The role of the antimicrobial compound 2,4-diacetylphloroglucinol in the impact of biocontrol *Pseudomonas fluorescens* F113 on *Azospirillum brasilense* phytostimulators

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Pseudomonads producing the antimicrobial metabolite 2,4-diacetylphloroglucinol (Phl) can control soil-borne phytopathogens, but their impact on other plant-beneficial bacteria remains poorly documented. Here, the effects of synthetic Phl and Phl+ *Pseudomonas fluorescens* F113 on *Azospirillum brasilense* phytostimulators were investigated. Most *A. brasilense* strains were moderately sensitive to Phl. *In vitro*, Phl induced accumulation of carotenoids and poly-β-hydroxybutyrate-like granules, cytoplasmic membrane damage and growth inhibition in *A. brasilense* Cd. Experiments with *P. fluorescens* F113 and a Phl− mutant indicated that Phl production ability contributed to *in vitro* growth inhibition of *A. brasilense* Cd and Sp245. Under gnotobiotic conditions, each of the three strains, *P. fluorescens* F113 and *A. brasilense* Cd and Sp245, stimulated wheat growth. Co-inoculation of *A. brasilense* Sp245 and *Pseudomonas* resulted in the same level of phytostimulation as in single inoculations, whereas it abolished phytostimulation when *A. brasilense* Cd was used. *Pseudomonas* Phl production ability resulted in lower *Azospirillum* cell numbers per root system (based on colony counts) and restricted microscale root colonization of neighbouring *Azospirillum* cells (based on confocal microscopy), regardless of the *A. brasilense* strain used. Therefore, this work establishes that Phl+ pseudomonads have the potential to interfere with *A. brasilense* phytostimulators on roots and with their plant growth promotion capacity.

INTRODUCTION

Microbial interactions in the rhizosphere are of paramount importance for plant growth and health (Barea et al., 2005; Raaijmakers et al., 2009). Many strains of fluorescent *Pseudomonas* are effective at colonizing plant roots and have been extensively studied for their plant-beneficial effects (Couillerot et al., 2009). *Pseudomonas* strains may benefit the plant directly, via induction of systemic resistance to pathogens (Bakker et al., 2007), deamination of the ethylene precursor 1-aminocyclopropane-1-carboxylate (Hontzeas et al., 2004), auxin production (Picard & Bosco, 2005) and/or associative nitrogen fixation (Mirza et al., 2006). Plant-beneficial effects by fluorescent pseudomonads may also include the inhibition of soil-borne phytoparasitic micro-organisms, which often involves production of siderophores (Lemanceau et al., 1992) and especially antimicrobial secondary metabolites (Raaijmakers et al., 2002; Haas & Défago, 2005).

2,4-Diacetylphloroglucinol (Phl), pyoluteorin, pyrrolnitrin, hydrogen cyanide and viscosinamide are some examples of well-studied antimicrobial metabolites produced by biocontrol strains of fluorescent pseudomonads (Haas & Keel, 2003). The ability to produce Phl was shown, both *in vitro* and *in vivo*, to be a particularly important biocontrol trait by comparing wild-types and non-producing mutants (Vincent et al., 1991; Fenton et al., 1992; Keel et al.,

Abbreviations: CLSM, confocal laser scanning microscopy; LC50, Phl concentration needed to abolish growth in at least 50% of cases; UHPLC, ultra-high-pressure liquid chromatography.

A supplementary figure is available with the online version of this paper.
In addition to microbial inhibition, Phl can also affect root fixation (Bally & Elmerich, 2007), production of nitric hormones, especially auxins (Costacurta & Vanderleyden, 1995; Dobbelaere et al., 2002). However, many other rhizobacterial taxa are also important to consider, because they can occur in the same rhizosphere as Phl+ pseudomonads (Barea et al., 2005; Kyselková et al., 2009) and may have positive effects on the host plant. Whether these rhizobacteria can be inhibited by Phl (and Phl+ pseudomonads) on roots is not known.

In addition to microbial inhibition, Phl can also affect root physiology (Brazelton et al., 2008), which in turn may influence the conditions in which saprophytic rhizobacteria colonize the rhizosphere. Indeed, Phl (or the presence of Phl+ pseudomonads) can elicit induced systemic resistance in the plant (Iavicoli et al., 2003), but the consequences are probably negligible when considering root colonization by other saprophytic bacteria. Much more significantly, roots exposed to Phl display enhanced exudation of amino acids (Phillips et al., 2004), and this may enhance the ability of saprophytic bacteria to colonize roots. Therefore, it can be anticipated that Phl+ pseudomonads may have negative, neutral or positive effects on other root-colonizing saprophytic bacteria, depending on whether or not the latter (i) are sensitive to Phl and/or (ii) can benefit from Phl-driven amino acid root exudation.

The aim of this study was to assess whether the ability of root-associated pseudomonads to produce Phl could have an impact on growth and root colonization by other saprophytic rhizobacteria. This possibility was investigated in the case of Azospirillum brasilense, one of the most important species of plant-growth-promoting rhizobacteria. The phytostimulatory effects of A. brasilense on cereals are extensively documented (Dobbelaere et al., 2001; Fuentes-Ramírez & Caballero-Mellado, 2006) and their plant-beneficial traits include associative nitrogen fixation (Bally & Elmerich, 2007), production of nitric oxide (Creus et al., 2005; Pothier et al., 2007) and phytohormones, especially auxins (Costacurta & Vanderleyden, 1995; Dobbelaere et al., 1999). This may stimulate root growth, which in turn can lead to a better uptake of nutrients and water by the plant, and thus to better plant health and development (Reid & Renquist, 1997; Dobbelaere et al., 2003; Richardson et al., 2009). Interestingly, Azospirillum and Pseudomonas indigenous populations have been found together in the rhizosphere of tobacco (Kyselková et al., 2009) and wheat (Sanguin et al., 2009).

In this work, A. brasilense strains from different geographical origins and host plants were exposed in vitro to synthetic Phl to determine whether this compound had any deleterious effect. A Phl+ Pseudomonas fluorescens strain and its Phl− mutant were then compared for their effect on cells of A. brasilense Cd and Sp245, both in vitro and in planta. The co-inoculation experiments were done under gnotobiotic conditions using autofluorescent bacterial derivatives to assess whether colonization patterns of particular root zones (and phytostimulatory properties) of A. brasilense can be affected in the presence of a Phl+ pseudomonad.

**METHODS**

**Strains and culture conditions.** All bacterial strains (Table 1) were routinely grown at 28 °C with shaking in Luria–Bertani medium (LBm; Sambrook et al., 1989) containing 5 g NaCl l−1. The other media were N-free medium NfB (Nelson & Knowles, 1978) supplemented with Congo red (0.25 % w/v) when in plates, AB medium (Chilton et al., 1974) containing 5 g malic acid l−1 as carbon source (ABmal), King’s B (King et al., 1954) and sucrose asparagine (Scher & Baker, 1982) supplemented with 100 μM FeCl₃ (SA-Fe; Cronin et al., 1997a). Antibiotics were used at the following concentrations: ampicillin, 40 μg ml⁻¹ (Amp40); chloramphenicol, 15 and 30 μg ml⁻¹ (Cm15 and Cm30, respectively); gentamicin, 25 μg ml⁻¹ (Gm25).

**Effect of synthetic Phl on growth and cell morphology of A. brasilense Cd.** The effect of synthetic Phl (Toronto Research Chemicals) on A. brasilense Cd was investigated in Petri dishes, where the compound was spotted onto water agar containing Cd cells, as follows. Strain Cd was grown for 48 h in liquid NFb supplemented with 1/40 (v/v) LBm. The cells were washed twice in 10 mM MgSO₄ and adjusted to 4 × 10⁶ cells ml⁻¹ (based on optical density). Five millilitres of cell suspension was then mixed with 5 ml molten water agar (1.5 % w/v), and the mixture was immediately poured onto SA-Fe or LBm agar. Phl was dissolved in methanol (to reach 0.1–100 mM Phl) and 15 μl was spotted onto the Cd agar layer. Methanol was used as a Phl-negative control. For SA-Fe and LBm sublayers, the effect of each of the 10 Phl concentrations studied and the Phl-negative control were analysed on two occasions, using two independent Cd cell cultures at each time. The plates were incubated for 72 h at 28 °C.

To assess the impact of Phl on growth, the diameter of the inhibition zone was measured. To assess the impact of Phl on cell morphology, small pieces of water agar were cut off at different locations in the plate. Samples were then fixed with 2 % osmium tetroxide, contrasted with 1 % uranyl acetate, dehydrated in a graded ethanol series and embedded in Epon. Ultra-thin sections (0.1 μm) were cut using a Reichert ultramicrotome, contrasted with lead citrate and observed by using a CM 120 transmission electron microscope (Philips) at 100 kV. Several dozen cells were examined in each of the two replicates.

**Effect of synthetic Phl on carotenoid production by A. brasilense Cd.** The effect of synthetic Phl on the production of carotenoids by A. brasilense Cd was studied by using ultra-high-
pressure liquid chromatography (UHPLC), after growing cells in 24-well microtitre plates (Nalgene NUNC). Phl was dissolved in methanol (0.0001–1 mM Phl) and 15 μl was added to 1.5 ml ABM medium in each well. Methanol (1 % v/v) was used as a Phl-negative control. Cd was inoculated at a 1.5 × 10^6 cells ml^-1. At least two replicates were prepared per treatment. After 7 days of growth at 28 °C, Cd cells were recovered from each well, and cell density was estimated by measuring the OD_{500}. Cells were pelleted, lyophilized and extracted with 1 ml pure methanol by ultrasonication for 15 min. After centrifugation (10 min at 16,000 g), the supernatant was collected. Another extraction with 0.75 ml pure methanol was performed on the pellet. Both supernatants were pooled, filtered and concentrated to 1 mg ml^-1.

UHPLC analysis of the extracts was performed with Agilent 1290 series coupled with a G4212A DAD (Agilent Technologies). The system was managed by the Mass Hunter (Agilent Technologies). Separation was carried out by using a Hypersil Gold-C18 column (100 × 1 mm, 1.9 μm particle size; Thermo Scientific), set at 40 °C. The mobile phase was a linear gradient of 1 % formic acid in water (solvent A) and acetonitrile (solvent B). The linear gradient at the flow rate 0.15 ml min^-1 was 0–0.5 min, 50 % solvent B; 0.5–7 min, 50–100 % solvent B; 7–8 min, 100 % solvent B. Ten microlitres of each sample was injected. Spectra were recorded between 200 and 600 nm and the response at 450 nm was used for quantification of the area under the curve (AUC) for each distinct carotenoid metabolite. The latter was calculated as the ratio of the peak area under the curve (AUC) of all characteristic peaks. The latter was subsequently divided by the AUC at 450 nm and the response at 450 nm was used for quantification of the total content of carotenoids.

Sensitivity of A. brasilense strains to synthetic Phl. Overnight liquid NFb cultures of 11 A. brasilense strains (Table 1) were used to inoculate NFb liquid medium in 96-well microtitre plates (50 μl inoculum into 150 μl medium). Each strain was inoculated in at least six wells (i.e. six replicates). After a 24 h incubation at 28 °C (without agitation), each liquid culture was drop-inoculated onto solid LBm or SA-Fe medium containing Phl at final concentrations of 5, 10, 20, 50, 100, 200, 500 or 1000 μM (previously dissolved in methanol and resulting in 1 % v/v methanol in media), and methanol (1 % v/v) was used in the Phl-negative control. The plates were incubated for 72 h at 28 °C. Colony growth was scored visually, and the Phl concentration needed to abolish growth in at least 50 % of the cases (LC_{50}) was determined. The Phl-negative control and each of the eight Phl concentrations studied were investigated using 6–22 replicates (Table 1).

A. brasilense in vitro inhibition experiments using Phl* pseudomonads and mutants. A. brasilense strains Sp245 and Cd were exposed in vitro to the Phl* sugar beet isolate P. fluorescens F113 (Shanahan et al., 1992), its Tn5: lacZY-induced Phl* biosynthetic derivative F113G22 (Shanahan et al., 1992), and the Phl* complemented mutant F113G22(pCU203) (Fenton et al., 1992). Strains F113 and F113G22 were grown in LBm and F113G22(pCU203) was grown in LBm Cm30. The cells were then washed twice in a 10 mM MgSO4 solution and adjusted to 10^6 cells ml^-1 (based on OD). Each A. brasilense strain was prepared in water agar, which was poured on LBm or SA-Fe medium, as described above, and 15 μl cell suspension of each pseudomonad was spotted. For each A. brasilense strain, growth inhibition tests were performed in duplicate and the whole experiment was done twice. After 72 h incubation at 28 °C, the width (radius) of the inhibition zone surrounding the Pseudomonas colonies was measured. Conversely, the potential inhibitory activity of A. brasilense Cd and Sp245 strains against P. fluorescens F113 was checked, using a similar protocol.

Growth chamber experiments. The effect of P. fluorescens F113, F113G22 and F113G22(pCU203) on growth and root colonization of the wheat isolate A. brasilense Sp245 and the wheat-adapted strain A. brasilense Cd strains was assessed on wheat (Triticum aestivum) under gnotobiotic conditions. The P\textsubscript{uu}–rffy plasmid pMP2444 was introduced (as described by Pothier et al., 2007) into the two A. brasilense strains retained and the P\textsubscript{uu}–rffy plasmid pMP6466 into P. fluorescens F113 and F113G22, in order to monitor cells by fluorescence microscopy (Bloomberg et al., 2000). Both plasmids derive from the broad-host-range vector pBBR1MCS-5 and confer gentamicin resistance. The inoculants were obtained after overnight growth in liquid LBm Cm30 for strain F113G22(pCU203) or LBm Gm25 for all

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host plant</th>
<th>Origin</th>
<th>Phl LC\textsubscript{50}*</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
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<td>France</td>
<td>50–100</td>
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<td>France</td>
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<td>Wheat</td>
<td>Pakistan</td>
<td>200</td>
<td>Blaha et al. (2006)</td>
</tr>
<tr>
<td>Wb3</td>
<td>Wheat</td>
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<td>200–500</td>
<td>Blaha et al. (2006)</td>
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<td>Pakistan</td>
<td>200–500</td>
<td>Blaha et al. (2006)</td>
</tr>
<tr>
<td>Sp245</td>
<td>Wheat</td>
<td>Brazil</td>
<td>200–500</td>
<td></td>
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<tr>
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<td>Cynodon dactylon</td>
<td>USA</td>
<td>200–500</td>
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<tr>
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<td>Japan</td>
<td>200–500</td>
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<td>500–1000§</td>
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<td>ZN1</td>
<td>Maize</td>
<td>Pakistan</td>
<td>200–500</td>
<td>500–1000§</td>
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</table>

*Minimal concentration of synthetic Phl to inhibit growth in at least 50 % of the replicates. Concentrations tested were 5, 10, 20, 50, 100, 200, 500 and 1,000 μM Phl. Where two Phl concentrations are indicated, LC_{50} was between the two values.

†Growth inhibition test performed on six replicates.
‡Growth inhibition test performed on 22 replicates.
§Growth inhibition test performed on 14 replicates.
||Colony pigmentation enhanced with increasing Phl quantities.

100, 200, 500 or 1,000 SA-Fe medium containing Phl at final concentrations of 5, 10, 20, 50, 100, 200, 500 or 1000 μM (previously dissolved in methanol and
other strains. The cells were washed twice in a 10 mM MgSO₄ solution and adjusted to 2 × 10⁷ c.f.u. ml⁻¹ for co-inoculation and 10⁷ c.f.u. ml⁻¹ for single inoculation (based on OD).

Seeds of spring wheat (cv. Fiorina), obtained from Florimond-Desprez, were surface-disinfected, as described by Pothier et al. (2007). They were placed on water agar (15 % w/v) and incubated for 48 h in the dark at 28 °C to enable germination. The plants were then co-inoculated using one Pseudomonas strain and one Azospirillum strain, and in the controls they were inoculated using only one strain or were not inoculated. This was done by treating pre-germinated seeds with 50 μl containing 10⁶ c.f.u. Azospirillum and 50 μl containing 10⁶ c.f.u. Pseudomonas or with 100 μl containing 10⁶ c.f.u. of each strain. Plants were placed near one edge of a square plate (12 × 12 cm) containing water agar (15 % w/v). Each A. brasilense strain was studied in a distinct experiment carried out independently. Four plates (containing four plants each) were used per treatment. The plates were placed standing in a growth chamber at 75 % relative humidity, with 16 h of light (63 μmol m⁻² s⁻¹) at 26 °C and 8 h of dark at 18 °C, and they were sampled 7 days later.

Root development at 7 days after inoculation was assessed by measuring root biomass and characterizing root system architecture using WinRHIZO (Régent Instruments). Four plants, each from a different plate, were studied per treatment.

Analysis of bacterial root colonization in the growth chamber experiments. Confocal laser scanning microscopy (CLSM) observations were done at 7 days using two plants, each from a different plate, per treatment. Samples of 1–2 cm in length were cut from different root zones (root apex, hair root zone and older root parts) and mounted in Aqua-Poly/Mount (Polysciences). A 510 Meta microscope (Carl Zeiss) equipped with argon–krypton and helium–neon lasers was used for analysis of green fluorescence (excitation at 488 nm and detection at 510–531 nm) and red fluorescence (excitation at 543 nm and detection at 563–628 nm). After acquisition of transmitted (in bright-field mode) and/or reflected (in blue, detection at 456–499 nm) lights, the single-colour images were overlaid into a single image using LSM software release 3.5 (Carl Zeiss).

For quantification of the inoculum, four plants were sampled per treatment at 7 days. Bacteria were extracted by vortexing each root system for 5 min in a 15 ml Falcon tube containing 5 ml 10 mM MgSO₄. A serial dilution was prepared in the same solution, and six 10 μl drops from each dilution were spotted onto King’s B Amp40 Cm30 to quantify F113G22(pMP4661), King’s B Amp40 Cm30 to quantify F113G22(pCU203), and NFb Cm15 Gm25 to quantify F113G22(pMP4661), and King’s B Amp40 Cm30 to quantify F113G22(pCU203), and NFb Cm15 Gm25 to quantify Cd(pMP2444) or Sp245(pMP2444). Colonies were not found on plates in the absence of inoculation of seedlings with the main model strains of this species (i.e. Cd, Sp245 and Sp7), provided that the added Phl exceeded 150 nmol (Fig. 1b). Photonic microscopy indicated that cell population biomass was higher in the dark pink halo compared with normal growth areas of the plate located further away (Fig. 1c).

In addition to growth inhibition, electron microscopy indicated that cell morphology of A. brasilense Cd was also affected upon exposure to Phl. In comparison with areas of the plate with normal growth (Fig. 1a), cells in the dark pink halo accumulated carbon storage material (Fig. 1e), presumably poly-β-hydroxybutyrate. In the inhibition zone, the cytoplasmic membrane of the few Cd cells present was physically damaged (Fig. 1f).

Inhibition zones and microscopic observations in the two independent repetitions were similar, and none of the effects of Phl was observed when the Phl solvent (i.e. methanol) was added alone. This indicates that these effects were solely due to Phl.

Effect of synthetic Phl on carotenoid production by A. brasilense Cd in liquid broth

The pink pigmentation of A. brasilense Cd is linked to the synthesis of bacterioruberin-type carotenoids (Nur et al., 1981; Thirunavukkarasu et al., 2008). UHPLC analysis showed that A. brasilense Cd produced four distinct carotenoids in liquid minimal broth (Supplementary Fig. S1, available with the online version of this paper). When 1–200 μM Phl was added, the same carotenoids were produced as well as one (at 1 and 10 μM Phl) to five other carotenoids (between 50 and 200 μM Phl), all displaying typical UV-visible spectra (i.e. 3 λmax between 400 and 550 nm, Supplementary Fig. S1). At 500 and 1000 μM Phl, the growth of Cd was strongly affected and no carotenoids could be detected. The total content of carotenoids was higher at 100 and especially 50 μM Phl compared with the control (Fig. 2).

LC₅₀ of A. brasilense strains to synthetic Phl in complex and defined media

There was some fluctuation in Phl sensitivity in vitro from one A. brasilense strain to the next (Table 1). For the three main model strains of this species (i.e. Cd, Sp245 and Sp7), LC₅₀ (concentration necessary for at least 50 % growth inhibition) was (i) between 200 and 500 μM Phl on LBm and (ii) 500 μM Phl (for Cd and Sp245) or between 500 and 1000 μM Phl (for Sp7) on SA-Fe agar, on which Azospirillum growth is slower (data not shown).
For *A. brasilense* Cd and Sp245 (which have been used hereafter), Phl sensitivity was also observed in liquid cultures. Strain Cd reached $9.2 \pm 1.6 \times 10^7$ c.f.u. ml$^{-1}$ in SA-Fe and $4.7 \pm 1.0 \times 10^8$ c.f.u. ml$^{-1}$ in LBm, but numbers decreased to $0.9 \pm 0.3 \times 10^7$ c.f.u. ml$^{-1}$ in SA-Fe and $0.4 \pm 0.1 \times 10^8$ c.f.u. ml$^{-1}$ in LBm 1 day after exposure to 1000 mMPhl. Similar results were obtained with *A. brasilense* Sp245 (not shown).

Phl sensitivity levels in *A. amazonense*, *A. doebereinerae* and *A. halopraeferens* (LC$_{50}$ on LBm between 200 and 500 mMPhl depending on the strain) were similar to those in *A. brasilense* (not shown). *A. irakense* still grew at 1000 mMPhl on both media, whereas a higher proportion of *A. lipoferum* strains had an LC$_{50}$ $\leq$ 200 mMPhl on LBm and LC$_{50}$ $<$ 500 mMPhl on SA-Fe than did *A. brasilense* strains (not shown).

**Effect of Phl$^+$ P. fluorescens F113 and mutants on growth of A. brasilense Cd and Sp245 in vitro**

Whether Phl production by *Pseudomonas* could have an effect on *A. brasilense* growth was determined by comparing the *in vitro* effects of the Phl$^+$ strain *P. fluorescens* F113, its Phl$^-$ mutant F113G22 and the complemented
derivative F113G22(pCU203) on growth of _A. brasilense_ Cd and Sp245, using the two previously used solid media, which are conducive to Phl production by F113. Strain F113 inhibited the growth of the two _A. brasilense_ strains within 2 days on both types of media (Table 2), and inhibition was greater on complex medium LBm compared with defined medium SA-Fe. Similar strain differences were observed at 7 days, with wider inhibition zones especially on SA-Fe agar (data not shown). Conversely, the _Azospirillum_ strains did not inhibit _P. fluorescens_ F113 even at 7 days.

Compared with the wild-type F113, the Phl" biosynthetic mutant F113G22 clearly failed to inhibit the two _Azospirillum_ strains. Mutant complementation partially restored the _Azospirillum_ inhibition phenotype of F113 (Table 2).

**Effect of Phl" _P. fluorescens_ F113 and mutants on _Azospirillum_ phytostimulation**

To establish whether Phl" _Pseudomonas_ could interfere with _Azospirillum_ phytostimulation ability, wheat seedlings were co-inoculated with both _Azospirillum_ and _Pseudomonas_ strains. When inoculated alone on wheat, after 7 days, _P. fluorescens_ F113 had improved (i) the total root length and total root surface in both wheat experiments and (ii) the root dry weight and the number of roots (only in the Cd experiment) or total root volume (in the Sp245 experiment) (Table 3). With the Phl" biosynthetic mutant F113G22, these phytostimulatory effects were either not found or of a lesser magnitude. The effects of the complemented derivative F113G22(pCU203) were intermediate between those of strains F113 and F113G22. Indeed, strain F113G22(pCU203) fared like F113 for total root length and total root surface (in both experiments), but rather like F113G22 for other root parameters especially in the Cd experiment.

Single inoculation of wheat with _A. brasilense_ Cd resulted in higher root dry weight and total root length (Table 3). However, in comparison with the non-inoculated control, plants co-inoculated with strain Cd and _P. fluorescens_ F113 (i) did not benefit from any increase in root dry weight, total root length or number of roots and (ii) had a lower total root surface. Plants co-inoculated with _A. brasilense_ Cd and either _P. fluorescens_ F113G22 or F113G22(pCU203) were comparable to non-inoculated plants, except that those inoculated with F113G22(pCU203) had greater root dry weight.

Single inoculation of wheat with _A. brasilense_ Sp245 resulted in higher total root length, total root volume and total root surface (Table 3). Compared with the non-inoculated control, plants co-inoculated with _A. brasilense_ Sp245 and _P. fluorescens_ F113 displayed enhanced total root length, total root volume and total root surface, similar to plants inoculated singly. A similar trend was also observed when _A. brasilense_ Sp245 was co-inoculated with F113G22 or F113G22(pCU203).

**Effect of Phl" _P. fluorescens_ F113 and mutants on _Azospirillum_ cell numbers in planta**

When co-inoculated with _P. fluorescens_ F113, _A. brasilense_ Sp245 reached lower cell numbers on wheat roots at 7 days compared with those attained when inoculated singly, but the decrease was only about 1 log c.f.u. g\(^{-1}\) (Fig. 3a). Unlike F113, the Phl" biosynthetic mutant F113G22 had no negative effect on Sp245 at 7 days, whereas the complemented derivative F113G22(pCU203) decreased Sp245 cell numbers to the same extent as the wild-type. Unexpectedly, the Phl" biosynthetic mutant F113G22 facilitated root colonization by _A. brasilense_ Sp245, which reached higher levels compared with those attained 7 days after single inoculation. Similar findings were made with _A. brasilense_ Cd, except that several of these differences were not significant (\(P<0.05\)) because of higher data fluctuation levels (Fig. 3b).

**Effect of Phl" _P. fluorescens_ F113 and mutants on root colonization patterns of _A. brasilense_ strains**

To establish whether Phl" _Pseudomonas_ could also change the microscale root colonization patterns of _Azospirillum_ strains, distinct molecular tags were used for both types of bacteria. CLSM observations 7 days after wheat roots were made with _A. brasilense_ Cd, except that several of these differences were not significant (\(P<0.05\)) because of higher data fluctuation levels (Fig. 3b).

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**Table 2. Growth inhibition of _A. brasilense_ Cd and Sp245 by Phl" _P. fluorescens_ F113, its Phl" mutant F113G22 and the complemented derivative F113G22(pCU203) after 2 days on LBm and SA-Fe agar**

Data shown are the width (mm) of the inhibition zone around the _Pseudomonas_ colony (mean ± SEM). In the absence of _Pseudomonas_, no growth inhibition of _Azospirillum_ strains was observed. For each medium and each _Azospirillum_ strain, letters a and b are used to indicate statistical relations between treatments based on ANOVA and Fisher’s LSD tests (\(P<0.05\)).

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<tr>
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<th>LBm agar</th>
<th>SA-Fe agar</th>
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<tr>
<td></td>
<td>F113</td>
<td>F113G22 (pCU203)</td>
</tr>
<tr>
<td><em>A. brasilense</em> Cd</td>
<td>2.8 ± 0.9 a</td>
<td>0 b</td>
</tr>
<tr>
<td><em>A. brasilense</em> Sp245</td>
<td>3.0 ± 0.0 a</td>
<td>0.8 ± 0.5 b</td>
</tr>
</tbody>
</table>
Table 3. Effect of single and co-inoculation on root system properties of wheat

Data shown are mean ± SEM; n=4 plants. Units for each plant parameter: root dry weight, mg per plant; total root length, cm per plant; total root volume, mm³ per plant; total root surface, mm² per plant. For each root system parameter studied, statistical differences between treatments are indicated with letters a–e (ANOVA and Fisher LSD tests; P<0.05). For the *A. brasilense* Sp245 experiment, root dry weight and number of roots, and for the *A. brasilense* Cd experiment, total root volume, were not significantly different between treatments.

<table>
<thead>
<tr>
<th>Plant parameters</th>
<th>Control</th>
<th>Single inoculation</th>
<th>Pseudomonas co-inoculated with <em>Azospirillum</em></th>
<th>Pseudomonas co-inoculated with <em>Pseudomonas</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Azospirillum</td>
<td>F113</td>
<td>F113G22 (pCU203)</td>
<td>F113G22 (pCU203)</td>
</tr>
<tr>
<td><em>A. brasilense</em> Sp245</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root dry weight</td>
<td>7.2 ± 0.9</td>
<td>7.3 ± 0.4</td>
<td>6.2 ± 0.3</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td>Total root length</td>
<td>10 ± 1 c</td>
<td>19 ± 2 ab</td>
<td>19 ± 2 ab</td>
<td>16 ± 1 b</td>
</tr>
<tr>
<td>Total root volume</td>
<td>13 ± 2 b</td>
<td>83 ± 18 a</td>
<td>73 ± 3 a</td>
<td>63 ± 13 a</td>
</tr>
<tr>
<td>Total root surface</td>
<td>122 ± 14 c</td>
<td>440 ± 65 ab</td>
<td>417 ± 21 ab</td>
<td>347 ± 41 b</td>
</tr>
<tr>
<td>No. of roots</td>
<td>20 ± 6</td>
<td>17 ± 2</td>
<td>17 ± 2</td>
<td>16 ± 4</td>
</tr>
<tr>
<td><em>A. brasilense</em> Cd</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root dry weight</td>
<td>5.5 ± 0.5 c</td>
<td>6.8 ± 0.3 b</td>
<td>8.0 ± 0.4 a</td>
<td>6.8 ± 0.4 b</td>
</tr>
<tr>
<td>Total root length</td>
<td>19 ± 2 c</td>
<td>33 ± 3 b</td>
<td>46 ± 3 a</td>
<td>31 ± 3 b</td>
</tr>
<tr>
<td>Total root volume</td>
<td>92 ± 9</td>
<td>76 ± 3</td>
<td>102 ± 8</td>
<td>83 ± 10</td>
</tr>
<tr>
<td>Total root surface</td>
<td>565 ± 48 cd</td>
<td>557 ± 33 bc</td>
<td>763 ± 55 a</td>
<td>564 ± 31 bc</td>
</tr>
<tr>
<td>No. of roots</td>
<td>25 ± 5 bc</td>
<td>39 ± 3 b</td>
<td>69 ± 11 a</td>
<td>35 ± 8 b</td>
</tr>
</tbody>
</table>

expressing the red fluorescent protein DsRed) showed that each pseudomonad formed large patches of cells, which were of moderate thickness (maximum 5 µm thick) (Fig. 4a, b). These cell patches were found throughout the root system (i.e. at the apex, in the root hair zones and on older parts of the roots), but were mainly located in the grooves between epidermal cells (Fig. 4b). This colonization all along the root system of *P. fluorescens* F113 and F113G22 was not significantly altered when these pseudomonads were co-inoculated with an *A. brasilense* strain.

*A. brasilense* Cd and Sp245 (both marked with a plasmid constitutively expressing the enhanced green fluorescent protein) were found as single cells as well as large, thick (more than 20 µm high) clumps of cells forming biofilm-like structures on wheat roots (Fig. 4c, f). Both strains colonized the root hair zone extensively (root surface and root hairs), but much less the older parts of the wheat root system. In the presence of F113 or its Phl− mutant F113G22, *A. brasilense* Cd and Sp245 still formed cell clumps on wheat roots, and some of them were very close to the cell clumps produced by F113 or F113G22 (Fig. 4d, e, g, h). However, in the case of *A. brasilense* Sp245, a distinct distribution pattern of *Azospirillum* and *Pseudomonas* strains was observed when comparing co-inoculation with F113 and F113G22 (Fig. 4g, h). Indeed,
**DISCUSSION**

Phl⁺ pseudomonads have been extensively studied for their ability to influence plant development and physiology (Iavicoli *et al.*, 2003; Phillips *et al.*, 2004; Brazelton *et al.*, 2008) and to inhibit a large variety of soil-borne phytopathogenic fungi and bacteria (Weller *et al.*, 2002; Haas & Défago, 2005; Couillerot *et al.*, 2009). In contrast, their capacity to affect other saprophytic microbial inhabitants in the rhizosphere has received much less attention (Natsch *et al.*, 1998; Girlanda *et al.*, 2001; Johansen *et al.*, 2002), and in particular their impact on most genera of plant-beneficial micro-organisms is largely unknown, except for *Rhizobium* (Walsh *et al.*, 2003). In this work, the effect of Phl⁺ fluorescent *Pseudomonas* strains on root-colonizing *A. brasilense* phytostimulators was investigated, and *Pseudomonas* mutants were used to assess the role of Phl production ability.

*A. brasilense* strains differed in Phl sensitivity, but most were moderately sensitive to Phl. They were more sensitive to Phl than many other saprophytic rhizobacteria (Keel *et al.*, 1992; Moënne-Loccoz *et al.*, 2001; Walsh *et al.*, 2003) or phytopathogenic root-associated micro-organisms (Keel *et al.*, 1992; Cronin *et al.*, 1997a; Schouten *et al.*, 2004), and the strain to strain fluctuation within *A. brasilense* was comparable to the fluctuation documented among isolates of the same species or genus in other taxa.

*Pseudomonas* cells were found around Sp245 cell clumps in the case of F113G22, whereas Sp245 and F113 were often observed on distinct areas within the same root zone. In addition, colonization by Sp245 cells within a given root zone was more extensive when colonization by F113 cells was of a lesser extent, a trend not found for F113G22.

Fig. 4. CLSM observations 7 days after colonization of wheat roots by *A. brasilense* and/or *P. fluorescens* strains. Single inoculations were performed with *P. fluorescens* F113 (a) or F113G22 (b), or *A. brasilense* Cd (c) or Sp245 (f). *A. brasilense* strains were coinoculated with the wild-type strain F113 [Cd in (d), Sp245 in (g)] or the Phl⁻ mutant F113G22 [Cd in (e), Sp245 in (h)]. *Azospirillum* cells constitutively expressing enhanced green fluorescent protein are green, *Pseudomonas* cells constitutively expressing DaRed are red, whereas yellow indicates mixed cell clumps (mcc), and white/grey and blue backgrounds correspond to the root image formed by the transmitted light and the reflected light, respectively. rs, Root surface; rh, root hair; cc, cell clump.
The mode of action of Phl is poorly understood. This phenolic metabolite, which inhibits both prokaryotic and eukaryotic organisms, can probably diffuse through the cytoplasmic membrane. On one hand, Phl is involved in cross-communication between Phl-producing *Pseudomonas* strains (Maurhofer et al., 2004), and can enhance the expression of a wide range of *A. brasilense* Sp245 genes, including genes involved in phytostimulation, at very low concentrations (Combes-Meynet et al., 2011). On the other hand, Phl induces the synthesis of ABC-transporter efflux pumps in *Botrytis cinerea* (Schouten et al., 2008) and impairs mitochondrial functioning in yeast by depolarization of the mitochondrial membrane (Gleeson et al., 2010). In *Pythium ultimum*, it affects the plasma membrane (de Souza et al., 2003). Here, a similar effect of Phl on the cytoplasmic membrane of *A. brasilense* Cd was observed in areas of the plates where Phl had been deposited and where *Azospirillum* cells were sparse (Fig. 1f).

Further away from the Phl deposit, Cd cells became more numerous, resulting in even higher cell population biomass (pink concentration halo) compared with plates without Phl. This was also found when Phl + strains F113 or Q2-87 were used instead of synthetic Phl (not shown). In the pink concentration halo, where the higher bacterial biomass might have resulted in carbon source depletion, Cd cells displayed cytoplasmic accumulation of poly-β-hydroxybutyrate-like granules. These granules favour the survival of *A. brasilense* under stress conditions such as carbon starvation (Tal & Okon, 1985; Kadouri et al., 2002, 2003). In another experiment, visual observation of *A. brasilense* Cd liquid cultures revealed an enhanced pink pigmentation when added Phl was increased from 50 to 200 μM (Fig. 2). This is consistent with the increase in carotenoid content found in the presence of 50 or 100 μM Phl (Fig. 2). In *A. brasilense* Cd, carotenoids are involved in protection against oxidative damage (Hartmann & Hurek, 1988; Nur et al., 1981; Thirunavukkarasu et al., 2008), and future work with mutants impaired in carotenoid synthesis will be needed to decipher the role of carotenoids in the resistance of *A. brasilense* Cd to Phl. However, no correlation was found between colony pigmentation and resistance to Phl when assessing a collection of *A. brasilense* strains (Table 1).

The Phl + strain *P. fluorescens* F113 inhibited the growth on plates of the two moderately sensitive *A. brasilense* strains Cd and Sp245. For *A. brasilense* Cd, the extent of inhibition implemented by F113 was equivalent to that caused by 13 nmol Phl on LBm and 8 nmol Phl on SA-Fe. These amounts of Phl require ~5 × 10^8 c.f.u. F113 on rich medium (Duffy & Defago, 1999), which is comparable to the inoculum level in the inhibition bioassay. Indeed, experiments performed with the Phl + biosynthetic mutant F113G22 and its Phl + complemented derivative F113G22(pCU203) indicated that the ability of F113 to produce Phl was involved in its growth inhibition effect on *Azospirillum* strains. Inhibition was only partially restored with F113G22(pCU203), in accordance with the fact that it produces less Phl than F113 under laboratory conditions (Fenton et al., 1992; Cronin et al., 1997a). The effect of Phl production ability in vitro was confirmed with another Phl + pseudomonad strain Q2-87 (Vincent et al., 1991) which, like F113, inhibited growth of the two *A. brasilense* strains within 2 days on both media tested (data not shown). Compared with Q2-87, the Phl + biosynthetic mutant Q2-87::Tn5-1 (Vincent et al., 1991) was less able to inhibit growth of the two *A. brasilense* strains (not shown), confirming the importance of Phl production in *Azospirillum* inhibition by *P. fluorescens*.

Concerning plant growth assays, there was no phytostimulation in plants co-inoculated with *A. brasilense* Cd and Phl + *P. fluorescens* F113. The significance of Phl production ability was not clear cut, because an unexpected phytostimulation effect (not found in previous work done on other crop plants) was seen with F113, but results were essentially similar with F113G22 or F113G22(pCU203). This phytostimulatory potential might be linked to 1-aminocyclopropane-1-carboxylate deaminase activity (Blaha et al., 2006). This potential could have masked some of the inhibition effects on co-inoculated *Azospirillum*, as co-inoculation of wheat phytostimulator *A. brasilense* Sp245 with Phl + *P. fluorescens* F113 led to similar levels of wheat stimulation as did single inoculations. The reduction of the total root surface of plants, when *A. brasilense* Cd and *P. fluorescens* F113 were co-inoculated, suggests that strain Cd might affect *Pseudomonas* strains and their plant-growth-promotion properties. The growth of *P. fluorescens* F113 was, however, not inhibited by *A. brasilense* Cd in vitro. Future work will be needed to better understand the interaction mechanisms between *Azospirillum* and *Pseudomonas* strains.

Root colonization is a property that may vary according to the plant species (Michiels et al., 1989; Bashan et al., 1991) as well as the plant-beneficial strain (Aßmus et al., 1997; Schlöter & Hartmann, 1998). Here, CLSM observations showed that *A. brasilense* Cd and Sp245 did not differ from each other in their patterns of wheat root colonization, whereas they displayed a spatially distinct distribution compared with *Pseudomonas* cells. Co-inoculation of *Azospirillum* and *Pseudomonas* strains did not change their overall cell distribution patterns on root zones, except at a microscale level where the local distribution of *Azospirillum* cells in the vicinity of *Pseudomonas* cell clusters seemed to depend on Phl production ability. Thus, especially in the case of Sp245, *Azospirillum* and Phl + *Pseudomonas* cells were often observed on distinct areas within the same root zone. Plating of wheat root samples indicated that *A. brasilense* Cd and Sp245 were approximately 10 times less abundant in the presence of the Phl + strain *P. fluorescens* F113, and comparison with F113 mutants showed that this effect was due to Phl production ability. The inhibition of *Azospirillum* growth on roots could be explained (i) by a direct impact of Phl on *Azospirillum* growth in the context of a competitive interaction and/or (ii) by a possible indirect effect as Phl can change root exudation patterns (Phillips et al., 2004).
In conclusion, the results suggest that Phl production ability could interfere, at a microscale level, with root colonization of \textit{A. brasilense} strains and their phytostimulation potential. Thus, bacterial interactions in the rhizosphere appear to be key ecological regulators of plant growth and health, and they need be taken into account when developing bio-inoculants for crops.

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