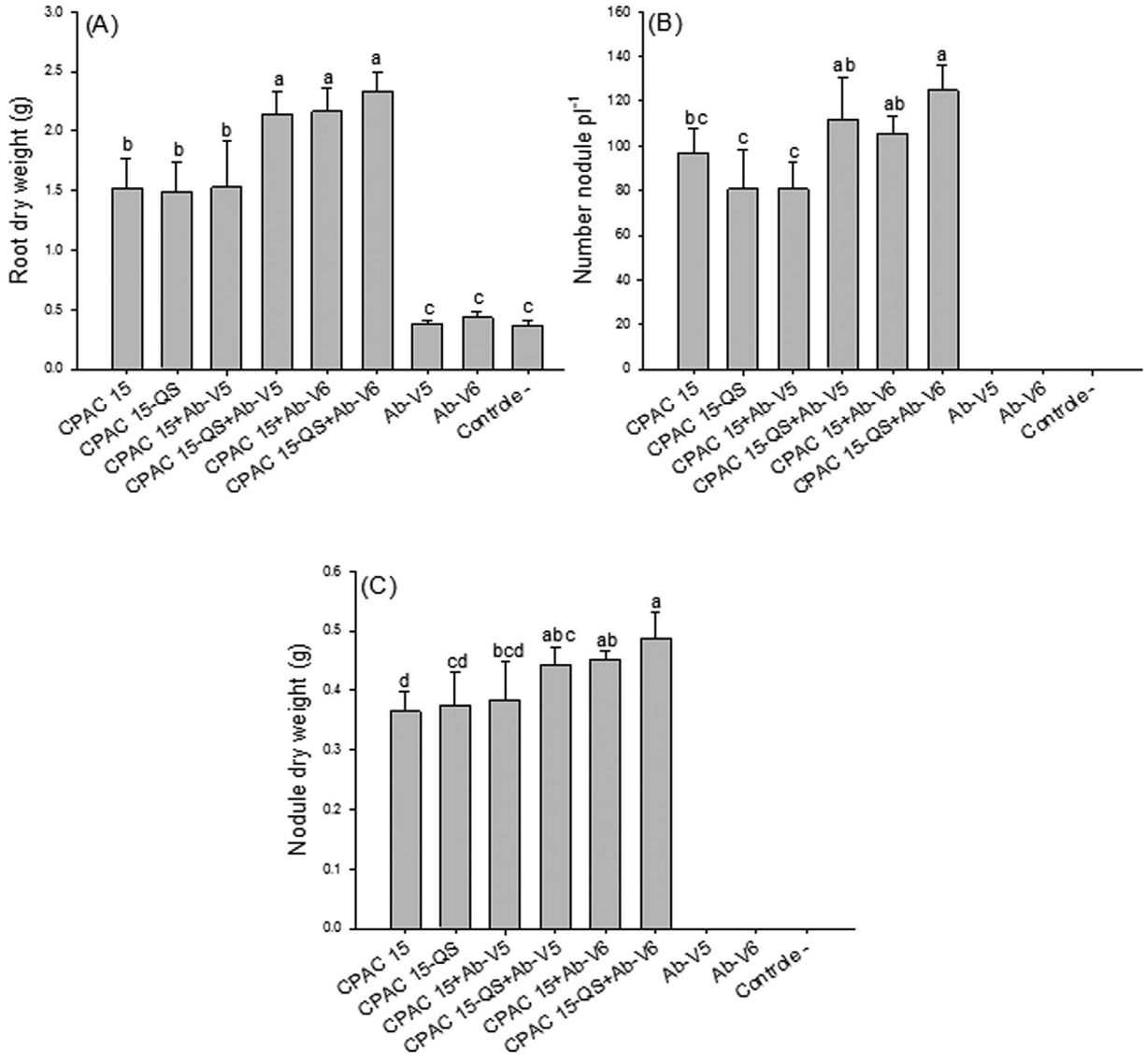

FIGURE 7 (A) Root dry biomass, (B) Nodule number, and (C) Nodule dry weight of soybean cultivar BRS 1010 when single and co-inoculated with *Bradyrhizobium japonicum* strains CPAC 15 and CPAC 15-QS and *Azospirillum brasilense* strains Ab-V5 and Ab-V6. Plants grown under greenhouse conditions and evaluated at 35 days after emergence. Error bars are shown and means followed by the same letter do not differ by the Tukeýs test at 5% probability

It is known that the formation of bacterial biofilms is dependent on QS mechanisms and reports show that the nodulation process depends on biofilm [5,28]. Cross-talk signals determining nodulation also involve plant compounds as flavonoids and bacterial molecules known as LCOs [6,24,30,39]. In our study, in *B. japonicum* alone or co-cultured with *A. brasilense*, we have not detected differences in biofilm formation between the wild-type and the transconjugant *B. japonicum* strains, or by the addition of genistein, LCO, or of soybean-seed exudates. Therefore, for the symbiosis of CPAC 15 with soybean, apparently, the biofilm formation via QS does not seem to play a main role. *Azospirillum* also did not respond to any of the external molecules, or Ab-V5 to the co-culture with the wild-type or the transconjugant *B. japonicum*. In general, we observed an increase in biofilm formation when both wild-type and transconjugant *B. japonicum* strains were co-cultured with *A. brasilense* Ab-V6, another indicator of interspecies communication with strain specificity. However, another hypothesis could be of a synergic, additive effect of extracellular polymeric substances (EPS) present in the biofilm matrix and produced by each individual strain. Anyway, the differences observed in relation to induction of biofilm synthesis were not consistent enough to indicate this property in pre-selection strain programs.

The last step of our study included studies *in vivo*, starting with a greenhouse experiment, where, by analyzing plant parameters, we did not observe differences in symbiotic

TABLE 1 Nodule number (NN), nodule dry weight (NDW), shoot dry weight (SDW), total shoot N content (TSNC) at 35 days after sowing and grain yield (Yield) and total *N* in grains (TNG) of soybean plants (BRS 360RR) in response to seed inoculation consisting of single inoculation with *Bradyrhizobium japonicum* strain CPAC 15, or of co-inoculation with *B. japonicum* and *Azospirillum brasilense* strains Ab-V5 and Ab-V6

Treatment	NN (n ° pl^{-1})	NDW (mg pl^{-1})	SDW (g pl^{-1})	TSNC (mg N pl^{-1})	Yield (kg ha^{-1})	TNG (kg N ha^{-1})
Non-inoculated	25 a[a]	55 b	1.72 a	36.6 b	2512 c	117 b
Non-inoculated + N-fertilizer (200 kg N ha^{-1})	14 b	33 c	2.03 a	60.2 a	2556 bc	147 a
Inoculated with *Bradyrhizobium*	26 a	79 a	1.89 a	55.4 a	2580 bc	148 a
Co-inoculated with *Bradyrhizobium* + *Azospirillum* Ab-V5	24 a	80 a	1.90 a	55.1 a	2720 ab	150 a
Co-inoculated with *Bradyrhizobium* + *Azospirillum* Ab-V6	27 a	85 a	2.00 a	56.7 a	2770 a	155 a

[a]Means ($n = 6$) followed by different letters on the same column are significantly different from one another ($p \leq 0.05$, Tukey test). Experiments performed in Londrina-PR, Brazil.

performance in the comparison of CPAC 15 and CPAC 15-QS. Another interesting observation is that, as an effect of QS, the induction of *nod* genes in USDA 110 in symbiosis with soybean is higher with low culture density and repressed at high densities [39], an important finding vis-à-vis the commercial use of high-density inoculants. However, as in our study, the QS-mechanism apparently did not affect the symbiosis; therefore, inoculants with high concentration should not represent a limitation for strain CPAC 15. Perez-Montaño et al. [28] also did not find significant differences in shoot or nodule dry weight between a wild-type strain of *Sinorhizobium fredii* and its transconjugant containing the same plasmid used in our study, but differences were detected in nodule number.

The best symbiotic performance was achieved with the co-inoculation of CPAC 15-QS with Ab-V5 or Ab-V6, and of CPAC 15 with Ab-V6. Therefore, our results indicate that the AHL molecules of CPAC 15 affect negatively the benefits of co-inoculation with *A. brasilense* Ab-V5; in this case, the removal of the QS of *B. japonicum* affected positively the development of soybean plants. On the contrary, strain Ab-V6 seemed not to perceive these molecules – or it could inactivate them – such that the symbiotic performance of soybean was not affected. Not being negatively affected by the AHL of CPAC 15 might also have contributed to the best performance under field conditions, such that the co-inoculation with Ab-V6 resulted in the highest grain yield. From these observations we may conclude that the lack of perception of AHL molecules by *Azospirillum* might be an interesting property to be searched for in strain selection programs for co-inoculation with soybean *Bradyrhizobium*.

Benefits of co-inoculation of soybean with *Bradyrhizobium* spp. and *Azospirillum* spp. have been broadly reported [40,15,19–21]. In our field experiment, the benefits were particularly relevant for the co-inoculation with *A. brasilense* Ab-V6, which resulted in an additional increase of 190 kg ha^{-1} in comparison to the single inoculation with *B. japonicum* CPAC 15. Among other effects, strain Ab-V6 might be benefited because it does not suffer any physiological limitation by the AHL of the *Bradyrhizobium* CPAC 15.

In conclusion, the analyses and experiments performed in vitro and in vivo confirmed the expression of *luxR* and *luxI* in *B. japonicum* CPAC 15 and that at least three AHL molecules are synthesized by this bacterium. Several putative homologues of *luxR* genes were detected in the genomes of *A. brasilense* strains Ab-V5 and Ab-V6, but no *luxI* gene. Interspecies communication via QS between *Bradyrhizobium* and *Azospirillum* was confirmed in several of the evaluations performed, but it also proved to be strain-specific, or that at least the impact of the signaling is strain-specific, such as Ab-V5, but not Ab-V6, responded to the AHL signals released by *B. japonicum* CPAC 15.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest or ethical problems.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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