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Research Article

RESEARCH ARTICLE

Friends or foes in the rhizosphere: traits of fluorescent *Pseudomonas* that hinder *Azospirillum brasilense* growth and root colonization

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ABSTRACT

Bacteria of the *Azospirillum* and *Pseudomonas* genera are ubiquitous members of the rhizosphere, where they stimulate plant growth. Given the outstanding capacity of pseudomonads to antagonize other microorganisms, we analyzed the interaction between these two bacterial groups to identify determinants of their compatibility. We could establish that, when in direct contact, certain *Pseudomonas* strains produce lethality on *Azospirillum brasilense* cells using an antibacterial type 6 secretion system. When analyzing the effect of *Pseudomonas* spp. diffusible metabolites on *A. brasilense* growth on King's B medium, we detected strong inhibitory effects, mostly mediated by siderophores. On Congo Red medium, both inhibitory and stimulatory effects were induced by unidentified compounds. Under this condition, *Pseudomonas protegens* CHA0 produced a Gac/Rsm-regulated antibiotic which specifically inhibited *A. brasilense* Sp7 but not Sp245. This effect was not associated with the production of 2,4-diacetylphloroglucinol. The three identified antagonism determinants were also active *in vivo*, producing a reduction of viable cells of *A. brasilense* in the roots of wheat seedlings when co-inoculated with pseudomonads. These results are relevant to the understanding of social dynamics in the rhizosphere and might aid in the selection of strains for mixed inoculants.

Keywords: *Azospirillum*; *Pseudomonas*; type VI secretion system; siderophores; Gac/Rsm; plant growth-promoting rhizobacteria

INTRODUCTION

The progressive increase of synthetic chemicals applications in agriculture to fight out pests and diseases has adverse effects on human health. As a green alternative to these practices, the use of inoculants for crops is in continuous growth and research. Of particular interest are the so-called 'plant growth-promoting rhizobacteria' (PGPR) that associate with plants in a mutualistic relationship. Within the different bacterial groups considered as PGPR, *Azospirillum* spp. are well known for their growth stimulation capacity on crops while fluorescent *Pseudomonas* are particularly attractive as biological control agents due to their

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capacity to produce a wide array of natural compounds with antibiotic effects (Lucy, Reed and Glick 2004). The rich arsenal of biocontrol factors that can be produced by pseudomonads includes physical weapons such as contact-dependent growth inhibition (CDI) toxins (Mercy *et al.* 2016) and type VI secretion systems (T6SS) (Bernal, Llamas and Filloux 2018), as well as diffusible compounds with antibacterial, antifungal, entomotoxic, nematotoxic and phytotoxic activity (Gross and Loper 2009). In the past two decades, a wide array of antibiotic metabolites produced by the model species *Pseudomonas protegens* (ex-*fluorescens*) have been identified and extensively characterized in terms of their genetic regulation (Lapouge *et al.* 2008). However, genome mining strategies have shown that PGPR might still hold a rich source of natural compounds with potential technological applications (Paterson *et al.* 2017).

For decades, *Azospirillum* and *Pseudomonas* strains have been successfully commercialized as separate crop inoculants. More recently, mixed inoculants were developed, which combine these two PGPR to potentiate their effect on plants, although their compatibility has not been properly studied (Valverde, Gonzalez Anta and Ferraris 2015). The pioneering work carried out by Couillerot *et al.* (2011) proved that biocontrol metabolites produced by certain fluorescent pseudomonads can indeed interfere with *Azospirillum brasilense* growth and root colonization. Notably, they showed that *A. brasilense* can either be inhibited or stimulated by the antibiotic 2,4-diacetylphloroglucinol (DAPG) produced by *Pseudomonas kilonensis F113* (ex- fluorescens), depending on the dose of the compound (Combes-Meynet *et al.* 2011). More recently, Pagnussat *et al.* (2016) analyzed the *in vitro* interaction between *A. brasilense* Sp245 and *P. protegens* CHA0 during biofilm formation. Unexpectedly, it was found that these bacteria can stimulate each other's growth in a cooperative way and that they co-exist separately in structured dual biofilms when growing together in static liquid cultures. However, when growing on solid media, diffusible metabolites produced by CHA0 can be detrimental to the growth of Sp245 colonies (Pagnussat *et al.* 2016).

The understanding of the social dynamics in microbial consortia and the molecular factors that regulate them is of utmost importance for the development of sustainable and more efficient biotechnologies for crop production (Babu 2011). In this work, we broadened the study of the interactions between different *A. brasilense* and fluorescent *Pseudomonas* strains. Using an *in vitro* approach at different growing conditions, we aimed to identify molecular processes and factors that define the outcome of this interaction and further analyze their relevance during seed colonization. The gathered information is not only relevant for understanding the rhizosphere ecology but also to maximize the compatibility of strains in mixed inoculants.

MATERIAL AND METHODS

Bacterial strains and growth

Bacterial strains used in this work (Table 1) were routinely cultured on King's B (KB) (King, Ward and Raney 1954) in the case of *Pseudomonas* spp. or Congo Red Medium (RC) (Rodriguez Cáceres 1982) for *A. brasilense*. In all cases, broth cultures were started in 3 mL of Nutrient Broth (NB, Laboratorios Britania, Argentina) from fresh streaks and incubated overnight (ON) at 28°C and 150 rpm shaking. Cultures were centrifuged at 3500 × *g* for 10 min and cells were resuspended in sterile saline solution (SS; 0.85% NaCl) adjusting their optical densities at 600 nm (OD_{600mn}) with a spectrophotometer (SmartSpec3000, Biorad).

Recombinant strains

To obtain *A. brasilense* Sp245R, a red fluorescent recombinant variant of Sp245, the *mob* region of plasmid pME3280a (Zuber *et al.* 2003) was inserted as a *Bam*HI fragment into the *Bgl*II site of pME7134 (Pagnussat *et al.* 2016). The resulting conjugative plasmid pME7134mob was mobilized into strain Sp245 by triparental mating (Pagnussat *et al.* 2016) and recombinant clones were recovered from RC medium supplemented with 10 μg/mL tetracycline and 20 μg/mL trimethoprim for counter-selection, since *A. brasilense* is naturally resistant to this antibiotic.

The gentamycin-resistant variants of *A. brasilense*, Sp245Gm and Sp7Gm, were obtained by inserting a Gm^r determinant into the chromosome using a miniTn*7* system. Plasmids pME3280a and pUX-BF13 (Bao *et al.* 1991) were mobilized into strains Sp245 and Sp7, and recombinant clones were recovered in RC medium supplemented with 10 μg/mL gentamycin and 20 μg/mL trimethoprim for counter-selection.

Direct bacteria-bacteria interaction

The growth of *A. brasilense* colonies in direct contact with *Pseudomonas* was studied on Nfb-Fe-NO_3 (NNF) medium (Pagnussat *et al.* 2016). First, 1 mL of *Pseudomonas* suspension containing approximately 1×10^9 CFU ($OD_{600nm} = 1$) was spread onto an NNF plate. After drying, the plates were seeded drop-wise with 10 μL of serial dilutions of an *A. brasilense* suspension that contained approximately 5×10^8 CFU·mL^{-1} ($OD_{600nm} = 2$). The plates were incubated at 28°C for 3 days or until colonies reached the desired size. Colony number and their morphology were analyzed with an optical magnifier (Olympus SZX12) at 7× magnification. NNF plates not seeded with *Pseudomonas* cells were used as a control for colony counts in the absence of interaction. In competition assays, RC plates were spotted in triplicate with 10 μL of a 1:1 mixed suspension of *A. brasilense* Sp245R and *Pseudomonas* sp. containing 5×10^8 CFU·mL^{-1} of each bacterium. After 48 h of incubation at 28°C, plates were visualized and photographed on a UV-transilluminator (ImageQuant 400, GE Healthcare) and the fluorescence intensity of each drop was estimated. Individual strain suspensions containing 5×10^8 CFU·mL^{-1} were used as a control.

Bacteria–bacteria interaction at a distance

The effect of fluorescent *Pseudomonas* diffusible metabolites on *A. brasilense* colonies growing nearby was studied on RC or King's B solid medium. After spreading 1 mL of a suspension of *A. brasilense* containing approximately 5×10^3 CFU, plates were left to dry, spotted with 5 μL of *Pseudomonas* suspensions containing 10^9 CFU·mL^{-1} and incubated at 28°C for 48–72 h. The size and morphology of *A. brasilense* colonies were analyzed with 7× magnification or a transparency scanning unit (UMAX Astra 2400S). To quantify the intensity of inhibition, the diameter of colonies within a radius of 1 to 4 mm from the pseudomonad drop, as well as peripheral colonies located at a >5mm radius in the same frame, were measured. The relative colony size value was then obtained as the ratio between diameters of inhibited *vs* peripheral colonies. Each value represents an average of all colonies within a field, and at least four fields were examined per treatment.

Bacteria–bacteria interaction during seed colonization

Bacterial inocula were prepared in Erlenmeyers containing 25

Table 1. Bacterial strains used in this work.

Strain	Description	Reference
A. brasilense		
Az39	Wild-type strain, isolated from wheat roots, Argentina	(Rodriguez Caceres, Di Ciocco and Carletti 2008)
Sp245	Wild-type strain, isolated from disinfected wheat roots, Brazil	(Baldani, Baldani and Döbereiner 1983)
Sp245R	Red fluorescent variant of Sp245	This work
Sp245Gm	Gm^r variant of strain Sp245	This work
$Sp7^T$	Wild-type strain, isolated from *Digitaria* sp. roots, Brazil	(Tarrand, Krieg and Dobereiner 1978)
Sp7Gm	Gm^r variant of strain Sp7	This work
P. protegens		
$CHA0^T$	Wild-type strain isolated from tobacco roots, Switzerland	(Stutz, Défago and Kern 1986)
CHA19	CHA0 Δ*gacS*	(Zuber *et al.* 2003)
CHA631	CHA0 Δ*phlA*	(Schnider-Keel *et al.* 2000)
Pf-5	Wild-type strain isolated from cotton rhizosphere, USA	(Howell 1979)
JL4806	Pf-5 Δ*pvdL*	(Hartney *et al.* 2011)
LK078	Pf-5 Δ*pchA*	(Hartney *et al.* 2011)
LK032	Pf-5 Δ*pvdL* Δ*pchA*	(Hartney *et al.* 2011)
P. brassicacearum Q8r1–96	Wild-type strain isolated from wheat rhizosphere, USA	(Raaijmakers and Weller 1998)
P. fluorescens		
Pf0–1	Wild-type strain isolated from sandy-loam soil, USA	(Compeau *et al.* 1988)
A506	Wild-type strain isolated from pear phyllosphere, Holland	(Wilson and Lindow 1993)
MME1	Wild-type strain isolated from tomato endosphere, Argentina	(Maroniche *et al.* 2016)
MME3	Wild-type strain isolated from tomato endosphere, Argentina	(Maroniche *et al.* 2016)
TAE4	Wild-type strain isolated from wheat endosphere, Argentina	(Maroniche *et al.* 2016)
TAR5	Wild-type strain isolated from wheat rhizosphere, Argentina	(Maroniche *et al.* 2016)
ZME4	Wild-type strain isolated from maize endosphere, Argentina	(Maroniche *et al.* 2016)
P. putida		
LSR1	Wild-type strain isolated from lettuce rhizosphere, Argentina	(Maroniche *et al.* 2016)
MTR4	Wild-type strain isolated from tomato rhizosphere, Argentina	(Maroniche *et al.* 2016)
KT2440R	Rif^r variant of KT2440	(Bernal *et al.* 2017)
KT2440R ΔtssA1	KT2440R mutant deficient in K1-T6SS	(Bernal *et al.* 2017)
KT2440R Δt6ss	KT2440R mutant deficient in all three T6SS	(Bernal *et al.* 2017)

mL of NB and cultivated at 28°C with 100 rpm orbital shaking. When the cultures reached the stationary phase, the cells were collected by centrifugation at 5000 × *g* and carefully resuspended in sterile saline solution to an $OD_{600nm} = 2$. Suspensions of *A. brasilense* and *Pseudomonas* were mixed in equal parts to obtain co-inocula with 1×10^9 CFU·mL^{-1} of each strain. The individual inoculum of *A. brasilense* was prepared by diluting the suspension with 1 vol of SS. Wheat seeds (cv Buck SY300) were superficially disinfected with 1% HClO for 5 min, thoroughly washed 6 times with sterile distilled water and incubated with 100 μL of inoculum per seed for 1 h at 28°C, obtaining a final inoculation dose of 10^8 CFU·seed^{-1}. After removing the excess of inoculum, the seeds were placed over wet filter paper inside a germination container and incubated on a growth chamber at 22°C and 16/8 h light/dark photoperiod. Each treatment consisted of four replicates (containers) with 15 seeds each. After 5 days, the roots of five representative plantlets from each container were crushed in a mortar with 10 vol of SS and the homogenate was used for counting *Pseudomonas* or *A. brasilense* CFUs on Gould's S1 (Gould *et al.* 1985) or RC media plates, respectively. Antibiotics were added to the plates as required.

Data analysis

The software ImageJ (https://imagej.nih.gov/ij/) was used for the measurement of distances and pixel intensities in the pictures. All plots and statistical analyses were carried out with GraphPad Prism 6 (GraphPad Software Inc., California, USA) and Infostat 2017 (http://www.infostat.com.ar), respectively. Plots depict average values and error bars indicate standard deviation. Data were analyzed by one-way ANOVA with Geisser-Greenhouse correction, plus Tukey´s post-test for multiple comparisons. The t-test was employed for paired analyses (Fig. 2E). The Kruskal–Wallis non-parametric test plus Dunn's post-test was used when the data did not fit the requirements of parametric tests (Figs. 2G and 6). In Fig. 1B, Sp245 and Az39 datasets were analyzed by a Linear Mixed Model (LMM) (fixed effects under a model of independent variances) and a Generalized Linear Mixed Model (GLMM) (negative binomial distribution / logarithmic function), respectively, followed by multiple comparisons with the DGC test (Di Rienzo, Guzmán and Casanoves Test; Di Rienzo, Guzman and Casanoves 2002). The A506 treatment was excluded from these analyses due to insufficient replicates. In all cases, differences were considered significant at $P < 0.05$. The statistical grouping resulting from multiple comparison analyses according to Infostat is indicated with letters in each figure. Null values

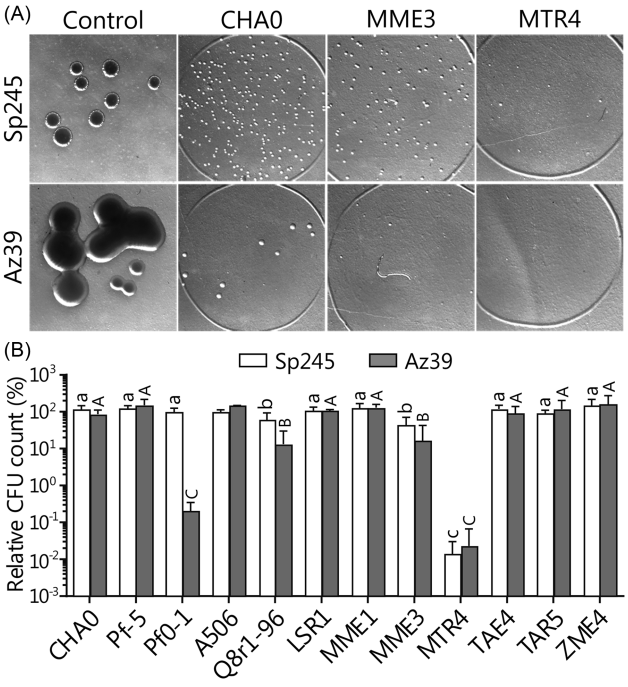

Figure 1. Development of *A. brasilense* colonies in direct contact with fluorescent *Pseudomonas*. **(A)** Colony morphology of two *A. brasilense* strains growing on NNF media plates alone (Control) or alongside representative pseudomonad strains (CHA0, MME3 and MTR4), analyzed under 7× magnification. **(B)** Viability of *A. brasilense* Sp245 or Az39 grown in contact with 12 different fluorescent *Pseudomonas* strains, expressed as the percentage of CFU count on each treatment in comparison to control plates. The average values of four independent experiments, or two in the case of A506 treatment, are plotted in log10 scale. Data were analyzed by LMM (Sp245) or GLMM (Az39) and statistically grouped according to the DGC post-test.

were included in the statistical analyses (Figs. 2G, 6B and Table 2).

RESULTS

The growth of *A. brasilense* on solid media is differentially affected by distinct strains of fluorescent *Pseudomonas*

Previous results indicated that, when co-cultured on solid media, *P. protegens* CHA0 inhibits the growth of *A. brasilense* Sp245 colonies both when in direct contact and in proximity (Pagnussat *et al.* 2016). Here, we carried out a more comprehensive analysis of the interaction between different strains of *A. brasilense* and fluorescent *Pseudomonas* spp. on solid media.

Discrete colonies of *A. brasilense* that grew in direct contact with a lawn of fluorescent *Pseudomonas* showed a reduced size (Fig. 1A). However, some of the *Pseudomonas* strains (i.e. Q8r1–96, MME3 and MTR4) also affected *A. brasilense* viability as evidenced by a reduction in CFU count. The most aggressive strain was *Pseudomonas putida* MTR4, which sharply decreased *Azospirillum* viability (Fig. 1B). *A. brasilense* Az39 was more sensitive to the presence of *Pseudomonas* spp. than strain Sp245 (Fig. 1).

In parallel, the effect of *Pseudomonas* diffusible metabolites on *A. brasilense* Sp245 colonies was analyzed on RC and KB media. All the *Pseudomonas* produced an inhibitory effect over Sp245 colonies, which was dependent on the radial distance between both species. Strain-by-strain variations in the intensity of inhibition were observed (Fig. 2). On RC media, strains

Table 2. Effect of DAPG from *P. protegens* CHA0 on *A. brasilense*.

	Radius of the exclusion halo (mm)	
Strain	*A. brasilense* Sp245	*A. brasilense* Sp7
CHA0	0	7.76 ± 0.30 a
CHA19	0	0 b
CHA631	0	8.26 ± 0.35 a

Pf0–1, MME3 and ZME4 were particularly inhibitory (Fig. 2, panels A and F). The diameter of colonies growing in proximity to Pf0–1 was reduced to ca. 40% of the normal size (Fig. 2E). On the other hand, strains A506, MME1, TAR5 and MTR4 produced only mild effects on the growth of *A. brasilense* Sp245 colonies (Fig. 2, panels A and F). Most notably, a dual and opposite effect was observed with some pseudomonads (*e.g. P. fluorescens* A506 and *P. putida* LSR1) which stimulated the growth of *A. brasilense* colonies in close proximity while inhibiting more distant ones (Fig. 2D). Colonies growing in a radius of up to 1 mm from *P. fluorescens* A506 biofilm were of a normal size, while more distant ones showed a diameter reduction of ca. 30% (Fig. 2E).

On KB rich media, the inhibition was overall stronger and led to the formation of exclusion halos (Fig. 2, panels B and G). There was some degree of coincidence between the aggressiveness of the different *Pseudomonas* strains on both media, with the exception of Pf0–1 and MME3 that were most aggressive on RC but slightly inhibitory on KB (Fig. 2, panels F and G). A correlation was found between the intensity of the inhibition on RC and KB that was significant according to Spearman's nonparametric test ($r = 0.84$, $P = 0.0037$), but only when Pf0–1 and MME3 were excluded from the analysis (Fig. 2H).

Altogether, these evidences suggest that, when co-cultured on solid media, pseudomonads produce a diverse array of inhibitory and stimulatory factors that affect the growth of *A. brasilense* colonies in a strain-dependent fashion.

A. brasilense is vulnerable to the class IV T6SS of *Pseudomonas*

As *P. putida* MTR4 induces lethality on *A. brasilense* when in physical contact but produces only a weak inhibition of colonies growing in proximity (Fig. 1B and 2A), we tested if the contact-dependent effect is caused by a T6SS. We used a rifampicin-resistant variant of *P. putida* KT2440 (which displays 99% *rpoD* nucleotide identity to MTR4) and its isogenic mutants lacking the antibacterial K1-T6SS or all three T6SS (Bernal *et al.* 2017) in direct interaction assays with *A. brasilense* Sp245. As expected, wild-type KT2440R produced a decrease of 3-log on *A. brasilense* CFU number while the T6SS mutants did not affect colony count (Fig. 3A). The same result was obtained in competition assays where equal cell number of each strain were mixed and seeded on RC medium, i.e. *A. brasilense* Sp245 growth was affected in the presence of wild-type but not T6SS mutants (Fig. 3B).

Pseudomonas siderophores are strong inhibitors of *A. brasilense* growth

It was shown that inhibition of *A. brasilense* growth by the presence of pseudomonads was overall stronger on KB, a medium that was developed to enhance the production of *Pseudomonas* siderophores (King, Ward and Raney 1954). Thus, we asked if these compounds were responsible for the enhanced growth

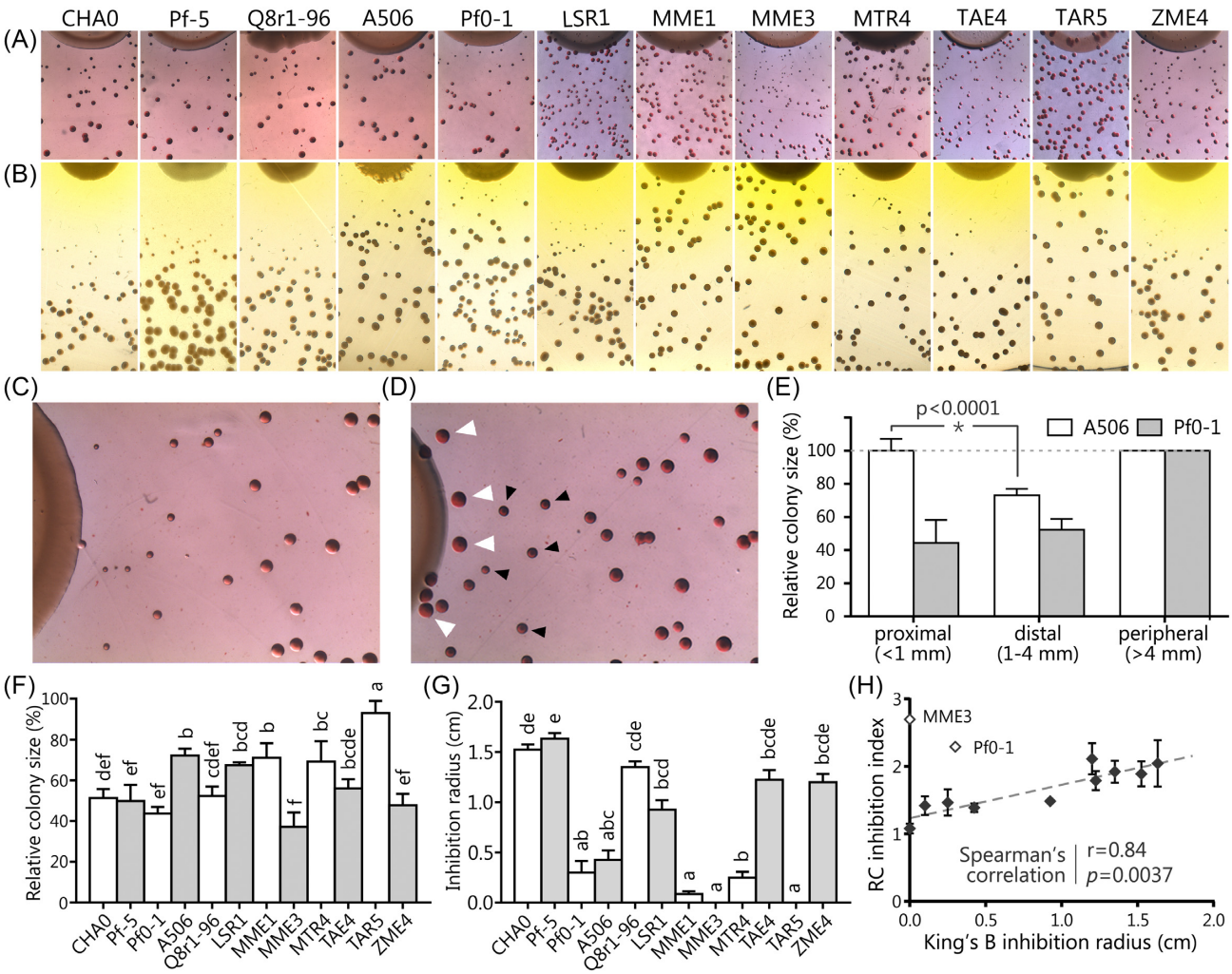

Figure 2. Effect of diffusible metabolites produced by fluorescent *Pseudomonas* spp. on the growth of *A. brasilense* colonies. **(A)** Colonies of *A. brasilense* Sp245 developed at different distances of drop-seeded biofilms from different *Pseudomonas* strains growing on RC media. Plates were photographed under 7× magnification. **(B)** Growth inhibition produced by the *Pseudomonas* strains on *A. brasilense* Sp245 growing on King's B media. **(C)** Zoom of *A. brasilense* Sp245 colonies growing next to *P. fluorescens* Pf-0. **(D)** Zoom of *A. brasilense* Sp245 colonies growing next to *P. fluorescens* A506. Representative colonies with enhanced and diminished growth are indicated with white and black arrows, respectively. **(E)** Relative colony size of *A. brasilense* Sp245 growing at different distances from the drops of *Pseudomonas* strains A506 and Pf0–1. Plotted values are an average of three independent experiments, each one consisting of three visual fields from which all colonies were measured. **(F)** Quantitative analysis of the inhibitory effects on RC medium as the relative size of colonies within the radius of inhibition in comparison with unaffected colonies. Bars depict an average of three values. **(G)** Quantitative analysis of inhibition on KB medium as the size of the exclusion halos. Bars depict an average of four values. **(H)** Regression analysis of growth inhibition in RC vs KB media. Plotted values were subjected to linear regression (black diamonds). Strains Pf0–1 and MME3 (white diamonds) were excluded from the analysis.

inhibition of *Pseudomonas* strains on neighboring *A. brasilense* Sp245 colonies. We repeated the analysis using *P. protegens* Pf-5 isogenic mutants impaired in the production of pyoverdine (Δ*pvdL*) and/or enantio-pyochelin (Δ*pchA*), the two major siderophores produced by this strain. The exclusion halo was significantly reduced in plates seeded with a Pf-5 pyochelin mutant or a pyoverdine-pyochelin double mutant, but was not modified in the single pyoverdine mutant (Fig. 4, panels A and C). A weaker inhibition was still produced by the double mutant (Fig. 4A). Results on RC medium did not differ between wild-type and Pf-5 mutant strains (Fig. 4, panels B and C), suggesting that siderophore production in this condition is negligible. Indeed, when these plates were subjected to O-CAS assay (Pérez-Miranda *et al.* 2007), no typical orange halos were detected around the drops of the different *Pseudomonas* strains (data not shown).

A Gac/Rsm-regulated metabolite of *P. protegens* inhibits *A. brasilense* Sp7 growth

Previous evidences indicated that the metabolite DAPG produced by *P. kilonensis* F113 inhibits the growth of *A. brasilense* Sp7 on solid media (Couillerot *et al.* 2011). In *P. protegens*, the production of this and several other antimicrobial metabolites is upregulated by the Gac/Rsm pathway (Lapouge *et al.* 2008). We tested if any of the observed inhibitory effects of *P. protegens* CHA0 over *A. brasilense* is produced by DAPG by analyzing the effect of isogenic mutants CHA19 (Δ*gacS*) and CHA631 (Δ*phlA*) on *A. brasilense*. Strains CHA19 and CHA631 maintained the capacity to affect colony growth of *A. brasilense* Sp245 both in direct contact (data not shown) and in close proximity on RC medium (Fig. 5A). The same result was obtained with *A. brasilense* Az39 (data not shown). However, *P. protegens* CHA0 produced a much

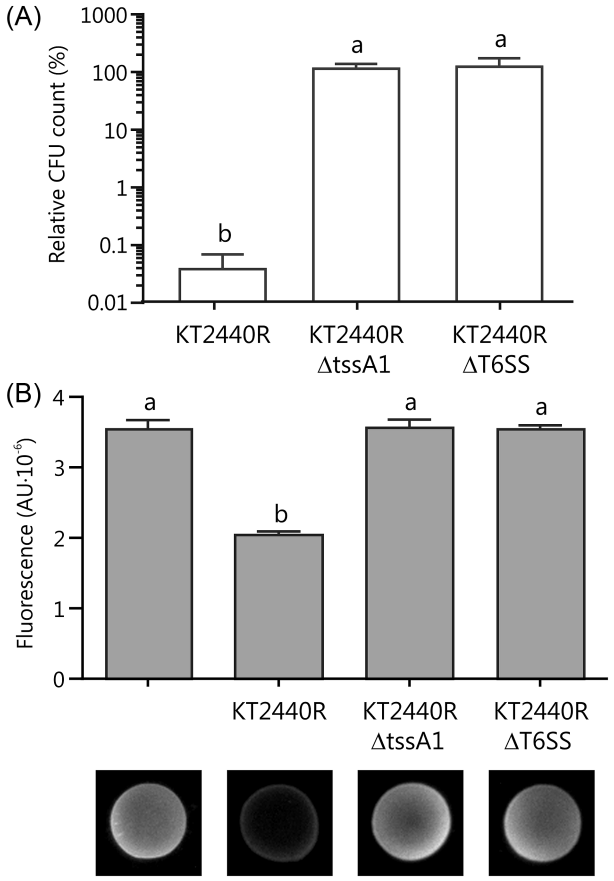

Figure 3. Effect of the T6SS from *P. putida* KT2440R on the viability of *A. brasilense* Sp245. **(A)** Relative CFU count of *A. brasilense* Sp245 grown alongside wild-type strain KT2440R or mutants lacking the K1-T6SS (ΔtssA1), or all three T6SS systems (ΔT6SS). The percentage of CFU count relative to the control plate, averaged from two independent experiments, is plotted in log10 scale. **(B)** Competition assay between the red fluorescent variant Sp245R and KT2440R or T6SS mutants. The red fluorescence on biofilms originated from drops of 1:1 mixtures of *A. brasilense* and *P. putida* on RC media (lower panel), was registered and quantified by densitometry in arbitrary units (upper plot). Bars depict the average of three replicates.

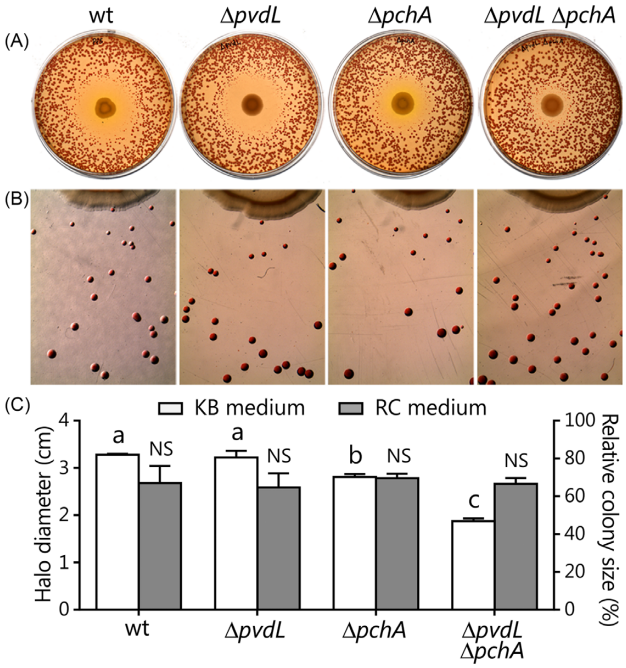

Figure 4. Effect of *P. protegens* Pf-5 siderophores on the growth of *A. brasilense* colonies. **(A)** King's B medium plates simultaneously seeded with a discrete lawn of *A. brasilense* Sp245 and central drops of *P. protegens* Pf-5 (wt), JL4806 (Δ*pvdL*), LK078 (Δ*pchA*), or LK032 (Δ*pvdL* Δ*pchA*). After 48 h of growth at 28°C, the plates were scanned. **(B)** RC medium plates were treated as explained above and examined under 7× magnification. **(C)** Quantification of the inhibitory effects on KB and RC media as the diameter of the inhibition halo (right axis, white bars) or the inhibited colony size relative to unaffected colony size (left axis, stripped bars), respectively. Bars depict the average of two independent experiments. No significant differences were found in RC medium inhibition (NS).

stronger inhibition on *A. brasilense* Sp7 than on the other *A. brasilense* strains, and this effect required the Gac/Rsm pathway regulation since the inhibition halo was not observed with CHA19 (Fig. 5B). Unexpectedly, the absence of DAPG production in CHA631 did not result in any reduction of Sp7 growth inhibition (Fig. 5B and Table 2). The same results were obtained with the DAPG-overproducer strain CHA638 (Schnider-Keel *et al.* 2000) and with strain CHA1018 impaired in the production of both DAPG and pyoluteorin (data not shown), which were tested to rule out a possible interference of the higher levels of pyoluteorin produced by CHA631 (Baehler *et al.* 2005).

In vitro antagonism determinants of *Pseudomonas* also interferes with *A. brasilense* root colonization

To test the relevance of our results in an *in vivo* system, we analyzed the influence of the T6SS, siderophores or Gac/Rsm-regulated metabolites on *Pseudomonas–Azospirillum* compatibility during wheat seed colonization under axenic conditions. The influence of *Pseudomonas* T6SS on *A. brasilense* Sp245 root colonization was assessed by co-inoculating it with *P. putida* KT2440

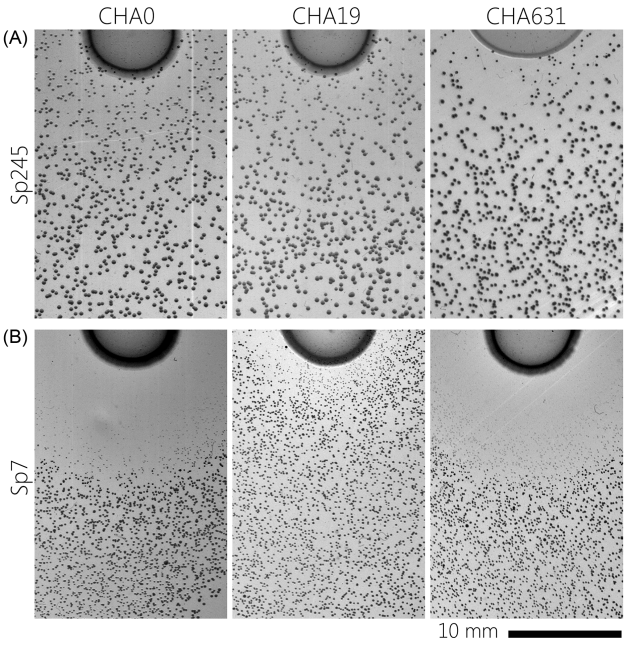

Figure 5. Effect of *P. protegens* CHA0 Gac/Rsm diffusible exoproducts on *A. brasilense*. Discrete lawns of *A. brasilense* strains Sp245 **(A)** or Sp7 **(B)** were developed on RC plates containing drop-seeded biofilms of *P. protegens* CHA0, CHA19 (Δ*gacS*) or CHA631 (Δ*phlA*). Plates scanned after 48 h of incubation at 28°C are shown.

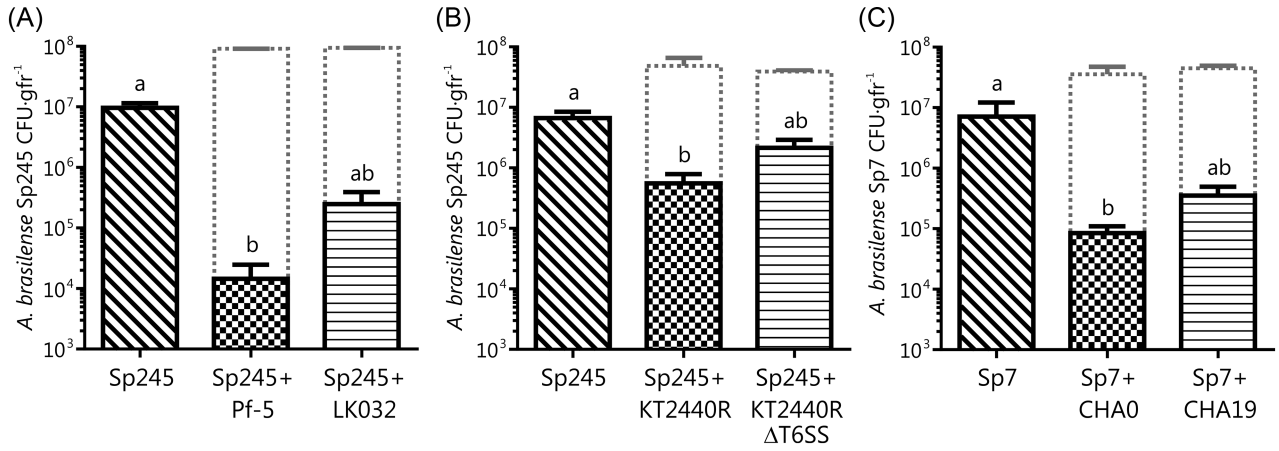

Figure 6. Effect of T6SS, siderophores or Gac/Rsm-regulated metabolites of *Pseudomonas* on *A. brasilense* wheat root colonization. The graphs show the number of *A. brasilense* cells recovered from the roots of wheat seedlings co-inoculated with *A. brasilense* Sp245Gm in the presence of *P. putida* KT2440R or T6SS mutants (**A**), *A. brasilense* Sp245Gm in the presence of *P. protegens* Pf-5 or LK032 (**B**), or *A. brasilense* Sp7Gm in the presence of *P. protegens* CHA0 or *gac*S mutant CHA19 (**C**). The number of CFU recovered per gram of fresh roots (gfr) is plotted in log10 scale. White dotted bars indicate the CFU number of *Pseudomonas* in wheat roots.

or the mutant strain KT2440ΔT6SS that is devoid of all three T6SS clusters. Similarly, the role of *Pseudomonas* siderophores on root colonization of *A. brasilense* Sp245 was analyzed by co-inoculation with *P. protegens* Pf-5 or the mutant strain LK032 that do not produce pyoverdine nor enantio-pyochelin. In third place, *A. brasilense* Sp7 was co-inoculated with *P. protegens* CHA0 or the mutant strain CHA19 that lacks the central regulator *gacS* to determine if Gac/Rsm-regulated exoproducts interfere with Sp7 root colonization. Gentamycin-variants of *A. brasilense* were used to facilitate UFC count from root extracts. We found that the number of *A. brasilense* Sp245Gm cells recovered from the roots of wheat plantlets was significantly lower by 1-log unit when co-inoculated with wild-type KT2440R, but when all the T6SS were non-functional the reduction of CFU was not significant in comparison to single-inoculated plants (Fig. 6A). The same result was obtained with the K1-T6SS mutant (data not shown). Similarly, *A. brasilense* Sp245Gm suffered a significant 3-log reduction in its colonization capacity when co-inoculated with *P. protegens* Pf-5 whereas the trend for an almost 2-log decrease was not statistically significant at $P < 0.05$ when co-inoculated with the isogenic mutant strain LK032 unable to produce siderophores (Fig. 6B). Finally, strain Sp7Gm co-inoculated with *P. protegens* CHA0 showed a significantly lower number of cells in wheat roots when compared to single-inoculation; co-inoculation with the Gac/Rsm deficient mutant strain CHA19 did not reduce this parameter significantly (Fig. 6C). In all three experiments, the root colonization by *Pseudomonas* mutant and wild-type strains was comparable as evidenced by similar CFU values (Fig. 6).

Although the loss of competitiveness in the mutant strains is evident, a systematic (not significant) reduction of *A. brasilense* CFU values was anyway observed when mutant strains were present. This was also evidenced by the lack of statistical significant differences between both co-inoculation treatments in each experiment (Fig. 6).

DISCUSSION

Azospirillum and *Pseudomonas* are within the most extensively studied genera of PGPR. However, basic studies on their compatibility are scarce despite the capacity of pseudomonads to antagonize other microorganisms, or even azospirilla (Loaces, Ferrando and Scavino 2011; Abo–baker Basha and Khalaphallah 2017). Here, by following an explorative approach, we uncovered an unexpected complexity of responses during the encounter of *Pseudomonas* and *A. brasilense* on solid media. We observed a reduction in *A. brasilense* viability and inhibition of colony growth by some strains of *Pseudomonas* (Figs. 1 and 2). Notably, stimulatory effects could also be detected in some conditions (Fig. 2, panels A and D). Overall, we conclude that several simultaneous mechanisms shape the outcome of these *in vitro* interactions. We next took the challenge of trying to identify some of the factors that underlie these phenomena.

In direct-contact experiments, and in spite of being outnumbered, small *A. brasilense* colonies were able to slowly develop within lawns of all the *Pseudomonas* strains (Fig. 1A). This phenotype, first observed by Pagnussat *et al.* (2016) when analyzing *A. brasilense* Sp245 and *P. protegens* CHA0 interaction, might be caused by generic factors present in pseudomonads. However, unlike CHA0, other strains of *Pseudomonas* decreased the number of Sp245 colonies (Fig. 1B). Using strain KT2440 mutants, we demonstrated that this lethal effect was caused by the action of the antibacterial K1-T6SS that belongs to the cluster or subclass 4B (Fig. 3) (Barret *et al.* 2011). Our evidences suggest that *A. brasilense* is particularly sensitive to an effector secreted by the class 4 T6SS from *Pseudomonas*, being subclass 4B more effective than 4A in killing *A. brasilense* prey cells. Interestingly, it has been shown that *Pseudomonas aeruginosa* counterattacks in response to T6SS firing of nearby aggressive cells from other bacterial species, a mechanism that is called 'T6SS dueling' (Basler, Ho and Mekalanos 2013). Since *A. brasilense* is predicted to encode a subtype 4A T6SS (Li *et al.* 2015), we are tempted to think that a similar process is somehow responsible for inciting the attack of type 4 T6SS from pseudomonads. It is also intriguing if this sensitivity is extended to effectors secreted by class 4 T6SS of other bacterial genera.

We also analyzed the effect of *Pseudomonas* diffusible metabolites on *A. brasilense* colonies growing on two different media. As expected, *A. brasilense* growth was much less affected by pseudomonads on RC medium, where radial growth inhibition allegedly caused by a diffusible metabolite resulted in the reduction of Sp245 colonies final size (Fig. 2, panels A and F). The diffusible factor underlying this general inhibitory effect might be common to fluorescent pseudomonads but not regulated in

the same manner in different strains. The strong inhibitory factor produced by Pf0–1 and MME3 on RC, and that did not have a correlation in KB (Fig. 2H), might be a particular trait of the *koorensis* phylogenetic group to which both strains are closely related (Gomila *et al*. 2015; Maroniche *et al*. 2016). The acidification of the medium has been pointed out as a possible antimicrobial trait of fluorescent *Pseudomonas* (Cheng, van der Voort and Raaijmakers 2015), but it may not be the case in our experiments since the mutant strain CHA1198, which is impaired in the production of gluconic acid (de Werra *et al*. 2009), produced the same results than CHA0 (results not shown).

It was unexpected to find that, simultaneously, some strains (*e.g*. A506) were able to stimulate the growth of nearby colonies (distance < 1 mm), which were dark-reddish and of the same size than control colonies (Fig. 2D and 2E). Three possible scenarios could explain this observation: (i) the presence of both inhibiting and stimulating compounds with differential diffusion patterns, (ii) the 'neutralization' of the inhibitory effect at closer distances or (iii) the same compound acting as a stimulator and inhibitor depending on its concentration. Stimulatory effects have already been described during the interaction of *Azospirillum* and *Pseudomonas* growing in biofilms. For example, the growth of *A. brasilense* Cd biofilms on solid media can be enhanced at sub-inhibitory concentrations of DAPG, an antibiotic produced by certain pseudomonads (Couillerot *et al*. 2011). Also, the growth of *A. brasilense* Sp245 biofilms on static liquid culture is stimulated in the presence of *P. protegens* CHA0 (Pagnussat *et al*. 2016).

The production of siderophores by pseudomonads is a major trait determining their competence in the rhizosphere (Ghirardi *et al*. 2012; Butaite *et al*. 2018). Previously, the negative influence of *Pseudomonas* siderophores on *A. brasilense* growth was inferred from indirect results by us and other researchers (Maroniche *et al*. 2016; Rariz *et al*. 2017). Here, we present solid evidence of the harmful effects of pyoverdine and enantio-pyochelin on *A. brasilense* (Fig. 4). Unchanged inhibition by the pyoverdine single mutation might be explained by a compensation mechanism, since the double mutant showed the strongest reduction of inhibition towards *A. brasilense* (Fig. 4C). Notably, when produced by *P. fluorescens* BBc6R8, these same two siderophores are strong inhibitors of *Laccaria bicolor* and *Streptomyces ambofaciens in vitro* growth (Deveau *et al*. 2016). Nevertheless, *A. brasilense* Sp245 might not be equally sensitive to all classes of pyoverdines because other highly fluorescent strains of *Pseudomonas* on KB are poorly inhibitory. The siderophore salicylate that is also produced by pseudomonads (Meyer, Azelvandre and Georges 1992) was not tested in this work but, since it can also be exploited by *Azospirillum* (Tortora, Díaz-Ricci and Pedraza 2011), inhibitory effects of this compound on *A. brasilense* are unlikely.

Both *P. protegens* CHA0 and Pf-5 strains are known to produce a wide array of secondary products with biocontrol properties, including the antibiotic DAPG, under the fine-tuning of the Gac/Rsm regulatory pathway (Dubuis, Keel and Haas 2007). However, the diffusible metabolites produced on RC media by CHA0 and Pf-5, as well as by the DAPG-producer *Pseudomonas brassicacearum* Q8r1–96, were not particularly inhibitory towards *A. brasilense* Sp245 (Fig. 2A) and Az39 (data not shown). These results were unexpected, since Couillerot *et al*. (2011) demonstrated that the DAPG-producer *P. kilonensis* F113, but not a DAPG$^-$ mutant, inhibits *A. brasilense* Cd and Sp245 growth *in vitro*. In the light of this inconsistency, we expanded RC experiments to include *A. brasilense* Sp7, closely related to strain Cd (Maroniche *et al*. 2017). We found that, unlike Sp245 and Az39, strain Sp7 is indeed sensitive to a Gac-regulated metabolite produced by *P. protegens* CHA0 which, surprisingly, is not DAPG (Fig. 5). The apparent lack of effect of DAPG on *A. brasilense* might be explained by a low-level production of this antibiotic on RC medium. The identification of the anti-Sp7 Gac-regulated metabolite remains as a challenge for future studies.

It has been argued that biocontrol traits identified by *in vitro* methods often fail to manifest under real conditions, possibly by the lack of expression of the required factors or inability to compete with natural microorganisms (Campbell 1986; Babu 2011). Thus, we aimed to test the relevance of our results *in vivo*. We demonstrated that wheat root colonization by *A. brasilense* Sp245 can be hampered in the presence of pseudomonads and that this interference is not significant if the T6SS machinery, siderophores synthesis or Gac/Rsm pathway are inactivated(Fig. 6), validating what was observed *in vitro* on solid media. The number of *A. brasilense* cells recovered from roots was always higher in single-inoculated plants than in co-inoculated plants, even with mutant strains of *Pseudomonas*, suggesting that multiple factors from pseudomonads are in action during seed colonization to outcompete azospirilla. In line with our findings, co-inoculation with *P. kilonensis* F113 or the mutant strain F113G22 (DAPG-) proved that DAPG production by pseudomonads decreases *A. brasilense* Sp245 and Cd cell number in wheat roots (Couillerot *et al*. 2011). Additional evidences of compatibility issues were reported after co-inoculating *A. brasilense* Az39 with *Pseudomonas oryzihabitans* on rice (Rariz *et al*. 2017), or *A. lipoferum* with *P. fluorescens* on wheat (Abo–baker Basha and Khalaphallah 2017). Taken together, all these evidences highlight the need of assessing the compatibility of rhizobacteria before combining them in mixed inoculants. It is important to note that, even when the axenic system used in this work was useful for visualizing the action of antagonistic factors during seed and root colonization, the expression of these characteristics might be supressed in the presence of other ecological factors like the complex composition of the rhizosphere microbiome.

Spatial segregation of *A. brasilense* and fluorescent *Pseudomonas* has been observed when they co-exist in mixed biofilms *in vitro* (Pagnussat *et al*. 2016) and in the roots (Couillerot *et al*. 2011); the possibility of this acting as a barrier that diminishes the potential detrimental effects of their encounter in the rhizosphere on *Azospirillum* is a hypothesis that needs further research. Even when the factors analyzed in this work can outcompete *A. brasilense* in the rhizosphere, they are also part of an arsenal that *Pseudomonas* spp. deploy to combat phytopathogens and other undesirable microorganisms (Lucy, Reed and Glick 2004). Inactivating those factors to improve the compatibility with co-inoculated partners might have unpredictable consequences in their effectiveness as biocontrol agents or PGPR. For example, disabling the Gac/Rsm pathway is adverse to the biocontrol capacity of *P. protegens* (Laville *et al*. 1992) but, on the contrary, increases biocontrol properties of *P. fluorescens* SBW25 (Cheng *et al*. 2013). Rather, the information on potential antagonists could be used for screening natural PGPR strains that are non-competing or mildly aggressive towards its partner, before combining them in a mixed inoculant. It could also guide the design of more suitable media or support for co-inoculant formulation, in which those factors might be repressed or neutralized. For example, *Pseudomonas* strains with T6SS lethal to *A. brasilense* might not be suitable partners for mixed inoculants based on solid carriers in which bacteria are sustained as mixed biofilms. Or high-iron media should be used to combine *Pseudomonas* strains that produce siderophores harmful to *A. brasilense*.

In conclusion, the exploratory approach followed in this work allowed us to establish a role for the T6SS, siderophores and Gac/Rsm pathway in the interaction of *A. brasilense* and fluorescent *Pseudomonas*. Additional evidences of unidentified factors with stimulatory and inhibitory activity were presented. Future studies will hopefully unveil the identity of these compounds and their ecological relevance in the field.

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