Phytobeneficial traits of rhizobacteria under the control of multiple molecular dialogues

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Summary

Pseudomonads play crucial roles in plant growth promotion and control of plant diseases. However, under natural conditions, other microorganisms competing for the same nutrient resources in the rhizosphere may exert negative control over their phytobeneficial characteristics. We assessed the expression of phytobeneficial genes involved in biocontrol, biostimulation and iron regulation such as, *phlD*, *hcnA*, *acdS*, and iron-small regulatory RNAs *prrF1* and *prrF2* in *Pseudomonas brassicacearum* co-cultivated with three phytopathogenic fungi, and two rhizobacteria in the presence or absence of *Brassica napus*, and in relation to iron availability. We found that the antifungal activity of *P. brassicacearum* depends mostly on the production of DAPG and not on HCN whose production is suppressed by fungi. We have also shown that the two-competing bacterial strains modulate the plant growth promotion activity of *P. brassicacearum* by modifying the expression of *phlD*, *hcnA* and *acdS* according to iron availability. Overall, it allows us to better understand the complexity of the multiple molecular dialogues that take place underground between microorganisms and between plants and its rhizosphere microbiota and to show that synergy in favour of phytobeneficial gene expression may exist between different bacterial species.

Received 21 October, 2021; revised 14 February, 2022; accepted 14 February, 2022.

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Microbial Biotechnology (2022) **15**(7), 2083–2096
doi:10.1111/1751-7915.14023

Funding information

AL has a researcher fellowship from the ANRT (Agence Nationale de la Recherche et de la Technologie) (CIFRE N° 2011/1473) and the InVivo company.

Introduction

The microbiota is thought to provide the host with valuable abilities that influence its physiology and improve its fitness (Zilber-Rosenberg and Rosenberg, 2008). The rhizosphere microbiota may play an important role in plant nutrition and protection against pathogens. Many bacteria are able to lower the level of the ethylene precursor, 1-aminocyclopropane-1-carboxylate (ACC) and consequently the production of ethylene by plants, thanks to the bacterial enzyme ACC deaminase (Glick, 2014). Bacteria producing *acdS* are capable of increasing plant tolerance to salinity (Heydarian *et al*., 2021) and to improve plant root growth (Penrose *et al*., 2001).

Many fluorescent pseudomonads with biocontrol abilities protect plants from soil-borne diseases by producing antimicrobial secondary metabolites such as hydrogen cyanide (HCN) and 2,4-diacetylphloroglucinol (DAPG), which are important biocontrol determinants (Vincent *et al*., 1991; Fenton *et al*., 1992; Keel *et al*., 1992; Haas and Défago, 2005). The *phl* cluster that encodes DAPG synthesis is involved in the biocontrol of a broad spectrum of diseases by many antagonistic bacterial strains (Haas and Défago, 2005).

The involvement of the *phl* cluster in biocontrol has been determined from studies of root-colonizing *Pseudomonas* in disease-suppressive and conducive soils (Raaijmakers and Weller, 1998; Weller *et al*., 2002). The presence of DAPG-producing strains does not necessarily guarantee disease suppression, as these strains have also been found in conducive soils (Ramette *et al*., 2003; Almario *et al*., 2013). Indeed, Rezzonico *et al*. (2007) reported a lack of correlation between the amounts of DAPG produced *in vitro* by bacteria having the *phl* cluster and their biocontrol efficacy *in planta*. The reason for the discrepancy between these studies has not yet been established.

Several studies have reported the benefit of introducing particular bacterial strains with *in vitro* antifungal activity in field conditions, as a strategy for disease control (Nelson, 2004). Effective application of this approach in the field requires knowledge of the biotic and abiotic factors that influence the functions of the introduced strain, especially under natural conditions. Biocontrol is

the exploitation of disease-suppressive microorganisms to improve plant protection (Oconnell *et al*., 1996). From this perspective, disease suppression through biocontrol involves plant–bacteria, bacteria–phytopathogen and bacteria–bacteria interactions, as well as interactions with their physicochemical environment. Although there is an increasing demand for biocontrol in the context of sustainable agriculture, biocontrol in field conditions still faces challenges before its practices can be widely accepted and optimally used (Meyer and Roberts, 2002; Bashan *et al*., 2014). The success of these biocontrol assays also depends on field conditions, where inter- and intra-species interactions can also impact the type and concentration of compounds produced by a microorganism (van Agtmaal *et al*., 2018).

The physicochemical soil properties and the interactions of biocontrol agents with root-colonizing microbiota may explain why many microorganisms suppressed diseases successfully under laboratory conditions, but failed in the field. Introduced biocontrol agents can be affected by other microbial communities, as well as inducing a change in the assembly of the microbiota leading to changes in interactions within the microbial community and, in some cases, they can work in synergy to suppress plant diseases. In other cases, multiple partners interact to regulate a single phytobeneficial trait, so that these complex relationships can have important ecological consequences (Hussa and Goodrich-Blair, 2013).

Abiotic factors may also be involved in the establishment of inoculated PGPR strains and in the expression of their phytobeneficial genes (Lim *et al*., 2012). Almario *et al*. (2013) hypothesized that the clay mineral composition of soils may impact on iron availability, which may confer disease suppressiveness in the rhizosphere, allowing the expression of biocontrol relevant genes in antagonistic *Pseudomonas protegens*.

Few studies, under controlled conditions, have reported interspecies interactions that could be based on quorum sensing that coordinates interactions both within a species and between species (Abisado *et al*., 2018), or in particular on competition for iron that plays a central role in microbe–microbe and microbe–host interaction. Ho *et al*. (2021) studied the interaction between pyochelin-producing bacteria and the plant pathogen *Phellinus noxius*. *P. noxius* converts pyochelin and ent-pyochelin from *Pseudomonas* and *Burkholderia* species to pyochelin-GA (and ent-pyochelin-GA), impairing their antifungal and iron chelation activities (Ho *et al*., 2021). Interspecies interactions occur between microorganisms occupying the same ecological niche, such as *Pseudomonas aeruginosa* and members of the *Burkholderia cepacia* complex, which often coexist in both the soil and the lungs of cystic fibrosis patients. Weaver and Kolter (2004) showed that ornibactin, a siderophore produced by nearly all *B. cepacia* strains, can induce the expression of *P. aeruginosa* PA4467 gene, indicating that ornibactin can be produced by *B. cepacia* and detected by *P. aeruginosa* when the two species coexist.

Pseudomonas brassicacearum was described as the major root-associated bacterium in the rhizosphere of *Arabidopsis thaliana* and *Brassica napus* (Achouak *et al*., 2000; Fromin *et al*., 2001) and displayed biological control, plant growth promotion and for some strains pathogenic traits (Belimov *et al*., 2007). Mutations in the *gacS-gacA* system have been shown to lead to drastic pleiotropic changes in *P. brassicacearum* (Lalaouna *et al*., 2012). The expression of secondary metabolites (*e.g*. the antifungal compounds DAPG and hydrogen cyanide), auxin, exoenzymes (*e.g*. lipase and protease), three different *N*-acyl-homoserine lactone molecules, the type VI secretion machinery and alginate synthesis was downregulated in variants with mutations in *gacS* or *gacA* genes, and biofilm formation ability was greatly reduced (Lalaouna *et al*., 2012). This indicates that the expression of the *phl* and *hcn* genes is under the positive control of the GacS/GacA system through the synthesis of *rsmX-1*, *rsmX-2*, *rsmY* and *rsmZ* (Lalaouna *et al*., 2021).

To study *in situ* gene expression of phytobeneficial bacteria, Haichar *et al*. (2013) investigated the expression of *phlD* by developing mRNA-stable isotope probing (mRNA-SIP). Their results have demonstrated that the *phlD* gene was expressed by bacteria inhabiting the rhizosphere soil that derive nutrients from the breakdown of organic matter and root exudates, whereas *phlD* gene expression appeared to be repressed on *A. thaliana* roots. *In vitro* expression of the *phlD* gene in *P. brassicacearum* was strongly activated by wheat and *Medicago truncatula* and to a lesser extent by *B. napus*, whereas it was downregulated by *A. thaliana* (Haichar *et al*., 2013). This regulation is probably mediated by plant root exudates (Haichar *et al*., 2013). In addition to other microorganisms, the plant can also be involved in modulating the expression of phytobeneficial genes. Little is yet known about the activity of the genes underpinning biocontrol and biostimulation under conditions of competition for scarce nutrients and essential elements such as iron, the lack of which has major consequences for the nature of interactions between organisms in the three kingdoms of life.

The objective of this work was therefore to better understand the behaviour of biocontrol agents by assessing the impact of biological factors on the expression of the *phlD*, *hcnA* and *acdS* genes, which encode two major biocontrol and a biostimulation traits. Here, we investigated the ability of the plant root-associated *P. brassicacearum* NFM421 to compete with two other rhizobacteria, *Kosakonia sacchari* NO9, a root-associated diazotroph (Bloch *et al*., 2020) and *Rhizobium alamii*

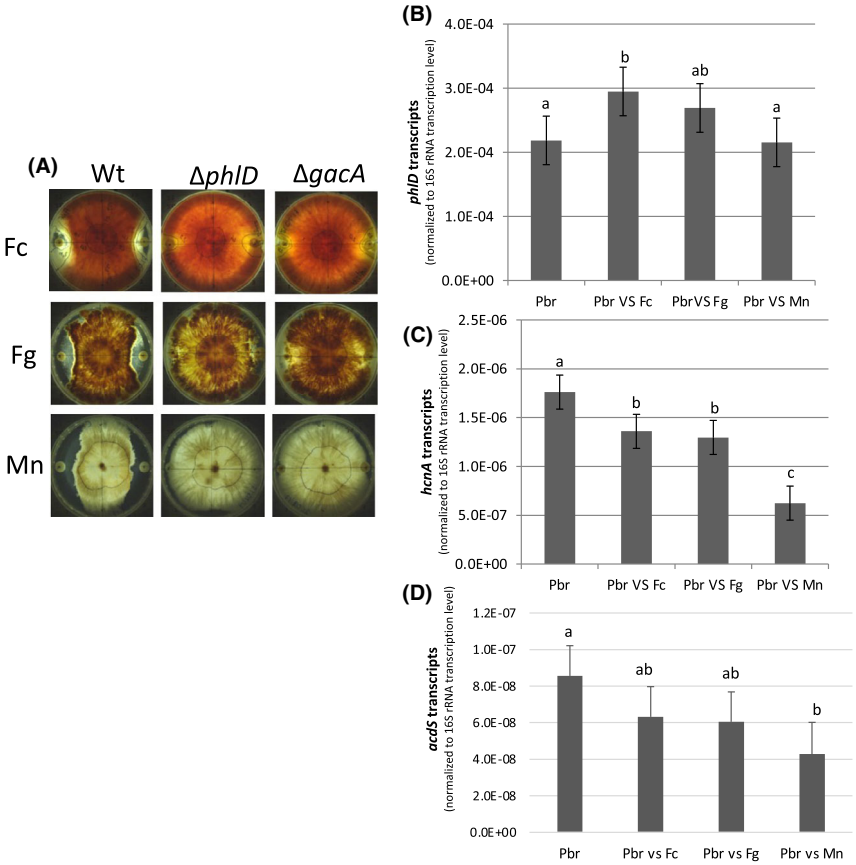

Fig. 1. Bacteria–fungi interaction. (A) Antifungal activity of *P. brassicacearum* NFM421wt, Δ*phlD* and Δ*gacA* towards *Fusarium culmorum* (Fc), *Fusarium graminearum* (Fg) or *Microdochium nivale* (Mn). Expression of the NFM421 strain genes *phlD* (B) *hcnA* (C) and *acdS* (D) grown alone (Pbr), or in the presence of *F. culmorum* (Pbr vs. Fc), *F. graminearum* (Pbr vs. Fg) or *M. nivale* (Pbr vs. Mn). Experiments were conducted in triplicates. Bars with different letters are significantly different at $P < 0.05$ according to the ANOVA test.

YAS34, known for its ability to produce exopolysaccharide and to contribute to soil stability in the rhizosphere of *Helianthus annuus* and *B. napus* (Alami *et al.*, 2000; Tulumello *et al.*, 2021), under conditions of iron depletion and iron repletion, and in the presence (or not) of *B. napus*. Under these different experimental conditions, we analysed the expression of the phytobeneficial genes *phlD*, *hcnA* and *acdS* and to assess the intracellular status of iron in *P. brassicacearum* NFM421, we analysed the expression of iron-regulatory RNAs *prrF* in this strain. We also tested the antagonism of *P. brassicacearum* NFM421 wt and the knockout mutants Δ*phlD* and Δ*gacA* against the soil-borne plant pathogens *Fusarium culmorum*, *Fusarium graminearum* and *Microdochium nivale*, to compare the antifungal activity of DAPG and HCN.

Results

Only DAPG is likely required for biocontrol activity in P. brassicacearum *NFM421*

We assessed the antagonistic activity of wild-type (wt) *P. brassicacearum* NFM421 and the knockout mutants Δ*phlD* and Δ*gacA* against the soil-borne phytopathogens *F. culmorum*, *F. graminearum* and *M. nivale*. The mutant Δ*gacA* did not produce DAPG or hydrogen cyanide, whereas the mutant Δ*phlD* was able to produce hydrogen cyanide but not DAPG. Only *P. brassicacearum* NFM421wt was able to inhibit the growth of the three assayed fungi, underscoring the role of DAPG produced by *P. brassicacearum* NFM421 in its antifungal activity (Table S1, Fig. 1A). *F. graminearum* and *M. nivale* displayed increased sensitivity to 2,4-DAPG in comparison with *F. culmorum*, as reflected in strong inhibition of their growth by *P. brassicacearum* NFM421 wt. The Δ*phlD* and Δ*gacA* mutants showed no antifungal activity, suggesting the involvement of DAPG (no fungal inhibition by Δ*phlD* mutant) and no conclusion for the role of HCN since the Δ*gacA* mutant is impaired in both DAPG and HCN production (Table S1, Fig. 1A).

Bacteria–fungi dialogue

To further investigate whether *phlD* (production of DAPG), *hcnA* (production of hydrogen cyanide) and

acdS (ACC deaminase activity) genes were expressed when bacteria were challenged with the fungi, we applied *P. brassicacearum* NFM421wt to the border of the Petri dish as well as the growing fungi at the centre of the dish (*F. culmorum*, *F. graminearum* and *M. nivale*) for 48 h. The expression of *phlD* and *hcnA* in *P. brassicacearum* NFM421wt was determined by RT-qPCR in PDA medium, and in the presence or absence of the three fungi. A significant increase in *phlD* expression was observed in presence of *F. culmorum* only (Fig. 1B). In contrast, *hcnA* and *acdS* expressions were significantly decreased especially by *M. nivale*, and to a lower extent by *F. culmorum* and *F. graminearum* (Fig. 1C and D). This indicates a fungal negative control of hydrogen cyanide production and ACC deaminase activity by *P. brassicacearum* NFM421wt. This result coupled with the lack of antifungal activity of the hydrogen cyanide-producing Δ*phlD* mutant (Fig. 1A) indicates the importance of DAPG produced by *P. brassicacearum* NFM421wt for the control of these three phytopathogen fungi, in contrast to HCN which does not appear to be involved in this biocontrol.

Population size of competing bacteria in vitro *and* in planta

Before studying the molecular dialogue between the bacteria, we established a quantitative method for the detection of the three interacting bacterial strains by quantitative PCR (q-PCR) of specific genes for each strain. For quantification, *phlD* (which encodes the production of DAPG) was used for *P. brassicacearum* NFM421wt, *hycE* for *K. sacchari* NO9 (Roumagnac *et al.*, 2012) and *gta* for *R. alamii* YAS34 (Santaella *et al.*, 2008; Schue *et al.*, 2011). Standard curves with DNA template plasmids containing the cloned target sequences of *phlD*, *hycE* and *gta* were created and used as standards in q-PCR.

Bacterial population size under competition in vitro: *P. brassicacearum* NFM421 was grown alone, or in co-culture with *R. alamii* YAS34 and/or *K. sacchari* NO9 in iron-depleted or iron-rich ($FeCl_3$ 300 μM) conditions in CAA medium. For interactions, an equal number of cells of each strain (10^7 cells ml^{-1}) were used to inoculate the culture media. The evolution of the relative abundance of each strain was determined after 48 h of co-culture (> 10^9 cells ml^{-1} in total) in order to assess the competitive interactions occurring between the *P. brassicacearum* NFM421 and the other two strains. *P. brassicacearum* NFM421 was significantly more abundant than the competing strains in all situations (Table 1). The population of *R. alamii* YAS34 did not exceed 3% when co-cultured with *P. brassicacearum* NFM421 and was less than 2% when co-cultured with the other two strains combined, regardless of iron availability in growth culture. The proportion of *K. sacchari* NO9 was higher, up to 17%, when competing with *P. brassicacearum* NFM421 alone, and up to 14% in the tripartite interaction (Table 1). No significant differences were observed with respect to iron availability.

Table 1. Population size (in percentage) of *P. brassicacearum* NFM421 grown alone, or with the competitors *K. sacchari* NO9 and *R. alamii* YAS34 (separately or together). Strains were grown in CAA medium supplemented with 300 μM $FeCl_3$ or not (denoted by a '−'). Values are the % mean ± SD.

Treatment	NFM421	YAS34	NO9
NFM421+$FeCl_3$	100.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
NFM421−	100.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
NFM421/YAS34+$FeCl_3$	97.8 ± 0.2	2.2 ± 0.2	0.0 ± 0.0
NFM421/YAS34−	96.8 ± 0.1	3.2 ± 0.1	0.0 ± 0.0
NFM421/NO9+ $FeCl_3$	83.2 ± 4.1	0.0 ± 0.0	16.8 ± 4.1
NFM421/NO9−	87.2 ± 2.1	0.0 ± 0.0	12.8 ± 2.1
NFM421/YAS34/NO9+$FeCl_3$	85.1 ± 3.8	1.6 ± 0.2	13.3 ± 3.7
NFM421/YAS34/NO9−	83.7 ± 1.4	1.9 ± 0.1	14.4 ± 1.3

Bacterial population size under in competition *in planta*: *B. napus* seeds was inoculated with an identical cell number of each strain and grown for three weeks. Compared to the *in vitro* conditions, *R. alamii* YAS34 displayed a higher capacity to compete *in planta* with *P. brassicacearum* NFM421 (mainly under iron-depleted conditions), with a population percentage reaching 18.5% (Table 2). However, considering the tripartite interaction, the proportion of *R. alamii* YAS34 was 2.7% under iron-rich conditions and 5.6% in iron-depleted conditions, which is comparable to the result obtained *in vitro* (Table 2). In competition with *P. brassicacearum* NFM421 alone, the proportion of *K. sacchari* NO9 was 8%, regardless of iron availability. In contrast, *K. sacchari* NO9 was much less competitive under iron-depleted condition in the tripartite interaction, representing less than 2% of the total bacteria interacting with the plant roots, but appears to be competitive in the presence of $FeCl_3$ (14.7%, Table 2). Regardless of the competing

Table 2. Population size (in percentage) of *P. brassicacearum* NFM421 grown alone, or with the competitors *K. sacchari* NO9 and *R. alamii* YAS34 (separately or together) in association with *B. napus*. Strains were supplemented with iron source (sequestrene) or not (denoted by a '−'). Values are the % mean ± SD.

Treatment	NFM421	YAS34	NO9
NFM421+Fe	100.0	0.0	0.0
NFM421−	100.0	0.0	0.0
NFM421/YAS34+Fe	96.0	4.0	0.0
NFM421/YAS34−	81.5	18.5	0.0
NFM421/NO9+ Fe	91.9	0.0	8.1
NFM421/NO9−	91.5	0.0	8.5
NFM421/YAS34/NO9+Fe	82.6	2.7	14.7
NFM421/YAS34/NO9−	92.6	5.6	1.8

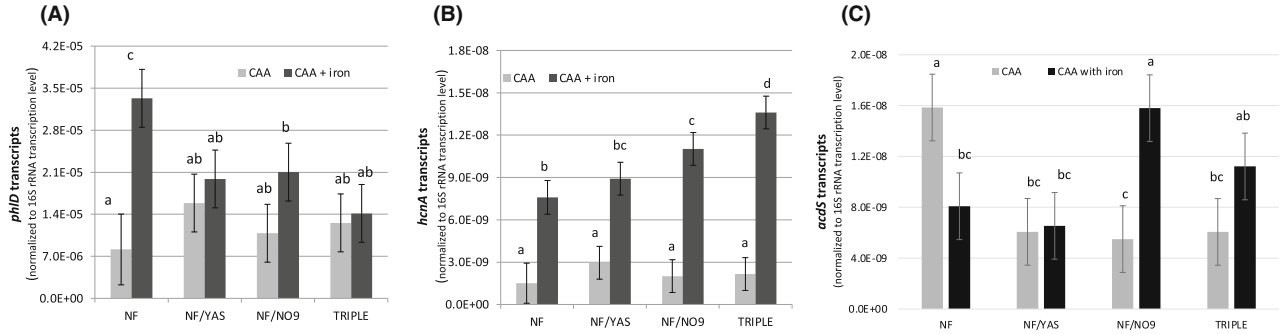

Fig. 2. Phytobeneficial gene expression of *P. brassicacearum* NFM421 in competition with two rhizobacteria *in vitro* in CAA medium. Expression of *phlD* (A), *hcnA* (B) and *acdS* (C) in *P. brassicacearum* NFM421 grown for 48 h alone, or in competition with *K. saccahari* NO9 (NF/NO9), *R. alamii* YAS34 (NF/YAS) or both competitors (Triple) in the absence (grey bars) or in the presence (black bars) of 300 μM $FeCl_3$. Bars with different letters are significantly different at $P < 0.05$ according to the ANOVA test.

strain or iron availability, *P. brassicacearum* NFM421 was the significantly dominant strain.

Bacteria–bacteria dialogue under in vitro *conditions*

The molecular dialogue between *P. brassicacearum* NFM421 and the two-competing bacterial strains (*K. sacchari* NO9 and *R. alamii* YAS34) was assessed after 48 h of co-culture by measuring their impact on the expression of the *phlD*, *hcnA* and *acdS* genes, which are involved in the biocontrol and biostimulation abilities of *P. brassicacearum* NFM421. The presence of the other two strains (separately or together) caused significant transcriptional changes in the expression of *phlD*, *hcnA* and *acdS* in *P. brassicacearum* NFM421 under iron-rich conditions (Fig. 2), despite their low abundance on the root system. (Table 2). In *P. brassicacearum* NFM421, *phlD* was positively regulated by iron, showing an almost fourfold increase under iron-rich conditions, when the bacterium was grown alone (Fig. 2A). However, the presence of the competitors in bipartite or tripartite co-cultures significantly decreased the *phlD* expression under iron-rich conditions (twofold), while no significant difference was observed under iron-depleted conditions (Fig. 2A). The *hcnA* gene was also upregulated by iron in *P. brassicacearum* NFM421 when grown alone, and its expression increased fivefold (Fig. 2B). This highly significant increase ($P < 0.05$) in the iron-rich condition was even observed when *P. brassicacearum* NFM421 was grown with *K. sacchari* NO9 and with both competitors together (Fig. 2B). Unlike the two genes involved in biocontrol (*phlD* and *hcnA*), the expression of the *acdS* gene was increased under iron-depleted conditions when *P. brassicacearum* NFM421 was grown alone. In the presence of competitors and under iron-depleted conditions, the *acdS* expression significantly decreased (Fig. 2C). However, as with *hcnA*, the expression of *acdS* was significantly increased in co-culture with *K. sacchari* NO9 under iron-rich conditions (Fig. 2C). Despite their limited growth in the presence of *P. brassicacearum* NFM421 (Table 2), the presence of both competitors had significant impact on the expression of three genes, two of which are involved in biocontrol and one in biostimulation in *P. brassicacearum* NFM421.

As our results showed the impact of iron availability on phytobeneficial gene expression and the nature of interactions of *P. brassicacearum* NFM421 with its competitors *K. sacchari* NO9 and *R. alamii* YAS34, we assessed the intracellular status of iron in *P. brassicacearum* NFM421. To do so, we analysed the expression of the small regulatory RNAs, *prrF1* and *prrF2*, whose expression is a powerful indicator of intracellular iron status (Wilderman *et al.*, 2004). In the presence of iron, the Fur regulator binds Fe and represses the expression of *prrF1* and *prrF2*. Under iron-depleted condition, these two sRNAs are expressed and prevent the translation of messenger RNAs encoding iron-requiring proteins, thus allowing the cell to save the little available iron (Wilderman *et al.*, 2004). *prrF1* and *prrF2* were expressed only when the medium was iron-depleted, as expected (Fig. 3A and B). Surprisingly, the expression level of *prrF1* and *prrF2* was significantly reduced in the presence of the competing strains NO9 and YAS34, suggesting that *P. brassicacearum* NFM421 was able to detect some amount of iron in the medium (Fig. 3A and B).

Although the expression profiles of both *prrF1* and *prrF2* RNAs showed the same trend, the expression of *prrF1* was more affected by co-culture with competitors and more importantly by *R. alamii* YAS34 in iron-depleted medium (Fig. 3A and B).

Plant–bacteria–bacteria dialogue

We investigated the transcriptional changes of plant growth promotion and biocontrol related genes in *P. brassicacearum* NFM421 in the presence of the two-competing bacterial strains (NO9 and YAS34) during

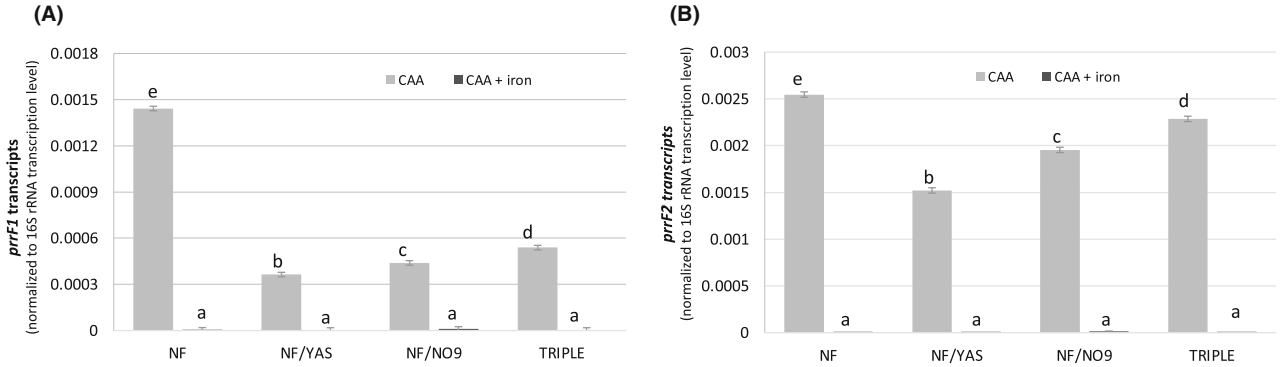

Fig. 3. Iron status of *P. brassicacearum* NFM421 in competition with two rhizobacteria *in vitro*. Expression of *prrF1* (A) and *prrF2* (B), in *P. brassicacearum* NFM421 grown for 48 h alone, or in competition with *K. saccahari* NO9 (NF/NO9), *R. alamii* YAS34 (NF/YAS), or both competitors (Triple) in the absence (grey bars) or in the presence (black bars) of 300 μM $FeCl_3$. Bars with different letters are significantly different at $P < 0.05$ according to the ANOVA test.

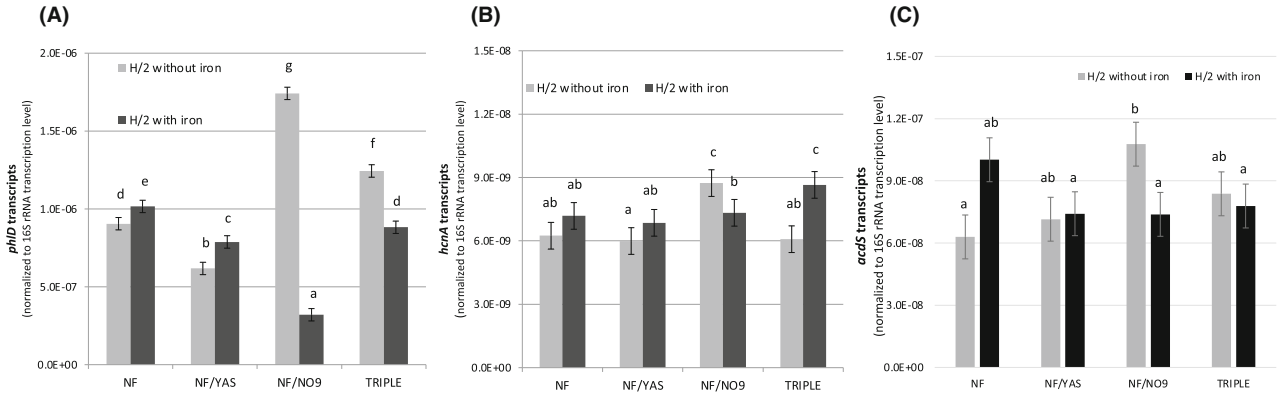

Fig. 4. Phytobeneficial gene expression of *P. brassicacearum* NFM421 in competition with two rhizobacteria *in planta*. Expression of *phlD* (a), *hcnA*(b) *and acdS* (c) in *P. brassicacearum* NFM421 grown for three weeks alone, or in competition with *K. sacchari* NO9 (NF/NO9), *R. alamii* YAS34 (NF/YAS), or both competitors (Triple) on twofold diluted Hoagland's medium (H/2) in the absence (grey bars) or in the presence (black bars) of sequestrene. Bars with different letters are significantly different at $P < 0.05$ according to the ANOVA test.

their interaction with *B. napus* root system. Contrary to what was observed under *in vitro* conditions, *phlD* expression in *P. brassicacearum* NFM421 did not change in response to iron availability when grown alone (Fig. 4A). The presence of *K. sacchari* NO9 significantly altered *phlD* expression of *P. brassicacearum* NFM421 in an iron-dependent manner: iron limitation caused a twofold increase, whereas there was a threefold decrease induced by addition under iron-rich medium (Fig. 4A). In comparison, the presence of *R. alamii* YAS34 slightly but significantly decreased *phlD* expression of *P. brassicacearum* NFM421, regardless of iron availability. When the three competitors interacted *in planta*, the impact of the *K. sacchari* NO9 was attenuated, although it was still significant in a way that was similar to its bipartite interaction with *P. brassicacearum* NFM421. The expression of *hcnA* was much less altered by iron availability, whether *P. brassicacearum* NFM421 was grown alone *in planta* or interacted with a combination of the two competitors (Fig. 4B). Expression of the *acdS* gene *in planta* followed the same pattern as *phlD* expression, with a reduction in expression under iron-depleted conditions when grown alone and an increase in competition with *K. sacchari* NO9 under iron-depleted condition (Fig. 4C).

Evaluation of the intracellular iron status of *P. brassicacearum* NFM421 *in planta* confirmed the significant decrease in the expression of *prrF* sRNAs upon interaction with both competitors and particularly in the presence of *R. alamii* YAS34. Surprisingly, the level of expression of *prrF1* and *prrF2* in the presence of sequestrene (490 μM EDDHA-NaFe) was not negligible (Fig. 5A and B). No correlation was observed between the level of intracellular iron and the expression of *phlD*, *hcnA* and *acdS*.

Proximity favours communication

Monitoring the proportion of each competing bacteria revealed an enrichment of specific microbial populations

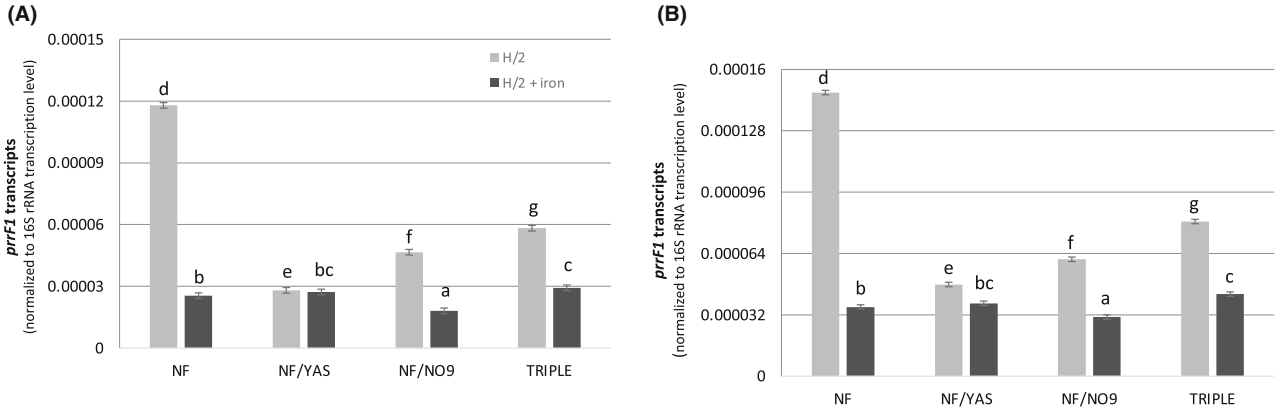

Fig. 5. Iron status of *P. brassicacearum* NFM421 in competition with two rhizobacteria *in planta*. Expression of *prrF1* (A) and *prrF2* (B), in *P. brassicacearum* NFM421 grown for three weeks alone, or in competition with *K. sacchari* NO9 (NF/NO9), *R. alamii* YAS34 (NF/YAS), or both competitors (Triple) in the absence (grey bars) or in the presence (black bars) of sequestrene. Bars with different letters are significantly different at $P < 0.05$ according to the ANOVA test.

that have evolved to colonize the rhizoplane. Colocalization of competing bacteria was performed to determine whether they share the same microniche on the root surface or whether they inhabit different sites (Fig. 6). Although *P. brassicacearum* NFM421 colonized *B. napus* roots more efficiently, we were able to locate competing strains *in planta*. As illustrated in Fig. 6A, the few sites colonized by *R. alamii* YAS34 were not colocalized with those of *P. brassicacearum* NFM421. When *K. sacchari* NO9::gfp was localized, it was often associated with *P. brassicacearum* NFM421::*rfp* colonizing the same sites on the rhizoplane (the yellow patch is due to mixed expression of the two colours of *rfp* and *gfp*) (in Fig. 6A).

Discussion

Although certain pseudomonads play a crucial role in promoting plant growth and controlling plant diseases, other living organisms such as bacteria, fungi and plants may interfere with their biocontrol efficiency. Most fluorescent pseudomonads promote plant growth and produce antimicrobial secondary metabolites including hydrogen hydrogen cyanide (HCN) and 2,4-diacetylphloroglucinol (DAPG). Here, we assessed the expression of *hcnA*, *phlD* and *acdS* in *P. brassicacearum* during co-culture with phytopathogenic fungi and during co-culture with non-pathogenic plant root-associated bacteria. This work demonstrates the modulation of phytobeneficial genes by other microorganisms, depending on the presence of competing bacteria as well as plant and iron availability. Host colonization by biocontrol and biostimulant agents is essential for biocontrol but not sufficient for plant protection against pathogens and plant growth promotion. In addition to competing for C and energy resources in the rhizosphere and of the rhizoplane sites to be colonized, the biocontrol agent must be able to express antimicrobial genes and produce the corresponding compounds. Genes that are modulated during interspecific bacterial interactions often include genes involved in the production of antibiotics, as a defensive or offensive strategy in

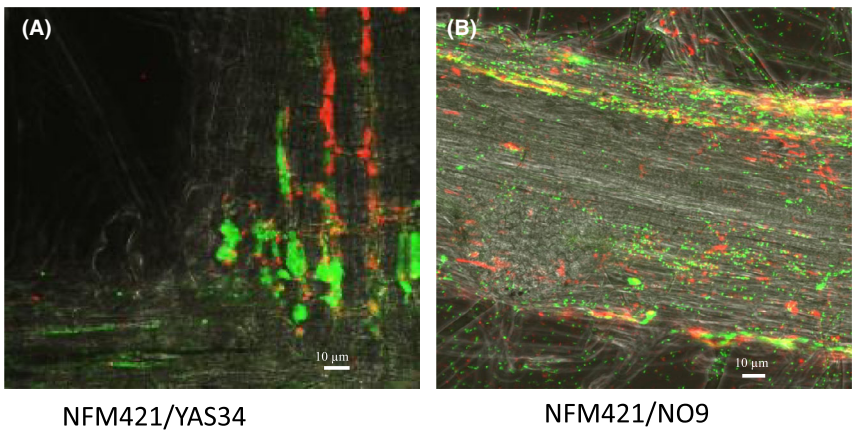

Fig. 6. Colocalization of competing bacteria. *In planta* colocalization of *rfp*-tagged *P. brassicacearum* NFM421 with: *gfp*-tagged *R. alamii* YAS34 (a) and *gfp*-tagged *K. sacchari* NO9 (b). Scale bars correspond to10 µm.

microbial interactions (Fong *et al*., 2001; Harrison *et al*., 2008; Garbeva and de Boer, 2009). Rhizosphere-associated bacteria are always encountered as mixed populations of numerous species in the environment, and they have to cope with other microorganisms, the host and environmental cues. These associations are the result of coevolution leading to the implementation of strategies for adaptation to specific ecological niches.

Antifungal activity of P. brassicacearum *is DAPG-dependent*

When iron is scarce, bacteria produce siderophores that may act as antifungals by depriving fungi of iron. In the presence of iron, other antifungal metabolites intervene like DAPG and phenazines among others. Interaction assays of *P. brassicacearum* NFM421 with *Fusarium* head blight fungi, carried out without addition of iron to determine the role of DAPG and HCN, clearly show the role of DAPG in inhibiting the growth of the tested fungi. Using the two mutants, Δ*gacA* and Δ*phlD*, we demonstrated that DAPG, but not HCN, seems to be primarily responsible for the antifungal activity.

We observed a repression of *hcnA* expression in the presence of the three phytopathogenic fungi *F. culmorum*, *F. graminearum* and *M. nivale*, whereas Barret *et al*. (2009) reported an increase in the expression of hydrogen cyanide synthesis genes with *Pseudomonas fluorescens* strain Pf29Arp, in the presence of the phytopathogenic fungus *Gaeumannomyces graminis var. tritici*. Co-culture of *P. brassicacearum* NFM421 with *M. nivale* species did not alter *phlD* expression, while we noticed a significant increase in *phlD* expression in the presence of *F. culmorum* and *F. graminearum*, whereas these fungi are known to produce fusaric acid, which has been reported to repress *phlD* expression (Notz *et al*., 2002). These bacterium–fungus interactions do not appear to be generalizable, and these interactions may be species- or even strain-dependent.

The expression of *phlD* was one thousand times higher than that of *hcnA*, indicating that even if HCN is known to be effective against pathogenic fungi, the levels of its expression by *P. brassicacearum* NFM421 are too low to inhibit fungal growth. Therefore, the mode of biocontrol of plant pathogenic fungi used in this study, which seems plausible in *P. brassicacearum* NFM421, most likely involves DAPG.

Pseudomonas brassicacearum *outcompetes* K. sacchari *and* R. alamii

Our results showed the competitiveness of *P. brassicacearum* NFM421 when grown with *K. sacchari* NO9 and *R. alamii* YAS34, under iron-depleted and iron-rich conditions via a higher growth rate. The total population size evolved from 3 10^7 cells ml^{-1} to at least 10^9 cells ml^{-1}, suggesting that the three bacterial strains could proliferate, and their population size increased at least tenfold. *P. brassicacearum* produces at least two types of siderophores, which facilitate the recovery of small amounts of iron in aerobic environments (Matthijs *et al*., 2016), and at least two antimicrobial compounds, DAPG and HCN (Lalaouna *et al*., 2012).

These observations are consistent with previous studies showing that batch cultures of *Pseudomonas aeruginosa* grown in a defined medium produce one or more secreted factors that are stimulated by iron limitation and inhibits *Agrobacterium tumefaciens* biofilms (Hibbing and Fuqua, 2012). Surprisingly, *R. alamii* YAS34 was more efficient *in planta* at competing with *P. brassicacearum* NFM421 under iron-depleted conditions than under iron-rich conditions, where they reached up to 18 and 4% of the total population, respectively. This finding suggests that the growth rate of *P. brassicacearum* NFM421 is probably much higher at the expense of root exudates and under iron-rich conditions.

Expression of phlD*,* hcnA *and* acdS *is not exclusively controlled by iron*

Both competitors (*K. sacchari* NO9 and *R. alamii* YAS34) mostly downregulated *phlD* expression under iron-rich conditions when co-cultured with *P. brassicacearum*. These results suggest that these competing bacterial strains may produce specific signals that are perceived by *P. brassicacearum*, which act directly or indirectly at the transcriptional or post-transcriptional level. They may produce certain metabolites that might activate the repressor *phlF*. Bacterial extracellular metabolites such as salicylate and pyoluteorin as well as fusaric acid, a toxin produced by the pythopathogen fungus *Fusarium*, strongly repressed DAPG synthesis in *Pseudomonas protegens* strain CHA0 (Schnider-Keel *et al*., 2000). The expression of *phlD* and *hcnA* genes was remarkably increased by the addition of iron, unlike *P. fluorescens* Pf-5 in which Lim *et al*. (2012) reported overexpression of DAPG-encoding genes and downregulation of hydrogen cyanide-encoding genes under iron-depleted conditions.

In contrast to the interaction with fungi, competition with bacteria led to a significant decrease in *phlD* expression in the presence of iron and conversely a significant increase in *hcnA* expression.

Analysis of the *prrf* sRNAs expression indicated that it was likely that the presence of competitors allowed *P. brassicacearum* NFM421 to increase its intracellular iron level. It is difficult to imagine how Fur could be activated other than by complexing iron, as it is the only

regulator known to repress the expression of the small *prrF* regulatory RNAs in the presence of iron (Wilderman *et al*., 2004; Liu *et al*., 2016). Even genes that are finely regulated seemed to be modulated by the co-culture of bacteria.

In addition to the indirect competition for iron involving siderophores, it has been reported that *P. aeruginosa* was able to kill *Staphylococcus aureus* and utilized the released iron (Mashburn *et al*., 2005). Competitive behaviours between bacteria are partly controlled by iron availability (Andrews *et al*., 2003). Numerous mechanisms may be involved in bacterial exchange, such as secondary metabolites and quorum-sensing quenching system. Bacteria are known to communicate with each other and modulate their gene expression through quorum-sensing signalling molecules (Lowery *et al*., 2008).

Contrary to *phlD*, whose expression is tenfold higher in CAA medium enriched with iron than *in planta*, *acdS* expression is tenfold higher *in planta* than CAA medium enriched with iron (Fig. 3A and C vs Fig. 4A and C). The production of ACC by the plant must probably activate the expression of *acdS*, as indicated by genes transcript level normalized to 16S rRNA transcription level. We previously reported the impact of the plant *via* root exudates on the expression of the non-coding RNAs *rsmZ*, *acdS* gene encoding 1-aminocyclopropane-1-carboxylate (ACC) deaminase and *nosZ* gene encoding nitrous oxide reductase, and evidenced their expression in the root-adhering soil and on the roots of *A. thaliana* by using mRNA-SIP approach (Haichar *et al*., 2012). However, *phlD* gene expression was shown to be highly activated by root exudates of wheat and that of *Medicago truncatula* and to a lesser extent by that of *B. napus*, while it was strongly suppressed by root exudates of *A. thaliana* suggesting that the signals for downregulation of *phlD* gene expression may originate from *B. napus* and *A. thaliana* root exudates (Haichar *et al*., 2013).

We observed no effect of iron on *phlD* or *hcnA* expression, with the exception of co-inoculation with *K. sacchari* NO9 which induced a strong increase in *phlD* and *hcnA* expression in the absence of iron and a significant decrease of *phlD* in the presence of iron. The triple inoculation also led to an increase of *phlD* expression in the absence of iron. During *in planta* interspecies interaction, *P. brassicacearum* NFM421 antifungal genes were positively modulated by *K. sacchari* NO9 denoting a synergy between these two strains in favour of the use of *P. brassicacearum* NFM421 as a biocontrol agent, in combination with *K. sacchari* NO9. However, *acdS* expression was reduced in *P. brassicacearum* NFM421 grown alone on CAA or in monoxenic condition *in planta*, whereas the presence of *K. sacchari* NO9 seems to induce an increase in *acdS* expression, *in planta* under iron-depleted condition. This increase in *acdS* expression did not support the need for increased ethylene production by the plant under Fe deficiency, which, in the roots of several plant species, led increased ethylene production contributing to the regulation of subapical root hairs and Fe acquisition genes (Angulo *et al*., 2021). Our results show an *in planta* modulation of the expression of phytobeneficial genes in *P. brassicacearum* NFM421, essentially in the presence of *K. sacchari* NO9 suggesting a signalling exchange between these strains on the plant root surface. For this reason, we colocalized the competing strains *in planta*. Colocalization imagery showed that *K. sacchari* NO9 strain occupied the same sites as *P. brassicacearum* NFM421 on the rhizoplane, as we consistently observed their colocalization (Fig. 6B). Figure 6B shows a strong colonization by the NO9 strain at the apical part of the roots; however, they are less abundant at the basal part (Fig. S1), while *R. alamii* YAS34 did not exhibit a significant impact on the expression of antifungal genes, probably because they inhabited niches on the roots that did not overlap with those of *P. brassicacearum* NFM421 (Fig. 6A).

It is likely that inhabiting separate niches may lead to a weaker interaction and exchange. The type and quantity of compounds produced by one bacterial strain can probably vary when it interacts directly with another, especially when they compete for space and resources in the same ecological niche. It is this proximity and exchange that should shape microbial assemblages and interaction networks within microbial communities. The interactions, we observed in a very simplified *in vitro* system with only three strains, hint at the complexity of what could happen *in situ* in real soil with thousands of bacterial species.

Scheme for multiple dialogues that may modulate phytobeneficial gene expression

To illustrate our results, we propose a scheme targeting the modulation of *phl* gene expression by other microorganisms and the plant, as well as the role of iron and its regulation by *prrF* regulatory non-coding RNAs (Fig. 7). The expression of *hcnA* and *acdS* also seems to depend on the biotic and abiotic environment of the bacterium. This scheme of interactions in a simplified *in vitro* system of bacteria with their biotic and abiotic environment and their potential impact on the expression of phytobeneficial traits hints at the complexity of these relationships under natural conditions.

Conclusions

Our results suggest that it is difficult to predict the behaviour of an introduced strain, since the expression of its

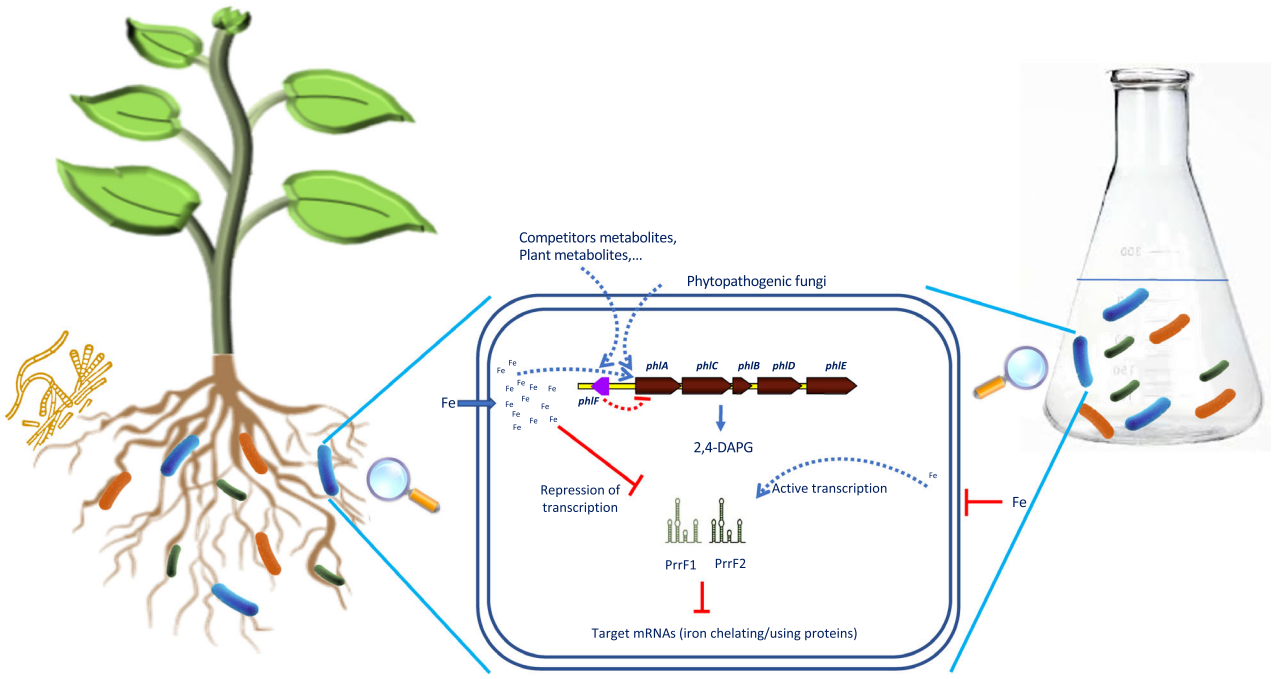

Fig. 7. Scheme for multiple dialogues that may modulate phytobeneficial gene expression. This model illustrates the modulation of *phl* gene expression by other microorganisms, the plant and iron, and the role of iron and its regulation by *prrF* regulatory non-coding RNAs. These interactions are most likely even more complex under natural conditions in the rhizosphere in the presence of a very diverse microbiota producing a multitude of metabolites. This is also valid for the other genes *hcnA* and *acdS*. ↓, positive effect; ⊥, negative effect; dotted lines, indirect effects. The conclusion of this study is that the regulation of phytobeneficial gene expression is under the control of different biotic and abiotic factors.

phytobeneficial traits will depend on several factors, including pedoclimatic factors not addressed here, interaction with other microorganisms, the plant and availability of nutritive resources including essential elements such as iron.

The expression of antifungal genes was modulated by interspecies interactions, confirming that rhizosphere bacteria can compete with each other through a range of antibacterial agents and quorum-sensing (as well as presumably unknown signalling molecules). The mechanisms by which one bacterial population interferes with the gene expression of another population are not yet fully understood. Interkingdom and interspecies interactions, as well as external factors, may affect the success of introduced beneficial microorganisms. Future research must continue to characterize the mechanisms by which these interactions occur and evolve in the rhizosphere.

The introduction of a bacterial strain into an ecological niche may have an impact on the assembly of the microbiota and in particular on the interaction networks within the microbial community. These interactions can be synergistic by acting positively directly or indirectly on the expression of genes of inoculated strain or conversely prevent the inoculated strain (biointrant) to express its phytobeneficial effect.

In conclusion, the successful development of smart biocontrol not only requires that bacteria carry the appropriate genes, but also that they are expressed in a coordinated and dynamic manner.

Experimental procedures

Bacterial growing and co-cultures conditions

Pseudomonas brassicacearum NFM421 was isolated from the *A. thaliana* rhizosphere (Achouak *et al.*, 2000), *Kosakonia sacchari* NO9 isolated from the rice rhizosphere (formerly *Enterobacter cloacae*, Omar *et al.*, 1989), and *Rhizobium alamii* YAS34 isolated from the sunflower rhizosphere (Alami *et al.*, 2000; Berge *et al.*, 2009).

Fungal–bacteria interaction experiments were performed using potato dextrose agar (PDA, Difco™) medium. PDA plates were inoculated with 5-mm agar plugs of the fungi *Fusarium culmorum*, *Fusarium graminearum* and *Microdochium nivale* (kindly provided by Biovitis, France) and incubated at 25°C for at least 48 h, to ensure bacterial growth at this temperature. Bacterial inocula, consisting of two spots of 10 μl containing 10^7 cells washed in a 110 mM KCl solution, were applied on either side of fungi at 2 cm from the fungal mycelium border. Two 10-μl spots of KCl were also applied on either side of fungi as control treatment, and all plates were incubated at 25°C for three more days.

The bacterial co-culture experiments were performed using an iron-limited medium (iron poor casamino acids

medium, CAA, Difco™) in the presence or not of $FeCl_3$ (300 μM). Bacterial inocula consisting of 10^7 cells of *P. brassicacearum* NFM421 were grown in pure culture, or co-culture with equal cell numbers of *K. sacchari* NO9 and/or *R. alamii* YAS34, in a 10-ml final volume of CAA or iron-supplemented CAA medium. Bacterial cultures were incubated at 28°C for 48 h, and bacterial cells were collected for DNA and RNA extraction.

Construction of the ΔphlD *mutant*

Upstream and downstream regions of *phlD* were amplified from *P. brassicacearum* NFM421 genomic DNA using the MD-*phlD*-1/MD-*phlD*-2 and MD-*phlD*-3/MD-*phlD*-4 primers respectively (Table S2). In a separate PCR, the overlap between the MD-*phl* -2 and MD-*phlD*-3 primers enabled amplification of a 2-kb fragment, in which the upstream and downstream regions were ligated together. This fragment was then cloned into the suicide vector pME3087 (Voisard *et al.*, 1994) and introduced into *E. coli* S17-1 by transformation. The recombinant plasmid was subsequently introduced into the wild type by conjugation and mutants obtained as previously described (Lalaouna *et al.*, 2012).

In planta B. napus *co-inoculation assays*

In order to determine the impact of the plant on the expression of phytobeneficial genes, we selected *B. napus* which is a plant highly colonized by *P. brassicacearum* (Achouak *et al.*, 2000) and protected against water stress by *R. alamii* (Tulumello *et al.*, 2021). Canola seeds (*B. napus* cv. Sensation) were selected between 1.6 and 2.0 mm in diameter, surface-sterilized as previously described (Achouak *et al.*, 2004). A 10^2–10^3 CFU suspension of *P. brassicacearum* NFM421 was used alone or in combination with equivalent suspension of *K. sacchari* NO9 and/or *R. alamii* YAS34. Bacterial suspensions were added to 1 ml of 3.5 g l^{-1} agar containing half-strength Hoagland's medium (H/2) (Arnon and Hoagland, 1940) and poured as a band on the surface of H/2 medium solidified with 0.4% phytagel (Sigma; St. Louis, MO, USA). Then, the seeds were sown on the surface of the agar band. The square dishes (15 cm × 15 cm) were sealed with Micropore™ tape (3 M; St. Paul, MN, USA) and incubated vertically for three weeks under a daily regimen of 22°C for 16 h in the light and 18°C for 8 h in the dark (approximately 100 photons m^{-2} s^{-1}).

To count and localize *P. brassicacearum* NFM421, we used *rfp*-tagged bacteria (Achouak *et al.*, 2004), *R. alamii* YAS34 *gfp*-tagged (Santaella *et al.*, 2008) and a *gfp*-tagged *K. sacchari* NO9 obtained by inserting the *pnpt2* promoter and *gfp* gene from plasmid p519ngfp (Matthysse *et al.*, 1996) between the *HindIII* and *EcoRI* restriction sites of plasmid pBBR1MCS-2 (Kovach *et al.*, 1995). Dual colour confocal images were acquired by sequential scanning, with settings that were optimal for green (excitation at 480 nm and emission at 520 nm) and red fluorescence (excitation at 587 nm and emission at 661 nm).

Quantification of bacteria

Single copies of genes specific to *P. brassicacearum* NFM421 (*phlD*), *K. sacchari* NO9 (*hycE*, hydrogenase 3, Roumagnac *et al.*, 2012) and *R. alamii* YAS34 (*gta*, glycosyltransferase, Schüe *et al.*, 2011), which, respectively, encode DAPG, the large subunit of hydrogenase and glycosyltransferase, were identified, amplified from the genomic DNA of each bacterial species, cloned into TopoXL plasmids and used as standards in quantitative PCR. A serial dilution of the plasmid DNA was performed, and the copy number of each specific gene from the three bacteria species was determined in the different culture combinations. Calculations were performed to determine the mass of plasmids containing cloned target sequences (plasmid + insert) corresponding to copy numbers of target nucleic acid sequences. DNA was extracted from pure cultures or co-cultures using the phenol/chloroform/isoamyl alcohol extraction protocol for bacterial culture.

phlD, hcnA, acdS, prrF1 *and* prrF2 *gene expression*

RNA was extracted from bacteria grown in co-culture in CAA medium supplemented or not with iron (300 μM $FeCl_3$), or in PDA medium in the presence of fungi using the RNeasy Kit (Qiagen, Hilden, Germany).

For *in planta* experiments, bacteria were collected from growth medium and from roots by vortexing, pelleted, and then, RNA was extracted according to manufacturer instruction (Qiagen RNasy kit, France). Quantitative reverse transcription PCR (RT-qPCR) assays were performed to quantify *phlD*, *hcnA*, *acdS* and *prrF1* and *prrF2* expression in *P. brassicacearum* NFM421 alone, or interacting with *K. sacchari* NO9 and/or *R. alamii* YAS34 by using the Transcriptor first strand cDNA synthesis kit (Roche, Manheim, Germany). Gene-specific primers for real-time PCR were designed based on *P. brassicacearum* NFM421 *phlD*, *hcnA*, *acdS* and *prrF1* and *prrF2* sequences to obtain predicted PCR products of 200-250 bases (Table S2). Amplifications were performed according to the real Q-PCR Light Cycler 480 SYBR Green I Master kit instructions for the Light Cycler 480 Real Time PCR System (Roche). Real-time PCR was performed in triplicate, and mRNA relative expression was normalized to the 16S reference gene.

Statistical analysis

Triplicates were used for each experiment. Statistical analyses were performed on q-PCR data from the three replicates per treatment in each experiment. Gene transcript levels were normalized to 16S rRNA transcription level. The significance of the results was examined by one-way analysis of variance (ANOVA) followed by post hoc tests (Student–Newman–Keuls test). Analyses were performed using STATGRAPHICS Centurion XVI.II. $P < 0.05$ was considered to be statistically significant.

Acknowledgement

AL had a researcher fellowship from the ANRT (Agence Nationale de la Recherche et de la Technologie) (CIFRE N° 2011/1473) and the InVivo company.

Author contributions

WA and TH conceived the experiments and coordinated the research. AL and SF performed the experiments. DL realized genetic constructs. JT and MB performed bioinformatics analysis. AL, SF, TH and WA discussed and interpreted the results. WA and TH wrote the manuscript. All authors read and approved the manuscript.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supporting information

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

Table S1. Antifungal activity of *P. brassicacearum* NFM421 towards phytopathgenic fungi.

Table S2. Strains, plasmids and primers used in this study.

Fig. S1. Colocalization of competing bacteria. *In planta* colocalization of *rfp*-tagged *P. brassicacearum* NFM421 with: *gfp*-tagged tagged *K. sacchari* NO9.

