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Compatibilité des bactéries phytobénéfiques *Azospirillum* et *Pseudomonas* dans la rhizosphère

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Résumé

Les bactéries rhizosphériques qualifiées de PGPR (*Plant Growth-Promoting Rhizobacteria*) forment des symbioses associatives avec les plantes, stimulant la croissance de ces dernières. Les PGPR présentent différents mécanismes phytobénéfiques (production de phytohormones, fixation non symbiotique de l'azote, etc.). Plusieurs PGPR sont susceptibles d'interagir avec la même plante hôte, et il est possible que leurs effets phytobénéfiques soient influencés par les interactions qu'elles auront les unes avec les autres. L'objectif de cette thèse était de caractériser la compatibilité des PGPR dans la rhizosphère d'une même plante hôte, dans le cas de modèles bactériens appartenant aux genres *Azospirillum* et *Pseudomonas*. Certains *Pseudomonas* phytobénéfiques produisant des métabolites antimicrobiens, comme le 2,4-diacétylphloroglucinol (DAPG), nous avons tout d'abord examiné si la capacité à produire du DAPG pouvait inhiber *Azospirillum*. Les expériences de confrontation réalisées *in vivo* avec *P. fluorescens* F113 et un mutant DAPG-négatif, en système gnotobiotique, ont montré que la colonisation racinaire et l'activité phytostimulatrice de certaines PGPR *Azospirillum* pouvaient effectivement être diminuées en présence de *Pseudomonas* producteurs de DAPG. Pour évaluer la colonisation racinaire par *Azospirillum* en sol non stérile, des outils de PCR quantitative en temps réel ont été développés et validés pour trois souches de premier plan (*A. lipoferum* CRT1, *A. brasilense* UAP-154 et CFN-535). L'utilisation de ces outils a permis la comparaison de ces trois souches d'*Azospirillum*, chacune co-inoculée avec la souche *P. fluorescens* F113 productrice de DAPG, sur du maïs cultivé en sol non stérile. Les niveaux de colonisation racinaire différaient selon la souche d'*Azospirillum*, et la combinaison de microorganismes phytobénéfiques conduisait à une meilleure croissance du maïs par comparaison avec des plantes non inoculées. Les résultats suggèrent que des PGPR des genres *Pseudomonas* et *Azospirillum* peuvent être compatibles dans la rhizosphère d'une même plante, même si les premiers ont le potentiel d'inhiber certains des seconds par la production de métabolites secondaires antimicrobiens.

Mots clefs : *Azospirillum*, *Pseudomonas*, PGPR, DAPG, Interaction, Rhizosphère, PCR quantitative en temps réel.

Abstract

Plant Growth-Promoting Rhizobacteria (PGPR) can form an associative symbiosis with plants, which results in stimulation of plant growth. PGPR harbour different phytobeneficial mechanisms (non-symbiotic nitrogen fixation, phytohormone synthesis, etc.). Various PGPR can interact with the same host plant, and it is possible that their phytobeneficial effects will be influenced by the interactions between these PGPR. The objective of this doctoral work was to characterize PGPR compatibility in the rhizosphere of the same host plant, in the case of model bacteria belonging to the genera *Azospirillum* and *Pseudomonas*. Because certain phytobeneficial *Pseudomonas* produce antimicrobial metabolites, such as 2,4-diacetylphloroglucinol (DAPG), we have first examined if DAPG production capacity could be involved in *Azospirillum* inhibition. *In vivo* experiments, performed with *P. fluorescens* F113 and a DAPG-negative mutant in gnotobiotic systems, showed that root colonization and phytostimulation activity of certain *Azospirillum* PGPR was indeed affected in the presence of DAPG-producing *Pseudomonas*. In order to evaluate *Azospirillum* root colonization in non-sterile soil, real-time quantitative PCR tools were developed and validated for three prominent *Azospirillum* strains (*A. lipoferum* CRT1, *A. brasilense* UAP-154 and CFN-535). The use of these real-time PCR tools enabled the comparison of the three *Azospirillum* strains, each co-inoculated with the DAPG-producing strain *P. fluorescens* F113, in the rhizosphere of maize grown in non-sterile soil. Root colonization levels differed according to the *Azospirillum* strain, and the combination of phytobeneficial microorganisms led to enhanced maize growth in comparison with non-inoculated plants. These results suggest that PGPR belonging to the genera *Pseudomonas* and *Azospirillum* may be compatible in the rhizosphere of a same plant, even if the former have the potential to inhibit some of the latter by producing antimicrobial secondary metabolites.

Keywords : *Azospirillum*, *Pseudomonas*, PGPR, DAPG, Interaction, Rhizosphere, Real-time quantitative PCR.

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Introduction générale

Plus de quatre décennies d'études de phylogénie moléculaire ont abouti à un arbre de classification du vivant où les microorganismes occupent une part importante (Lòpez-Garcia et Moreira 2008; Bertrand *et al.* 2009). Ils sont présents dans les trois "règnes" actuellement reconnus: les Archaeabactéries, les Bactéries, et les Eucaryotes (Pace 1997). Les microorganismes ont colonisé tous les types d'environnements, des plus extrêmes (comme les sources d'eau chaude des dorsales océaniques), aux plus communs tels que l'eau, l'air et le sol. Ils y assurent des fonctions importantes dans les cycles biogéochimiques, et lors d'interactions avec les macroorganismes animaux et végétaux (Atlas et Bartha 1998; Bertrand *et al.* 2009). Les microorgansimes ont notamment un rôle important dans le fonctionnement du système sol-plante (Franche *et al.* 2009; Lemanceau *et al.* 2009).

Le végétal, par le biais de ses racines, va puiser dans le sol les ressources minérales et l'eau nécessaires à sa croissance, mais aussi y libérer via le phénomène de rhizodéposition des composés organiques issus de la photosynthèse (Nguyen 2003; Somers et Vanderleyden 2004). Ces rhizodépôts, constitués d'acides aminés, de sucres, d'acides organiques, de métabolites secondaires, etc., vont stimuler la croissance et l'activité d'une partie des microorganismes situés dans la rhizosphère (Hartmann *et al.* 2009). Le terme Rhizosphère a été introduit il y a plus d'un siècle (Hiltner 1904; Hartmann *et al.* 2008). "Rhizo" vient du grec "rhiza" signifiant racine et "Sphère" vient du grec ancien "sfaira" (signifiant balle, ballon ou globe). La rhizosphère définit donc la zone du sol entourant les racines et qui est directement sous leur influence physique, chimique et biologique (Hinsinger *et al.* 2009). C'est un site d'interactions intenses entre le sol, le végétal et les microorganismes (Lambers *et al.* 2009). Cet environnement est l'un des plus riches en termes d'effectifs et de diversité microbiens (Gans *et al.* 2005; Roesch *et al.* 2007). Par exemple, un gramme de sol peut contenir plus de 10^{10} bactéries correspondant à plus de 4000 génomes différents (Torsvik *et al.* 1990). Dans la rhizosphère, les interactions entre plantes et microorganismes sont très nombreuses. Elles peuvent être bénéfiques (symbioses, commensalisme), neutres ou délétères (parasitisme, prédation, antagonisme) (Raaijmakers *et al.* 2009).

On peut distinguer deux types de symbioses plantes-microorganismes. La symbiose mutualiste est souvent considérée comme une interaction obligatoire entre les deux partenaires. Elle s'accompagne généralement d'un spectre d'hôte relativement étroit et / ou d'une différenciation morphologique chez l'un ou les deux partenaires de la symbiose (Bertrand *et al.* 2009). Les symbioses mutualistes plantes-microorganismes les plus étudiées sont les symbioses fixatrices d'azote impliquant des Fabacées (avec par exemple *Rhizobium*) ou des plantes actinorhiziennes (avec *Frankia*), ainsi que les symbioses mycorhiziennes des

végétaux supérieurs avec les champignons (*Hebeloma*, *Glomus*, etc.) (Marmeisse *et al.* 2004; Normand *et al.* 2007; Franche *et al.* 2009). La symbiose associative (synonyme de coopération) est également une interaction à bénéfices réciproques entre les deux partenaires. Elle est habituellement considérée comme une interaction facultative, à plus large spectre d'hôte, et avec peu ou pas de différenciation des partenaires. L'exemple le mieux connu est celui des bactéries rhizosphériques stimulatrices de la croissance des plantes, ou *Plant Growth-Promoting Rhizobacteria* (PGPR) (Dobbelaere *et al.* 2001; Franche *et al.* 2009; Richardson *et al.* 2009).

Les PGPR présentent un intérêt agronomique important, car leur utilisation pourrait permettre de diminuer les apports d'engrais ou de pesticides chimiques (Morrissey *et al.* 2002; Fuentes-Ramirez et Caballero-Mellado 2006). On peut en effet distinguer deux conditions d'utilisation des PGPR: la phytostimulation (appelée quelquefois biofertilisation), lorsque que la PGPR stimule directement la croissance du végétal, et la phytoprotection (appelée aussi biocontrôle), lorsqu'elle conduit à l'inhibition du développement d'organismes phytopathogènes (Lucy *et al.* 2004; Richardson *et al.* 2009). Les bactéries phytostimulatrices, dont de nombreuses souches étudiées appartiennent au genre *Azospirillum*, stimulent directement la croissance de la plante par la synthèse de phytohormones ou de signaux comme NO, la fixation non-symbiotique de l'azote, et / ou en interférant avec le métabolisme végétal de l'éthylène (Glick *et al.* 1998; Dobbelaere *et al.* 2003; Creus *et al.* 2005). Les premières études sur ces bactéries se sont intéressées à la fixation libre de l'azote (gène *nifH*) (James 2000). Les recherches ont ensuite et surtout concerné les effets hormonaux. C'est ainsi que différents gènes phytobénéfiques ont pu être identifiés, comme *ipdC*, impliqué dans la production de l'auxine acide indole-acétique (AIA) (Lambrecht *et al.* 2000); et *acdS*, impliqué dans la désamination du 1-aminocyclopropane-1-carboxylate (ACC), précurseur direct de l'éthylène chez la plante (Prigent-Combaret *et al.* 2008). Des études sur la diversité et les mécanismes de régulation de ces gènes ont été menées chez *Azospirillum* (Vande Broek *et al.* 1999; Bashan *et al.* 2004; Nukui *et al.* 2006). Quant aux bactéries phytoprotectrices, les souches les plus étudiées appartiennent au groupe des *Pseudomonas* fluorescents (Haas et Défago 2005; Raaijmakers *et al.* 2009). Leur principal mode d'action est l'inhibition de microorganismes phytopathogènes par la production d'antibiotiques (Raaijmakers *et al.* 2002) et l'induction de résistance systémique chez la plante (Bakker *et al.* 2007). L'étude des propriétés antagonistes des PGPR phytoprotectrices a concerné principalement les interactions avec les phytopathogènes fongiques. Parmi les antibiotiques, le plus étudié est le 2,4-diacétylphloroglucinol (DAPG), dont la production constitue une composante majeure de

l'effet phytoprotecteur de nombreuses souches de *Pseudomonas* (Haas et Défago 2005; Weller *et al.* 2007). Le DAPG est un antibiotique à large spectre qui a principalement des propriétés antifongiques. L'opéron de biosynthèse du DAPG a été caractérisé (Moynihan *et al.* 2009), ainsi que les mécanismes de régulation de son expression (Duffy et Défago 1999; Haas et Keel 2003) et plusieurs études se sont intéressées au gène *phlD* de cet opéron afin de mettre en évidence la diversité génétique des souches productrices de DAPG (Ramette *et al.* 2006; Frapolli *et al.* 2008).

Les modes d'action, directs et indirects, des PGPR sur le partenaire végétal sont nombreux. Certains de ces modes d'action sont d'ailleurs présents chez différents types de PGPR. Par exemple, les gènes phytobénéfiques *acdS*, *nifH*, et *ipdC* ont été identifiés à la fois chez des PGPR des genres *Pseudomonas* et *Azospirillum* (Blaha *et al.* 2006; Mirza *et al.* 2006; Spaepen *et al.* 2007). Il est donc vraisemblable qu'en plus de PGPR typiquement phytoprotectrices ou phytostimulatrices, il existe également des souches avec à la fois des propriétés phytoprotectrices et phytostimulatrices (Bashan et Holguin 1998). C'est d'ailleurs le cas de *P. fluorescens* F113 (résultat non publié), une souche utilisée dans ce travail.

Les PGPR ont été isolées de racines de nombreuses espèces végétales, sauvages ou cultivées, dans les zones tropicales, subtropicales et tempérées, et semblent être ubiquistes (Bashan et Levanony 1990; Wang *et al.* 2001; Lucy *et al.* 2004). Lors de la mise en place de la symbiose associative entre les PGPR et leur plante hôte, le processus de rhizodéposition va fournir des substrats nutritifs qui vont stimuler la croissance des PGPR et leur colonisation du système racinaire (Hartmann *et al.* 2009). La colonisation de la plante hôte à des effectifs suffisamment élevés est un prérequis pour l'expression des effets phytobénéfiques des PGPR, qu'il s'agisse de PGPR phytostimulatrices ou phytoprotectrices (Weller 1988; Jacoud *et al.* 1999). En raison de leur caractère ubiquiste, ces PGPR sont sans doute présents conjointement dans une même rhizosphère (Kyselkovà *et al.* 2009). Si les PGPR phytostimulatrices et phytoprotectrices colonisent le même habitat rhizosphérique, elles sont vraisemblablement amenées à interagir entre elles. Ces interactions pourraient être positives (par exemple via le *quorum sensing*) (Wei et Zhang 2006; Vial *et al.* 2006a) ou négatives (comme la compétition pour les exsudats racinaires) (Kamilova *et al.* 2008). Certaines de ces interactions négatives pourraient même impliquer des mécanismes phytobénéfiques, comme la production de DAPG antifongique par les PGPR phytoprotectrices. En effet, le DAPG présente également des propriétés antibactériennes (Keel *et al.* 1992; Johansen *et al.* 2002). Or, très peu d'études se sont intéressées jusqu'ici aux interactions entre les PGPR phytoprotectrices et les PGPR phytostimulatrices, chacune étant étudiée séparément au sein de la rhizosphère. On peut

néanmoins penser que les relations coévolutives entre la plante et sa cohorte de bactéries PGPR ont conduit à une situation de compatibilité des différents types de PGPR dans la rhizosphère (Hartmann *et al.* 2009; Lambers *et al.* 2009). Dans ce contexte, **l'objectif général** de cette thèse était de caractériser le niveau de compatibilité de différents types de PGPR dans la rhizosphère d'une même plante hôte.

Le premier objectif était d'évaluer l'impact, sur les PGPR phytostimulatrices *Azospirillum* et leur capacité de phytostimulation, de la capacité des *Pseudomonas* fluorescents à produire du DAPG (**Partie expérimentale 1**). *Azospirillum* est-il sensible au DAPG ? Le DAPG est-il impliqué dans les relations sociales entre *Pseudomonas* et *Azospirillum* sur une même plante hôte ? Les deux types de PGPR colonisent-ils les mêmes zones racinaires ou bien des sites différents sur une même racine ? Ces études ont été effectuées sur des plantes de blé (*Tricitum aestivum*) ou de riz (*Oryza sativa*) cultivées en conditions contrôlées (*in vitro*). Les plantes ont été inoculées par la PGPR DAPG$^+$ *P. fluorescens* F113 ou un mutant DAPG$^-$ (Fenton *et al.* 1992), en confrontation avec les PGPR phytostimulatrices *A. irakense* KBC1 ou *A. lipoferum* 4B (riz), *A. brasilense* Sp245 ou Cd (blé). L'utilisation de systèmes gnotobiotiques a permis de dénombrer les PGPR par des techniques de microbiologie classique de dilution-étalement sur milieux sélectifs, et a facilité les observations microscopiques des souches marquées avec des gènes codant des protéines autofluorescentes (Bloemberg *et al.* 2000).

Le deuxième objectif était de caractériser, cette fois-ci en conditions naturelles, la compatibilité entre les deux types de PGPR en termes de coexistence des populations et d'effets bénéfiques sur la plante. Les relations entre différents types de PGPR sont-elles influencées par l'habitat rhizosphérique, c'est-à-dire par la présence du sol et d'une communauté microbienne indigène ? L'hétérogénéité spatiale de la rhizosphère à microéchelle facilite-t-elle la coexistence de PGPR différentes sur une même racine ? Les conditions écologiques de la rhizosphère sont-elles favorables à l'utilisation conjointe de différents microorganismes phytobénéfiques pour améliorer les résultats de la phytostimulation ? Cependant, aucune technique permettant un suivi satisfaisant des inoculats d'*Azospirillum* en sol naturel n'étant disponible, nous avons tout d'abord choisi de développer des outils de PCR quantitative en temps réel, à partir d'amorces correspondant à des séquences nucléotidiques souche-spécifiques issues d'amplifications PCR aléatoires (Fani *et al.* 1993) (**Partie expérimentale 2**). La compatibilité des PGPR a ensuite été analysée lors d'expériences de co-inoculation réalisées en serre, dans un contexte agronomique (**Partie expérimentale 3**). Ces études ont été effectuées sur maïs (*Zea mays*), dont la culture nécessite

des apports importants en fertilisants chimiques. Différentes souches PGPR de *Pseudomonas* et *Azospirillum* adaptées au maïs ont été utilisées, à savoir *P. fluorescens* F113 et Pf-153, *A. lipoferum* CRT1, et *A. brasilense* UAP-154 et CFN-535.

Ce manuscrit comprend quatre chapitres : une synthèse bibliographique et trois parties expérimentales. La synthèse bibliographique traite des modes d'action et de colonisation des PGPR phytoprotectrices et phytostimulatrices, et de la compatibilité des PGPR dans la rhizosphère d'une même plante hôte. La première partie expérimentale porte sur l'étude de l'impact sur *Azospirillum* de la capacité de *Pseudomonas* à produire du DAPG. La deuxième partie expérimentale présente la mise au point d'un outil de PCR quantitative permettant le suivi de PGPR d'*Azospirillum* inoculées en sol non stérile. La troisième partie expérimentale correspond à l'étude de la compatibilité des PGPR en sol non stérile et l'impact sur la phytostimulation.

Synthèse bibliographique

Introduction

La Rhizosphère est le volume de sol entourant les racines et placé sous leur influence directe (Hiltner 1904). Cet environnement constitue un écosystème complexe où de multiples interactions s'établissent entre la plante, le sol, les microorganismes et la micro-faune. Ces interactions conditionnent le développement et la croissance de la plante. Dans la rhizosphère, les bactéries sont sans doute les microorganismes les plus abondants, et nous nous intéresserons plus particulièrement à celles qui favorisent la croissance des plantes en établissant une symbiose associative avec leur plante-hôte, c'est-à-dire les PGPR.

Les PGPR sont donc des bactéries phytobénéfiques interagissant avec la plante sous la forme d'une symbiose associative, c'est-à-dire une interaction facultative, sans processus de différenciation morphologique des deux partenaires. Leur habitat privilégié est la rhizosphère, même si certaines souches sont capables de coloniser l'intérieur des plantes (endophytes, Sturz *et al.* 2000) ainsi que les parties aériennes (épiphytes, Gnanamanickam et Immanuel 2006). Au niveau de la rhizosphère, les PGPR vont pouvoir bénéficier des exsudats racinaires et autres composés issus de la photosynthèse relargués par la plante au niveau de ses racines (rhizodépots). En retour, les PGPR vont avoir un effet bénéfique sur les plantes colonisées. Cet effet peut impliquer différents modes d'action, et nous pouvons classer la plupart des PGPR en deux groupes selon leurs modes d'action sur les plantes (Bashan et Holguin 1998). D'un coté nous avons les PGPR phytoprotectrices, qui protègent les plantes et stimulent donc indirectement leur croissance. D'un autre coté, nous avons les PGPR phytostimulatrices, qui stimulent directement la croissance racinaire via la synthèse d'hormones ou la fixation d'azote, ce qui augmente les capacités d'absorption d'eau et de composés minéraux nécessaires à la plante. La distinction entre ces deux groupes fonctionnels n'est cependant pas toujours nette, et certaines PGPR peuvent appartenir aux deux groupes. Ainsi, certaines souches de *Pseudomonas*, genre représentatif des PGPR phytoprotectrices, peuvent interagir avec le métabolisme hormonal de la plante (activité ACC désaminase ; Blaha *et al.* 2006) afin de stimuler la croissance racinaire, alors que certaines souches d'*Azospirillum* ont des d'activités phytoprotectrices (Miché *et al.* 2000).

De nombreuses PGPR phytoprotectrices protègent la plante en inhibant la croissance de phytopathogènes, via la production de métabolites secondaires antimicrobiens. Ces composés peuvent avoir un effet négatif sur les PGPR phytostimulatrices qui vont coloniser le même habitat que constitue la rhizosphère. La question de la compatibilité entre ces deux types de PGPR doit donc être abordée. Au cours de cette synthèse bibliographique, nous

résumerons les observations et résultats soutenant ou pas l'hypothèse d'une compatibilité entre ces deux types de PGPR, en nous intéressant dans un premier temps au genre *Pseudomonas*, avec un intérêt particulier pour le rôle du 2,4-diacétylphlroglucinol (DAPG). Nous nous intéresserons ensuite au genre *Azospirillum*, en mettant l'accent sur les conditions nécessaires à la phytostimulation des plantes. Enfin, dans un dernier temps, nous traiterons de la compatibilité entre les deux types de PGPR, dans le contexte d'interactions multi-partites avec d'autres microorganismes de la rhizosphère.

I/ Pseudomonas

1.1. *Pseudomonas fluorescens* and closely-related fluorescent pseudomonads as biocontrol agents of soil-borne phytopathogens

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Abstract

Many strains of *Pseudomonas fluorescens* show potential for biological control of phytopathogens especially root pathogens. In taxonomic terms, several of them are indeed *P. fluorescens sensu stricto*, while others belong in fact to neighbouring species of the '*P. fluorescens*' complex or to ill-defined related species within the fluorescent *Pseudomonas* spp.. These bacteria have become prominent models for rhizosphere ecological studies and analysis of bacterial secondary metabolism, and in recent years knowledge on their plant-beneficial traits has been considerably enhanced by widening the focus beyond the case of phytopathogen-directed antagonism. Current genomic analyses of rhizosphere competence and biocontrol traits will likely lead to the development of novel tools for effective management of indigenous and inoculated *P. fluorescens* biocontrol agents and a better exploitation of their plant-beneficial properties for sustainable agriculture.

Table 1 Exemples of well-studied biocontrol agents presented as *P. fluorescens* in the literature, and belonging to *P. fluorescens* or closely-related fluorescent *Pseudomonas* species within or outside the '*P. fluorescens*' species complex

Strains	Origin[a]	Molecular characterization	Plant protection[f]	Plant-beneficial traits documented[g]	Reference[h]
DR54	Sugarbeet (Denmark)	BC-C[b]	Sugarbeet (Pu, Rs)	**Viscosinamide**, chitinase	Sanguin *et al.* 2008
F113	Sugarbeet (Ireland)	BC-D[b], B[c], Hcn-1[d], BOX K[e]	Sugarbeet (Pu), potato (Pc)	**DAPG**, HCN, pyoverdine, ACC deaminase, T3SS	Moënne-Loccoz *et al.* 1998
KD	Wheat (China)	BC-G[b]	Cucumber (Pu), tomato (FORL)	**T3SS**, HCN, pyoverdine	Rezzonico *et al.* 2005
Pf29A	Wheat (France)	BC-G[b]	Wheat (Ggt)	Pathogen growth inhibition, ISR	Barret *et al.* 2009
Q2-87	Wheat (WA)	BC-G[b], C[c], Hcn-3[d], BOX B[e]	Wheat (Ggt), tomato (FORL)	**DAPG**, HCN, ACC deaminase	Weller 2007
Q8r1-96	Wheat (WA)	DGGE genotype G, BOX D[e]	Wheat (Ggt)	DAPG	Mavrodi *et al.* 2006
SBW25	Sugarbeet (UK)	BC-C[b]	Pea (Pu)	T3SS, competition, pyoverdine	Sanguin *et al.* 2008
WCS365	Potato (The Netherlands)		Tomato (FORL)	**ISR**, T3SS, siderophore, competition	de Weert *et al.* 2004
WCS374	Potato (The Netherlands)		Radish (FOR)	**ISR**, T3SS, pseudoverdine, pseudomonine, salicylate	Pieterse *et al.* 2003
2P24	Wheat (China)	BC-F[b]	Wheat (Ggt), tomato (Ras), cotton (Rs)	DAPG, HCN, pyoverdine	Sanguin *et al.* 2008
2-79	Wheat (WA)		Wheat (Ggt, Rs), Kentucky bluegrass (Mp)	**Phenazine-1-carboxylate**, pyoverdine, T3SS, anthranalate	Cook *et al.* 1995
CHA0	Tobacco (Switzerland)	Outside of '*P. fluorescens*' complex, BC-A[b], F[c], Hcn-4[d], BOX A[e]	Tobacco (Tb), wheat (Ggt), cucumber (Pu)	**DAPG**, **HCN**, **pyoluteorin**, **pyoverdine**, salicylate, pyrrolnitrin, ISR	Haas et Défago 2005
Pf-5	Cotton (Texas)	Outside of '*P. fluorescens*' complex, BC-A[b], F[c], Hcn-4[d], BOX A[e]	Cotton (Pu, Rs), cucumber (Pu), bluegrass (Dp, Sh)	DAPG, HCN, **pyoluteorin**, **pyoverdine**, pyrrolnitrin	Loper *et al.* 2007

[a]UK, United Kingdom; WA, Washington State.
[b]Phylogenetic cluster based on 16S rRNA gene *rrs* (Sanguin *et al.* 2008).
[c]Multilocus group determined based on phylogenetic analysis of concatenated sequences for 10 housekeeping genes (Frapolli *et al.* 2007
[e]Genotype defined based on BOX PCR (Weller 2007).
[f]The corresponding pathogens are Dp, *Drechslera poae*; FORL, *Fusarium oxysporum* f. sp. *radicis-lycopersici*; FOR, *F. oxysporum* f. sp. *raphani*; Ggt, *Gaeumannomyces graminis* var. *tritici*; Mp, *Magnaporthe poae*; Pc, *Pectobacterium carotovorum* (previously *Erwinia carotovora* subsp. *carotovora*); Ps, *Phomopsis sclerotioides*; Pu, *Pythium ultimum*; Ras, *Ralstonia solanacearum*; Rs, *Rhizoctonia solani*; Sh, *Sclerotinia homoeocarpa*; St, *Septoria tritici*; Tb, *Thielaviopsis basicola*.
[g]Traits shown to actually contribute to biocontrol in this strain are in bold.
[h]Reference from the current article, from which further information can be accessed on earlier work on these strains.

Introduction

The *Pseudomonas* (γ-Proteobacteria subclass, Pseudomonadales order, Pseudomonadaceae family) are motile (one or several polar flagella), non sporulating rods with Gram-negative reaction and 58-69% GC content (Palleroni 2008). They are catalase positive and chemo-organotrophic, with a strictly respiratory metabolism (using oxygen and in some cases nitrate as terminal electron acceptor). Within the *Pseudomonas sensu stricto*, which corresponds to the rRNA group I (Palleroni 2008), the fluorescent pseudomonads include all *Pseudomonas* species with the ability to produce fluorescent pyoverdine siderophore(s), noticeably *P. aeruginosa*, *P. syringae*, *P. putida* and *P. fluorescens* (Bossis *et al.* 2000).

P. fluorescens is adapted to survival in soil and colonization of plant roots (Kiely *et al.* 2006), and this applies also to the particular case of biocontrol agents from this species. Biocontrol strains have noticeably been observed at the root surface, the rhizoplane, often forming microcolonies or discontinued biofilms in the grooves between epidermal cells (Fig. 1). Certain strains are also capable of endophytic colonization. Within root tissues, they are mostly found in the intercellular spaces of the epidermis and the cortex (Duijff *et al.* 1997). They are effective at utilizing seed and root exudates for growth and can colonize the rhizosphere aggressively. Strains with biocontrol ability may represent in the order of 10% of all rhizosphere strains, and they have been isolated from a very wide range of soils, climatic regions and host plants (Rezzonico *et al.* 2007).

Biocontrol agents from *P. fluorescens* are rather non-specific in their ability to protect plants from soil phytopathogens. Indeed, each biocontrol strain can typically act in more than one pathosystem (Table 1), i.e. protect more than one plant species from often distinct pathogens, provided the rhizosphere is successfully colonized. They have been mostly studied for protection of crop plants from phytopathogenic oomycetes (particularly *Pythium* spp.) and fungi (*Fusarium oxysporum*, *Gaeumannomyces graminis*, *Rhizoctonia solani*, etc.), and to a lesser extent bacteria (e.g. *Pectobacterium carotovorum*) and nematodes (e.g. *Meloidogyne* spp.). Disease suppression by these bacteria often entails inhibition of phytopathogens in soil or on roots, by competition and/or antagonism (Haas et Défago 2005). Plant protection may also result from direct interactions with the host plants, especially in the case of induced systemic resistance (ISR) (Bakker *et al.* 2007).

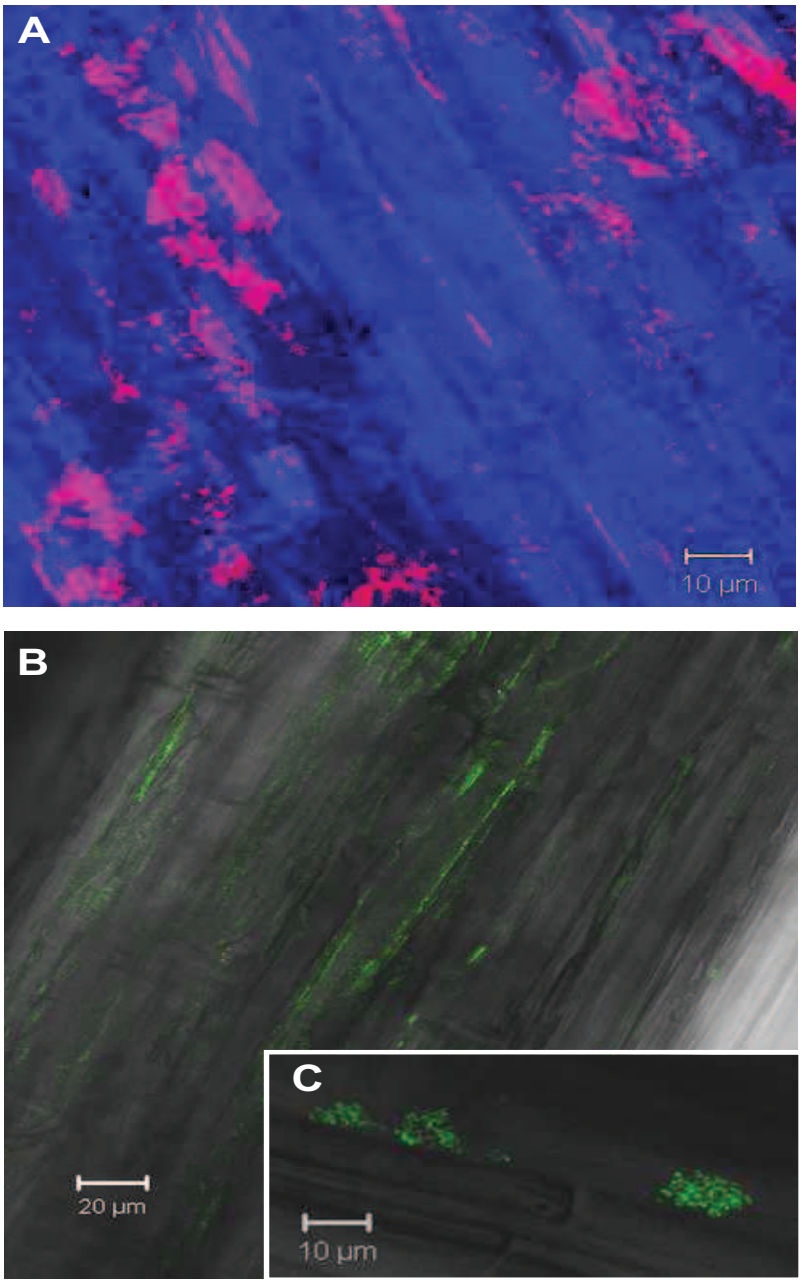

Fig. 1 Colonization of rice and wheat roots respectively by the biocontrol strains *Pseudomonas fluorescens* F113 (A) and fluorescent *Pseudomonas* sp. CHA0 (B,C). The pseudomonads express autofluorescent red (A) and green (B,C) proteins following marking with P_{lac}-*dsRed* and P_{lac}-e*gfp* plasmid fusions, respectively. Plants were grown under gnotobiotic conditions and observations were done using confocal laser scanning microscopy. Blue (A) and white/grey (B,C) backgrounds correspond to the root images formed by transmitted and reflected light, respectively. A three-dimensional reconstruction of colonized roots is shown in A. Bacterial cells form thin discontinued biofilms (see enlargement in C) located mainly in the intercellular spaces between epidermal cells.

Taxonomy and distribution

A large number of strains with disease suppression potential are presented as *P. fluorescens* in the literature, but only some of these biocontrol strains actually belong to this species (Sanguin *et al.* 2008). Many of the other strains correspond in fact to closely-related species from the same '*P. fluorescens*' complex, noticeably *P. kilonensis*, *P. aurantiaca*, *P. thivervalensis* and *P. brassicacearum* (Frapolli *et al.* 2007), which are often difficult to distinguish from *P. fluorescens*. In addition, a few strains described as *P. fluorescens* belong to a separate fluorescent *Pseudomonas* lineage, taxonomically ill-defined and usually referred to as ARDRA-1 based on 16S rRNA gene restriction profiling (Keel *et al.* 1996). Therefore, in this review, we are dealing with *P. fluorescens* biocontrol agents in the wider sense, i.e. by considering also those present in closely-related taxa of fluorescent pseudomonads and presented as *P. fluorescens* in the literature.

The current state of the *Pseudomonas* taxonomy makes it difficult to assess the phylogenetic distribution of biocontrol agents within *P. fluorescens* and closely-related fluorescent pseudomonads (Bossis *et al.* 2000). However, it seems clear that these taxa include both biocontrol agents and strains without any obvious biocontrol potential, regardless of whether only true *P. fluorescens* or also related fluorescent pseudomonads are considered (Sanguin *et al.* 2008). It is important to note that *P. fluorescens* and neighbouring species are thought to include also strains with human pathogenicity potential (Wei *et al.* 20022; Bodilis *et al.* 2004), but the evidence to date is not fully convincing in the current taxonomic context and this issue deserves further clarification.

P. fluorescens and closely-related fluorescent pseudomonads appear to be predominantly clonal (Frapolli *et al.* 2007). Yet, horizontal gene transfer may take place, and such a possibility has been raised for genes involved in the interaction with the plant and/or phytopathogens (Ramette *et al.* 2003; Blaha *et al.* 2006). This includes also the hypothesis that genes involved in the synthesis of biocontrol compounds might have been acquired from the plant itself (Cook *et al.* 1995; Ramette *et al.* 2001).

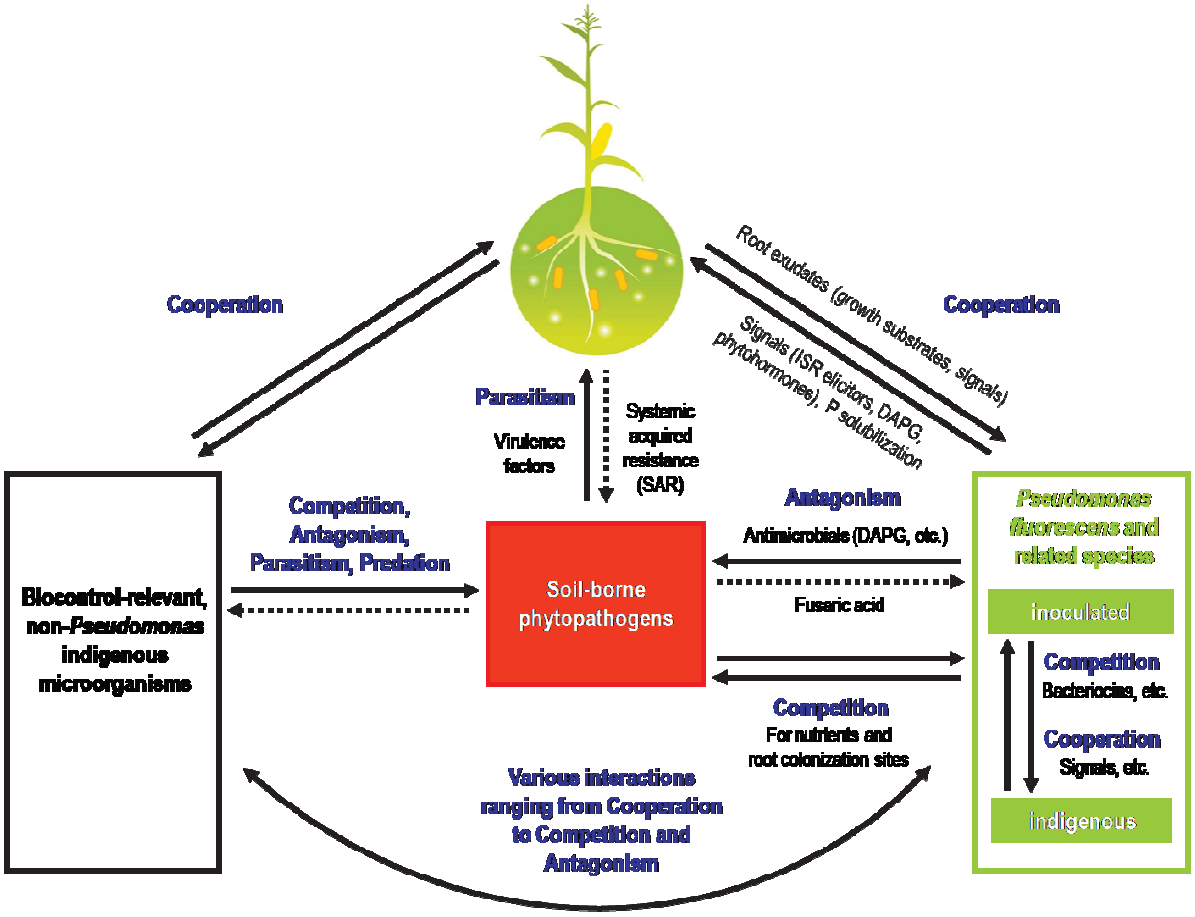

Fig. 2. Overview of plant-protection mechanisms in biocontrol agents from *P. fluorescens* and closely-related species of fluorescent *Pseudomonas*. These pseudomonads may act directly on the plant, noticeably via production of various signals (DAPG, phytohormones, etc.) and/or induction of ISR pathways, and the plant provides them with organic exudates and molecular signals. They may also inhibit the phytopathogens by competition and/or antagonism mediated by secondary metabolites such as DAPG. In addition, these effects are modulated by the action of certain non-*Pseudomonas* members of the microbial community, which may also have direct or indirect (i.e. via the plant) biocontrol effects and/or interfere with the functioning of biocontrol agents from *P. fluorescens* and related species. As for *Pseudomonas* inoculants, their ecology and plant-beneficial properties can be influenced positively (via signalling and cooperation) or negatively (via competition) by indigenous root-colonizing pseudomonads. Dashed lines are used to indicate possible feed-back responses of partners subjected to negative interactions, noticeably inhibition of DAPG production in *Pseudomonas* by fusaric acid from *F. oxysporum* phytopathogens, and systemic acquired resistance in plant in response to infection.

Modes of action

Many biocontrol agents from *P. fluorescens* and closely-related species are well characterized for their ability to produce antimicrobial compounds, including 2,4-diacetylphloroglucinol (DAPG), phenazines, hydrogen cyanide and surfactants (Haas et Défago 2005) (Fig. 2; Table 1). The biosynthetic pathways involved in their production, as well as their regulation and the signals involved have received extensive attention (Baehler *et al.* 2006; Dubuis *et al.* 2007), and these bacteria have become prominent models for analysis of bacterial secondary metabolism. These secondary metabolites inhibit various phytopathogens *in vitro* and some of these metabolites have been detected in the rhizosphere by chemical means (Raaijmakers *et al.* 2002). Their importance in the antagonistic properties of biocontrol agents was evidenced from the comparison of wild-type strains, non-producing insertion or deletion mutants, and complemented derivatives (Raaijmakers *et al.* 200202; Haas et Défago 2005). In addition, transfer of genes encoding the synthesis of these antimicrobial compounds may confer or enhance biocontrol potential to non-producing pseudomonads and strains already producing them, respectively. This mode of action is effective in various pathosystems, even though certain pathogens can fight back, e.g. *F. oxysporum* may produce fusaric acid, which represses DAPG synthesis in *Pseudomonas* sp. CHA0 (Notz *et al.* 2002). Furthermore, certain antimicrobial secondary metabolites (e.g. DAPG) are involved in protection of different plant species, from different phytopathogens, and by different biocontrol strains (Rezzonico *et al.* 2007; Weller 2007). Other antagonistic modes of action are also documented but to a much lesser extent. First, many *Pseudomonas* biocontrol strains produce extracellular lytic enzymes (Diby *et al.* 2005), but genetic evidence for an actual role in biocontrol is lacking and/or formal membership to *P. fluorescens* needs clarification. Second, a functional type III protein secretion (T3SS) gene *hrcV* is needed in *P. fluorescens* KD to effectively reduce polygalacturonase activity in *Pythium ultimum* and protect cucumber from *P. ultimum*-mediated damping-off (Rezzonico *et al.* 2005), suggesting that effectors (not identified so far) secreted via this system could be involved in biocontrol.

Since strains from *P. fluorescens* and related species colonize the rhizosphere aggressively, competition with root pathogens for nutrients and root surface colonization has been proposed as an important trait for biological control (Fig. 2; Table 1), although in recent years its significance has been debated (Haas et Défago 2005). Competition may concern the acquisition of organic substrates released by seeds and roots (Kamilova *et al.* 2005), as well as micronutrients such as soluble iron, which is often in limiting amounts in soil. Iron acquisition

entails the production of iron transporters (siderophores), noticeably fluorescent pyoverdines. Once complexed to ferric iron in soil or the root zone, the siderophores are then taken up using outer membrane receptors. In a context of biological control, competition for iron involves the synthesis of siderophores of higher affinity compared with siderophores used by phytopathogens (Lemanceau *et al.* 1992). Interestingly, siderophore-mediated iron competition by *P. fluorescens* may also be useful to prevent growth of human pathogen *Escherichia coli* O157:H7 on food products (McKellar 2007).

In addition to antagonism and competition, biocontrol strains from *P. fluorescens* and related species may also act directly on the growth, physiology and health of the plant they colonize (Fig. 2; Table 1). First, several strains can induce an ISR response in the plant, which makes the plant more efficient in fighting back against pathogens (Bakker *et al.* 2007). In *P. fluorescens* WCS417r, ISR involves the phytohormones jasmonate and ethylene as signals (Pieterse *et al.* 2003). It does not lead to major transcriptomic changes in the plant, but activates plant genes involved in defence mechanisms. ISR can be triggered by contact of the plant to certain cell surface components of biocontrol strains, such as lipopolysaccharides and flagella, or exposure to biocontrol metabolites including pyoverdine and DAPG (Pieterse *et al.* 2003; Bakker *et al.* 2007). Second, deamination of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) can diminish the quantity of plant ACC left for ethylene synthesis (Glick 2005), and the introduction of the ACC deaminase locus into the fluorescent *Pseudomonas* strain CHA0 improved suppression of Pythium damping-off of cucumber (Wang *et al.* 2000; Blaha *et al.* 2006). Since many biocontrol strains of *P. fluorescens* harbour ACC deaminase activity (Blaha *et al.* 2006), it raises the possibility that this property could be important for biological control.

Biocontrol strains from *P. fluorescens* or related species may vary from one another in terms of their mode(s) of action, as well as their efficacy at protecting plants (Haas et Défago 2005; Rezzonico *et al.* 2005). It is important to note that several metabolites play a role in different plant-protection mechanism, e.g. pyoverdine in ISR and competition, and DAPG in ISR and antagonism. In addition, certain strains display multiple plant-beneficial traits, e.g. *P. fluorescens* F113 exhibits ACC deaminase activity and produces the phytohormone indole-acetic acid (Caballero-Mellado *et al.* unpublished result), pyoverdine siderophore(s), and the antimicrobials DAPG and hydrogen cyanide (Table 1).

Biocontrol in practice

There are two contexts in which biological control mediated by *P. fluorescens* strains and related pseudomonads has important practical implications. The first context corresponds to the use of biocontrol agents as inoculants of soil or plants, which has been successfully implemented in agronomic field trials (Amein *et al.* 2008; Karthikeyan et Gnanamanickam 2008). The use of *P. fluorescens* biocontrol agents is thought to have a limited ecological impact on indigenous saprophytic populations and to take place without negative side-effects on rhizosphere functioning (Moënne-Loccoz *et al.* 1998, Mark *et al.* 2006). Many inoculation products are commercially available (Mark *et al.* 2006), but *Pseudomonas* biocontrol strains may lose cell viability during biomass stabilisation or subsequent storage of the inoculant product (Haas et Défago 2005). However, recent advances show that *Pseudomonas* formulation can be improved for long term storage (Guo *et al.* 2004) and efficient antagonistic activity (Wiyono *et al.* 2008).

In addition, *Pseudomonas* inoculants may perform inconsistently from one field to another and/or from one year to the next, as a consequence of variability in root colonization (Weller 2007) or in expression of biocontrol traits (Mark *et al.* 2006). Therefore, superior root colonization and effective functioning in the rhizosphere are key criteria when selecting strains, and research aims at better understanding the molecular basis of these traits (Mark *et al.* 2005; Mavrodi *et al.* 2006) and the signalling processes regulating the ecology of *P. fluorescens in situ* (Kiely *et al.* 2006; Dubuis *et al.* 2007; Barret *et al.* 2009). Other studies have focused on the possibility of promoting microevolution of biocontrol strains to enhance their rhizosphere competence (de Weert *et al.* 2004). Promising results were also obtained with the development of genetically-improved strains with higher plant protection ability, either by reprogramming the regulation of existing biocontrol traits (Mark *et al.* 2006) or the introduction of novel mechanisms such as the degradation of pathogen quorum-sensing molecules (Molina *et al.* 2003) or ACC deaminase activity (Wang *et al.* 2000). Another way to seek more effective biocontrol treatments is to inoculate consortia of *P. fluorescens* biocontrol agents, sometimes in mixture with other plant-beneficial microbes (Karthikeyan et Gnanamanickam 2008). However, the compatibility of these inoculants (despite possible bacteriocin-mediated competition; Validov *et al.* 2005), their antimicrobial metabolites and their extracellular signals needs to be considered (Molina *et al.* 2003; Dubuis *et al.* 2007), especially when synergistic effects are sought. Compatibility with indigenous pseudomonads is also a relevant issue (Fig. 2). It is interesting to note that certain non-biocontrol and biocontrol pseudomonads produce signals activating the Gac/Rsm cascade in biocontrol

Pseudomonas strain CHA0, which is important for expression of its biocontrol traits (Dubuis *et al.* 2007).

The second context in which biological control by *P. fluorescens* strains and related pseudomonads is important corresponds to disease-suppressive soils, in which disease-susceptible plants can grow without being extensively damaged by virulent root pathogens (Janvier *et al.* 2007). In contrast, non-suppressive soils (i.e. conducive soils) allow plant infection and spread of the disease. Suppressive soils are extensively documented in the case of fungal soil-borne pathogens, noticeably *Gaeumannomyces graminis* var. *tritici* (take-all of wheat), *Fusarium oxysporum* (wilt diseases of several crop plants), *Rhizoctonia solani* (seedling damping-off of various crops) and *Thielaviopsis basicola* (black root rot of tobacco and other species), and to a lesser extent phytoparasitic oomycetes e.g. *Phytophthora cinnamoni* (root rot of eucalyptus), nematodes e.g. *Meloidogyne incognita* (root-knot galls on several tropical and subtropical crops) and bacteria e.g. *Streptomyces scabies* (potato scab) and *Ralstonia solanacearum* (bacterial wilt of several crops) (Janvier *et al.* 2007; Weller 2007). *P. fluorescens* strains and related pseudomonads are thought to be responsible for soil suppressiveness to take-all of wheat, which is induced by crop monoculture. This decline of take-all disease requires that $DAPG^+$ pseudomonads exceed a minimum threshold population density, which will determine DAPG concentration in the rhizosphere (Raaijmakers et Weller 1998; Weller 2007). $DAPG^+$ pseudomonads are also present in high number in the rhizosphere of tobacco grown in soils naturally suppressive to black root rot, where suppressiveness does not require monoculture (Ramette *et al.* 2006). However, these bacteria are also found in neighbouring disease-conducive soils, often in lower numbers but not always.

Conclusion and future prospects

Biocontrol agents from *P. fluorescens* and closely-related species have become prominent models for analysis of plant protection mechanisms and secondary metabolism. This has resulted in a good understanding of their direct effects on the pathogens as well as the triggering of ISR pathways in plants. It is likely that these biocontrol agents are also able to interfere with the functioning of the rest of the rhizosphere microbial community, either directly via antimicrobial compounds or indirectly by modulating rhizodeposition patterns (Phillips *et al.* 2004). Whether these effects can, in turn, have an impact on plant health remains to be clarified.

Significant advances have been made in recent years concerning the biotechnology of *P. fluorescens* biocontrol agents for effective inoculation and protection of crops (Wiyono *et al.* 2008). Progress is now needed to better integrate these biocontrol treatments with the other farming practices. However, in certain countries (e.g. those in the European Union), the main limitation to the practical use of these biocontrol agents is rather the current regulatory framework controlling commercialisation of microbial strains for agricultural applications (Mark *et al.* 2006). The exploitation of indigenous *Pseudomonas* biocontrol agents in disease-suppressive soils seems more complicated, because of the lack of guidelines for assessment of soil health and suppressiveness (Janvier *et al.* 2007). However, management of indigenous plant-beneficial *Pseudomonas* populations, via farming practices or the choice of crop varieties, offers considerable potential for sustainable agriculture (Picard et Bosco 2006).

Whole genome sequences are now available for two biocontrol strains belonging to the *P. fluorescens* lineage (strain SBW25) or a closely related species (strain Pf-5), as well as non-biocontrol *P. fluorescens* Pf0-1. Comparative genomics (Loper *et al.* 2007), gene array-based expression studies (Barret *et al.* 2009) and integrated, *in situ* molecular analyses of microbe-host interactions (Kiely *et al.* 2006) have started to provide advanced knowledge on plant-protection properties and rhizosphere competence of these biocontrol agents. It is expected that gene arrays and metagenomics will provide additional light on naturally-occurring biocontrol populations of pseudomonads and other taxa in the rhizosphere (including in disease-suppressive soils). Transcriptomics will be useful to decipher molecular dialogs and interactions in the root zone (including between *Pseudomonas* biocontrol agents, phytopathogens and plants). Thus, the forthcoming developments in environmental omics technology will help us understand further the establishment, rhizosphere functioning and performance of biocontrol strains from *P. fluorescens* and related species, and overcome current bottlenecks restricting their commercial use.

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1.2. Impact du DAPG sur la plante et les microorganismes non-cibles de la rhizosphère

Introduction

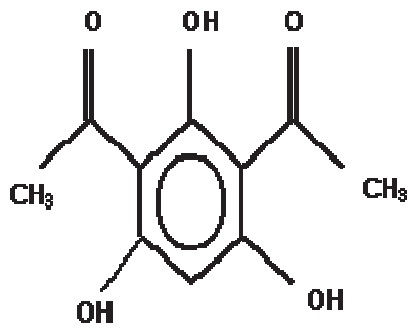
Le sol contient de nombreux phytopathogènes qui infectent les racines des plantes et causent des maladies. Il existe néanmoins quelques sols résistants à ces maladies, où des microorganismes phytobénéfiques présents dans le sol limitent l'infection de la plante par certains pathogènes (Raaijmakers *et al.* 1997; Ramette *et al.* 2003). Des populations spécifiques de microorganismes comme des *Pseudomonas* fluorescents producteurs de 2,4-diacétylphloroglucinol (DAPG, Figure 1) contribuent à la résistance de ces sols (Weller *et al.* 2007; Frapolli *et al.* 2008). Le DAPG inhibe en effet des phytopathogènes, comme *Chalara elegans* (pourriture noire du tabac) et *Gaeumannomyces graminis var. tritici* (piétin échaudage du blé) (Keel *et al.* 1992). Ces bactéries antagonistes, productrices de DAPG, font partie du groupe des PGPR, qui colonisent la plante dans un contexte de symbiose associative (Landa *et al.* 2002). L'inoculation de souches productrices de DAPG dans des sols non résistants aux maladies permet ainsi de protéger la plante (Keel *et al.* 1990).

La production de DAPG constitue une composante majeure de l'effet phytoprotecteur des *Pseudomonas* fluorescents (Bonsall *et al.* 1997; Raaijmakers *et al.* 1999; Weller *et al.* 2007). Le DAPG a des effets directs sur les pathogènes (Keel *et al.* 1990; Keel *et al.* 1992), mais aussi des effets indirects sur ces derniers en induisant une résistance systémique (*Induced Systemic Resistance*, ISR) chez certaines plantes (Iavicoli *et al.* 2003), qui vont ainsi devenir partiellement résistantes. Mais le DAPG est un antimicrobien à large spectre, qui a aussi bien des propriétés antifongiques, antibactériennes, qu'antihelminthiques (Keel et Haas 2003). Il pourrait aussi avoir des effets sur d'autres organismes, non pathogènes, présents dans la rhizosphère.

Un impact direct du DAPG ne peut être clairement identifié que suite à une expérience *in vitro* de confrontation entre l'organisme et du DAPG synthétique, mais les observations *in vitro* n'intègrent aucun des nombreux facteurs biotiques ou abiotiques qui peuvent influencer la production de DAPG (Shanahan *et al.* 1992; Naseby et Lynch 1999a; Notz *et al.* 2001; Raaijmakers *et al.* 2002; Baehler *et al.* 2005). Il est donc important d'effectuer des expériences *in situ*, tout en restreignant les conclusions à l'impact de la capacité de production de DAPG. L'objectif de cette partie sera d'effectuer une synthèse des connaissances actuelles sur les effets non-cibles du DAPG, qu'ils soient positifs ou négatifs. On verra que ce dernier

peut avoir un impact non cible à la fois sur la plante, et sur l'ensemble de la communauté microbienne rhizosphérique.

Figure 1 Structure du DAPG.



Impact sur la plante

La plante est confrontée directement au DAPG produit dans la rhizosphère. Il va avoir des effets stimulants sur l'exsudation racinaire et des effets bénéfiques sur la croissance des racines des plantes. Mais il pourra aussi avoir des effets phytotoxiques à la fois au niveau de la germination des graines et de la croissance des plantes.

Augmentation de l'exsudation racinaire

Les exsudats racinaires constituent une source de composés carbonés pour les microorganismes rhizosphériques. Des expériences de confrontation *in vitro* entre la plante et du DAPG synthétique (Phillips *et al.* 2004) démontrent que le DAPG, à une concentration de 200 µM, augmente l'exsudation racinaire de plus de 20 fois. A une concentration de 100 µM de DAPG, cette augmentation de l'exsudation racinaire a été observée sur trois espèces de plantes différentes, avec un effet particulièrement prononcé sur le maïs. Des expériences *in vitro* avec des acides aminés et des plantes marqués au ^{15}N indiquent que cette augmentation était à la fois le résultat d'une augmentation de l'efflux racinaire, et d'un blocage de l'influx racinaire. Les concentrations de DAPG ayant un impact dans cette étude sont du même ordre que celles mesurées dans la rhizosphère (Bonsall *et al.* 1997) ; en tenant compte du fait que ces concentrations mesurées *in situ* peuvent être affectées par l'instabilité chimique de ce composé et les liaisons qui pourraient s'établir entre le DAPG et les colloïdes ou la matière organique du sol (Figure 3). Le DAPG permettrait ainsi aux microorganismes producteurs de DAPG d'augmenter les ressources carbonées que constituent les exsudats racinaires. Cette augmentation de l'exsudation racinaire sera aussi positive pour la plante car elle va stimuler la croissance des populations bénéfiques, comme celle des *Pseudomonas* fluorescents producteurs de DAPG. Ces exsudats seront cependant disponibles pour l'ensemble de la communauté rhizosphérique, et cette augmentation pourrait donc aussi avoir un impact sur les populations délétères comme les phytopathogènes. Aucune étude ne s'est encore intéressée à cet impact non-cible du DAPG.

En observant les effets possibles d'une augmentation de l'exsudation racinaire, d'autres études, effectuées *in situ*, apportent des preuves indirectes de cette stimulation. Ainsi, la capacité de *P. fluorescens* F113 à produire du DAPG entraîne une augmentation significative du contenu protéique total et des concentrations en certains acides organiques mesurés dans la rhizosphère (Naseby et Lynch 2001). Cette capacité à produire du DAPG semble aussi augmenter le taux de nodulation des racines par *Rhizobium* (nombre de nodosités par gramme de racine) ainsi que la taille des nodosités formées (Andrade *et al.* 1998; De Leij

et al. 2002). Ces deux derniers résultats pourraient en effet être la conséquence d'une augmentation de l'exsudation : des composés impliqués dans la mise en place de la nodulation de *Rhizobium*, tels que les flavonoïdes, seraient exsudés en plus grande quantité, ce qui expliquerait le taux de nodulation plus élevé. Mais ces observations pourraient aussi être la conséquence d'un impact combiné du DAPG sur la communauté microbienne rhizosphérique et sur la plante.

Augmentation de la croissance des racines

Pour évaluer les conditions de croissance des plantes, on utilise souvent le rapport (masse des parties aériennes/masse des racines). La production de DAPG entraîne une diminution significative de ce rapport (Naseby et Lynch 1998; Naseby *et al.* 1999b; Naseby et Lynch 2001; De Leij *et al.* 2002). Cette diminution s'explique par une tendance à l'augmentation de la biomasse racinaire (cependant significative que dans les études de Naseby et Lynch 2001; De Leij *et al.* 2002), ainsi que par une tendance à la diminution de la biomasse aérienne. Dans l'une de ces études (Naseby et Lynch 2001), où l'augmentation de la biomasse racinaire était significative, aucune variation de la longueur des racines principales n'a été observée, mettant ainsi en évidence une stimulation de la ramification. L'étude de De Leij *et al.* 2002 enregistre à la fois une augmentation du nombre de racines secondaires, mais aussi de la longueur moyenne des racines. Une étude récente a mis en évidence des changements morphologiques et physiologiques des racines après application de DAPG à des concentrations qui sont celles mesurées dans la rhizosphère (Brazelton *et al.* 2008). Ces résultats indiquent donc que le DAPG a une action directe sur la rhizogénèse en stimulant la croissance et la ramification des racines, ce qui peut être considéré comme un avantage dans l'acquisition de l'eau et des nutriments. Cette possibilité est renforcée par l'observation des effets directs du DAPG sur l'exsudation racinaire des plantes (Phillips *et al.* 2004) et dans l'induction de résistance systémique (Iavicoli *et al.* 2003).

Effets phytotoxiques

Des expériences *in vitro* sur la germination et la croissance de huit espèces de plantes ont montré que le DAPG pouvait avoir un effet phytotoxique (Keel *et al.* 1992). En général, le DAPG est plus toxique avec les dicotylédones qu'avec les monocotylédones, mais les concentrations de DAPG qui entraînent un effet phytotoxique varient beaucoup en fonction des espèces concernées. De plus, lorsque l'on compare ces concentrations phytotoxiques avec

celles impliquées dans les effets phytoprotecteurs (c'est-à-dire dans l'inhibition des phytopathogènes), des incohérences apparaissent : (i) le cresson est sensible à des concentrations de DAPG de 8 à 16 µg/ml, alors que ces concentrations n'ont quasiment aucun effet inhibiteur sur les champignons phytopathogènes et (ii) les concentrations de DAPG requises pour l'inhibition de *Thielaviopsis basicola*, un pathogène du tabac, entraînent des effets toxiques sur la germination et la croissance du tabac (Keel *et al.* 1990). Ces résultats suggèrent que la production de DAPG par les *Pseudomonas* fluorescents ne pourrait protéger les plantes que par l'induction de résistance systémique.

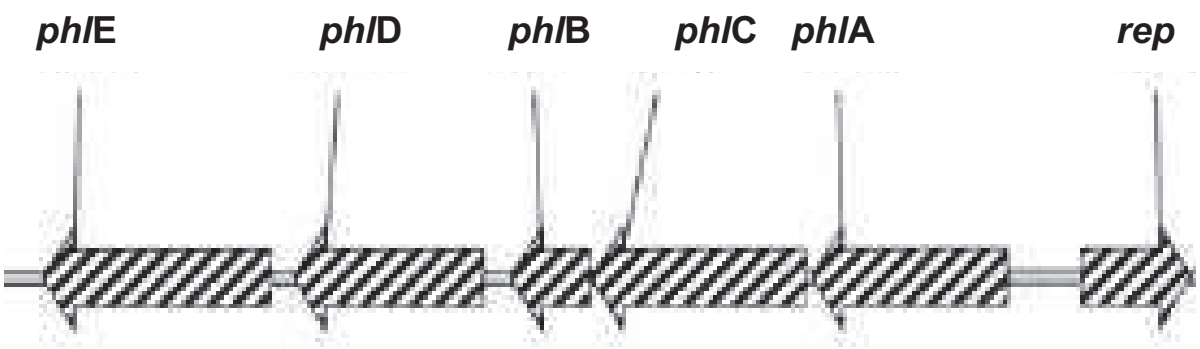
Impact sur la communauté microbienne rhizosphérique.

Impact sur les populations indigènes de *Pseudomonas*.

L'inoculation de souches de *Pseudomonas* fluorescents productrices de DAPG dans la rhizosphère de plante peut avoir un impact, par la compétition trophique, sur les populations qui sont succeptibles de partager la même niche écologique. Les populations indigènes de *Pseudomonas* sont donc celles qui devraient être les plus concernées. Parmi les populations de *Pseudomonas* fluorescents indigènes, il peut y avoir une forte proportion de souches productrices de DAPG : 144 souches de *Pseudomonas* fluorescents producteurs de DAPG ont été isolées d'un sol sous monoculture de maïs (Picard et Bosco 2003). De plus, des analyses de sols résistants ont révélé des effectifs de *Pseudomonas* fluorescents $DAPG^+$ allant de 5.10^5 à 2.10^6 CFU par gramme de racine (Raaijmakers et Weller 2001; Ramette *et al.* 2003). Des expériences de génétique (Schnider-Keel *et al.* 2000) ont montré que le DAPG stimulait l'expression du gène *phlA* (Figure 2), le premier gène de l'opéron qui dirige la biosynthèse du DAPG. Dans un sol artificiel, deux souches de *Pseudomonas* fluorescents producteurs de DAPG (CHA0 et Q2-87) ont été capables d'induire leur propre biosynthèse de DAPG, mais aussi d'influer l'une sur l'autre via le DAPG (Maurhofer *et al.* 2004). En effet, la production de DAPG par la souche CHA0 semble stimuler la biosynthèse de DAPG chez la souche Q2-87, et réciproquement. Ces résultats suggèrent que les populations de *Pseudomonas* productrices de DAPG seraient capables de percevoir le DAPG exogène, et donc de communiquer dans la rhizosphère. Cette autoinduction du DAPG pourrait favoriser son accumulation dans la rhizosphère, et il pourrait ainsi atteindre une concentration qui permette d'inhiber les phytopathogènes. Le DAPG a donc un impact particulier sur les populations indigènes de *Pseudomonas* producteurs de DAPG.

Mais qu'en est-il de l'ensemble des populations de *Pseudomonas* de la rhizosphère ? Les résultats disponibles indiquent que l'inoculation de la souche *Pseudomonas fluorescens* F113 productrice de DAPG a un impact sur la composition de la communauté indigène des *Pseudomonas* fluorescents cultivables (De Leij *et al.* 1995; Natsch *et al.* 1997; Moënne-Loccoz *et al.* 2001). Se pose alors la question du rôle du DAPG dans ce contexte de compétition entre l'inoculum et l'ensemble des populations indigènes. Le DAPG pourrait ainsi avoir un rôle dans la compétition directe (trophique). Mais l'inactivation de la production de DAPG dans cette même souche F113 n'affecte pas sa capacité à coloniser les racines (Carroll *et al.* 1995), montrant ainsi que le DAPG ne jouait pas de rôle déterminant dans la compétitivité de F113, en tout cas sur betterave. D'ailleurs, les souches de *P. fluorescens* productrices ou non de DAPG sont résistantes à des concentrations assez élevées de DAPG (Keel *et al.* 1992; Natsch *et al.* 1997; Moënne-Loccoz *et al.* 2001 ; Figure 3), et l'inoculation d'une souche productrice de DAPG n'a pas eu d'influence sur le pourcentage des *Pseudomonas* fluorescents indigènes résistants au DAPG (Moënne-Loccoz *et al.* 2001).

Figure 2: Schéma du locus de biosynthèse du DAPG chez *Pseudomonas fluorescens* Q2-87 (Bainton *et al.* 2004).
Le gène *phlE* code pour une pompe à efflux putative, le gène *phlD* pour une polyketide synthase nécessaire à la productionde monoacétylphloroglucinol (MAPG), tandis que les gènes *phlC*, *phlB* et *phlA* codent pour des protéines necessaires à la transformation du MAPG en DAPG.



Ces résultats sont cohérents avec ceux de l'étude de Phillips et collaborateurs en 2004, qui ont montré que 87% des 568 isolats, issus d'un sol non traité, étaient résistants à des concentrations de DAPG de 100 µg/ml. D'autres expériences (Naseby et Lynch 1999a; Naseby *et al.* 1999b; Naseby et Lynch 2001), avec la souche F113 et son mutant F113G22 ($DAPG^-$), ont aussi montré l'absence d'impact du DAPG sur les effectifs des populations indigènes de *Pseudomonas* fluorescents. Tous ces résultats indiquent donc que la production de DAPG joue un rôle mineur dans les interactions entre les souches productrices de DAPG introduites dans l'écosystème et les populations indigènes de *Pseudomonas*. Le DAPG ne semble donc pas avoir d'impact négatif sur la sous-communauté des *Pseudomonas* fluorescents.

Impact sur la communauté microbienne rhizosphérique.

L'absence d'impact négatif de la production de DAPG sur les populations indigènes de *Pseudomonas* fluorescents s'explique par leur capacité à résister à ce composé. Mais qu'en est-il de la communauté microbienne rhizosphérique dans son ensemble ? Cette question peut être considérée au niveau des effectifs, de la diversité et de l'activité de la communauté microbienne rhizosphérique.

Le DAPG inhibe de nombreux microorganismes *in vitro* : (i) il inhibe la croissance de champignons phytopathogènes et champignons saprophytes (Keel *et al.* 1992; Bakker *et al.* 2002), (ii) il inhibe le développement du mycélium du champignon mycorhizien *Glomus mossae* à des doses relativement faibles (10 µM ; Barea *et al.* 1998), et (iii) il inhibe la croissance de nombreuses bactéries, y compris des non-phytopathogènes (Keel *et al.* 1992; Walsh *et al.* 2003), et notamment les bactéries Cytophaga-like (CLB). Les CLB représentent une grande partie des bactéries isolées de la rhizosphère de l'orge, et leur croissance est inhibée en présence de la souche $DAPG^+$ CHA0 (Johansen *et al.* 2002). Les résultats des expériences en sol montrent que les effectifs des communautés de champignons et bactéries n'ont pas été affectés par la production de DAPG (Bakker *et al.* 2002; Johansen *et al.* 2002) ; même en pratiquant des expériences d'inoculations répétées de souches productrices de DAPG pendant deux années (Bakker *et al.* 2002).

La structure de la communauté microbienne rhizosphérique cultivable (Naseby et Lynch 1998; Shaukat et Siddiqui 2003; Mazzola *et al.* 2004) ou totale (Bakker *et al.* 2002) peut être affectée par la production de DAPG. Mais l'impact, observé lors de la première année, a disparu au cours de la deuxième année d'étude (Bakker *et al.* 2002). Cet impact

transitoire sur la structure de la communauté bactérienne était plus durable que celui sur la communauté fongique. Au niveau des microorganismes indigènes cultivables, la capacité à produire du DAPG a eu un impact significatif sur la structure des communautés fongiques (Shaukat et Siddiqui 2003), et bactérienne (Naseby et Lynch 1998; Mazzola *et al.* 2004). La pression de sélection qu'exercerait le DAPG aurait pour conséquence de favoriser les bactéries à stratégie K par rapport aux autres (Naseby et Lynch 1998). Cependant, une monoculture répétée sur un même sol peut avoir un impact plus fort que le DAPG sur cette structure en favorisant le développement d'une microflore indigène propre à la rhizosphère de la plante cultivée (Girlanda *et al.* 2001; Bakker *et al.* 2002). Au niveau des populations, l'inoculation avec une souche $DAPG^+$ a eu un impact sur la diversité génétique de l'espèce *Rhizobium leguminosarum* (Walsh *et al.* 2003). Cependant, le pourcentage de *Rhizobium* sensibles au DAPG ne semble pas varier, et l'absence de témoin négatif (une souche $DAPG^-$) dans cette dernière étude ne nous permet pas de conclure à un effet de la production de DAPG.

Ces résultats suggèrent que le DAPG possède un potentiel d'inhibition *in vitro* mais qui ne s'exprime pas systématiquement *in situ*. En effet, la production *in situ* de DAPG est fortement liée à la capacité des souches introduites à coloniser les racines, cette production étant constante par unité de population (0,62 ng/10^5 CFU de *Pseudomonas* producteurs de DAPG ; Raaijmakers *et al.* 1999). Or cette capacité de colonisation varie beaucoup au sein de la population des *Pseudomonas* fluorescens (Raaijmakers et Weller 2001; Landa *et al.* 2002). De plus, de nombreux facteurs de la rhizosphère, biotiques et abiotiques, influent sur la production de DAPG (Shanahan *et al.* 1992; Naseby et Lynch 1999a; Raaijmakers *et al.* 2002). La Figure 3 récapitule ainsi le potentiel d'action du DAPG à différentes concentrations testées aussi bien *in vitro* qu'*in situ.* L'impact non-cible du DAPG sur les effectifs et la structure de la communauté microbienne rhizosphérique semble donc être modéré.

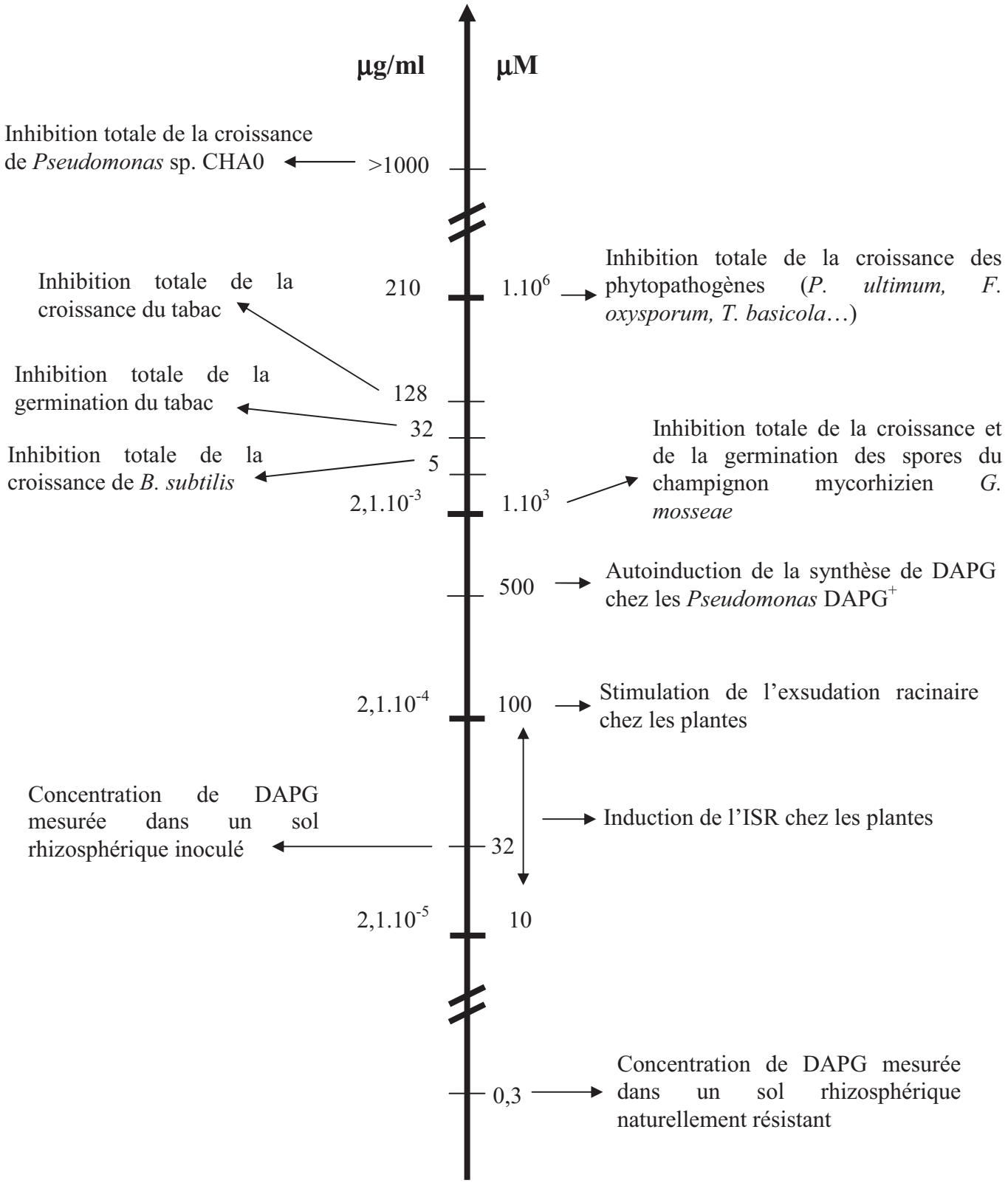
Impact sur les fonctions de la communauté microbienne rhizosphérique.

Le suivi des activités enzymatiques des microorganismes rhizosphériques peut permettre de mettre en évidence des perturbations de fonctionnement de l'écosystème rhizosphérique. Ce type de mesure a été utilisé par l'équipe de Lynch afin d'évaluer l'impact de la production de DAPG sur l'écosystème rhizosphérique (Naseby et Lynch 1998; Naseby *et al.* 1999b; Naseby et Lynch 2001). Des effets significatifs ont ainsi été observés : les activités β-glucosidase et *N*-acétyl glucosaminidase ont diminué, alors que les activités phosphodiestérase et aryl sulfatase ont augmenté. Ces enzymes sont respectivement

impliquées dans les cycles du carbone, de l'azote, du phosphate et du soufre. Les variations de ces activités enzymatiques indiquent donc un impact de la capacité de production de DAPG sur les fonctions de la communauté microbienne rhizosphérique. On peut alors proposer deux hypothèses: le DAPG pourrait avoir un impact indirect via l'exsudation racinaire (Phillips *et al.* 2004), et/ou direct sur la communauté microbienne rhizosphérique (Naseby *et al.* 1999b).

De plus, la capacité de la souche *P. fluorescens* F113 à produire du DAPG peut affecter la mise en place des symbioses mycorhizienne (Barea *et al.* 1998) et fixatrice d'azote (Andrade *et al.* 1998). Ainsi, la capacité à produire du DAPG augmente le taux de nodulation de *Rhizobium leguminosarum* bv. *viciae* de plus de quatre fois (Andrade *et al.* 1998). Si l'inoculation de cette souche productrice de DAPG ne semble pas avoir d'impact négatif sur le champignon mycorhizien *G. mossae* (l'inoculation semble même avoir un effet positif sur la colonisation mycorhizienne et le développement du mycelium), la production de DAPG par la souche surproductrice *P. fluorescens* F113(pCU203) a tendance à avoir un effet inhibiteur sur *G. mossae* (Barea *et al.* 1998). Le DAPG pourrait avoir un impact indirect sur la nodulation des plantes par *Rhizobium*, via la stimulation de la croissance et de l'exsudation racinaire (De Leij *et al.* 2002). On peut remarquer que l'impact sur la structure de l'espèce *Rhizobium leguminosarum* n'a pas affecté le fonctionnement de la symbiose *Rhizobium*-trèfle (Andrade *et al.* 1998). Mais il semble avoir un impact direct sur le champignon *G. mossae*, comme le prouvent les résultats *in vitro* (Barea *et al.* 1998).

Figure 3 : Effet du DAPG *in vitro* ou *in situ* selon la concentration.



Conclusion

Différents effets non cibles du DAPG ont été mis en évidence. *In vitro*, et selon sa concentration (Figure 3), le DAPG peut avoir des effets non-cibles très variés, positifs ou négatifs, sur la communauté microbienne rhizosphérique. Ainsi, le DAPG peut jouer le rôle de molécule signal en autoinduisant sa biosynthèse. A de fortes concentrations, il a un impact négatif sur la croissance et la germination de certaines plantes, ainsi que sur la croissance de champignons et bactéries (phytopathogènes, saprophytes ou symbiotiques). *In situ,* par contre, aucun impact significatif sur les effectifs de la communauté microbienne rhizosphérique n'a pu être montré. Par contre, la structure et les fonctions de la communauté peuvent être affectées par la production de DAPG des *Pseudomonas*, mais cet impact reste modéré comparé à celui du développement des plantes. Néanmoins, l'impact résultant du développement des plantes pourrait ainsi être considéré comme un impact indirect du DAPG sur la communauté microbienne rhizosphérique. Au niveau du végétal, le DAPG stimule la ramification et la croissance racinaire de certaines plantes cultivées en milieu non stérile, et cet impact est positif pour les plantes et pour les populations de *Pseudomonas* fluorescents producteurs de DAPG. Le DAPG pourrait donc agir directement sur le développement des plantes, et/ou par un effet indirect sur les populations de *Pseudomonas* producteur de DAPG, avec pour conséquence un effet phytoprotecteur accru envers la plante. Cet impact positif sur la physiologie de la plante est donc à considérer en plus des effets de type ISR et de l'inhibition des phytopathogènes.

Finalement, les expériences *in situ* sont importantes car de nombreuses interactions biotiques ou abiotiques peuvent influencer les souches de *Pseudomonas* fluorescents producteurs de DAPG. Cependant, les études *in situ* sont difficiles à interpréter en raison des multiples effets (positifs/négatifs) du DAPG, sur des organismes en relation étroite les uns avec les autres dans l'écosystème rhizosphérique (impacts directs/indirects). De plus, il faut souligner que les études de l'impact du DAPG restent incomplètes. Certains aspects, comme par exemple les effets du DAPG sur d'autres PGPR que les *Pseudomonas* fluorescents, restent inexplorés. Ainsi, *Azospirillum* (PGPR phytostimulateur) et *Pseudomonas* colonisent tous deux la rhizosphère dans le cadre d'une symbiose associative avec la plante ; mais on ne sait pas si la présence de *Pseudomonas* producteurs de DAPG conduit à l'exclusion d'*Azospirillum*.

II/ Azospirillum

Les bactéries du genre *Azospirillum* appartiennent à la sous-division α des Protéobactéries. La première espèce de ce genre, appelée à l'origine *Spirillum lipoferum*, a été isolée d'un sol des Pays-Bas en 1925 (Beijerinck 1925). Mais elle n'ont été redécouvertes que dans les années 1970 au cours de recherche de fixateurs libres d'azote atmosphérique au Brésil (von Bulow et Döbereiner 1975; Day et Döbereiner 1976). Elles ont depuis été isolées de la rhizosphère de graminées (surtout de céréales) dans le monde entier, tant sous des climats tropicaux que tempérés (Döbereiner et Day 1976; Patriquin *et al.* 1983), et on dénombre à l'heure actuelle 13 espèces au sein du genre *Azospirillum* (Tableau 1). Elles semblent donc ubiquistes de la rhizosphère, et sont devenues l'un des genres de PGPR les plus étudiés et les mieux caractérisés (Holguin *et al.* 1999; Steenhoudt et Vanderleyden 2000; Bashan *et al.* 2004). Nous nous intéresserons dans un premier temps aux principaux mécanismes mis en évidence chez *Azospirillum* et impliqués dans son effet phytobénéfique, puis dans un deuxième temps à l'utilisation de ces bactéries en tant qu'inocula bactériens dans un contexte agronomique.

Tableau 1 : Espèces d'*Azospirillum* identifiées

Espèces identifiées	Reférence
lipoferum	Tarrand *et al.* 1978
brasilense	Tarrand *et al.* 1978
amazonense	Magalhaes *et al.* 1983
halopraeferans	Reinhold *et al.* 1987
irakense	Khammas *et al.* 1989
largimobile	Ben Dekhil *et al.* 1997
doebereinerae	Eckert *et al.* 2001
oryzae	Xie et Yokota 2005
melinis	Peng *et al.* 2006
canadense	Mehnaz *et al.* 2007a
rugosum	Young *et al.* 2008
zea	Mehnaz *et al.* 2007b
picis	Lin *et al.* 2009

2.1. Mécanismes phytobénéfiques

Cette partie fait la synthèse des principaux mécanismes phytobénéfiques identifiés chez *Azospirillum*. Outre les effets phytostimulateurs, nous nous intéresserons aussi aux effets phytoprotecteurs d'*Azospirillum*, encore peu documentés à l'heure actuelle. L'ensemble des pricipaux mécanismes est représenté sur la Figure 1.

Fixation biologique de l'azote atmosphérique (BNF)

La fixation de l'azote atmosphérique était le premier mécanisme identifié chez *Azospirillum* comme ayant potentiellement un impact sur la croissance des plantes (Döbereiner et Day 1976). En condition de microaérobie, les bactéries du genre *Azospirillum* convertissent l'azote atmosphérique en ammoniaque grâce à l'action de la nitrogénase. Chez *Azospirillum*, ces gènes peuvent être plasmidiques ou chromosomiques (Blaha *et al.* 2005). Cependant, une grande variabilité dans la capacité à fixer l'azote a pu être observée entre différents isolats d'*Azospirillum* étudiés *in vitro*, mais aussi entre les taux de fixation mesurés *in vitro* et *in planta* (Han et New 1998). Plusieurs études d'inoculation de plantes d'intérêt agronomique se sont intéressées au rôle de la fixation d'azote atmosphérique dans la phytostimulation par *Azospirillum* (Barbieri *et al.* 1986; Rao *et al.* 1998; James 2000; Dobbelaere *et al.* 2003). Toutes ces études montrent que : (i) la fixation biologique de l'azote ne joue pas de rôle majeur dans la stimulation de la croissance des plantes (Barbieri *et al.* 1986), (ii) les niveaux d'azote transférés vers la plante sont très faibles (Rao *et al.* 1998) et (iii) les taux de fixation d'azote atmosphérique sont très variables en fonction des plantes et des conditions environnementales (Dobbelaere *et al.* 2003). Deux hypothèses peuvent être formulées pour expliquer ces taux de fixation variables et faibles (par rapport aux taux de fixation mesurés chez les symbioses fixatrices d'azote ayant lieu dans les nodosités ; Mylona *et al.* 1995) : (i) les conditions de microaérobie rencontrées dans la rhizosphère ne seraient pas suffisantes pour un fonctionnement optimal de la nitrogénase, qui est inhibée en présence d'oxygène et (ii) les plantes hôtes ne libèreraient pas suffisamment de carbone dans la rhizosphère pour permettre un développement de symbioses associatives efficaces en terme de fixation d'azote (Wood *et al.* 2001).

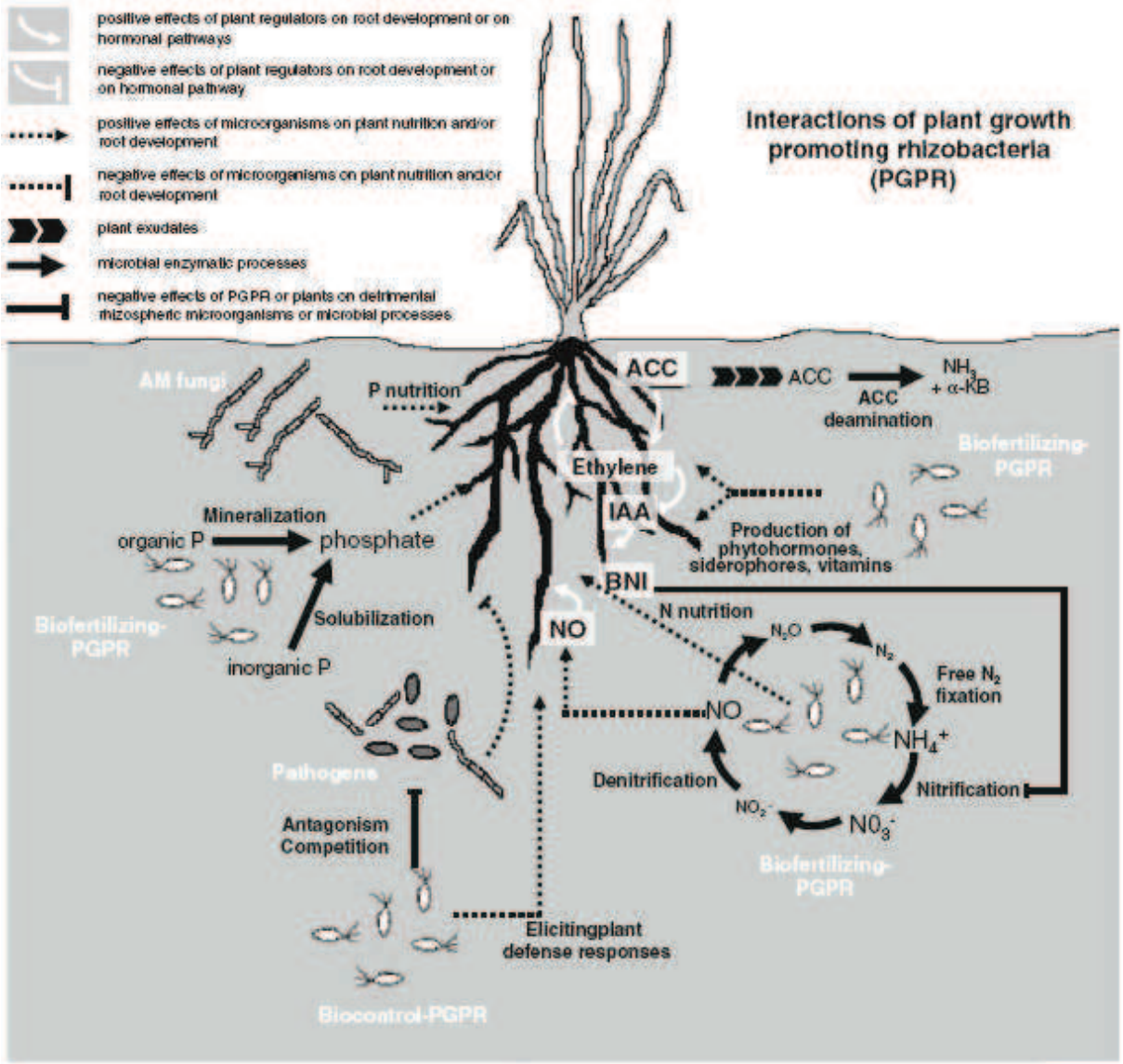


Figure 1 : Principaux mécanismes phytobénéfiques identifiés chez *Azospirillum* (Richardson *et al.* 2009).

Production de phytohormones

La synthèse d'hormones de plantes, de polyamines et d'acides aminés identifiés comme des phytohormones a été démontrée *in vitro* sur des cultures d'*Azospirillum* (Thuler *et al.* 2003). Ces phytohormones sont des substances organiques connues pour influencer les processus physiologiques des plantes à très faibles concentrations. Elles sont chimiquement identiques ou analogues aux hormones synthétisées par les plantes (auxines, cytokinines et gibbérellines). Parmi ces substances, nous nous intéresserons plus particulièrement aux auxines qui semblent jouer un rôle majeur dans la phytostimulation.

Parmi les auxines, l'acide indole-3-acétique (AIA) est la plus répandue (on estime que 80 % des bactéries isolées de la rhizosphère sont capables de la synthétiser ; Patten et Glick 2002) et la mieux caractérisée des auxines connues. Elle est connue pour induire une stimulation de l'élongation racinaire, de la division et de la différentiation cellulaire (Aloni *et al.* 2006; Fukaki *et al.* 2007). Chez les bactéries et les plantes, la conversion du tryptophan (Trp) en AIA est effectuée via l'une des trois voies identifiées, à savoir (i) la voie de l'indole-3-acétamide, (ii) la voie de l'acide indole-3-pyruvique et (iii) la voie de la tryptamine. Chez *Azospirillum*, la biosynthèse de l'AIA se fait principalement par la voie de l'acide indole-3-pyruvique (IPyA ; Spaepen *et al.* 2007). L'implication de la biosynthèse d'AIA par *Azospirillum* dans la phytostimulation a été confirmée par l'utilisation de mutants qui ne produisait presque plus d'auxine (Barbieri et Galli 1993; Dobbelaere *et al.* 1999) ou qui en surproduisait (Harari *et al.* 1988).

La biosynthèse d'autres phytohormones telles que les cytokinines, les gibbérellines, l'acide abscissique (ABA) et l'éthylène a aussi été rapportée chez *Azospirillum* (Perrig *et al.* 2007), mais les gènes impliqués sont peu caractérisés et aucune mise en évidence directe du rôle de la production de ces composés dans la phytostimulation n'a encore pu être faite. Il a aussi récemment été mis en évidence qu'une production d'oxyde nitrique (NO, molécule signal régulatrice de la croissance et du développement des plantes) était responsable de la formation de racines latérales lors de l'association d'*Azospirillum* avec la tomate (Creus *et al.* 2005).

Synthèse d'enzymes modulant le développement des plantes

L'éthylène est une phytohormone gazeuse jouant plusieurs rôles tels que la stimulation de la formation des racines adventives et des poils absorbants, la levée de la dormance des graines, ou le contrôle de certains mécanismes systémiques de défense vis-à-vis de phytopathogènes. A fortes concentrations, l'éthylène inhibe l'élongation racinaire. Il a ainsi été proposé

qu'*Azospirillum* puisse stimuler la croissance des plantes en diminuant les taux d'éthylène via l'activité 1-aminocyclopropane-1-carboxylate (ACC) désaminase. Cette enzyme hydrolyse l'ACC qui est le précurseur immédiat de l'éthylène chez les plantes, diminuant ainsi la synthèse d'éthylène et stimulant donc indirectement l'élongation racinaire (Glick *et al.* 1998; Glick 2005). Ce modèle a été validé chez *Azospirillum* (Holguin et Glick 2003), et le gène *acdS* codant pour l'enzyme ACC désaminase a été caractérisée chez plusieurs souches d'*Azospirillum* (Blaha *et al.* 2006; Prigent-Combaret *et al.* 2008).

Effets phytoprotecteurs

Azospirillum n'est généralement pas reconnue comme une PGPR phytoprotectrice au sens strict, car elle ne semble pas produire de composés antimicrobiens qui pourraient être impliqués dans l'inhibition des phytopathogènes par antagonisme. Cependant, plusieurs observations ont montré que certaines infections occasionnées par des phytopathogènes se trouvaient réduites par l'inoculation d'*Azospirillum*. Ainsi l'inoculation des plantes avec *Azospirillum* (en inoculation simple ou en inocula mixtes) est impliquée dans la réduction (i) des populations de nématodes parasites tels que *Meloidogyme incognita* (Ramakrishnan *et al.* 1997; Ismail et Hasabo 2000; Khan et Kounsar 2000), *Pratylenchus zeae* (Babu *et al.* 1998), et *Heterodera avenae* (Bansal *et al.* 1999), (ii) des symptômes de la maladie causée par la mouche *Atherigona soccata* (Kishore 1998a; Kishore 1998b), (iii) des symptômes de la maladie causée par les champignons phytopathogènes *Sclerospora graminicola* (Gupta et Singh 1999) et *Pythium aphanidermatum* (Kavitha *et al.* 2003). D'autres méthodes d'application ont aussi fait leurs preuves, avec notamment l'application d'*Azospirillum* en aérosols foliaires qui a permis l'inhibition partielle de champignons phytopathogènes (*Phyllactania corylea*, *Pseudocercospora mori* et *Cerotelium fici*) et l'inhibition totale de la bactérie phytopathogène *Pseudomonas mori* (Sudhakar *et al.* 2000a; Sudhakar *et al.* 2000b). La combinaison de méthodes chimiques (telles que l'épandage de cuivre et l'utilisation de pesticides) avec l'inoculation d'*Azospirillum* a permis de réduire significativement les symptômes de la maladie causée par *Pseudomonas syringae* pv. *tomato* (PST ; Bashan et de-Bashan 2002a). *Azospirillum* inhibe aussi la germination de la plante parasite *Striga* (*Striga hermonthica* ; Bouillant *et al.* 1997), et cette inhibition est médiée par des composés lipophiles relargués dans le milieu de culture (Miché *et al.* 2000).

Des tests *in vitro* ont aussi démontré la capacité d'*Azospirillum* à inhiber plusieurs phytopathogènes tels que *Fusarium oxysporum* f. sp. *lycopersici*, *Rhizoctonia solani*, *Sclerotinia* et *Pythium* sp. (Hassouna *et al.* 1998). Les mécanismes à l'origine de ces

inhibitions n'ont pas encore été identifiés, mais des expériences de co-inoculation d'*Azospirillum* avec le phytopathogène PST montrent que des mécanismes tels que la compétition trophique et / ou spatiale pourraient être impliqués (Bashan et de-Bashan 2002a). La synthèse de bactériocines, de sidérophores avec des activités antimicrobiennes, et d'acide cyanhydrique (HCN) a aussi été mise en évidence *in vitro* chez plusieurs souches d'*Azospirillum* (Saxena *et al.* 1986; Tapia-Hernández *et al.* 1990; Shah *et al.* 1992; Gonçalves et de Oliveira 1998). Ces molécules pourraient être impliquées dans des interactions compétitives et / ou antagonistes avec les phytopathogènes. A l'heure actuelle, l'induction d'une résistance systémique (ISR) chez la plante n'a pas été démontrée chez *Azospirillum* (Bashan et de-Bashan 2002b).

2.2. Inoculation dans un contexte agronomique

L'inoculation d'*Azospirillum* dans un contexte agronomique a fait l'objet d'études depuis l'isolement de cette bactérie afin de caractériser les conditions favorables à l'expression de son potentiel phytostimulateur. L'une des conditions principales identifiée est la survie de l'inoculum dans la rhizosphère afin qu'une colonisation racinaire efficace par *Azospirillum* puisse avoir lieu (Dobbelaere *et al.* 2002). En effet, une colonisation inappropriée résulte généralement en une phytostimulation marginale voire inexistante (Hecht-Buchholz 1998; Benizri *et al.* 2001). Cette partie fait donc dans un premier temps la synthèse des connaissances actuelles sur la colonisation racinaire par *Azospirillum*, avant d'aborder les applications agronomiques de l'inoculation des plantes par *Azospirillum*.

Colonisation racinaire

La colonisation racinaire par *Azospirillum* est un prérequis à l'expression de son potentiel de phytostimulation. Le processus de colonisation peut être décomposé en plusieurs étapes identifiées comme déterminantes (migration vers la racine, adhésion, croissance et survie).

Migration vers la racine

La première étape clé du processus de colonisation racinaire par *Azospirillum* repose sur sa capacité à se déplacer vers la racine. Cette capacité va dépendre à la fois de sa mobilité, mais aussi de sa perception de la plante, via les composés relargués par cette dernière. La mobilité est une des caractéristiques taxonomiques principales d'*Azospirillum* (Tarrand *et al.* 1978). En

effet, toutes les espèces semblent avoir des mégaplasmides et le séquençage du p90 propre à l'espèce *brasilense* a permis de mettre en évidence la présence de trois loci impliqués dans la mobilité, et notamment dans la synthèse des flagelles polaires et latéraux (Vande Broek et Vanderleyden 1995).

Des gènes homologues aux gènes *nod* (nodulation des plantes par *Rhizobium*) et aux gènes *chv* (attachement d'*Agrobacterium* aux racines et virulence) ont été identifiés sur le p90. La protéine codée par le gène similaire à *chv* chez *A. tumefaciens* a d'ailleurs pu être caractérisée comme étant impliquée dans le chimiotactisme d'*Azospirillum* vers différents sucres exsudés par les plantes (van Domelen *et al.* 1997) et sa synthèse est induite par des exsudats de blé (van Bastelaere *et al.* 1999). Le chimiotactisme permet donc à *Azospirillum* de répondre au gradient de métabolites exsudés par les racines des plantes, mais les bactéries du genre *Azospirillum* sont aussi capables de répondre à un gradient d'oxygène dissout (aérotactisme). Cette propriété permet à *Azospirillum* de se déplacer vers les concentrations en oxygène qui lui sont le plus favorable, c'est-à-dire les conditions de microaérobies qui sont favorables à la fixation d'azote (Zhulin *et al.* 1996). La combinaison de la mobilité associée aux capacités de chimiotactisme et aérotactisme permet donc à *Azospirillum* de se déplacer vers la racine.

Adhésion aux racines

L'adhésion d'*Azospirillum* aux racines est un processus en deux étapes (Michiels *et al.* 1991). La première étape est la phase d'adsorption, qui correspond à un attachement faible, réversible, et non spécifique des bactéries aux racines. Cette étape implique des protéines de surface, principalement des polysaccharides capsulaires, et le flagelle polaire (Croes *et al.* 1993). La deuxième étape est la phase d'ancrage, qui semble correspondre à un attachement fort et irréversible aux racines. Cette étape implique des fibrilles extracellulaires qui emprisonnent les bactéries au sein d'un biofilm. La production de polysaccharides extracellulaires et d'une protéine membranaire (MOMP), tous deux impliqués dans le processus de floculation chez *Azospirillum*, pourraient aussi jouer un rôle dans le processus d'adhésion (Rodríguez-Navarro *et al.* 2007). L'adhésion aux racines des plantes permet ainsi à *Azospirillum* d'avoir un meilleur accès aux exsudats racinaires, mais cette adhésion est aussi bénéfique pour la plante en lui facilitant l'accès aux composés excrétés par les bactéries.

Azospirillum colonise majoritairement le rhizoplan, c'est-à-dire la surface racinaire, et seules quelques souches d'*A. lipoferum* et *A. brasilense* (Döbereiner *et al.* 1995) sont capables de pénétrer les tissus racinaires par l'intermédiaire de blessures ou de crevasses du cortex

(Elbertagy *et al.* 2001). Les zones pilifères sont les sites préférentiellement colonisés par *Azospirillum* (Pastorelli *et al.* 1995) et les patrons de colonisation racinaire semblent être souche et / ou plante-spécifiques. Ainsi, les cellules d'*A. irakense* sont préférentiellement localisées vers les zones pilifères du riz (Zhu *et al.* 2002), tandis que les cellules d'*A. brasilense* ont aussi pu être observées dans les zones d'élongation racinaire et d'émergence des poils absorbants des racines de blé (Assmus *et al.* 1995; Ramos *et al.* 2002). Cette spécificité dans la colonisation racinaire peut être expliquée par des variations dans le panel de composés impliqués dans le chimiotactisme chez *Azospirillum*, ce qui pourrait refléter une adaptation des bactéries aux conditions nutritives établies par chaque plante-hôte.

Etablissement et survie dans la rhizosphère

Azospirillum est retrouvée à de fortes densités dans la rhizosphère indépendamment du type et de l'origine des sols, mais semble survivre plus difficilement dans des sols non rhizosphériques (Bashan *et al.* 1995; Bashan 1999). *Azospirillum* a des capacités métaboliques particulières susceptibles de favoriser sa survie dans la rhizosphère, telles que le catabolisme des rhizopines (composés dont la synthèse est induite lors de l'infection des plantes par *A. tumefaciens* ; Gardener et de Bruijn 1998) et l'activité pectinolytique (Khammas et Kaiser 1991), lui ouvrant l'accès à de nouvelles sources de carbone. *Azospirillum* présente aussi des caractéristiques physiologiques particulières, comme la formation de cystes, la production de mélanine et de poly-hydroxy-butyrate (PHB), qui peuvent lui permettre de survivre à des conditions défavorables (Sadasivan et Neyra 1985; Sadasivan et Neyra 1987; Kadouri *et al.* 2002). En outre, le mécanisme de quorun sensing a pu être mis en évidence chez certaines souches de *lipoferum* (Vial *et al.* 2006a) et pourraient permettre ainsi à ces souches d'interagir entre elles et de coordonner l'expression de leurs propriétés phytobénéfiques en fonction de leur densité cellulaire. La variation de phase a aussi pu être identifiée chez certaines souches d'*Azospirillum* (Vial *et al.* 2006b), et pourrait représenter un avantage certain lors de la colonisation racinaire (Wisniewski-Dyé et Vial 2008).

Applications agronomiques

Azospirillum fait l'objet d'un intérêt croissant en recherche appliquée depuis le début des années 1970 (Bashan et Levanony 1990). Dans certains cas, l'inoculation de plantes d'intérêt agronomique par *Azospirillum* a ainsi amélioré les rendements (Charyulu *et al.* 1985; Okon et Labandera-Gonzalez 1994; Dobbelaere *et al.* 2001; Pedraza *et al.* 2009). Les chefs de file en

termes d'applications d'inocula d'*Azospirillum* sur des cultures céréalières, sont le Mexique, avec plus de 300 000 ha inoculés en 2007, et l'Argentine avec 200 000 ha de blé et de maïs inoculés avec *Azospirillum* en 2008 (Hartmann et Bashan 2009).

Dans cette partie, nous nous intéresserons plus spécialement aux facteurs pouvant avoir un impact sur l'établissement des inocula d'*Azospirillum*, avant de faire la synthèse des connaissances actuelles quant à la formulation des inocula d'*Azospirillum* et l'impact des inoculations sur les plantes.

Facteurs modulant l'inoculation d'*Azospirillum*

L'application pratique d'*Azospirillum* en tant qu'inocula bactériens a été quelque peu controversée à ses débuts, en raison de l'inconstance de la réponse des plantes, qui dépend de facteurs écologiques, agronomiques et techniques. De grandes avancées ont cependant été faites sur ces derniers points, ce qui a permis d'identifier quelques facteurs principaux affectant la colonisation racinaire par *Azospirillum*. Ainsi, l'acidité des sols, les hautes températures, des précipitations faibles ou de grandes variabilités climatiques contribuent à une mauvaise colonisation (Klein *et al.* 1990; Parke 1991; Okon et Labandera-Gonzalez 1994; Dobbelaere *et al.* 2001). Il a aussi été rapporté que l'effet phytostimulateur d'*Azospirillum* ne pouvait s'exprimer que dans des conditions limitantes en azote (Fallik et Okon 1996; Dobbelaere *et al.* 2001). L'effet phytostimulateur a cependant été plus souvent observé après addition d'engrais (Okon et Labandera-Gonzalez 1994). Dans certains cas, il a aussi été observé qu'une augmentation maximale de rendement est plus souvent obtenue par l'inoculation de souches d'*Azospirillum* indigènes à la rhizosphère de la plante inoculée (Fages et Arsac 1991).

Formulation des inocula d'*Azospirillum*

Un inoculum bactérien consiste en une formulation contenant une ou plusieurs espèces bactériennes dans un matériau de transport économique et surtout pratique d'utilisation pour l'agriculteur (Bashan 1998a). L'inoculum est donc le moyen de transport des bactéries jusqu'à la plante. Chez *Azospirillum*, la méthode d'inoculation la plus simple est l'application de la bactérie en suspension liquide directement dans le sol ou sur la graine. Cette technique est la plus couramment utilisée au cours des expériences en serre ou en laboratoire, mais n'est cependant pas appropriée à la survie d'*Azospirillum* dans le sol (Bashan et Levanony 1990). Les meilleurs résultats ont été obtenus avec une formulation à base de tourbe appliquée au moment de l'ensemencement (Okon et Hadar 1987). Une autre approche a aussi été développée par encapsulation dans des billes d'alginate, ce qui permet d'obtenir une

population homogène ainsi qu'une libération progressive des bactéries dans le sol (Bashan 1986).

La concentration de l'inoculum est un facteur crucial dans l'établissement d'*Azospirillum* et dans l'expression de son potentiel phytostimulateur (Dobbelaere *et al.* 2002). La concentration optimale pour l'inoculation de graines ou de semis pour la plupart des céréales et autres plantes cultivées oscille entre 10^5 et 10^7 CFU / ml, tandis que des concentrations plus élevées (10^8 – 10^{10} CFU / ml) inhibent généralement la croissance racinaire (Bashan et Levanony 1990). Ces concentrations d'inocula ne reflètent cependant pas la quantité de bactéries par graines nécessaires à l'expression du potentiel phytobénéfique d'*Azospirillum*. Il a aussi été montré que l'interaction précoce entre *Azospirillum* et les graines était primordiale à la phytostimulation (Jacoud *et al.* 1999).

Impacts de l'inoculation d'*Azospirillum* sur les plantes

L'inoculation des plantes par *Azospirillum* peut amener des changements significatifs de plusieurs paramètres morphologiques des plantes (Fig 2), pouvant avoir un impact sur le rendement. La plupart des études ont été réalisées sur des céréales et autres graminées, et ont mis en évidence des augmentations significatives des paramètres suivants : masse sèche totale, concentration en azote, nombre d'épis, graines par épis, hauteur des plantes, taille des feuilles, et taux de germination (Bashan et Levanony 1990). Les effets les plus marqués de l'inoculation par *Azospirillum* sont néanmoins observés au niveau du système racinaire, et notamment au niveau la longueur racinaire, du nombre de racines latérales, de la masse sèche, du nombre et de la densité des poils absorbants et de la surface racinaire (Bashan et Levanony 1990).

Cette stimulation de la croissance du système racinaire peut faciliter l'accès aux éléments nutritifs nécessaire à la plante (Okon et Kapulnik 1986; Jacoud *et al.* 1999). Ainsi, l'inoculation par *Azospirillum* pourrait être envisagée non pas dans un contexte d'amélioration de rendement, mais plutôt dans l'objectif de réduire les doses actuellement utilisées en engrais azotés, sans diminuer le rendement actuel (El Zemrany *et al.* 2006; Fuentes-Ramirez et Caballero-Mellado 2006; Adesemoye *et al.* 2009).

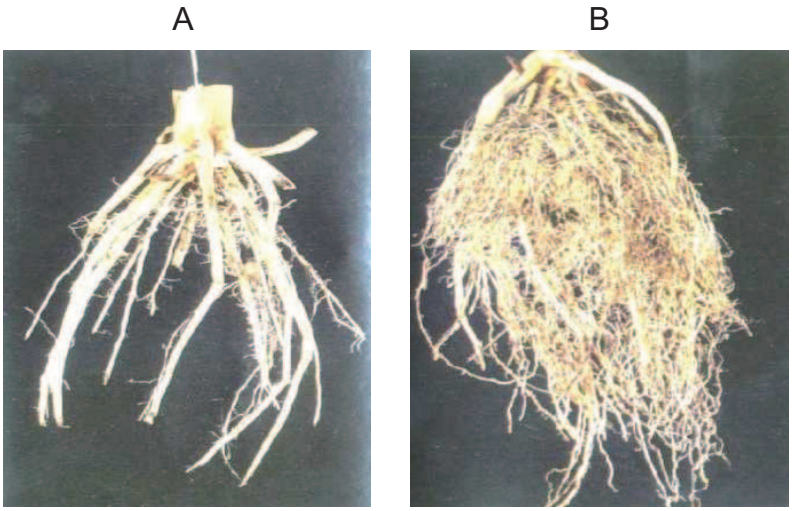


Figure 2 : Racines de maïs non inoculé (A) et inoculé par Azo-Green (B), inoculum d'*Azospirillum lipoferum* CRT1 anciennement commercialisé par Merck/Lipha (Meyzieu, France)

III/ Compatibilité des deux types de PGPR avec d'autres microorganismes rhizosphériques phytobénéfiques

La rhizosphère est un écosystème fortement colonisé par les microorganismes. On y dénombre entre 10^8 et 10^9 bactéries par gramme de sol, soient des effectifs jusqu'à 1.10^4 fois plus élevés que ceux dans un sol non rhizosphérique (Molina *et al.* 2000). Cet effet « Rhizosphère » correspond à un recrutement de communautés microbiennes telluriques par la plante (Smalla *et al.* 2001; Somers et Vanderleyden 2004; Haichar *et al.* 2008). Nous nous sommes intéressés dans un premier temps au rôle de médiateur que la plante pourrait jouer dans la compatibilité entre microorganismes phytobénéfiques, puis dans un deuxième temps aux interactions établies par les PGPR *Azospirillum* et / ou *Pseudomonas* avec les autres microorganismes phytobénéfiques.

3.1. Médiation de la compatibilité par la plante

Signalisation cellulaire

Azospirillum et *Pseudomonas* font sans doute partie des PGPR les mieux documentés en ce qui concerne leurs mécanismes d'interaction avec la plante (Vande Broek et Vanderleyden 1995; Holguin *et al.* 1999; Steenhoudt et Vanderleyden 2000; Lugtenberg *et al.* 2001; Somers et Vanderleyden 2004; Mercado-Blanco et Bakker 2007; Weller 2007). Cependant, les microorganismes rhizosphériques les plus documentés en ce qui concerne leurs mécanismes d'interaction avec la plante sont ceux qui établissent des symbioses, et comprennent entre autres *Rhizobium* et les champignons mycorhiziens (Schultze *et al.* 1994; van Rhijn et Vanderleyden 1995; Rillig 2004; Samac et Graham 2007; Martin *et al.* 2008). L'étude de ces interactions symbiotiques a mis en évidence l'importance de la communication par signaux chimiques entre les partenaires microbiens et leur plante-hôte (Somers et Vanderleyden 2004). Dans le cas de la signalisation entre les fabacées et *Rhizobium*, les racines sécrètent des flavonoïdes, qui vont induire la production de facteurs Nod chez *Rhizobium*, déclenchant en retour la formation de nodules sur les racines (Phillips *et al.* 1991). Mais ces flavonoïdes peuvent aussi agir en tant que molécules signal pour d'autres microorganismes rhizosphériques (Phillips et Tsai 1992) et des gènes homologues aux gènes *nod* de *R. meliloti* on été identifiés sur le plasmide p90, identifié chez la plupart des souches des espèces *A. brasilense* et *A. lipoferum* (Vieille et Elmerich 1992; Holguin *et al.* 1999). Ces résultats nous permettent donc de formuler l'hypothèse selon laquelle les plantes pourraient recruter une communauté microbienne spécifique avec les mêmes signaux moléculaires. La plante pourrait

donc médier la compatibilité entre microorganismes phytobénéfiques via un recrutement spécifique au niveau de la rhizosphère.

En outre, plusieurs mécanismes phytobénéfiques identifiés chez les PGPR *Azospirillum* et *Pseudomonas* sont régulés par quorum sensing (Venturi 2006; Vial *et al.* 2006a) ; et les plantes sont capables de produire et de sécréter des molécules interférant avec les voies de signalisation du quorum sensing (Teplitski *et al.* 2000; Gao *et al.* 2003; Bauer et Mathesius 2004). Les plantes pourraient ainsi médier la compatibilité entre microorganismes phytobénéfiques rhizosphériques en interférant avec leurs voies de régulation.

Sélection trophique

Dans la rhizosphère, la plante fournit des nutriments aux microorganismes sous la forme d'exsudats racinaires, de lysats, de mucus, et de cellules mortes (Lynch et Whipps 1990). La quantité de composés carbonés relâchée par ce processus de rhizodéposition pourrait ainsi avoisiner 40 à 50 % des photosynthétats produits quotidiennement par une plante (Lynch et Whipps 1990; Bottner *et al.* 1999). En stimulant ainsi la croissance des microorganismes rhizosphériques, la plante va pouvoir façonner la communauté microbienne colonisant ses racines (Haichar *et al.* 2008). La composition des exsudats racinaires variant en fonction de l'âge des plantes, la composition des communautés microbiennes stimulées au sein des biofilms est donc susceptible d'évoluer au cours du temps (Vancura 1980; Lugtenberg *et al.* 1999). En outre, les cellules périphériques de la coiffe, qui sont programmées pour se détacher de la périphérie des racines et être ainsi relâchées dans le milieu extérieur, pourrait aussi fournir à la plante un moyen de contrôler la dynamique des populations microbiennes rhizosphériques (Hawes *et al.* 1998). En fait, la colonisation racinaire peut être considérée comme un processus d'enrichissement en microorganismes les mieux adaptés à cette niche particulière (Del Gallo et Fendrik 1994). L'ensemble de ces observations montre donc que (i) la plante pourrait sélectionner les populations microbiennes colonisant ses racines et (ii) cette sélection évolue au cours de la croissance de la plante. Au sein de la rhizosphère, cette pression de sélection exercée par la plante est donc susceptible de moduler la compatibilité entre PGPR et avec les autres microorganismes phytobénéfiques.

Biofilms racinaires

La rhizosphere offre un environnement qui a tous les pré-requis à la formation de biofilms : des effectifs microbiens importants, une humidité suffisante et un apport de nutriments fournis par les plantes. La croissance bactérienne au sein de biofilms racinaires offre de multiples avantages tels que : (i) une résistance accrue aux stress environnementaux, (ii) une

tolérance accrue aux composés antimicrobiens, (iii) une protection supplémentaire contre la prédation par les protozoaires, (iv) la possibilité d'établir des consortia métaboliques, et (v) une probabilité accrue d'effectuer des transferts horizontaux de gènes (Danhorn et Fuqua 2007). Cet environnement complexe et dynamique (Ramey *et al.* 2004) pourrait favoriser la compatibilité de différentes populations microbiennes phytobénéfiques colonisant la même racine. De plus, les cellules au sein de biofilms sont physiologiquement différentes des cellules dispersées observées en culture liquides (Whiteley *et al.* 2001; Sauer *et al.* 2002) et le mécanisme de la variation de phase peut être impliqué (Wisniewski-Dyé et Vial 2008). Les biofilms sont aussi des sites préférentiels pour l'établissement de mécanismes de communication cellulaires tels que le quorum sensing, qui régule la colonisation racinaire ainsi que l'expression de certaines gènes phytobénéfiques (Fuqua et Greenberg 2002; Vittorio 2006; Wei et Zhang 2006). Ce mécanisme de communication cellulaire est très répandu chez les bactéries rhizosphériques (Elasri *et al.* 2001; Vial *et al.* 2006a), et certaines bactéries rhizosphériques sont aussi capables de dégrader les molécules signal du quorum sensing (Wang et Leadbetter 2005). L'ensemble de ces mécanismes de régulation et de communication pourrait donc favoriser une compatibilité au sein de la communauté fonctionnelle des microorganismes phytobénéfiques colonisant une même plante-hôte.

3.2. Interactions entre PGPR et les autres microorganismes phytobénéfiques

La majorité des études sur les interactions entre PGPR et les autres microorganismes rhizosphériques phytobénéfiques a été réalisée dans l'objectif d'identifier des interactions synergiques au niveau de leurs mécanismes phytobénéfiques respectifs. Ces recherches ont donc principalement concerné les interactions établies par les PGPR avec les microorganismes symbiotiques tels que *Rhizobium* et les champignons mycorhiziens, au sein d'inocula mixtes.

Interactions entre PGPR et *Rhizobium*

La fixation symbiotique de l'azote atmosphérique chez les fabacées est assurée par les bactéries symbiotiques du genre *Rhizobium* au sein des nodosités. L'étude des interactions entre les PGPR et ces bactéries symbiotiques pourrait permettre d'identifier des mécanismes

synergiques afin de stimuler la fixation d'azote atmosphérique au niveau de la rhizosphère des fabacées.

Ainsi la co-inoculation d'*Azospirillum* avec *Rhizobium* a conduit à une stimulation de la nodulation et du rendement de plusieurs types de fabacées tels que : (i) le soja (Singh et Subba Rao 1979; Iruthayathas *et al.* 1983), (ii) le pois (Iruthayathas *et al.* 1983; Sarig *et al.* 1986), et (iii) plusieurs fabacées fourragères (Sarig *et al.* 1986; Yahalom *et al.* 1987). Cependant, des effets négatifs de l'inoculation d'*Azospirillum* ont été observés sur la nodulation du trèfle par différentes souches de *R. leguminosarum* bv. *trifolii*, en conditions *in vitro* (Plazinski et Rolfe 1985a). L'inhibition observée dans cette étude semble être liée d'une part au ratio des effectifs d'*Azospirillum* et de *Rhizobium* inoculés (1:2000) et d'autre part au moment de l'inoculation d'*Azospirillum* (24h avant ou après *Rhizobium*). Une étude complémentaire (Plazinski et Rolfe 1985b) a permis de montrer qu'*Azospirillum* pouvait bloquer certaines souches de *Rhizobium* au niveau de la première étape de la nodulation (déformation des poils absorbants). Dans le cas d'interactions positives entre *Azospirillum* et *Rhizobium* avec une stimulation de la nodulation, une augmentation significative du nombre et de la longueur des poils absorbants a été observée, suggérant ainsi qu'*Azospirillum* pouvait créer de nouveaux sites potentiels d'infection colonisés ensuite par *Rhizobium*. Cette hypothèse a été étayée par l'observation de cellules d'*Azospirillum* localisées à proximité de nodules sur le trèfle après co-inoculation avec *Rhizobium* (Tchebotar *et al.* 1998).

Au cours d'expériences de co-inoculation de *P. fluorescens* avec *R. leguminosarum*, une stimulation de la germination, de la nodulation, de la fixation d'azote atmosphérique et de la masse racinaire a été observée sur les lentilles (*Lens culinaris*) mais pas sur le pois (*Pisum sativum*) (Chanway *et al.* 1989; Andrade *et al.* 1998; De Leij *et al.* 2002). Néanmoins, la co-inoculation de *Pseudomonas* et *Rhizobium* sur du pois, cultivé sur un sol infecté par le phytopathogène *F. oxysporum,* a montré une réduction des plantes infectées ainsi qu'une stimulation de la croissance via augmentation de la taille des parties aériennes et de la masse sèche totale (Kumar *et al.* 2001). Les souches de *Pseudomonas* utilisées dans cette étude avaient des propriétés antifongiques et la capacité à produire des sidérophores. La stimulation de la croissance du pois a donc été étudiée par la suite en co-inoculant *Rhizobium* avec une souche de *Pseudomonas* productrice de 2,4-diacétylphloroglucinol (DAPG) ou son mutant $DAPG^-$ (Andrade *et al.* 1998; De Leij *et al.* 2002). Les fortes concentrations en DAPG ont été détectées au niveau de la rhizosphère du pois, et corrélées à des taux de nodulation plus de deux fois supérieurs à ceux observés avec *Rhizobium* en inoculation simple. Ces résultats suggèrent que le DAPG pourrait agir en tant qu'hormone, induisant des changements

morphologiques et physiologiques chez la plante qui peuvent conduire à une augmentation des taux de nodulation par *Rhizobium*. D'autres expériences de co-inoculations sur du soja (*Glycine max*) ont mis en évidence un effet souche-dépendant dans les interactions positives entre *Pseudomonas* et *Bradyrhizobium* (Polonenko *et al.* 1987; Chebotar *et al.* 2001). La co-inoculation de souches de *Pseudomonas* antogonistes de champignons phytopathogènes avec *Mesorhizobium* sur le pois chiche (*Cicer arietinum*) a montré une forte stimulation de la nodulation (de plus de 100% par rapport à *Mesorhizobium* en inoculation simple) et de la croissance (Goel *et al.* 2002).

Enfin, des expériences de co-inoculations impliquant à la fois *Rhizobium*, *Pseudomonas* et une autre PGPR du genre *Bacillus*, ont montré l'efficacité d'un tel consortium sur la nodulation et le rendement du haricot urd (*Vigna mungo ;* Prasad et Chandra 2003). Une autre étude sur des inoculations mixtes (impliquant trois PGPR différentes, dont un *Pseudomonas*, avec *Sinorhizobium*) sur du soja, a montré l'importance du mode d'inoculation dans l'application de consortia complexes (Lucas García *et al.* 2004). En effet, la co-inoculation n'a eu aucun impact, alors que des rendements supérieurs ont pu être obtenus avec des inoculations différées (les PGPRs ou *Sinorhizobium* en premier). Des inoculations différées pourraient ainsi permettre d'éviter la compétition entre *Pseudomonas* et *Sinorhizobium* mise en évidence sur la luzerne (Villacieros *et al.* 2003).

L'ensemble des études présentées ici soutient l'hypothèse formulée par De Leij et collaborateurs en 2002 pour l'interaction *Pseudomonas-Rhizobium*, mais généralisable à l'ensemble des PGPR, selon laquelle l'interaction entre les PGPR et *Rhizobium* est densité-dépendante. Ainsi, à des concentrations relativement faibles de *Rhizobium*, le nombre de site d'infection n'est pas limitant et la co-inoculation avec une PGPR n'amènera donc vraisemblablement pas d'augmentation de rendement. Cependant, à de fortes concentrations en *Rhizobium*, le nombre de sites d'infections devient limitant et la co-inoculation avec une PGPR va créer de nouveaux sites d'infection pour *Rhizobium*, amenant ainsi une augmentation du rendement.

Interaction entre PGPR et champignons mycorhiziens

Les champignons mycorhiziens, et plus spécialement les champignons mycorhiziens à arbuscules (AMF) appartenant au phylum des Gloméromycètes, sont ubiquistes des écosystèmes terrestres et donc de la rhizosphère (Rillig 2004). Il est reconnu que le développement des symbioses mycorhiziennes a un impact sur la composition de la communauté microbienne rhizosphérique d'une plante-hôte (Bonfante et Anca 2009). Dans le

cas des PGPR, il a été montré que ces dernières stimulent le fonctionnement de cette symbiose et certaines ont d'ailleurs été identifiées comme des *Mycorrhiza helper bacteria* (ou bactéries auxiliaires de la mycorhization) (Frey-Klett *et al.* 1997; Hodge 2000; Frey-Klett et Garbaye 2005). Les PGPR ont été observées associées aux spores et aux hyphes des champignons mycorhiziens, ce qui pourrait favoriser leur dispersion dans la rhizosphère (Bianciotto et Bonfante 2002). Ainsi, il a été montré que des racines de tomates mycorhizées par *Glomus* avaient le potentiel de recruter des effectifs plus importants de *Pseudomonas* indigènes par rapport à des racines non mycorhizées (Sood 2003). Ce recrutement s'effectue par chimiotropisme : la mycorhization des racines entraîne une modification de la composition des exsudats racinaires, dont certains composés sont des chimioattractants spécifiques pour *Pseudomonas*. Cependant, la comparaison de différentes souches de *Pseudomonas* inoculées sur du blé a montré une inhibition de la colonisation par les mycorhizes indigènes, associée à une grande variabilité dans la réponse de la plante, en fonction de la souche et des paramètres morphologiques évalués (Germida et Walley 1996).

Des expériences de co-inoculation avec *Azospirillum* ou *Pseudomonas*, et des AMF ont montré une stimulation de la colonisation de la tomate par le champignon mycorhizien (Linderman et Paulitz 1990; Barea *et al.* 1998; Pulido *et al.* 2003), et ce indépendamment de la capacité à produire du DAPG (Barea *et al.* 1998). Cependant d'autres expériences ont montré que la colonisation mycorhizienne du blé et du maïs n'était pas affectée par la co-inoculation avec différentes souches d'*Azospirillum* (Russo *et al.* 2005). Les mêmes observations contradictoires ont été effectuées lors d'expériences de co-inoculations de PGPRs avec des champignons ectomycorhiziens (ECM). Ainsi, la co-inoculation de *Pseudomonas* avec le champignon ectomycorhizien *Laccaria bicolor* sur des pins Douglas-fir a montré des taux de mycorhization stimulés entre 45 et 77% en fonction des effectifs inoculés (Frey-Klett *et al.* 1999). A contrario, une autre étude a montré que la co-inoculation de *Pseudomonas* avec *Laccaria bicolor* sur de l'eucalyptus amenait une inhibition de la colonisation mycorhizienne (Dunstan *et al.* 1998).

En conclusion, l'ensemble de ces études met en évidence une grande variabilité dans les interactions établies entre PGPR et champignons mycorhiziens. Plusieurs facteurs sont susceptibles d'influer sur ces interactions. La production d'exopolysaccharides chez plusieurs souches de *Pseudomonas* a ainsi été identifiée comme jouant un rôle important dans les interactions établies avec les AMF (Bianciotto *et al.* 2001; Turnbull *et al.* 2001). Le chimiotropisme des PGPR envers les racines mycorhizées pourrait aussi être une étape déterminante dans les interactions entre PGPR et champignons mycorhiziens.

Partie Expérimentale 1
Rôle du 2,4-diacétylphloroglucinol (DAPG) dans la compatibilité entre *Pseudomonas* et *Azospirillum* en système gnotobiotique

Introduction

Les populations de *Pseudomonas* fluorescents productrices 2,4-diacétylphloroglucinol (DAPG) jouent un rôle clé dans la résistance des sols à certaines maladies comme le piétin échaudage du blé (cf. synthèse bibliographique). Le DAPG est un métabolite secondaire antimicrobien à large spectre, qui a des propriétés antifongiques, antibactériennes et antihelminthiques. Il pourrait donc être susceptible d'inhiber la croissance d'autres PGPR colonisant le même habitat rhizosphérique, comme *Azospirillum*. Nous nous sommes intéressés ici au rôle du DAPG dans la compatibilité entre les PGPR des genres *Pseudomonas* et *Azospirillum*. Afin de mettre en évidence l'impact direct éventuel du DAPG sur *Azospirillum*, l'ensemble des expériences de confrontation décrites dans cette partie a été réalisé en conditions *in vitro*, afin de s'affranchir des effets indirects du DAPG passant par le reste de la communauté microbienne rhizosphérique.

Dans un premier temps, nous avons étudié le rôle du DAPG dans l'impact des PGPR *Pseudomonas* sur *Azospirillum*. Pour ce faire, une collection de souches d'*Azospirillum* a été confrontée à du DAPG synthétique en concentrations croissantes, ce qui nous a permis d'identifier les concentrations minimales inhibitrices (CMI). Ces expériences ont été réalisées dans l'équipe au cours du stage de Master Recherche d'Elita Challita, et les résultats ont montré (i) un impact du DAPG sur la physiologie des cellules d'*Azospirillum*, ainsi que (ii) des niveaux de résistance très variables en fonction des espèces testées. Quatre souches d'*Azospirillum* représentatives des différents niveaux de sensibilité observés ont été ensuite choisies : *A. lipoferum* 4B (sensible au DAPG), *A. brasilense* Cd et Sp245 (résistance intermédiaire), et *A. irakense* KBC1 (résistante). Ces quatre souches ont ensuite été confrontées à la souche *P. fluorescens* F113 productrice de DAPG, un mutant ne produisant pas de DAPG, et un dérivé complémenté. Les souches d'*Azospirillum* spp. 4B, Cd, Sp245 et KBC1, ainsi que la souche *Pseudomonas* sp. F113 ont été marquées avec des plasmides codant des protéines fluorescentes de type GFP. Les observations en microscopie confocale ont ensuite été effectuées par Claire Prigent-Combaret. Ces expériences de confrontation, effectuées sur plantes en systèmes gnotobiotiques, nous ont permis d'étudier l'impact de la capacité de production de DAPG et de mettre ainsi en évidence une inhibition de la colonisation racinaire des souches d'*Azospirillum* sensibles au DAPG. Les effets de phytostimulation différaient lors de co-inoculations de souches sauvages, mais la comparaison avec les co-inoculations impliquant le mutant DAPG$^-$ indique que les effets négatifs

n'impliquent pas nécessairement le DAPG. L'ensemble de ces expériences a fait l'objet d'un manuscrit qui sera soumis dans un journal scientifique.

Dans un deuxième temps, une étude de l'impact du DAPG sur la souche *A. lipoferum* 4B, très sensible au DAPG, a été effectuée en prenant en compte la variation phénotypique. La souche 4B a en effet la capacité de générer *in vitro* un variant stable nommé $4V_I$. Nous avons donc étudié l'impact du DAPG sur *A. lipoferum* 4B et son variant $4V_I$. Cette étude a été effectuée en comparant (i) la physiologie des cellules de 4B et celle de son variant $4V_I$, et (ii) leurs colonisations racinaires respectives lors de confrontations avec la souche *P. fluorescens* F113 productrice de DAPG. Les résultats obtenus ont montré une plus forte résistance du variant $4V_I$, mais les mécanismes physiologiques impliqués dans cette résistance accrue restent à identifier.

L'ensemble de ces études *in vitro* nous a permis de montrer que l'impact des PGPR *Pseudomonas* productrices de DAPG sur les PGPR phytostimulatrices *Azospirillum* dépend en partie (i) de la sensibilité d'*Azospirillum* au DAPG, et (ii) de la nature de la plante-hôte. Mais ces observations n'intégrent pas des facteurs biotiques (présence d'une communauté microbienne indigène) et abiotiques (présence de sol) qui peuvent influencer la production de DAPG (cf. synthèse bibliographique). Des analyses en sol non-stérile seront nécessaires pour tirer des conclusions définitives.

I/ Role of the antimicrobial compound 2,4-diacetylphloroglucinol in the impact of biocontrol *Pseudomonas fluorescens* F113 on *Azospirillum* phytostimulators

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Abstract

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* phytostimulators were investigated. Phl induced accumulation of poly-β-hydroxybutyrate-like granules, cytoplasmic membrane damage and growth inhibition in *Azospirillum brasilense* Cd, but Phl sensitivity differed between *Azospirillum* strains. Experiments with *P. fluorescens* F113 and a Phl^- mutant indicated that Phl production ability contributed to *in vitro* growth inhibition of *Azospirillum* strains. Plate counts and confocal microscopy showed that *P. fluorescens* F113 had a strong deleterious impact on the Phl-sensitive strain *Azospirillum lipoferum* 4B *in planta*, abolishing its phytostimulatory effect on rice, but also that other *Pseudomonas* properties than Phl production were involved in this inhibition. In contrast, inhibition of the moderately Phl-sensitive strains *A. brasilense* Cd and Sp245 on roots implicated only Phl production ability in F113. Root colonization by the Phl-resistant strain *Azospirillum irakense* KBC1 was not affected by strain F113, and both bacteria readily formed mixed biofilms. Therefore, results suggest that Phl-production ability contributed to inhibition of *Azospirillum* phytostimulators by *Pseudomonas in planta*, which was of particular significance for strains with intermediate Phl sensitivity.

Additionnal Keywords: Antagonism, antibiosis, compatibility, competition, rhizosphere

Introduction

Microbial interactions in the rhizosphere are of paramount important 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 and Bosco 2005) and/or associative nitrogen fixation (Mirza et al. 2006). Plant-beneficial effects by fluorescent pseudomonads may also entail the inhibition of soil-borne phytoparasitic microorganisms, which often involves production of siderophores (Lemanceau et al. 1992) and especially antimicrobial secondary metabolites (Raaijmakers et al. 2002; Haas and 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 and Keel 2003). The ability to produce Phl was shown, both *in vitro* and *in vivo*, as a particularly important biocontrol trait by comparing wild-types and non-producing mutants (Vincent et al. 1991; Fenton et al. 1992; Keel et al. 1992). In addition, Phl^+ strains protected plants better than naturally non-producing counterparts when assessing collections of wild-type biocontrol pseudomonads (Rezzonico et al. 2007). The polyketide Phl inhibits the growth of several phytopathogenic bacteria (*Pectobacterium carotovorum*; Cronin et al. 1997a), oomycetes (*Pythium* spp.) and fungi (e.g. *Rhizoctonia solani*, *Thielaviopsis basicola*, *Gaeumannomyces graminis* var. *tritici*) (Howell and Stipanovic 1979; Keel et al. 1992; Shanahan et al. 1992), and is also active against nematodes (Cronin et al. 1997b).

The inhibitory properties of Phl are not restricted to phytopathogens, as non-pathogenic rhizosphere fungi (Girlanda et al. 2001) and bacteria (Natsch et al. 1998) might be inhibited as well. As far as saprophytic rhizobacteria are concerned, this possibility has been assessed in detail only for a very limited number of taxa, especially *Bacillus* (Natsch et al. 1998), *Rhizobium leguminosarum* (Walsh et al. 2003), and *Cytophaga*-like bacteria (Johansen 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) and may have positive effects on the host plant. Whether these rhizobacteria can be inhibited by Phl (and Phl^+ pseudomonads) on roots is usually unknown.

In addition to microbial inhibition, Phl can also have an effect on root physiology, which in turn may influence the conditions in which saprophytic rhizobacteria colonize the rhizosphere. Indeed, Phl (or the presence of Phl^+ pseudomonads) can elicit an 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 significant, 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,* one of the most important genera of plant growth-promoting rhizobacteria, whose phytostimulatory effects on cereals are extensively documented (Charyulu et al. 1985; Dobbelaere et al. 2001; El Zemrany et al. 2006). Plant-beneficial traits documented in *Azospirillum* strains include associative nitrogen fixation (Bally and Elmerich 2007), production of nitric oxide (Creus et al. 2005; Pothier et al. 2008) and phytohormones especially auxins (Costacurta and Vanderleyden 1995; Dobbelaere et al. 1999), and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity (Blaha et al. 2006, Prigent-Combaret et al. 2008). 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 and Renquist 1997; Dobbelaere et al. 2003; Richardson et al. 2009).

In this work, strains from different *Azospirillum* species, geographic origins and host plants were exposed *in vitro* to synthetic Phl to determine whether this compound had any deleterious effect. Phl^+ fluorescent *Pseudomonas* strains and Phl^- mutants were then compared for their effect on *Azospirillum* numbers, both *in vitro* and *in planta*. The co-inoculation experiments were done using autofluorescent bacterial derivatives to assess whether (i) colonization patterns of particular root zones by *Azospirillum* strains, and (ii) phytostimulatory properties of *Azospirillum* can be affected in the presence of a Phl^+ pseudomonad.

Table 1. Phl sensitivity of the 23 Azospirillum strains used in this study.

Species	Strain	Host plant	Geographic Origin	Reference	Phl LC_{50}[a]	
					LBmb	SA-Fe
Azospirillum amazonense	Y2	Hyparrhenia rufa	Brazil	Magalhaes et al., 1983	200-500[c]	500-1000[c,d]
Azospirillum brasilense	L4	Sorghum	France	Kabir et al., 1996	50-100[c]	200[d]
	Wb1	Wheat	Pakistan	Blaha et al., 2006	200	200-500[c,e]
	Wb3	Wheat	Pakistan	Blaha et al., 2006	200	500-1000[c,e]
	Sp245	Wheat	Brazil	Penot et al., 1992	200-500[c]	500[d]
	WS1	Wheat	Pakistan	Blaha et al., 2006	200-500[c]	500[e]
	WN1	Wheat	Pakistan	Blaha et al., 2006	200-500[c]	200-500[c,e]
	PH1	Rice	France	Rinaudo, 1982	200	500[d]
	B506	Rice	Japan	Elbeltagy et al., 2001	200-500[c]	500[b]
	Cd	Cynodon dactilon	USA	Eskew et al., 1977	200-500[c]	500[d]
	Sp7	Digitaria	Brazil	Tarrand et al., 1978	200-500[c]	500-1000[c,d]
	ZN1	Maize	Pakistan	Blaha et al., 2006	200-500[c]	500-1000[c,e]
Azospirillum doebereinerae	DSMZ13400	Miscanthus sinensis	Germany	Eckert et al., 2001	200	500-1000c[,e]
Azospirillum halopraeferens	DSMZ3675	Kallar grass	Pakistan	Reinhold et al., 1987	200	100[b]
Azospirillum irakense	KBC1	Rice	Iraq	Khammas et al., 1989	G	1000[e]
	RSB1	Rice	Pakistan	Blaha et al., 2006	G	G[d]
Azospirillum lipoferum	4B	Rice	France	Bally et al., 1983	50	200[d]
	B510	Rice	Japan	Elbeltagy et al., 2001	200	200-500[b,c]
	RSWT1	Rice	Pakistan	Blaha et al., 2006	200	200[b]
	TVV3	Rice	Vietnam	Tran Van et al., 1997	500	1000[e]
	N4	Cotton	Pakistan	Blaha et al., 2006	200	NA
	Br17	Maize	Brazil	Tarrand et al., 1978	200-500[c]	500-1000[b,c]
	CRT1	Maize	France	Fages and Mulard, 1988	500	200-500[c,d]

[a] 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 1000 μM Phl.
[b] Growth inhibition test performed using 6 replicates.
[c] LC50 was between the two Phl concentrations.
[d] Growth inhibition test performed on 22 replicates.
[e] Growth inhibition test performed on 14 replicates.
NA, not applicable (because too little growth on SA-Fe medium).
G, growth in more than 50% of the replicates at 1000 μM Phl.

Materials and Methods

Strains and culture conditions

All bacterial strains (Table 1) were routinely grown at 28°C with shaking in LBm, i.e. Luria-Bertani medium (Sambrook et al. 1989) containing only 5 g NaCl/L. The other media were the N-free medium Nfb (Nelson and Knowles 1978) supplemented with Congo red (0.25% w/v) when in plates, King's B (King et al. 1954), and SA-Fe (Cronin et al. 1997a), i.e. Sucrose Asparagine (Scher and Baker 1982) supplemented with 100 µM $FeCl_3$. Antibiotics were used at the following concentrations: ampicillin, 40 µg/mL (Amp40); chloramphenicol, 15 and 30 µg/mL (Cm15 and Cm30, respectively); gentamycin, 25 µg/mL (Gm25).

Effect of synthetic Phl on growth and cell morphology of *Azospirillum brasilense* Cd

The effect of synthetic Phl (Toronto Research Chemicals Inc., North York, Canada) 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 a 10 mM $MgSO_4$ solution and adjusted to 4×10^7 cells/mL (based on optical density). Five mL of cell suspension were then mixed with 5 mL of 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 to 100 mM Phl) and 15 µL were spotted onto the Cd agar layer. Methanol was used as Phl-negative control. For SA-Fe and LBm sublayers, the effect of each of the ten 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 72 h at 28°C.

To assess the impact of Phl on growth, the diameter of the inhibition zone was measured (using a ruler). 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 osmium tetroxide 2%, contrasted with uranyl acetate 1%, dehydrated in a graded ethanol series and embedded in Epon. Ultra-thin sections (0.1 µm) were cut using a Reichert ultramicrotone (Vienna, Austria), contrasted with lead citrate and observed using a CM 120 transmission electron microscope (Philips, Eindhoven, The Netherlands) at 100 kV. Several dozens of cells were examined.

Sensibility of *Azospirillum* spp. to synthetic Phl and LC_{50} determination on solid media

Overnight liquid Nfb cultures of 23 *Azospirillum* strains (Table 1) were used to inoculate Nfb liquid medium in 96-well microtiter plates (50 µL inoculum into 150 µL of 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. Growth of colonies was scored visually, and the Phl concentration needed to abolish growth in at least 50% of the case (LC_{50}) was determined. Each of the eight Phl concentrations studied and the Phl-negative control were investigated using 6 to 22 replicates.

Azospirillum in vitro inhibition experiments using Phl^+ pseudomonads and mutants

Four different *Azospirillum* strains (*A. lipoferum* 4B, *A. brasilense* Sp245 and Cd, and *A. irakense* KBC1) were exposed *in vitro* to the Phl^+ sugarbeet isolate *P. fluorescens* F113 (Shanahan et al. 1992), its Tn*5::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) in LBm Cm30. The cells were then washed twice in a 10 mM $MgSO_4$ solution and adjusted to 10^9 cells/mL (based on optical density). Each *Azospirillum* strain was prepared in water agar, which was poured on LBm or SA-Fe medium, as described above, and each pseudomonad was spotted as 15 µL of cell suspension. For each *Azospirillum* strain, growth inhibition tests were performed in duplicate and the whole experiment was done twice. After 72 h of incubation at 28°C, the width (radius) of the inhibition zone surrounding the *Pseudomonas* colonies was measured using a ruler.

Growth chamber experiments

The effect of *P. fluorescens* F113, F113G22 and F113G22(pCU203) on growth and root colonization of *Azospirillum* strains was assessed on rice (*Oryza sativa*; for the rice isolates *A. lipoferum* 4B and *A. irakense* KBC1) and wheat (*Triticum aestivum*; for the wheat isolate *A. brasilense* Sp245 and the wheat-adapted strain *A. brasilense* Cd), under gnotobiotic

conditions. The P_{lac}-*egfp* plasmid pMP2444 was introduced (as described in Pothier et al. 2007) into the four *Azospirillum* strains retained and the P_{lac}-*rfp* plasmid pMP4661 into *P. fluorescens* F113 and F113G22, so as to monitor cells by fluorescence microscopy (Bloemberg et al. 2000). Both plasmids derive from the broad host-range vector pBBR1MCS-5 and confer gentamycin resistance. The inoculants were obtained after overnight growth in liquid LBm Cm30 for strain F113G22(pCU203) or LBm Gm25 for all other strains. The cells were washed twice in a 10 mM $MgSO_4$ solution and adjusted to 2×10^7 CFU/mL for co-inoculation and 10^7 CFU/mL for single inoculation (based on optical density).

Seeds of spring wheat (cv. Fiorina) and rice (cv. Cigalon) were obtained respectively from Florimond-Desprez (Cappelle en Pévèle, France) and the Centre Français du Riz (Camargue, France), and they were surface-disinfected as described by Pothier et al. 2007. They were placed on water agar (15% w/v for wheat and 7.5% w/v for rice) and incubated 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 10^6 CFU of *Azospirillum* and 50 µl containing 10^6 CFU of *Pseudomonas* or with 100 µl containing 10^6 CFU of each strain. Plants were placed near one edge of square plates (12 cm × 12 cm) containing water agar (15% w/v for wheat and 7.5% w/v for rice). Each *Azospirillum* strain was studied in a distinct experiment. 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 $\mu E/m^2/s$) at 26°C and 8 h of dark at 18°C, and they were sampled seven days later.

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

Analysis of bacterial root colonization in the growth chamber experiments

CLSM observations were done at 7 d using two plants per treatment. Samples 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, Eppelheim, Germany). A 510 Meta microscope (Carl Zeiss, Le Pecq, France) equipped with argon-krypton and He-Ne 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 (in blue)

of reflected lights (detection at 456-499 nm), the three single-colour images were overlaid into a single image using LSM software release 3.5 (Carl Zeiss).

For quantification of inoculant bacteria, four plants were sampled per treatment at 7 d. Bacteria were extracted by vortexing each root system 5 min in a 15-mL Falcon tube containing 5 mL of 10-mM $MgSO_4$ solution. A serial dilution was prepared in the same solution, and six 10-µl drops from each dilution were spotted onto King's B Amp40 Cm15 Gm25 to quantify F113(pMP4661) or F113G22(pMP4661), King's B Amp40 Cm30 to quantify F113G22(pCU203), and Nfb Gm25 to quantify 4B(pMP2444), Cd(pMP2444), Sp245(pMP2444) or KBC1(pMP2444). Colonies were not found on plates in the absence of inoculation of seedlings with the corresponding strain(s).

Statistical analysis

CFU of root-colonizing inoculants were expressed per g of dry root and were log-transformed before analysis. All results were processed by analysis of variance (ANOVA), followed when appropriate with Fisher's least significant difference (LSD) tests. All analyses were conducted at $P < 0.05$, using S-plus software (Hearne Scientific Software, Kilkenny, Ireland).

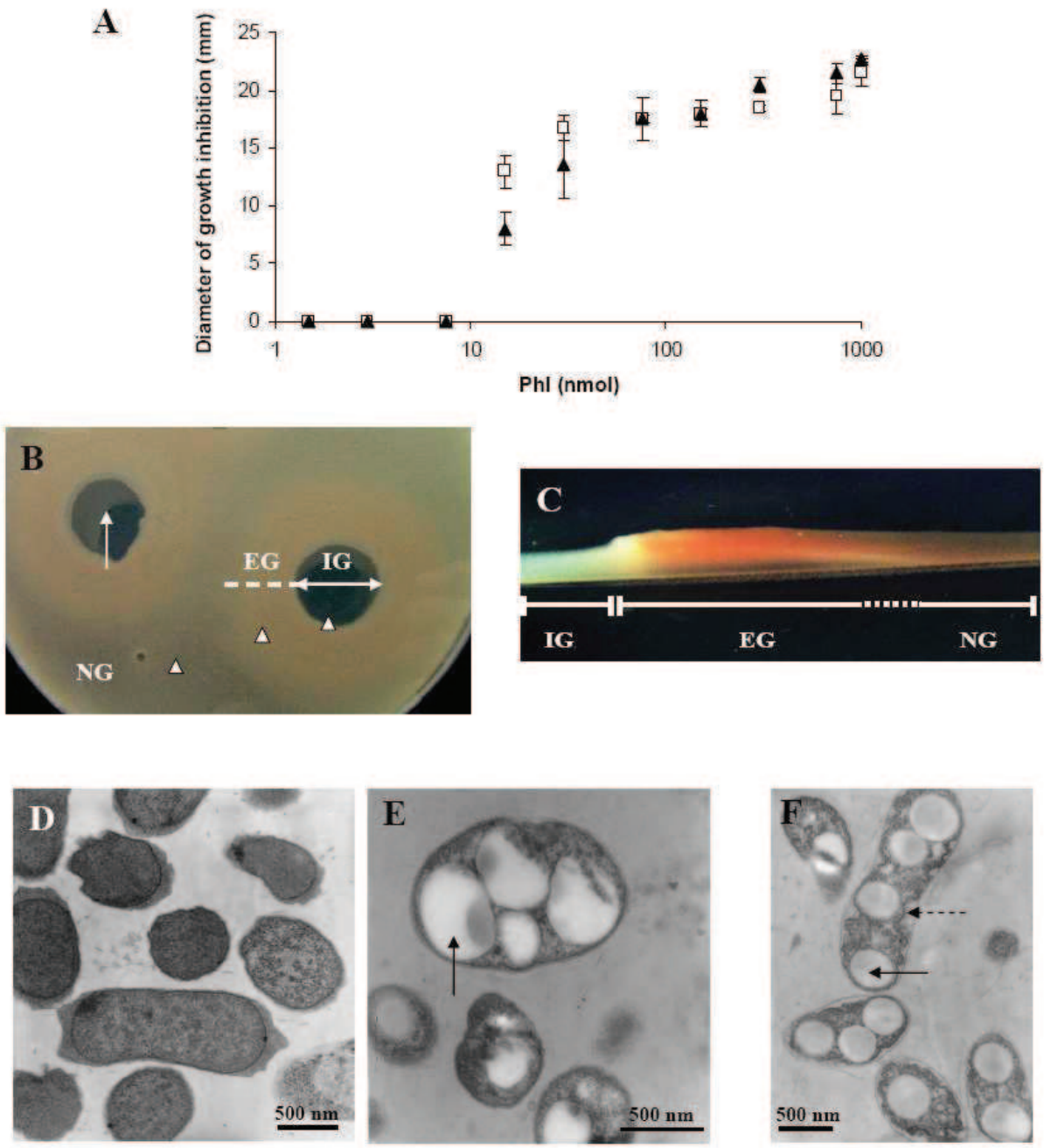


Fig. 1. Effect of synthetic Phl on *A. brasilense* Cd on SA-Fe (open squares) and LBm (filled squares) after 72 h incubation of plates at 28°C. **A**, Diameter of growth inhibition according to Phl amount in the 15-µL drops. **B**, A dark pink halo (10-20 mm wide) of enhanced growth (EG) was observed around the zone of inhibited growth (IG) when added Phl (arrow) exceeded 150 nmol, in comparison with normal growth conditions further away in the plate (NG). White triangles indicate locations in the plate where small pieces of water agar were cut off for scanning microscopy. **C**, Photonic microscopy (using a binocular loop) of a vertical section performed in the *A. brasilense* Cd cell layer across the zones of inhibited growth (IG), enhanced growth (EG) and normal growth (NG). **D-E,** Scanning electronic microscopy of *A. brasilense* Cd cells upon exposure to Phl in the zones of normal growth (D), enhanced growth (E) and inhibited growth (F). Poly-β-hydroxybutyrate-like granules (arrows) and damage to the cytoplasmic membrane (dashed arrow) of *A. brasilense* Cd are shown.

Results

Effect of synthetic Phl on *A. brasilense* Cd on plates

Synthetic Phl was used in a simple plate assay to determine if it had an effect on *Azospirillum*. A wide range of synthetic Phl concentrations (from 0 to 1500 nM) was tested with *A. brasilense* Cd as model strain. Growth of *A. brasilense* Cd in rich LBm medium and minimal SA-Fe medium was inhibited when at least 15 nmol of synthetic Phl was added (as 15 µL spot). With both media, the diameter of the inhibition zone increased with increasing Phl quantities (Fig. 1A). Only few cells were found by photonic microscopy in this inhibition zone.

Surprisingly, a dark pink halo (10-20 mm wide) of *Azospirillum* growth was observed around the inhibition zone when Cd was grown on SA-Fe (but not on LBm), provided that added Phl exceeded 150 nmol (Fig. 1B). Photonic microscopy indicated that cell population density 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, cell morphology of *A. brasilense* Cd was also affected upon exposure to Phl. In comparison with normal growth areas of the plate (Fig. 1D), cells in the dark pink halo displayed accumulation of 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).

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.

LC_{50} of different *Azospirillum* strains to synthetic Phl in complex and minimal media

The lethal concentration LC_{50} (i.e. concentration necessary for at least 50% of growth inhibition) of *A. brasilense* Cd was between 200 and 500 µM Phl on LBm, and 500 µM Phl on SA-Fe agar. To assess whether Phl had similar deleterious effects on other *Azospirillum* strains as well, their LC_{50} was determined for a collection of strains from different *Azospirillum* species, geographic origins and host plants (Table 1). This was done on LBm complex medium and SA-Fe minimal, on which *Azospirillum* growth is slower (data not shown). Most *Azospirillum* strains displayed some level of Phl sensitivity, which was often higher on LBm than on SA-Fe agar, and overall some species were most sensitive to Phl than others (Table 1), as follows.

Table 2. Growth inhibition of *A. lipoferum* 4B, *A. brasilense* Cd and Sp245, and *A. irakense* KBC1 by Phl$^+$ *P. fluorescens* F113, its Phl$^-$ mutant F113G22 and the complemented derivative F113G22(pCU203) at 2 days on LBm and SA-Fe agar, as indicated by the width (mm) of the inhibition zone around the *Pseudomonas* colony (mean ± standard error).

	LBm agar				SA-Fe agar			
	No pseudomonad	F113	F113G22	F113G22 (pCU203)	No pseudomonad	F113	F113G22	F113G22 (pCU203)
A. lipoferum 4B	0 c[a]	8.0 ± 0.4 a	1.8 ± 1 bc	3.3 ± 1.9 b	0 b	1.3 ± 0.5 a	0 b	0.3 ± 0.3 a
A. brasilense Cd	0 b	2.8 ± 1 a	0 b	1.0 ± 0.6 b	0	0.8 ± 0.3	0	1.0 ± 0.6
A. brasilense Sp245	0	2.5 ± 0.5	0.3 ± 0.3	1.8 ± 1.1	0 b	1.8 ± 0.6 a	0 b	0.1 ± 0.1 b
A. irakense KBC1	0 c	6.8 ± 0.6 a	2.5 ± 0.3 b	3.8 ± 0.5 b	0 b	6.5 ± 0.6 a	0.5 ± 0.0 b	0.5 ± 0.0 b

[a] For each medium and each *Azospirillum* strain, letters a-c are used to indicate statistical relations between treatments based on ANOVA and Fisher's LSD tests ($P < 0.05$).

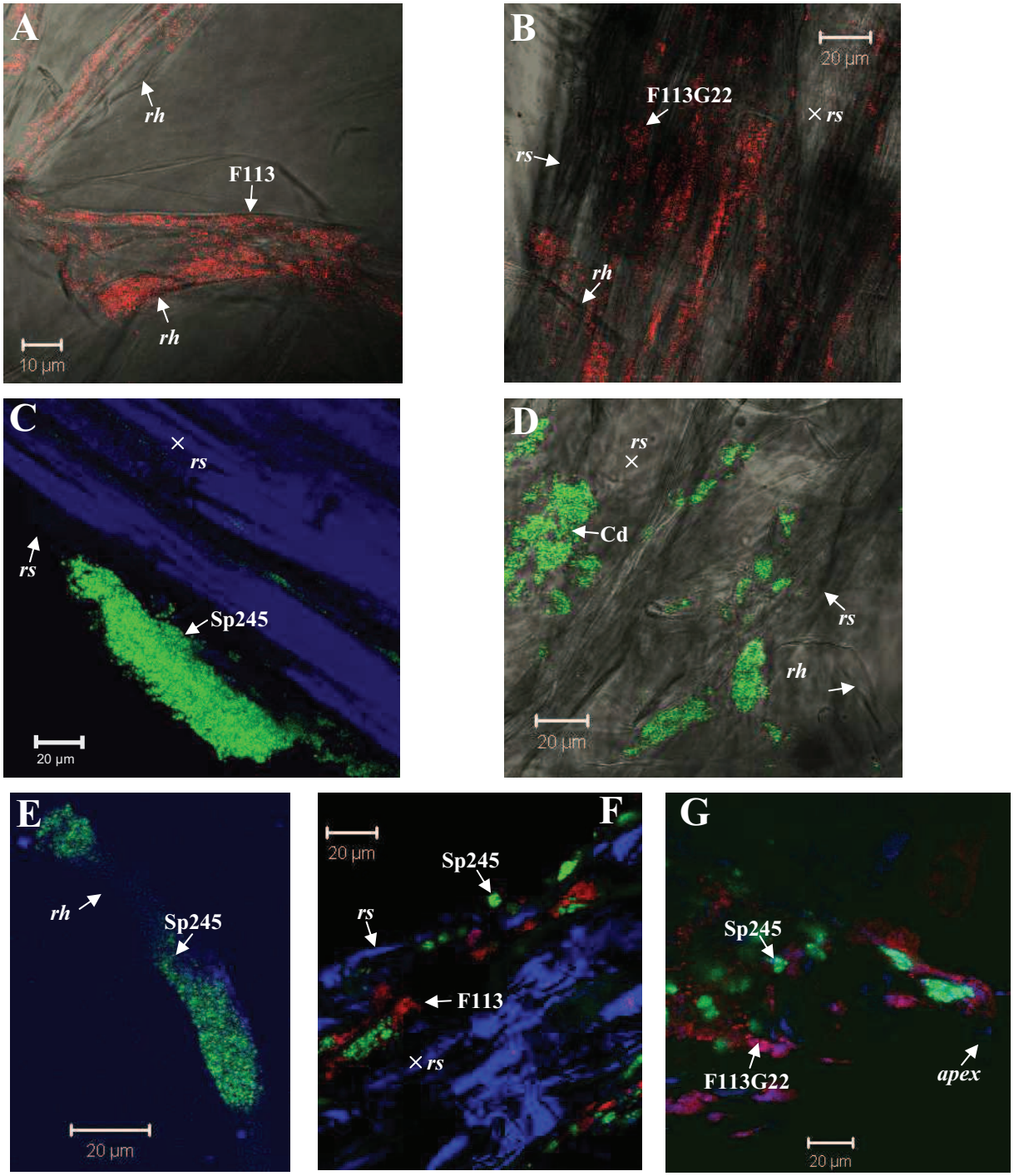
First, only the two *A. irakense* strains tested were not completely inhibited even at the very high concentration (1000 µM Phl), both on LBm and SA-Fe (Table 1), and on this basis they were considered Phl resistant. Second, a higher proportion of *A. lipoferum* strains than *A. brasilense* strains had a $LC_{50} \leq 200$ µM Phl on LBm (4 of 7 strains versus 4 of 11 strains) and $LC_{50} < 500$ µM Phl on SA-Fe (4 of 6 strains versus 3 of 11 strains). Third, strains of *Azospirillum amazonense*, *Azospirillum doebereinerae* and *Azospirillum halopraeferens* displayed intermediary behaviour, as their LC_{50} on LBm was 200-500 µM Phl depending on the strain. There was no obvious relation between Phl sensitivity and the geographic origin or the host plant of *Azospirillum* strains. For the rest of the study, four *Azospirillum* strains of contrasted Phl sensitivity were selected, i.e. (i) a sensitive strain (*A. lipoferum* 4B; LC_{50} of 50 µM on LBm and 200 µM on SA-Fe), (ii) two moderately-sensitive strains (*A. brasilense* Cd and Sp245; LC_{50} between 200 and 500 µM Phl on LBm and 500 µM Phl on SA-Fe), and (iii) a Phl-resistant strain (*A. irakense* KBC1; only weak growth inhibition at 1000 µM Phl on LBm and on SA-Fe).

Effect of Phl^+ *P. fluorescens* F113 and mutants on growth of *Azospirillum* strains *in vitro*

Whether Phl production by *Pseudomonas* could have an effect on *Azospirillum* 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. lipoferum* 4B (Phl sensitive), *A. brasilense* Cd and Sp245 (moderately sensitive to Phl), and *A. irakense* KBC1 (Phl resistant), using two solid media conducive to Phl production by strain F113. Strain F113 inhibited the growth of the four *Azospirillum* strains within 2 d, on one medium or both (Table 2), and inhibition was greater on complex medium LBm compared with minimal medium SA-Fe. The most sensitive strains were *A. lipoferum* 4B (Phl sensitive) and *A. irakense* KBC1 (Phl resistant) on LBm, and *A. irakense* KBC1 and to a lesser extent *A. brasilense* Sp245 (sensitive to intermediate Phl concentrations) on SA-Fe. Similar strain differences were observed at 7 d, with wider inhibition zones especially on SA-Fe agar (data not shown).

Compared with the wild-type F113, the ability of the Phl^- biosynthetic mutant F113G22 to inhibit the four *Azospirillum* strains was lower (Table 2). Inhibition depended on

the *Azospirillum* strain. *A. brasilense* Cd was the only strain not affected at all on LBm, whereas *A. lipoferum* 4B and the two *A. brasilense* strains were not inhibited at all on SA-Fe.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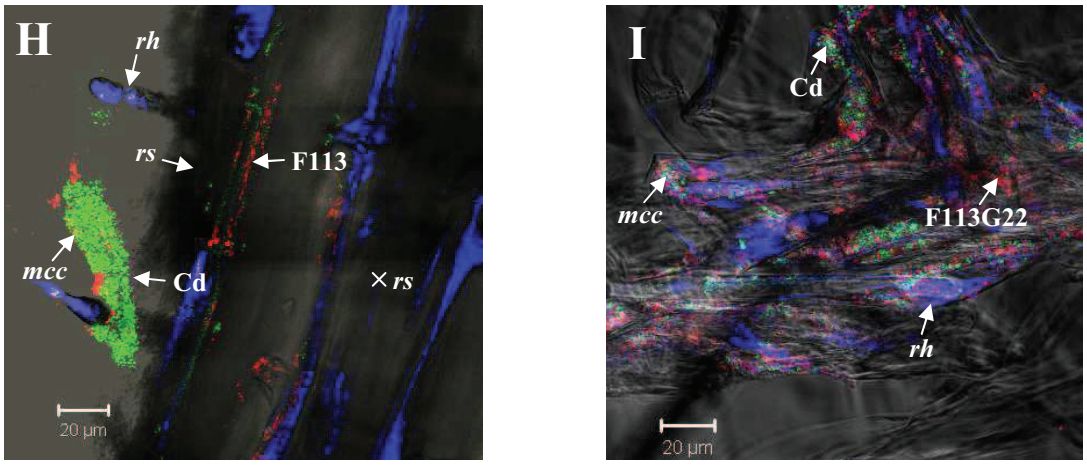


Fig. 2. CLSM observations at 7 d of wheat roots colonized by *A. brasilense* and/or *P. fluorescens* strains. Single inoculations were performed with *A. brasilense* Sp245 (A) or Cd (B), or *P. fluorescens* F113 (C) or F113G22 (D). *A. brasilense* strains were coinoculated with the wild-type strain F113 (Sp245 in E and F, Cd in H) or the Phl$^-$ mutant F113G22 (Sp245 in G, Cd in I). *Azospirillum* cells constitutively expressing EGFP are green, *Pseudomonas* cells constitutively expressing DsRed 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.

There was a trend for higher *Azospirillum* inhibition by the complemented mutant F113G22(pCU203) compared with F113G22, but this trend was statistically significant only for *A. lipoferum* 4B on SA-Fe.

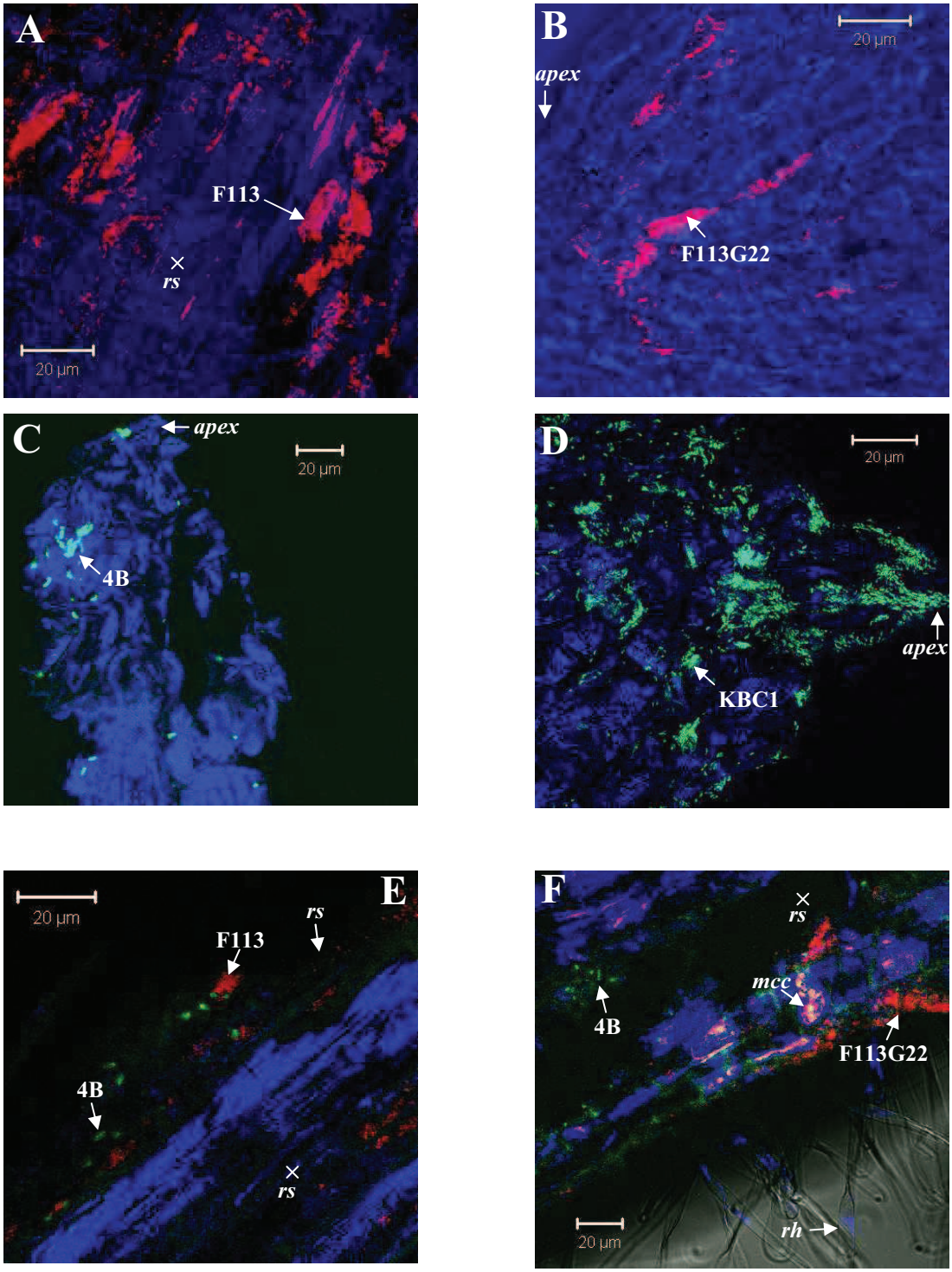
Effect of Phl$^+$ *P. fluorescens* F113 and mutants on root colonization patterns of *Azospirillum* strains

To establish whether Phl$^+$ *Pseudomonas* could change root colonization patterns of *Azospirillum* strains, plants were co-inoculated using distinct molecular tags for both types of bacteria. Confocal Laser Scanning Microscopy (CLSM) observations at 7 d of wheat and rice roots colonized by *P. fluorescens* F113 or its Phl$^-$ mutant F113G22 (both marked with a plasmid expressing constitutively the red fluorescent protein DsRed) evidenced that each pseudomonad formed large patches of cells, which were of moderate thickness (maximum 5 µm thick). 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, regardless whether wheat (Fig. 2AB) or rice was considered (Fig. 3AB). This root colonization pattern of *P. fluorescens* F113 and F113G22 was not altered when these pseudomonads were co-inoculated with an *Azospirillum* strain.

A. brasilense Cd and Sp245 (both marked with a plasmid expressing constitutively the enhanced green fluorescent protein EGFP) 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. 2CD). Both strains extensively colonized the root hair zone (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. 2E-I). Mixed biofilms of *A. brasilense* and *P. fluorescens* cells (appearing yellow) were observed on a few occasions, regardless of whether F113 or F113G22 was used (data not shown). Based on several microscopic observations, cells of *A. brasilense* Cd and Sp245 seemed more numerous in presence of F113G22 (Fig. 2GI) than of F113 (Fig. 2FH).

Unlike the *A. brasilense* strains on wheat, *A. lipoferum* 4B and *A. irakense* KBC1 (both marked with a plasmid expressing the enhanced green fluorescent protein EGFP) mainly colonized root growth areas on rice, i.e. the elongation zone (near the root apex) and zones where lateral roots emerge. In these areas, *Azospirillum* was found as a mixture of single cells and small cell aggregates (about 5 µm high). The two *Azospirillum* strains

colonized poorly the root hair zone (Fig. 3AB). In presence of *P. fluorescens* F113, only a few single cells of *A. lipoferum* 4B could be observed on root surfaces, sometimes close to *Pseudomonas* cells (Fig. 3E). In comparison, more cells of *A. lipoferum* 4B were visualised on rice roots when co-inoculated with F113G22, and mixed *Azospirillum-Pseudomonas* biofilms were even found (Fig. 3F). Unlike for *A. lipoferum* 4B, the colonization pattern of *A. irakense* KBC1 on rice roots was not affected by *P. fluorescens* F113 (or F113G22). The occurrence of mixed *Azospirillum-Pseudomonas* biofilms was high with *A. irakense* KBC1, regardless of whether F113 or F113G22 was used (Fig. 3G).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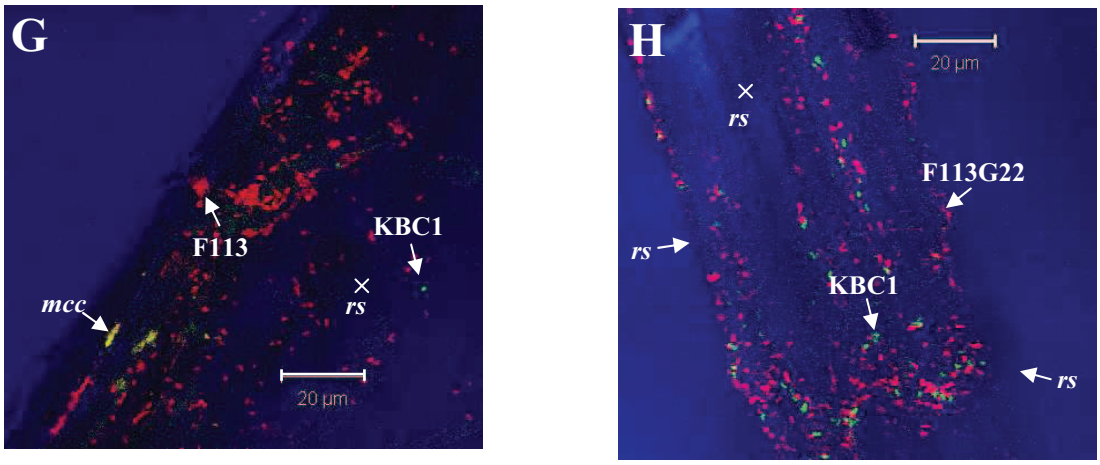


Fig. 3. CLSM observations at 7 d of rice roots colonized by *A. lipoferum*, *A. irakense* and/or *P. fluorescens* strains. Single inoculations were performed with *A. lipoferum* 4B (A) or A. irakense KBC1 (B), or *P. fluorescens* F113 (C) or F113G22 (D). *A. lipoferum* 4B and A. irakense KBC1 were coinoculated with the wild-type strain F113 (4B in E, KBC1 in G) or the Phl$^-$ mutant F113G22 (4B in F, KBC1 in H). *Azospirillum* cells constitutively expressing EGFP are green, *Pseudomonas* cells constitutively expressing DsRed 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.

Effect of Phl$^+$ *P. fluorescens* F113 and mutants on *Azospirillum* cell numbers *in planta*

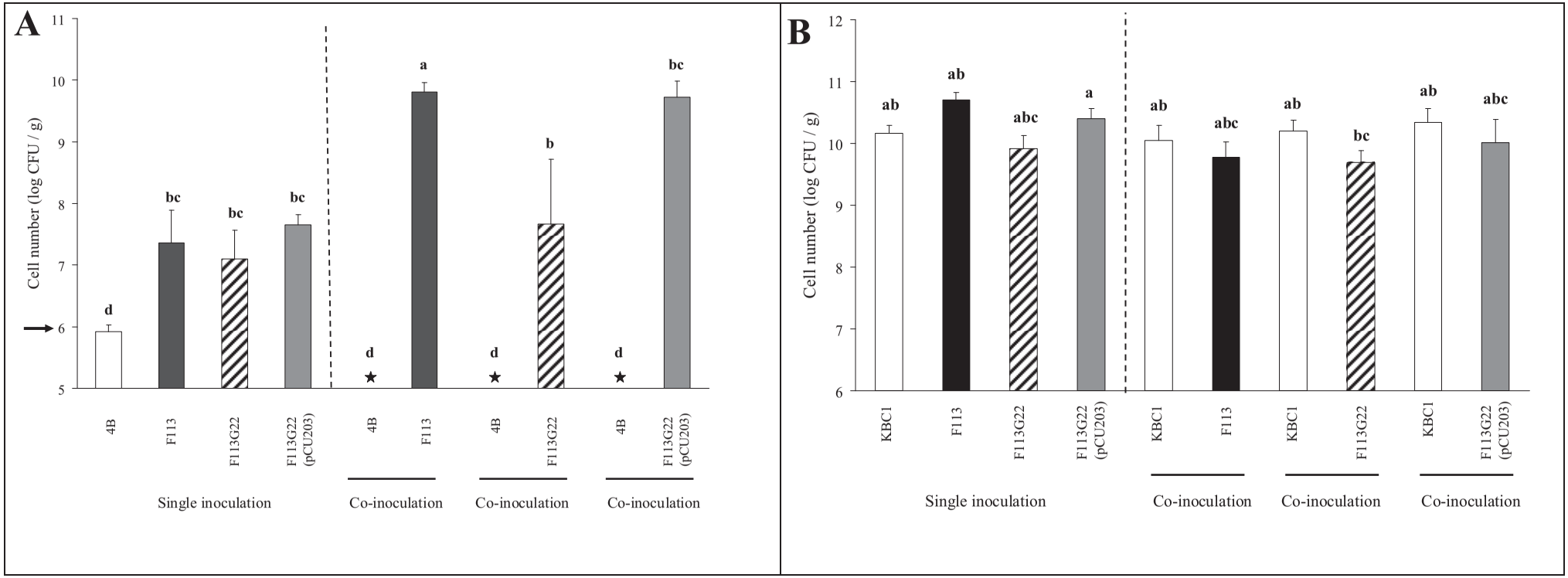
A. lipoferum 4B colonized rice effectively at 7 d when inoculated singly (10^6 CFU/g root). In contrast, the strain was below the detection limit in presence of *P. fluorescens* F113, its Phl$^-$ mutant F113G22 or the complemented derivative F113G22(pCU203) (Fig. 4A).

When co-inoculated with *P. fluorescens* F113, *A. brasilense* Sp245 reached lower cell numbers on wheat roots at 7 d compared to those attained when inoculated singly, but the decrease was about 1 log CFU/g only (Fig. 4C). Unlike F113, the Phl$^-$ biosynthetic mutant F113G22 had no negative effect on strain Sp245 at 7 d, whereas the complemented derivative F113G22(pCU203) decreased cell numbers of strain Sp245 as the wild-type did. Unexpectedly, the Phl$^-$ biosynthetic mutant F113G22 facilitated root colonization by *A. brasilense* Sp245, which at 7 d reached higher levels in comparison with those attained after single inoculation. Similar findings were made with *A. brasilense* Cd, except that several of these differences were not significant at $P < 0.05$ because of higher data fluctuation levels (Fig. 4D).

In contrast to the three other *Azospirillum* strains, root colonization by *A. irakense* KBC1 was not affected by strain F113 (Fig. 4B). Indeed, the same cell numbers of strain KBC1 were recovered from rice roots at 7 d, regardless of whether strain KBC1 was inoculated alone or in combination with F113, F113G22 or F113G22(pCU203).

Effect of Phl$^+$ *P. fluorescens* F113 and mutants on *Azospirillum* phytostimulation of rice

When inoculated alone on rice, *P. fluorescens* F113 (but not its Phl$^-$ biosynthetic mutant F113G22) had a negative effect at 7 d on (i) root dry weight in both rice experiments, and (ii) total root length, total root volume, total root surface and number of roots in experiment A (the trend was similar but not statistically significant in experiment D) (Table 3AD). The


A
Cell number (log CFU / g)
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4B
F113
F113G22
F113G22 (pCU203)
4B
F113
4B
F113G22
4B
F113G22 (pCU203)
Single inoculation
Co-inoculation
Co-inoculation
Co-inoculation
B
Cell number (log CFU / g)
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11
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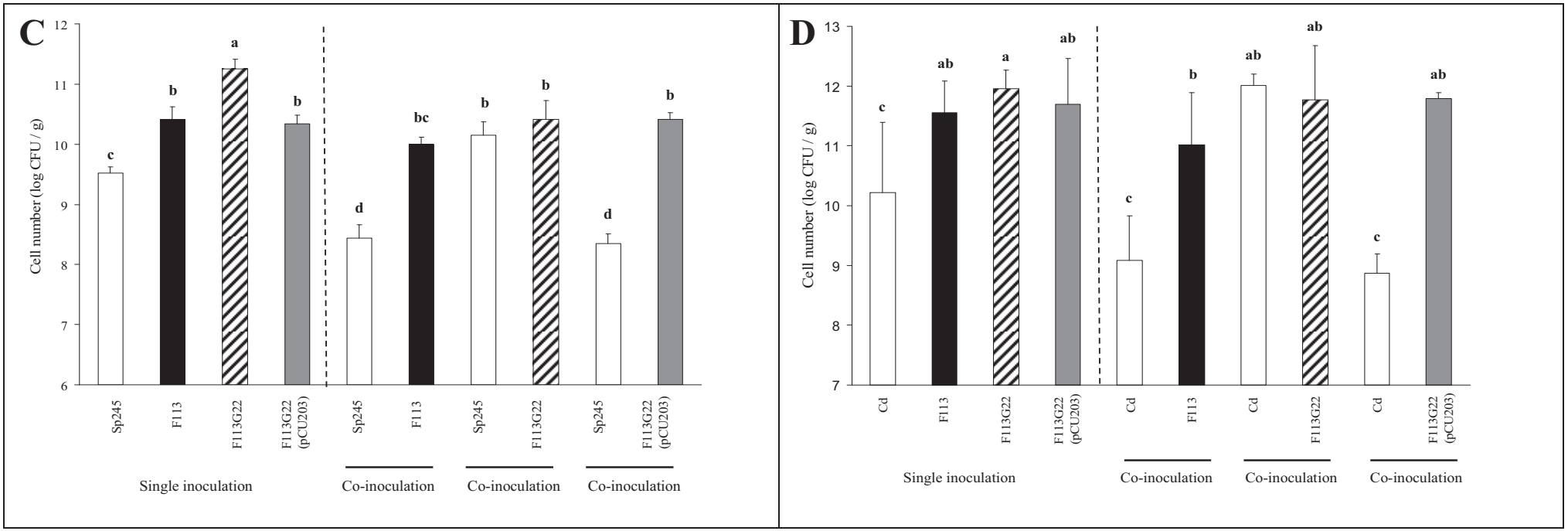

Fig. 4. Effect of Phl$^+$ *P. fluorescens* F113, its Phl$^-$ mutant F113G22 and the complemented derivative F113G22(pCU203) on cell numbers (means ± SE) of Phl-sensitive *A. lipoferum* 4B (A), moderately Phl-sensitive *A. brasilense* Sp245 (C) and Cd (D), and Phl-resistant *A. irakense* KBC1 (B). In each panel, letters a-d are used to indicate statistical differences between treatments based on ANOVA and Fisher's LSD tests ($P < 0.05$). Stars indicate cell numbers below detection limit (i.e. 10^5 CFU/g dry root) and the arrow (in A) the inoculation level.

complemented derivative F113G22(pCU203) had a small but significant negative effect on total root length, total root volume and total root surface in experiment A (the trend was similar but not statistically significant in experiment D).

Single inoculation of rice with *A. lipoferum* 4B resulted in higher root dry weight (i.e., a 2.5-fold increase) at 7 d. Rice plants co-inoculated with strain 4B and *P. fluorescens* F113 were comparable to those inoculated with strain F113 alone (Table 3A). Plants co-inoculated with strains 4B and either F113G22 or F113G22(pCU203) fared like (i) non-inoculated plants based on root dry weight and (ii) plants inoculated with strain F113 alone based on most parameters describing root system architecture.

A. irakense KBC1 had no significant effect on rice root system at 7 d when inoculated alone, but when co-inoculated with *P. fluorescens* F113 it alleviated the negative effect of the pseudomonad on root dry weight (Table 3D). In the latter case, it also resulted in enhanced total root length, total root surface and number of roots in comparison with the non-inoculated control. A similar trend on these three parameters was also observed when strain KBC1 was co-inoculated with F113G22 or F113G22(pCU203), but this trend was statistically significant only for the number of roots when F113G22 was used.

Effect of Phl$^+$ *P. fluorescens* F113 and mutants on *Azospirillum* phytostimulation of wheat

When inoculated alone on wheat, *P. fluorescens* F113 improved at 7 d (i) total root length and total root surface in both wheat experiments, and (ii) root dry weight and the number of roots (only in experiment B), or total root volume (in experiment C) (Table 3BC). With the Phl$^-$ biosynthetic mutant F113G22, these phytostimulatory effects were either not found or of less 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 experiment B.

Single inoculation of wheat with *A. brasilense* Cd resulted in higher root dry weight and total root length (Table 3B). 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 and the number of roots, and (ii) even displayed 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 they displayed enhanced root dry weight in the case of F113G22(pCU203).

Table 3. Effect of single and co-inoculation on root system of rice (experiments A and D) and wheat (experiments B and C ; mean ± SE; n = 4 plants).

Plant			Single inoculation				*Pseudomonas* co-inoculated with *Azospirillum*		
parameters [a]	Units	Control	*Azospirillum*	F113	F113G22	F113G22 (pCU203)	F113	F113G22	F113G22 (pCU203)
Experiment A (*A. lipoferum* 4B)									
Root dry weight	mg/plant	1.5 ±0.1 b	3.8 ±0.3 a	0.8 ±0.1 c	2.3 ±0.2 ab	1.8 ±0.2 ab	0.8 ±0.1 c	2 ±0.1 ab	2.2 ±0.2 ab
Total root length	cm/plant	13 ±3 ab	13 ±2 a	2.0 ±0.2 d	7.0 ±2.0 bc	6.0 ±2.0 cd	2.0 ±0.2 d	3.0 ±1.0 cd	3.0 ±0.2 cd
Total root volume	mm^3/plant	23 ±4 a	20 ±4 ab	5 ±1 d	17 ±4 abc	15 ±6 bcd	12 ±1 bcd	8 ±1 cd	1 ±1 bcd
Total root surface	mm^2/plant	190 ±40 a	180 ±40 ab	30 ±6 e	120 ±30 abc	100 ±40 bcd	50 ±3 de	50 ±10 cde	60 ±3 cde
Number of roots		24 ±4 ab	31 ±8 a	3 ±1 c	17 ±5 abc	13 ±5 bc	5 ±1 c	11 ±5 bc	7 ±1 c
Experiment B (*A. brasilense* Cd)									
Root dry weight	mg/plant	5.5 ±0.5 c	6.8 ±0.3 b	8.0 ±0.4 a	6.8 ±0.4 b	6.7 ±0.2 b	5.7 ±0.4 bc	6.0 ±0.3 bc	7.8 ±0.3 a
Total root length	cm/plant	19 ±2 c	33 ±3 b	46 ±3 a	31 ±3 b	43 ±7 a	12 ±2 c	14 ±1 c	18 ±2 c
Total root volume	mm^3/plant	92 ±9	76 ±3	102 ±8	83 ±10	92 ±7	54 ±14	77 ±14	103 ±17
Total root surface	mm^2/plant	565 ±48 cd	557 ±33 bc	763 ±55 a	564 ±31 bc	700 ±82 ab	284 ±48 e	359 ±38 de	473 ±37 cd
Number of roots		25 ±5 bc	39 ±3 b	69 ±11 a	35 ±8 b	39 ±11 b	11 ±2 c	12 ±2 c	16 ±3 c
Experiment C (*A. brasilense* Sp245)									
Root dry weight	mg/plant	7.2 ±0.9	7.3 ±0.4	6.2 ±0.3	5.7 ±0.7	6.9 ±0.8	6.3 ±0.3	6.9 ±0.2	7.7 ±0.4
Total root length	cm/plant	10 ±1 c	19 ±2 ab	19 ±2 ab	16 ±1 b	24 ±1 a	20 ±2 ab	18 ±2 b	15 ±3 b
Total root volume	mm^3/plant	13 ±2 b	83 ±18 a	73 ±3 a	63 ±13 a	78 ±9 a	66 ±8 a	70 ±16 a	71 ±9 a
Total root surface	mm^2/plant	122 ±14 c	440 ±65 ab	417 ±21 ab	347 ±41 b	484 ±39 a	406 ±39 ab	393 ±69 ab	368 ±58 ab
Number of roots		20 ±6	17 ±2	17 ±2	16 ±4	22 ±5	16 ±2	16 ±3	10 ±2
Experiment D (*A. irakense* KBC1)									
Root dry weight	mg/plant	2.6 ±0.1 ab	2.7 ±0.1 ab	0.8 ±0.1 c	2.3 ±0.1 ab	1.8 ±0.1 b	2.8 ±0.1 a	1.8 ±0.1 b	1.7 ±0.1 b
Total root length	cm/plant	9.0 ±2.0 bc	11 ±3 bc	2.0 ±0.2 c	7.0 ±2.0 bc	6.0 ±2.0 bc	26 ±8 a	16 ±6 ab	15 ±3 ab
Total root volume	mm^3/plant	19 ±4	16 ±2	6 ±1	17 ±4	15 ±6	30 ±7	20 ±5	20 ±5
Total root surface	mm^2/plant	143 ±31 bc	143 ±29 bc	33 ±6 c	124 ±31 bc	108 ±40 bc	316 ±76 a	188 ±64 ab	192 ±34 ab
Number of roots		14 ±4 b	25 ±10 ab	3 ±1 b	18 ±6 b	11 ±4 b	71 ±21 a	70 ±30 a	50 ±9 ab

[a] For each data row, statistical differences between treatments are indicated with letters a-e (ANOVA and Fisher LSD tests; $P < 0.05$).

Single inoculation of wheat with *A. brasilense* Sp245 resulted in higher total root length, total root volume and total root surface (Table 3C). In comparison with the non-inoculated control, plants co-inoculated with strain Sp245 and *P. fluorescens* F113 displayed enhanced total root length, total root volume and total root surface, similarly to plants inoculated singly. A similar trend was also observed when *A. brasilense* Sp245 was co-inoculated with F113G22 or F113G22(pCU203).

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 and Défago 2005; Couillerot et al. 2009). In contrast, their capacity to affect other saprophytic microbial inhabitants in the rhizosphere has received much less research attention (Natsch et al. 1998; Girlanda et al. 2001; Johansen et al. 2002), and in particular their impact on most genera of plant-beneficial microorganisms is largely unknown except for rhizobia (Walsh et al. 2003). In this work, the effect of Phl^+ fluorescent *Pseudomonas* strains on root-colonizing *Azospirillum* phytostymulators was investigated, and *Pseudomonas* mutants were used to assess the role of Phl production ability.

Results indicated that Phl could inhibit *Azospirillum* growth, but the 23 strains tested differed in Phl sensitivity. Similar results were also obtained in liquid media for the four strains studied in more details (not shown). The frequency of sensitive strains was higher in *A. lipoferum*, whereas the two resistant strains belonged to *A. irakense*, i.e. two contrasted species within the *Azospirillum* genus (Eckert et al. 2001; Young et al. 2008). Most *Azospirillum* strains were more sensitive to Phl than other saprophytic (Keel et al. 1992; Moënne-Loccoz et al. 2001; Walsh et al. 2003) or phytopathogenic root-associated microorganisms (Keel et al. 1992; Cronin et al. 1997a; Schouten et al. 2004), and the strain to strain fluctuation within *Azospirillum* was comparable to the fluctuation documented among isolates of a same species or genus in other taxa.

The mode of action of Phl is poorly understood. This phenolic metabolite inhibits both prokaryotic and eukaryotic organisms, and 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. At further distance from the Phl deposit, Cd cells were more numerous and displayed cytoplasmic accumulation of poly-β-hydroxybutyrate-like granules, which favor the survival of *A. brasilense* under stress conditions such as carbon starvation, ultraviolet irradiation, heat, osmotic shock, desiccation, and hydrogen peroxide (Tal and Okon 1985; Kadouri et al. 2002; 2003). Unexpectedly, Phl at even lower levels resulted in higher cell population density (concentration halo) compared with plates without Phl, which also took place when Phl^+ strains F113 or Q2-87 were used instead of synthetic Phl (not shown).

The Phl^+ strain *P. fluorescens* F113 inhibited the growth on plates of four *Azospirillum* strains from *A. lipoferum*, *A. brasilense* or *A. irakense* and selected based on their contrasted sensitivity to Phl. 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 in the order of 5×10^8 CFU of F113 on rich medium (Duffy and Défago 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). Despite not producing Phl, the mutant F113G22 could inhibit growth of *A. irakense* KBC1 on LBm, meaning that KBC1 is sensitive to other compounds secreted by F113 (and its derivatives), such as perhaps hydrogen cyanide (HCN).

The effect on *Azospirillum* of two other Phl^+ fluorescent *Pseudomonas* strains i.e. *Pseudomonas* sp. CHA0 (Stutz et al. 1986) and *P. fluorescens* Q2-87 (Vincent et al. 1991) was also checked *in vitro*. On both media tested, the two pseudomonads inhibited the growth of all four *Azospirillum* strains within 2 d (data not shown). The inhibition effect of strain Q2-87 was similar to that of strain F113, whereas strain CHA0 inhibited *Azospirillum* strains to a lesser extent. In comparison with strain Q2-87, the Phl^- biosynthetic mutant Q2-87::Tn*5-1* (Vincent et al. 1991) displayed a lower ability to inhibit growth of the four *Azospirillum* strains (not shown), confirming the importance of Phl production in *Azospirillum* inhibition by *P. fluorescens*. The Phl^- biosynthetic mutant CHA631 (Schnider-Keel et al. 2000) obtained from *Pseudomonas* sp. CHA0 retained the ability to inhibit the *Azospirillum* strains (data not shown), but inactivation of Phl production ability in *Pseudomonas* sp. CHA0 stimulates synthesis and leads to increased production of pyoluteorin, another antimicrobial metabolite

not found in *P. fluorescens* F113 and Q2-87 ((Schnider-Keel et al. 2000; Baehler et al. 2005). This suggests that *Azospirillum* inhibition by *Pseudomonas* sp. CHA0 could have involved pyoluteorin production, an issue that will be dealt with in future work.

The rice and wheat *Azospirillum* strains tested differed in their patterns of root colonization, a property that may vary according to the plant species (Michiels et al. 1989; Bashan et al. 1991) as well as the *Azospirillum* strain (Aβmus et al. 1997; Schloter and Hartmann 1998). Differences in root colonization patterns were perhaps not a major factor in the interaction with pseudomonads, as the latter colonized all root parts extensively. CLSM observations as well as plating of rice root samples indicated that Phl^+ strain *P. fluorescens* F113 had a strong inhibitory effect on the Phl-sensitive strain *A. lipoferum* 4B, but the Phl^- mutant F113G22 had similar effects. This suggests that strain 4B was also affected by other *Pseudomonas* metabolites than Phl. Sensitivity of this *Azospirillum* strain to other *Pseudomonas* metabolites on roots was suggested by preliminary results from a co-inoculation experiment performed with a derivative of *Pseudomonas* sp. CHA0 that overproduces pyoluteorin (data not shown). Here, cell numbers of the two moderately Phl-sensitive strains *A. brasilense* Cd and Sp245 on wheat roots were approximately 10 times lower in presence of Phl^+ strain *P. fluorescens* F113, and comparison with F113 mutants showed that this effect was due to Phl production ability. This was confirmed by CLSM observation of wheat roots. Colonization of rice roots by the Phl-resistant strain *A. irakense* KBC1 was not affected by *P. fluorescens* F113, regardless of whether samples were assessed by plating or CLSM observation, and mixed biofilms were more frequent than with any of the other *Azospirillum* strains (Fig. 3G). Therefore, the results obtained on roots with the four *Azospirillum* strains evidenced some relation between their level of Phl sensitivity, their inhibition by co-inoculated Phl^+ strain *P. fluorescens* F113, and the importance of Phl-production ability in inhibition effects. More specifically, results suggest that Phl-production ability contributed to the ability of *P. fluorescens* F113 to inhibit root colonisation mostly in the case of *Azospirillum* strains of intermediate Phl sensitivity.

These findings were substantiated by results from rice growth tests, in that (i) the phytostimulatory effects of Phl-sensitive strain *A. lipoferum* 4B was abolished by *Pseudomonas* co-inoculation (but Phl production ability was not the sole *Pseudomonas* factor implicated), and (ii) the Phl-resistant strain *A. irakense* KBC1 alleviated the negative effect (linked to Phl production ability) of *P. fluorescens* F113 on rice growth. The significance of Phl production ability was less clear cut on wheat, as the wheat phytostimulators *A. brasilense* Sp245 and Cd (both moderately sensitive to Phl) failed to stimulate wheat growth when in

presence of *P. fluorescens* F113, but results were essentially similar with F113G22 or F113G22(pCU203). Future work will make use of non-sterile soil conditions to enhance the ecological relevance of the findings.

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II/ Variation de phase chez *A. lipoferum* 4B et résistance au 2,4-diacétylphloroglucinol

Introduction

Les populations bactériennes peuvent être confrontées à de brusques changements des conditions de leur environnement. Le phénomène de variation de phase est l'un des procédés utilisé par les bactéries pour y faire face, car il permet d'opérer de rapides et parfois réversibles changements phénotypiques pléiotropes (Rainey et Rainey 2003; Wolf *et al.* 2005; Wisniewski-Dyé et Vial 2008). Ce procédé est ainsi utilisé par plusieurs espèces bactériennes et notamment par des PGPR (Alexandre et Bally 1999a; van den Broek *et al.* 2003; Vial *et al.* 2006b), afin de générer de la diversité au sein de leur population, ce qui leur permet d'augmenter leur capacité à coloniser différentes niches (Achouak *et al.* 2004; van den Broek *et al.* 2005). Contrairement aux mutations spontanées qui apparaissent à des fréquences de l'ordre de 10^{-8} à 10^{-6} par cellule et par génération, la variation de phase apparaît à des fréquences supérieures à 10^{-5} par cellule et par génération (Vial *et al.* 2004; Wisniewski-Dyé et Vial 2008).

Azospirillum lipoferum 4B, une souche isolée de la rhizosphère du riz (Bally *et al.* 1983), a la capacité de former *in vitro*, et à une fréquence de 10^{-3} à 10^{-4} par cellule et par génération, un variant stable nommé $4V_I$ (Alexandre et Bally 1999a; Alexandre *et al.* 1999b). Ce variant se différencie de la souche 4B par plusieurs phénotypes, tels que la perte de mobilité, une différence de fixation de certains colorants et l'incapacité à assimiler certains sucres (Alexandre *et al.* 1999b). Des réarrangements génomiques ont lieu lors de la variation de phase chez 4B, l'un des plus importants étant la perte d'un 10ème du génome de 4B, dont un plasmide de 750 kb portant le gène *acdS* codant une ACC désaminase (Vial *et al.* 2006b). Cependant le variant $4V_I$ ne semble pas perdre ses capacités de phytostimulation (Blaha *et al.* 2005). La mobilité des bactéries rhizosphériques est une propriété importante au cours des premières étapes d'interaction avec la plante, mais ne semble plus jouer de rôle prépondérant une fois que les bactéries ont adhéré aux racines (Vande Broek et Vanderleyden 1995). On peut alors penser que l'émergence d'un variant non-mobile pourrait être avantageuse lors des dernières étapes de la colonisation racinaire (Alexandre 1998).

A. lipoferum 4B est sensible au 2,4-diacétylphloroglucinol (DAPG), et des colonies de phénotype variant ont été obtenues à partir d'échantillons racinaires lors d'expériences de confrontation effectuées avec *P. fluorescens* F113 (non montré). La question du rôle de la

variation de phase dans la résistance au DAPG peut donc être posée. Cette étude a été effectuée de manière comparative, en évaluant l'impact du DAPG sur la souche 4B et sur son variant $4V_I$. Dans un premier temps, le niveau de résistance au DAPG de chacune des deux souches a été caractérisé par les concentrations seuils à partir desquelles leur croissance était entièrement inhibée (DL_{100}).

Dans un deuxième temps, les effets du DAPG sur la physiologie de la souche 4B et de son variant $4V_I$ ont été comparés. Pour cela, deux types de composants cellulaires ont été choisis, sur la base d'observations préliminaires: les granules de poly-β-hydroxybutyrate (PHB, composant intracellulaire) et les exopolysaccharides (EPS, composants extracellulaires). Le PHB est un polymère organique intracellulaire constituant une réserve de nutriments et d'énergie, et jouant un rôle écologique important lors de la colonisation de la rhizosphère (Okon et Itzigsohn 1992). Les bactéries du genre *Azospirillum* en produisent en conditions suboptimales de croissance, et il a été montré que ces composés jouaient un rôle dans la capacité de ces dernières à endurer différentes conditions de stress (Kadouri *et al.* 2003). De plus, lors des expériences de confrontations présentées précédemment, nous avons pu observer une accumulation de granules de PHB chez *A. brasilense* Cd dans les cellules en contact direct avec du DAPG. Quant aux EPS, ce sont des polymères sécrétés, de haut poids moléculaire et composés de résidus de sucres. Les EPS forment une matrice autour des cellules bactériennes, leur fournissant ainsi un microenvironnement qui va permettre une résistance accrue aux antibiotiques (Fux *et al.* 2005; Izano *et al.* 2007). Ces EPS pourraient donc moduler l'impact du DAPG.

Dans un troisième temps, nous avons effectué des expériences de confrontation sur plantes avec la souche $DAPG^+$ *P. fluorescens* F113, afin d'étudier l'impact de la capacité de production de DAPG sur les capacités phytostimulatrices des souches 4B et $4V_I$.

Matériels et méthodes

Souches bactériennes utilisées et conditions de cultures

Toutes les souches ont été cultivées en routine à 28°C avec agitation en milieu LBm (Pothier *et al.* 2007), i.e. milieu Luria-Bertani (Sambrook *et al.* 1989) contenant seulement 5 g NaCl L^{-1}. Les autres milieux utilisés ont été le milieu dépourvu d'azote NFb (Nelson et Knowles 1978), parfois supplémenté avec du Rouge Congo (0,25% v/v) en milieu solide ou avec 0,25% v/v de LBm (appelé alors milieu NFb*), le milieu King's B agar pour le dénombrement des *Pseudomonas* fluorescents (King *et al.* 1954), et enfin les milieux NAB (Alexandre 1998), et SA (Sucrose-Asparagine, Scher et Baker 1982) complémenté ou non par 100 µM de $FeCl_3$ (Cronin *et al.* 1997).

Sensibilité d'*A. lipoferum* 4B et $4V_I$ au DAPG

Un test de sensibilité au DAPG a été réalisé sur les deux souches d'*A. lipoferum* afin de caractériser leur DL_{50} et DL_{100}. 50 µl de pré-cultures en milieu NFb liquide âgées de 24 h ont été utilisées afin d'inoculer chacune des deux souches dans 40 puits d'une microplaque à 96 puits dont chaque puit contenait 150 µl de NFb liquide. Les 16 puits restants et non-inoculés constituaient les témoins. Après 24 h d'incubation, ces microcultures ont été répliquées sur milieux gélosés LBm, NAB, et SA, et SA-Fe, supplémentés par des concentrations croissantes en DAPG comprises entre 0 et 1000 µM. La solution mère de DAPG synthétique (Toronto Research Chemicals Inc., North York, Canada) a été préparée dans du méthanol à une concentration de 100 mM puis diluée successivement afin d'obtenir les différentes concentrations de DAPG voulues (10, 20, 50, 100, 200, 500 et 1000 µM). La croissance des colonies au niveau des 40 puits répliqués a été observée au bout de 72 h d'incubation à 28°C.

Cultures d'*A. lipoferum* 4B et $4V_I$ en présence de DAPG

L'effet du DAPG synthétique (Toronto Research Chemicals Inc.) sur la production de granules de PHB et la synthèse d'EPS chez *A. lipoferum* 4B et $4V_I$ a été étudié en milieu NFb. Une solution mère de DAPG a été préparée à une concentration de 100 mM dans du méthanol, puis diluée en série jusqu'à 100 µM. 500 µl de ces dilutions ont ensuite été inoculées dans 4 ml de NFb afin d'obtenir les concentrations suivantes : 10, 50, 100, 150, et 200 µM. 500 µl de méthanol ont été mis dans le témoin. 500 µl d'une même pré-culture de 24 h en NFb* ont ensuite été inoculées pour chacune des souches 4B et $4V_I$. Les cultures en

présence de DAPG ont été incubées pendant 24 h, avant d'être utilisées pour les observations physiologiques décrites par la suite.

Impact du DAPG sur la production de PHB

La production de granules de PHB chez *A. lipoferum* 4B et $4V_I$ a été étudiée à l'aide d'un fluorochrome, le Rouge du Nil (Sigma-Aldrich, Lyon, France). Le Rouge du Nil, 9-diethylamino-5H-benzo-α-phenoxazine-5-one, est un colorant qui se fixe spécifiquement aux composés intracellulaires lipidiques et donc aux granules de PHB (Degelau *et al.* 1995; James *et al.* 1999). 250 µl des cultures d'*A. lipoferum* 4B et $4V_I$ en présence de DAPG (décrites précédemment) ont été prélevés à 24 et 48 h puis mélangés à 2,5 µl de Rouge du Nil et incubés pendant 20 min à l'obscurité. La suspension a ensuite été centrifugée à 4000 *g* pendant 10 min et les culots on été repris dans du $MgSO_4$ à 10 mM afin d'éliminer l'excès de fluorochrome. 15 µl des suspensions ainsi obtenues ont été ensuite montées entre lames et lamelles et 6 champs par lamelles ont été observés afin d'avoir des observations représentatives de chaque échantillon. Les observations microscopiques ont été effectuées à l'aide d'un dispositif d'épifluorescence comprenant un filtre (Cy3) d'une longueur d'émission entre 610 et 685 nm et une longueur d'excitation entre 545 et 575 nm.

Impact du DAPG sur la production d'EPS

La synthèse d'EPS chez *A. lipoferum* 4B et $4V_I$ a été évaluée en utilisant (i) le Calcofluor, (ii) le Rouge Congo, et (iii) une méthode de floculation. Premièrement, les EPS peuvent être détectés à l'aide de Calcofluor (Sigma-Aldrich), qui se lie aux polysaccharides β-liés (Maeda et Ishida 1967) et peut donc être utilisé pour marquer les EPS chez *A. lipoferum* (Del Gallo *et al.* 1989; Michiels *et al.* 1990). Les cultures de 4B et $4V_I$ ont été marquées à l'obscurité avec du Calcofluor à 0,1% m/v (Del Gallo *et al.* 1989; Michiels *et al.* 1990) ou à 0,025% (Cowan *et al.* 2000) pendant 1 min, avant de rincer l'excès de colorant par une étape de centrifugation à 4000 *g* pendant 10 min suivie d'un rinçage au $MgSO_4$ 10 mM. Les observations microscopiques ont ensuite été effectuées à l'aide d'un dispositif d'épifluorescence comprenant un filtre (DAPI) d'une longueur d'émission de 420 nm et d'une longueur d'excitation de 365 nm.

Deuxièmement, de nombreux protocoles décrivent l'utilisation du Rouge Congo (RC) pour la détection d'EPS (Katupitiya *et al.* 1995; Merritt *et al.* 2007). En effet, la fixation de ce colorant est corrélée à la présence d'EPS chez *Azospirillum* (Rodriguez Caceres 1982;

Katupitiya *et al.* 1995; Pereg-Gerk *et al.* 1998) et plus spécialement l'espèce *lipoferum* (Del Gallo *et al.* 1989). Une gamme étalon standard de RC a été réalisée en mesurant la DO_{490} de solutions de RC aux concentrations suivantes : 0,005%, 0,004%, 0,003%, 0,002%, et 0,001%. Un aliquot de 0,5 ml de culture de 4B et $4V_I$ en présence de DAPG et âgées de 24 ou 48 h (décrites précédemment) a été prélevé afin de mesurer la DO_{600}. Le reste des cultures est centrifugé 10 min à 4000 *g*, et les culots sont re-suspendus dans 1 ml d'une solution de RC à 0.005% (w/v) puis incubées 3 h à 28°C sous agitation forte. Les cultures sont ensuite centrifugées 10 min à 4000 *g* pour culotter les bactéries et la DO_{490} du surnageant est mesurée. Les résultats sont ensuite exprimés en quantité de RC fixée par unité de DO_{600} (mg/DO_{600}).

Troisièmement, la capacité des cellules à floculer, liée à la capacité de production d'EPS (Kadouri *et al.* 2003), a été mesurée par la méthode de Madi et Denis avec quelques modifications (Burdman *et al.* 1998). Les cultures de 4B et $4V_I$ en présence de DAPG et âgées de 48 h (décrites précédemment) ont été transférées dans des tubes coniques et laissées à reposer. Après 30 min, les cellules agrégées avaient sédimenté au fond de chaque tube, et les suspensions étaient majoritairement composées de cellules libres non agrégées. La mesure de l'absorbance du surnageant (DOs) de la suspension bactérienne a alors été effectuée à 540 nm dans un spectrophotomètre. Les cultures ont ensuite été agitées au vortex afin de remettre le culot en suspension, et une deuxième lecture d'absorbance a été faite (DOt). Le pourcentage de floculation des bactéries est (DOt – DOs) × 100 / DOt.

Impact de *P. fluorescens* F113 DAPG$^+$ sur la colonisation

L'impact de la production de DAPG par *P. fluorescens* F113 sur la colonisation racinaire et les capacités phytostimulatrices de *A. lipoferum* 4B et $4V_I$ a été étudié sur riz (*Oryza sativa*) en conditions gnotobiotiques. Les inoculations sur plantes ont été réalisées à partir de cultures de 12 h en LBm (pour la souche *P. fluorescens* F113) ou en NFb* (pour les deux souches d'*Azospirillum*). Les graines de riz (cv. Cigalon) ont été fournies par le Centre Français du Riz (CFR, Camargue, France), et désinfectées en suivant le protocole décrit par Pothier *et al.* 2007 avec quelques modifications : les graines ont été immergées dans de l'éthanol à 70° pendant 3 min, avant d'être rincées par trois bains successifs d'eau déminéralisée stérile. Elles ont ensuite été immergées dans deux bains de 15 min d'hypochlorite de calcium 1%, avant d'être de nouveau rincées par trois bains successifs d'eau déminéralisée stérile. Les graines ont ensuite été plongées dans un bain de 30 min de thiosulfate de sodium 2% (Miché et Balandreau 2001). Trois bains de rinçage d'eau déminéralisée stérile ont été effectués, et les

graines ont été laissées dans le dernier bain pendant 4 h afin de favoriser leur germination. Les graines ont ensuite été placées sur des boîtes d'eau gélosée à 7,5% pendant 48 h, à l'obscurité et à 28°C. Les plantes ont ensuite été inoculées par une souche de *Pseudomonas* et une souche d'*Azospirillum*, une seule souche, ou bien n'ont pas été inoculées. Les inoculations d'*Azospirillum* ont été réalisée dans la gélose, en mélangeant 500 µl de suspension contenant 10^8 CFU avec 50 ml de gélose à 7,5% en surfusion, avant de couler les géloses dans des boites carrées (12 cm × 12cm). Les plantes germées ont ensuite été placées près du bord de ces boîtes, et les inoculations de *Pseudomonas* ont ensuite été réalisées par dépôt de 100 µl de suspension contenant 10^6 CFU. Six boîtes contenant chacune quatre plantes ont été utilisées par traitement. Les boîtes ont été placées dans un phytotron réglé à 75% d'humidité, avec 16 h de lumière (63 $\mu E/m^2/s$) à 26°C et 8 h d'obscurité à 18°C, et ont été échantillonnées 10 jours plus tard. Quatre plantes provenant chacune d'une boîte différente ont été utilisées pour les dénombrements par CFU pour chaque traitement. L'ensemble des plantes, soient 24 plantes par traitement, ont ensuite été analysées à l'aide du logiciel WinRhizo (Régent Instruments Inc., Québec, Canada), avant d'être placées 24 h dans une étuve réglée à 70°C pour déterminer les poids secs.

Tableau 1 : Sensibilité d'*A. lipoferum* 4B et son variant $4V_I$ au DAPG synthétique

	DL_{50}*				DL_{100}*			
Souches	**LBm**	**NAB**	**SA**	**SA-Fe**	**LBm**	**NAB**	**SA**	**SA-Fe**
A. lipoferum 4B	200-300	150-200	200-300	200-300	300	300	400	300
A. lipoferum $4V_I$	500	300-400	500-1000	500-1000	1000	400	1000	1000

* Concentration minimale de DAPG synthétiques nécessaire pour inhiber la croissance bactérienne dans au moins 50 % (DL_{50}) ou 100 % (DL_{100}) des réplicats. Les différentes concentrations testées sont : 10, 20, 50, 100, 200, 500 et 1000 µM.

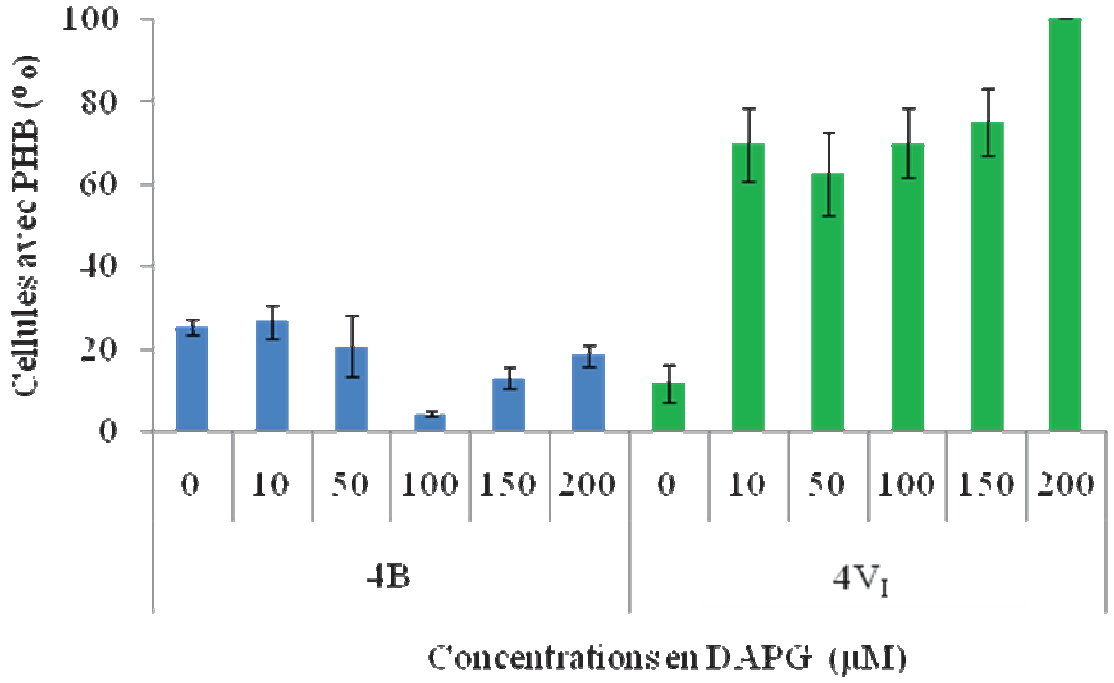

Figure 1 : Proportion de cellules présentant des inclusions de PHB (en % des cellules totales) chez *A. lipoferum* 4B et son variant $4V_I$ (moyenne +/- erreur standard, n = 6). Observations en microscopie à épifluorescence sur des cultures de 24h en présence de concentrations croissantes en DAPG (µM). Aucune différence significative entre les traitements selon le test ANOVA ($P > 0,05$).

Résultats

Sensibilité d'*A. lipoferum* 4B et $4V_I$ au DAPG

Les DL_{50} de 4B varient entre 200 et 300 µM, alors que celles de $4V_I$ varient de 500 à 1000 µM selon les milieux testés (i.e. LBm, NAB, SA et SA-Fe) (Tableau 1). Les mêmes différences sont observées en ce qui concerne les DL_{100}, qui sont plus de deux fois plus faibles chez 4B par rapport à $4V_I$ pour les milieux LBm, SA et SA-Fe (Tableau 1). Sur milieu NAB, la résistance de $4V_I$ est nettement atténuée (DL_{100} de 400 µM contre 1000 µM sur les trois autres milieux testés) et la différence de résistance entre $4V_I$ et 4B est donc beaucoup plus faible sur ce milieu (300 et 400 µM pour respectivement 4B et $4V_I$). La composition des milieux de culture semble aussi avoir une incidence sur la capacité de résistance de la souche 4B : la présence de fer dans le milieu SA diminue la résistance (DL_{100} de 400 µM sur SA contre 300 µM sur SA-Fe). Cet impact du fer n'a pas été observé pour le variant $4V_I$. Les résultats obtenus lors de ce test ont été confirmés par deux autres répétitions sur les milieux LBm et SA-Fe.

Impact du DAPG sur la production de PHB

Nous avons pu obtenir une mesure reproductible pour $4V_I$ mais pas pour 4B. Dans les trois répétitions, 100% des cellules de $4V_I$ confrontées à 200 µM DAPG pendant 24 h présentaient des granules de PHB (Figure 1). Ce pourcentage était plus faible à des concentrations moindres (Figure 1) ou si l'incubation était de 48 h.

Impact du DAPG sur la production d'EPS

Les observations réalisées à l'aide du Calcofluor à 0,1 ou 0,025% n'ont pas permis de mettre en évidence de différences entre 4B et son variant $4V_I$ aux différentes concentrations en DAPG testées, ce qui nous a conduit à utiliser le test de fixation du RC et le test de floculation.

Les résultats obtenus par la fixation du RC sur une culture de 24 h ont montré deux différences entre 4B et $4V_I$: (i) de 0 à 100 µM DAPG, aucun impact n'est observé chez $4V_I$ tandis que la fixation de RC est quasiment multipliée par deux dès 10 µM chez 4B, (ii) à 200 µM une diminution de la fixation du RC de plus de moitié a été observée chez $4V_I$ tandis que la croissance de 4B est déjà inhibée (Figure 2). Ces résultats n'ont cependant pas pu être confirmés lorsque l'expérience a été refaite. La seule observation reproductible a été

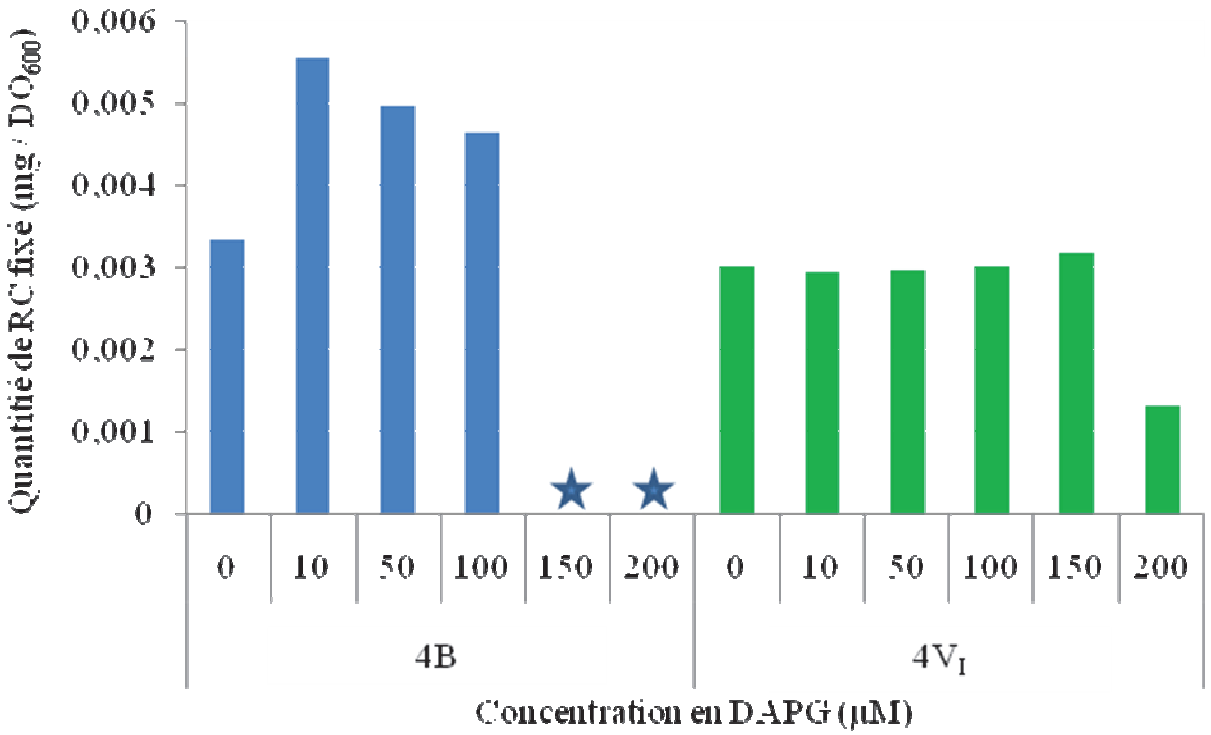

Figure 2 : Test de fixation du Rouge Congo (RC) réalisé sur des cultures d'*A. lipoferum* 4B et son variant $4V_I$, après 24h d'incubation en présence de différentes concentrations en DAPG synthétique (moyenne +/- erreur standard, n = 4). Les ★ marquent l'inhibition de croissance de 4B aux concentrations de 150 et 200 µM. Aucune différence significative entre les traitements selon le test ANOVA ($P > 0,05$).

l'inhibition de croissance de 4B à 150 et 200 µM DAPG (croissance 20 fois moins importante à ces concentrations). Cette inhibition, combinée à une fixation de RC légèrement diminuée par rapport aux autres conditions (quantité de RC fixé de 0,03 contre 0,04 pour les concentrations plus faibles), explique les pourcentages de fixation multipliés d'un facteur 10 observés chez 4B aux concentrations de 150 et 200 µM (données non montrées).

Lors des tests de floculation, des problèmes de reproductibilité ont aussi été rencontrés, mais nous avons néanmoins pu dégager deux observations reproductibles : aucune variation de la floculation n'est observée chez $4V_I$ aux différentes concentrations testées, et une augmentation de la floculation d'un facteur de 2 à 3 est observée chez 4B à 50 et 100 µM de DAPG (Figure 3). Aucune différence significative n'a pu être montrée entre 4B et $4V_I$, aux différentes concentrations en DAPG testées.

Impact de *P. fluorescens* F113 DAPG$^+$ sur la colonisation racinaire

La souche *P. fluorescens* F113 DAPG$^+$ n'a pas eu d'impact négatif significatif sur les populations racinaires des deux souches d'*Azospirillum* dénombrées 10 jours après co-inoculation. Un effet positif et significatif de la co-inoculation de 4B avec F113 a même été observé sur leurs populations respectives par rapport aux inoculations simples (Figure 4).

Concernant la phytostimulation des plantes co-inoculées, F113 n'a pas eu d'impact significatif sur les capacités phytostimulatrices de 4B sur l'ensemble des paramètres racinaires morphologiques (Figures 5 et 6) et sur les poids secs (Figure 7). Une légère augmentation (non significative) a été observée sur la longueur racinaire totale et le nombre des racines (Figure 5AB). Par contre, F113 a aboli l'effet phytostimulateur du variant $4V_I$ en terme de surface et de volume racinaires (Figure 6CD), et de poids sec des feuilles (Figure 7A). La présence de F113 a tendance à diminuer la dimension fractale des systèmes racinaires, mais cet effet n'était pas significatif (Figure 5C).

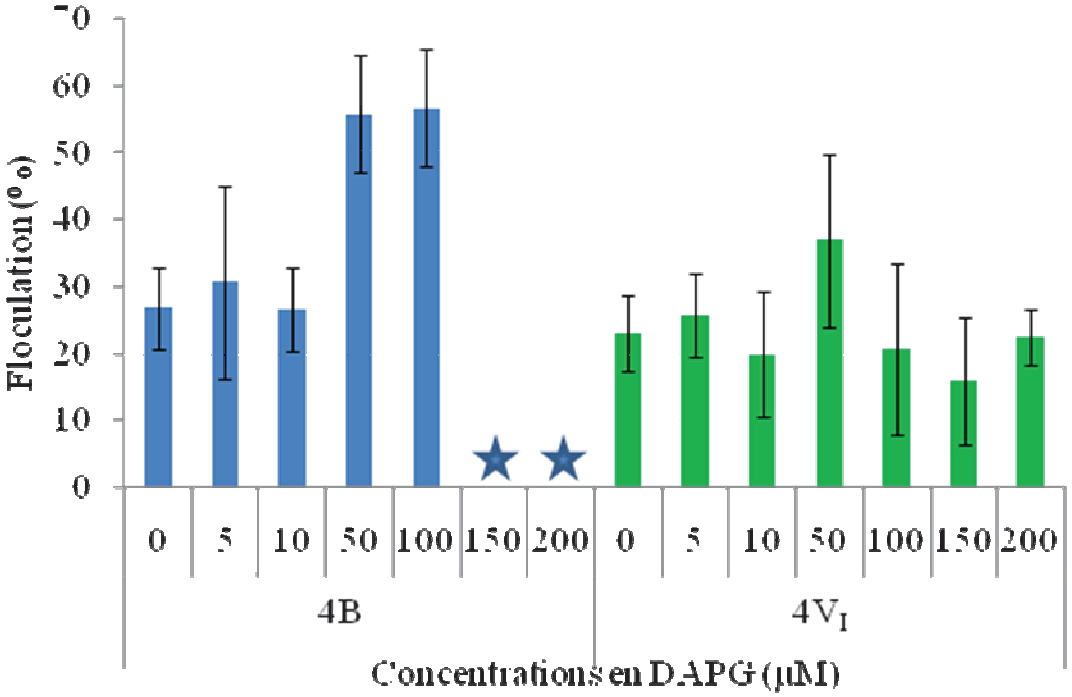

Figure 3 : Test de Floculation réalisé sur des cultures d'*A. lipoferum* 4B et de son variant $4V_I$ après 48 h en présence de différentes concentrations en DAPG (moyenne +/- écart type, n = 3). Les ★ marquent l'inhibition de croissance de 4B aux concentrations de 150 et 200 µM. Aucune différence significative entre les traitements selon le test ANOVA ($P > 0,05$).

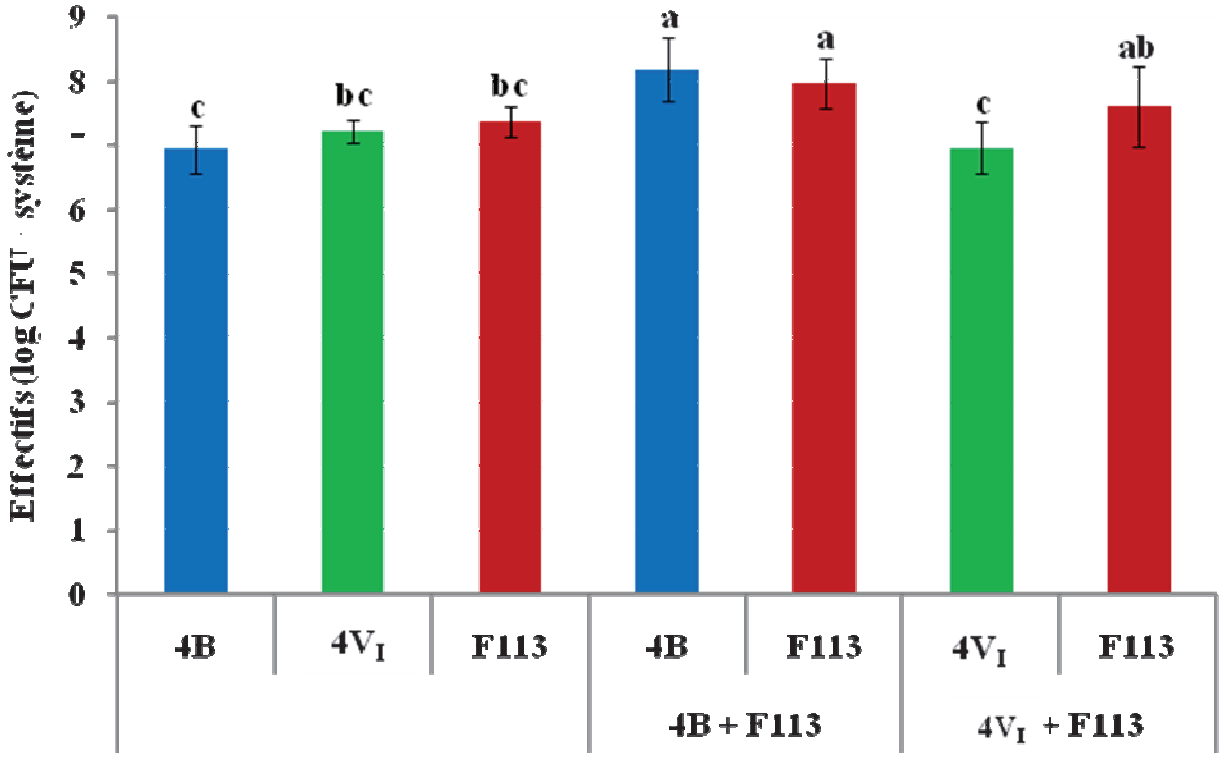

Figure 4 : Effet de *P. fluorescens* F113 DAPG$^+$ sur la colonisation racinaire du riz par *A. lipoferum* 4B et son variant 4V$_I$ (moyenne +/- erreur standard ; n = 4). Les lettres en gras indiquent les différences significatives entre traitements (ANOVA et test de Fisher LSD ; $P <$ 0,05).

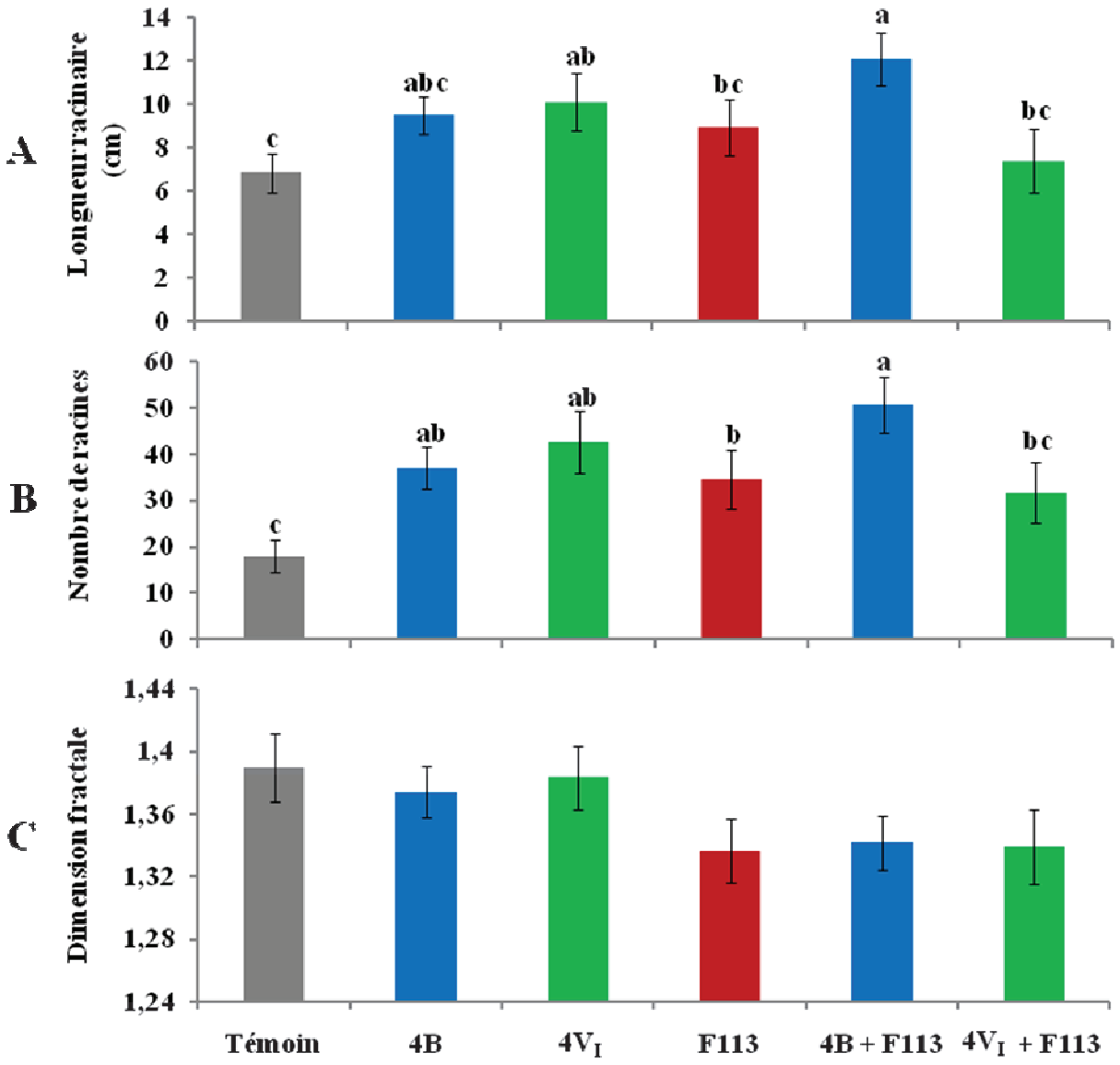

Figure 5 : Analyses morphologiques réalisées à l'aide du logiciel WinRhizo sur différents paramètres tels que: **A**. la longueur racinaire, **B**. Le nombre de racines et **C**. La dimension fractale (moyenne +/- erreur standard ; n = 24). Les lettres en gras indiquent les différences significatives entre traitements (ANOVA et test de Fisher LSD ; $P < 0,05$).

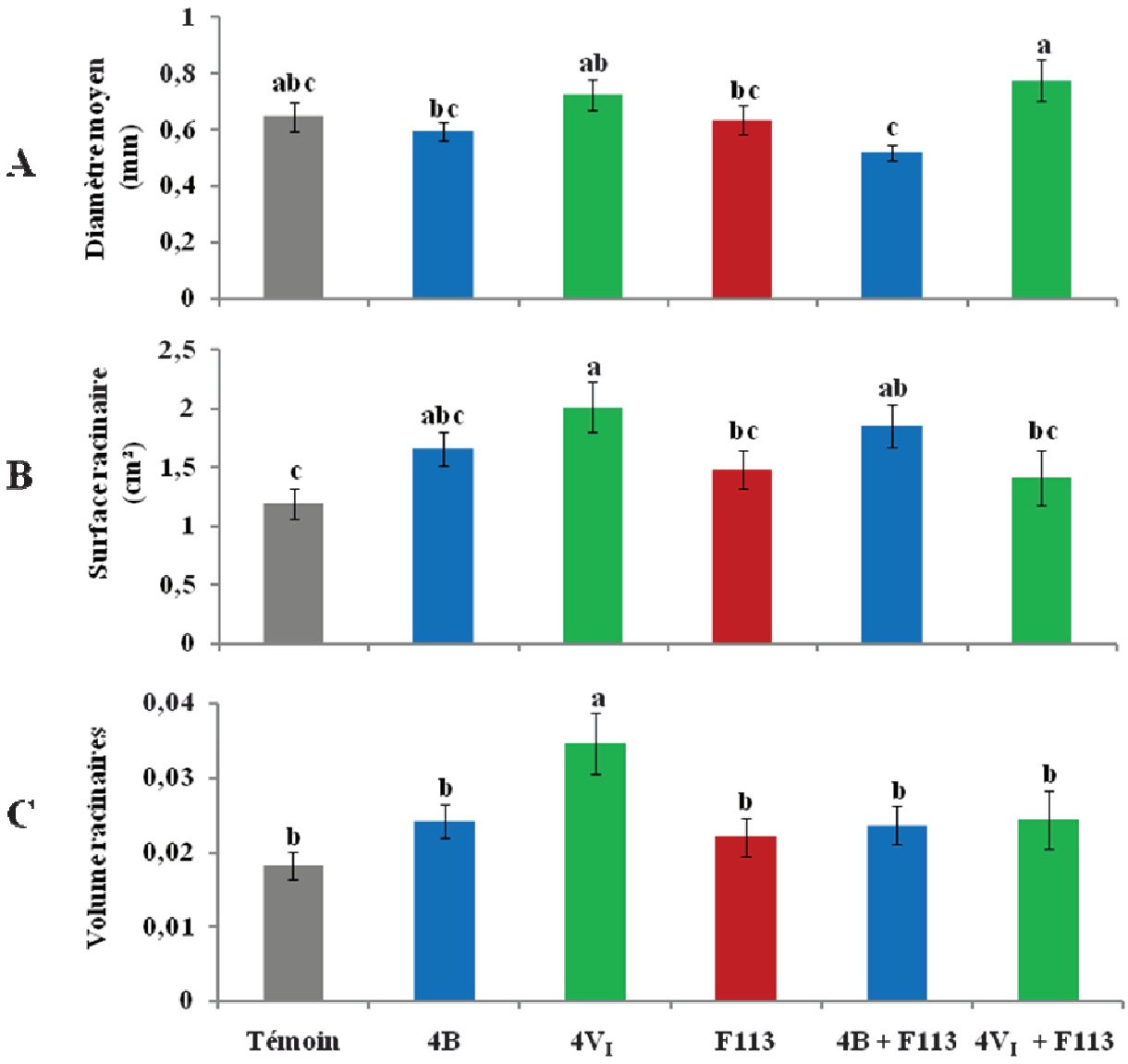

Figure 6 : Analyses morphologiques réalisées à l'aide du logiciel WinRhizo sur différents paramètres tels que: **A**. la diamètre moyen, **B**. la surface racinaire et **C**. le volume racinaire (moyenne +/- erreur standard ; n = 24). Les lettres en gras indiquent les différences significatives entre traitements (ANOVA et test de Fisher LSD ; $P < 0,05$).

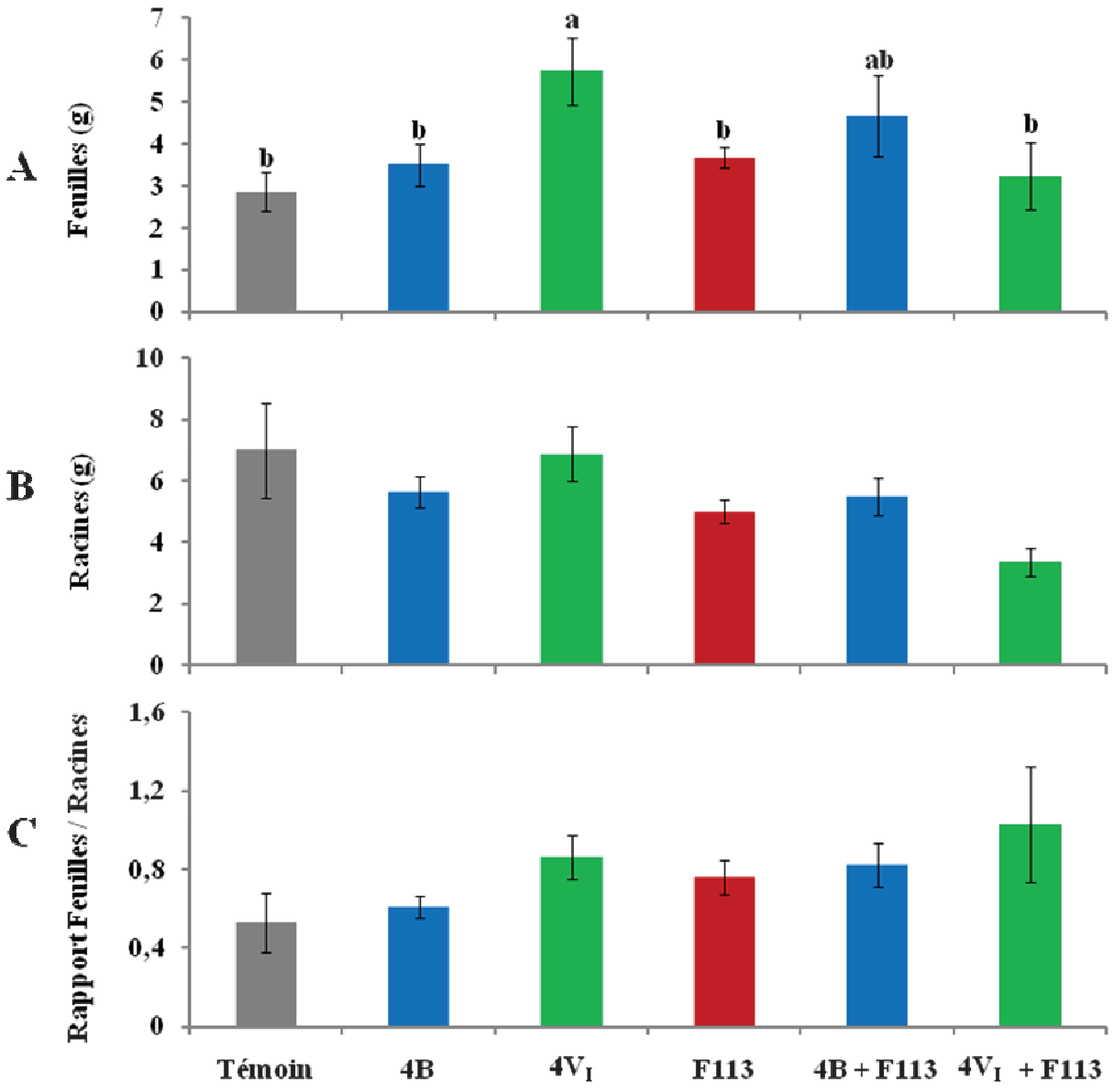

Figure 7 : Poids secs pesés après 24 h à 70°C pour **A**. les parties aériennes, **B**. les parties racinaires, et **C**. le rapport Feuilles / Racines. (moyenne +/- erreur standard ; n = 24). Les lettres en gras indiquent les différences significative entre traitements dans le cas des poids secs des parties aériennes (ANOVA et test de Fisher LSD ; $P < 0,05$).

Discussion

Les résultats indiquent que le variant *A. lipoferum* $4V_I$ présente une plus forte résistance au DAPG par rapport à la souche sauvage 4B sur l'ensemble des milieux testés. Il est cependant important de noter que les écarts importants entre les DL_{100} établies pour les deux souches sur les milieux LBm, SA, et SA-Fe s'expliquent en partie par le fait qu'aucune concentration intermédiaire n'a été testée entre 500 et 100 µM. L'origine de la résistance accrue du variant n'est pas connue.

Un des phénotypes distinguant le variant $4V_I$ de sa souche sauvage 4B est la différence de fixation de certains colorants (Alexandre *et al.* 1999b), qui peut être liée à la synthèse de composés extracellulaires tels que les EPS chez l'espèce *lipoferum* (Del Gallo *et al.* 1989), qui pourraient protéger physiquement la cellule bactérienne. La comparaison des niveaux de synthèse d'EPS entre 4B et $4V_I$ n'a bien fonctionné qu'avec la méthode utilisant le RC. Une diminution de la fixation du RC a été observée chez $4V_I$ à 200 µM de DAPG, ce qui suggère que le DAPG a diminué la production d'EPS chez $4V_I$. Néanmoins, cette diminution n'a pas été observée lors des tests de floculation. Or la fixation du RC est liée à une spécificité d'interaction entre certains EPS et le colorant, alors que la mesure de la capacité de floculation prend en compte l'ensemble des polysaccharides produits par les bactéries. Nous pouvons donc penser que la diminution de fixation du RC ne correspond pas à une diminution de production d'EPS, mais peut-être à un changement dans la nature des EPS. Une augmentation de la fixation de RC corrélée à une augmentation du pourcentage d'agrégation a pu être observée chez 4B en présence de 50 et 100 µM de DAPG. Cette augmentation correspond peut-être à un mécanisme de résistance, afin de diminuer la diffusion du DAPG. Aux concentrations de 150 et 200 µM, des augmentations de fixation du RC d'un facteur 10 ont été observées chez 4B, mais ces résultats n'ont pas pu être confirmés par le test d'agrégation.

Les mêmes problèmes de reproductibilité ont été rencontrés lors de l'étude des granules de PHB chez *A. lipoferum* 4B et $4V_I$. Les fluctuations d'une répétition à l'autre étaient déjà importantes après seulement 4 h d'incubation (données non montrées). Nous n'avons pas réussi à calibrer les conditions expérimentales des cultures sur lesquelles nous avons effectué nos observations, bien qu'elles aient été effectuées dans des conditions très proches (mêmes agitateurs, même chambre chaude, même lot de DAPG, etc.). La seule observation solide était une forte production de PHB chez $4V_I$ (100% des cellules ont présenté des inclusions de PHB) à 200 µM DAPG, qui n'a pas eu lieu chez 4B. Il est donc possible

d'affirmer que la production de PHB coïncide avec une résistance accrue au DAPG chez $4V_I$ comparé à 4B.

Malgré une plus forte résistance du variant $4V_I$ au DAPG synthétique par rapport à sa souche sauvage 4B, les résultats de confrontations sur plante indiquent que la souche 4B persiste mieux que son variant $4V_I$ en présence de la souche *P. fluorescens* F113 productrice de DAPG. Il faut cependant noter que le système expérimental utilisé ici est plus adapté à la mesure d'effets phytostimulateurs qu'à des expériences de confrontations entre souches bactériennes, *Azospirillum* ayant été inoculé à des effectifs environ cent fois plus importants que la souche F113 $DAPG^+$. Cette différence au niveau des effectifs inoculés pourrait expliquer les différences de résultats obtenus lors des expériences de confrontations présentées dans la partie précédente (où chaque souche était inoculée à des effectifs de 10^6 bactéries par système racinaire). Néanmoins, les résultats de dénombrement obtenus ici mettent en évidence une meilleure capacité de la souche 4B à cohabiter avec F113 que son variant $4V_I$. Cela est d'ailleurs confirmé par les résultats de phytostimulation.

Par ailleurs, on note que dans les conditions expérimentales utilisées ici, l'effet phytotoxique de *P. fluorescens* F113 sur le riz (présenté dans la partie précédente) ne s'est pas manifesté. L'analyse des données de dimension fractale indique une tendance à une moindre ramification des systèmes racinaires dans les traitements comprenant la souche F113, mais cette tendance n'était pas significative.

En conclusion, le variant $4V_I$ présente *in vitro* une plus grande résistance au DAPG par rapport à sa souche sauvage 4B, qui coïncide avec sa capacité à produire plus de granules de PHB à fortes concentrations en DAPG. Cette résistance accrue ne semble pas liée à la production d'EPS. Néanmoins, l'effet de F113 + 4B sur la plante était supérieur à celui de 4B seul, ce qui n'était pas le cas avec $4V_I$; une résistance accrue au DAPG ne semble donc pas une propriété importante dans les conditions expérimentales choisies.

Partie Expérimentale 2
Mise au point d'outils de PCR quantitative en temps-réel pour le suivi d'*Azospirillum*

Introduction

L'étude de la compatiblité des PGPR des genres *Pseudomonas* et *Azospirillum* dans les conditions naturelles de l'interaction entre les PGPR inoculées et leur plante-hôte nécessite l'utilisation d'outils permettant le suivi des souches introduites, lors de leur colonisation des plantes cultivées en sol agricole non stérile. Plusieurs articles ont fait la synthèse de l'ensemble des techniques disponibles pour la quantification d'inocula bactériens (marquage génétique, sonde moléculaire, PCR quantitative, etc. ; Jansson et Prosser 1997; Tebbe et Miethling-Graff 2006). Pour les PGPR du genre *Pseudomonas*, deux techniques de marquage ont permis la quantification de souches inoculées en sol non stérile : (i) en utilisant des mutants spontanés résistants à la rifampicine (Troxler *et al.* 1997; Barea *et al.* 1998; Moënne-Loccoz *et al.* 2001; Villacieros *et al.* 2003) ou (ii) en utilisant un système de marquage métabolique par insertion d'une cassette contenant les gènes *lacZY* (Andrade *et al.* 1998; De Leij *et al.* 2002). Ces techniques comportent cependant deux inconvénients: (i) des processus physiologiques majeurs peuvent être affectés lors de la mutation spontanée ou l'insertion des marqueurs et (ii) les marqueurs introduits peuvent constituer un fardeau métabolique qui va affecter le fonctionnement de la cellule bactérienne. Pour les PGPR du genre *Azospirillum*, différents anticorps et sondes moléculaires souche-spécifiques ont été utilisés au cours d'expériences d'hybridations fluorescentes *in situ* (technique FISH ; Aßmus *et al.* 1997; Rothballer *et al.* 2003). Ces techniques ne permettent cependant que la détection des inocula d'*Azospirillum* et leur quantification n'a pu être effectuée qu'en couplant l'utilisation de techniques de dénombrement classique sur milieux semi-sélectifs avec des techniques basées sur la PCR (ARDRA :Russo *et al.* 2005) ou l'hybridation de sondes nucléiques radio-marquées (El Zemrany *et al.* 2006).

Les méthodes de quantification souche-spécifique des PGPR *Pseudomonas* et *Azospirillum* reposent à l'heure actuelle principalement sur des techniques de dénombrement sur milieux sélectifs ou semi-sélectifs, qui sont lourdes et fastidieuses à mettre en place pour l'analyse à haut débit d'un grand nombre d'échantillons. Le dévelopement d'outils de PCR quantitative pour le suivi des souches des PGPR *Pseudomonas* et *Azospirillum* représente une alternative intéressante, spécialement pour des souches de PGPR utilisées dans un contexte d'applications agronomiques. Des outils de PCR quantitative de type compétitive ont déjà été développés pour *Pseudomonas* sp. CHA0 (Rezzonico *et al.* 2003) et *P. fluorescens* Pf153 (Gobbin *et al.* 2007). Pour la souche *P. fluorescens* F113 (utilisée dans la partie expérimentale 3), la mise au point et la validation d'un outil de PCR quantitative en temps réel a été

effectuée par Andreas von Felten, du laboratoire de G. Défago (ETH, Zurich), dans le cadre d'une collaboration au sein du projet MicroMaize.

Cette partie décrit la mise au point et la validation d'outils de PCR quantitative en temps réel pour trois souches d'*Azospirillum* d'intérêt agronomique : *A. lipoferum* CRT1, qui a été commercialisé sous le nom d'AZOGREEN-m® par la société Merck/Lipha (Meyzieu, France), et *A. brasilense* UAP-154 et CFN-535, toutes deux utilisées pour l'inoculation de centaines de milliers d'hectares de céréales au Mexique (Dobbelaere *et al.* 2001; Fuentes-Ramirez et Caballero-Mellado 2006). Le dévelopement des outils de PCR quantitative en temps réel passe par la recherche et le séquençage de marqueurs génétiques souche-spécifiques. Trois approches ont été successivement utilisées, qui font chacune l'objet d'un manuscrit: la première repose sur l'identification de marqueurs souche-spécifiques à partir de fragments générés aléatoirement par des PCR de types RAPD, BOX ou ERIC (Fani *et al.* 1993; Fancelli *et al.* 1998), la deuxième repose sur l'utilisation de la région intergénique située entre les ADN ribosomaux 16S et 23S, qui semble être suffisamment variable pour potentiellement discriminer des souches au sein d'une même espèce bactérienne (Gürtler et Stanisich 1996; Buchan *et al.* 2001; Sadeghifard *et al.* 2006), et la troisième repose sur l'utilisation d'un fragment souche-spécifique de 1,4 kb déjà utilisé pour l'identification de la souche CRT1 (Jacoud *et al.* 1998; Jacoud *et al.* 1999; El Zemrany *et al.* 2006).

La première approche a été appliquée avec succès pour les souches *A. brasilense* UAP-154 et CFN-535, et nous a permis de mettre au point des amorces souche-spécifiques ainsi que deux outils de PCR quantitative en temps réel, qui ont fait l'objet d'un manuscrit soumis dans la revue scientifique *Journal of Applied Microbiology*. Cette approche n'a cependant pas aboutie avec la souche *A. lipoferum* CRT1. Une deuxième approche a alors été suivie, et l'étude de l'intergène 16S-23S de l'ANDr pour la mise au point d'amorces souche-spécifiques a été effectuée dans notre équipe par Ezekiel Baudoin. Cependant, après le criblage d'une collection d'*Azospirillum* spp., nous avons montré des amplifications aspécifiques chez quelques souches. L'application de cette approche reste néanmoins possible pour la détection de la souche CRT1 inoculée en sol non stérile et a fait l'objet d'une publication dans la revue scientifique *Journal of Applied Microbiology* (Baudoin *et al.* 2009a ; Annexe 1). La troisième approche a finalement été envisagée et des amorces souche-spécifiques ont été conçues sur la base d'un fragment spécifique à la souche CRT1 identifié par Jacoud et collaborateurs en 1998. De nombreuses optimisations ont été nécessaires afin de rendre l'outil suffisamment spécifique en sol non stérile. Cette méthode fait l'objet d'un

manuscrit qui sera soumis dans une revue scientifique. Les manuscrits détaillant la première et la troisième approche sont présentés successivement dans cette partie.

I/ Assessment of SCAR markers to design real-time PCR primers for rhizosphere quantification of *Azospirillum brasilense* phytostimulatory inoculants of maize

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Abstract

Aims: To assess the applicability of Sequence Characterized Amplified Region (SCAR) markers obtained from BOX, ERIC and RAPD fragments to design primers for real-time PCR quantification of the phytostimulatory maize inoculants *Azospirillum brasilense* UAP-154 and CFN-535 in the rhizosphere.

Methods and Results: Primers were designed based on strain-specific SCAR markers and were screened for successful amplification of target strain and absence of cross-reaction with other *Azospirillum* strains. The specificity of primers thus selected was verified under real-time PCR conditions using genomic DNA from strain collection and DNA from rhizosphere samples. The detection limit was 60 fg DNA with pure cultures, and 4×10^3 (for UAP-154) and 4×10^4 CFU g^{-1} (for CFN-535) in the maize rhizosphere. Inoculant quantification was effective from 10^4 to 10^8 CFU g^{-1} soil.

Conclusion: BOX-based SCAR markers were useful to find primers for strain-specific real-time PCR quantification of each *A. brasilense* inoculant in the maize rhizosphere.

Significance and Impact of the Study: Effective root colonization is a prerequisite for successful *Azospirillum* phytostimulation, but cultivation-independent monitoring methods were lacking. The real-time PCR methods developed here will help understand the effect of environmental conditions on root colonization and phytostimulation by *A. brasilense* UAP-154 and CFN-535.

Keywords: PGPR; *Azospirillum*; Root colonization; SCAR markers; Inoculant quantification; Real-time PCR.

Introduction

Azospirillum strains are well known as Plant Growth-Promoting Rhizobacteria (PGPR). Phytostimulatory traits identified in these bacteria include free-living nitrogen fixation (James 2000), production of phytohormones (Dobbelaere *et al.* 2003) or nitric oxide (Creus *et al.* 2005), and deamination of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC; Prigent-Combaret *et al.* 2008). By stimulating root proliferation and elongation, *Azospirillum* PGPR may lead, in turn, to improved uptake of water and nutrients (Okon et Kapulnik 1986; Jacoud *et al.* 1999).

Plant growth-promoting effects of *Azospirillum* inoculants have been documented with different types of crops (often cereals), under different soil and climatic conditions, and they may lead to improved crop yield (Charyulu *et al.* 1985; Okon et Labandera-Gonzalez 1994; Dobbelaere *et al.* 2001; Pedraza *et al.* 2009). In certain countries, crop inoculation with *Azospirillum* phytostimulatory strains is receiving attention as a mean to enable a reduction in nitrogen fertilizer usage without compromising crop yield, in a context of lower-input agriculture (Fuentes-Ramirez et Caballero-Mellado 2006; El Zemrany *et al.* 2006).

One of the main conditions necessary for effective phytostimulation by *Azospirillum* is rhizosphere survival of the inoculant in sufficient numbers (Dobbelaere *et al.* 2002), even though the preliminary interaction between *Azospirillum* and seed is already important (Jacoud *et al.* 1999). This means that techniques are required for effective monitoring of inoculant cell number in the rhizosphere. However, very few tools are available to assess the fate of wild-type *Azospirillum* strains after inoculation. In the case of *Azospirillum lipoferum* CRT1, a PCR approach targeting the 16S-23S rDNA internal spacer region can be used for inoculant detection (Baudoin *et al.* 2009a). However, many important PGPR strains of *Azospirillum* belong to the *A. brasilense* species (Lucy *et al.* 2004). Strain-specific antibodies and/or molecular probes are available for identification of *A. brasilense* strains Wa3 (Aßmus *et al.* 1997), Sp7 and Sp245 (Rothballer *et al.* 2003) by Fluorescent In Situ Hybridisation (FISH), but these tools do not allow strain-specific quantification.

Quantitative PCR approaches are promising to quantify individual strains in complex environments, and in the rhizosphere they have been implemented in the case of MPN-PCR (Rosado *et al.* 1996; Mirza *et al.* 2006) and competitive PCR (Johansen *et al.* 2002; Mauchline *et al.* 2002; Rezzonico *et al.* 2005; Gobbin *et al.* 2007). More recently, real-time PCR has become the method of choice for quantifying rhizosphere populations since it enables high specificity, sensitivity, and speed (Sørensen *et al.* 2009). This method is based

on the measurement of fluorescence generated by a fluorochrome that binds to double-stranded DNA after each PCR cycle. The cycle at which the fluorescence crosses the threshold line, known as C_T, is directly proportional to the amount of DNA present in the sample. In the rhizosphere, however, real-time PCR has only been used so far for quantification of indigenous bacterial groups (Mavrodi *et al.* 2007), and its applicability to monitor bacterial inoculant strains remains to be assessed.

The objective of this study was to develop real-time PCR protocols for strain-specific quantification of two prominent phytostimulatory *A. brasilense* PGPR, i.e. strains UAP-154 and CFN-535, which have been used in hundreds of thousands hectares as inoculants for cereals (Dobbelaere *et al.* 2001; Fuentes-Ramirez et Caballero-Mellado 2006). To this end, Sequence Characterized Amplified Region (SCAR) markers (i.e. markers corresponding to PCR amplicons of known DNA sequence) that are strain-specific were sought by random or rep-PCR genomic fingerprinting and used to develop primers, and primer pairs were screened for specificity. The primer pairs obtained were further assessed for SYBR Green-based real-time PCR quantification of the two *A. brasilense* strains in soil and the maize rhizosphere.

Table 1 Strains used in this study

Species and strains	Host plant	Origin	Reference
Azospirillum brasilense			
UAP-154 ; CFN-535	Maize	Mexico	Dobbelaere *et al.* 2001
ZN1	Maize	Pakistan	Blaha *et al.* 2006
L4	Sorghum	France	Blaha *et al.* 2006
Sp245	Wheat	Brazil	Blaha *et al.* 2006
Wb1 ; Wb3; WS1 ; WN1	Wheat	Pakistan	Blaha *et al.* 2006
PH1	Rice	France	Blaha *et al.* 2006
R5(15)	Rice	Cuba	Blaha *et al.* 2006
Cd	*Cynodon dactilon*	USA	Blaha *et al.* 2006
Sp7	Digitaria	Brazil	Blaha *et al.* 2006
NC9	Soil	Mali	Blaha *et al.* 2006
NC16	Soil	Mali	Vial *et al.* 2006b
Azospirillum lipoferum			
CRT1	Maize	France	Blaha *et al.* 2006
Br17	Maize	Brazil	Vial *et al.* 2006b
B506 ; B510 ; B518	Rice	Japan	Blaha *et al.* 2006
RSWT1	Rice	Pakistan	Blaha *et al.* 2006
TVV3	Rice	Vietnam	Blaha *et al.* 2006
4B	Rice	France	Blaha *et al.* 2006
N4	Cotton	Pakistan	Blaha *et al.* 2006
Br10	Soil	Brazil	Vial *et al.* 2006b
NC4	Soil	Mali	Vial *et al.* 2006b
Pseudomonas fluorescens			
F113	Sugar beet	Ireland	Ramette *et al.* 2003
Pf-153	Tobacco	Switzerland	Gobbin *et al.* 2007
C10-186 ; S7-29	Tobacco	Switzerland	Ramette *et al.* 2003
Q37-87	Wheat	USA	Ramette *et al.* 2003
K94-41	Cucumber	Czech Republic	Wang *et al.* 2001
P97-1	Cucumber	Bhutan	Wang *et al.* 2001
Pseudomonas sp. CHA0	Tobacco	Switzerland	Ramette *et al.* 2003
Rhizobium etli CFN-42	Bean	Mexico	Romero *et al.* 1991
Agrobacterium tumefaciens C58	Prunus	USA	Blaha *et al.* 2006

Table 2 Primers used in the study

PCR type and primers	Sequence	Reference
BOX PCR		
BOX-A1R	CTACGGCAAGGCGACGCTGACG	Versalovic *et al.* 1998
ERIC PCR		
ERIC 1R	ATGTAAGCTCCTGGGGATTCAC	Rademaker *et al.* 1998
ERIC 2	AAGTAAGTGACTGGGGTGAGCG	Rademaker *et al.* 1998
RAPD analysis		
Primer 1253	GTTTCCGCCC	Fancelli *et al.* 1998
Real-time PCR CFN-535		
F12*	AAGCGATCCCGACCTTGAGGCA	This work
F24 *	TGTCGATGCCGACAGGCTTGACCA	This work
Real-time PCR UAP-154		
U2 *	TGACGGCCAACACCAACGACTC	This work
U7 *	TGCCGTCGATGAACGACGCCATCTG	This work

* Primers designed based on a BOX SCAR marker.

Material and Methods

Bacterial strains

All *Azospirillum* strains (Table 1) were routinely grown at 28°C with shaking in N-free NFb medium (Nelson et Knowles 1978) supplemented with 2.5% v/v LBm (i.e. Luria-Bertani medium containing only 5 g NaCl l^{-1} ; Pothier *et al.* 2007). *Pseudomonas* strains were grown in LBm, *Agrobacterium* in LPG (Roy *et al.* 1982) and *Rhizobium* in YEM (Vincent 1970). Colony counts of *Azospirillum* strains in media or gnotobiotic rhizosphere samples were performed after spreading dilutions on RC plates (Rodriguez Caceres 1982) and a 72-h incubation at 28°C.

DNA preparation

Three methods were used to obtain DNA. Genomic DNA from bacterial log cultures was extracted using Macherey & Nagel DNA Tissue kit (Düren, Germany) according to manufacturer's instructions. Rhizosphere DNA in the experiment where sterile soil was used was extracted by thermal shock (Baudoin *et al.* 2009a). The aliquots were heated for 10 min at 100°C and placed directly on ice for 5 min. DNA from the other rhizosphere samples and from bulk soil samples was extracted with the FastDNA® SPIN® kit (BIO 101 Inc., Carlsbad, CA). To this end, 250-300 mg samples (described below) were transferred in Lysing Matrix E tubes from the kit, and DNA was extracted and eluted in 50 µl of sterile ultra-pure water, according to the manufacturer's instructions. DNA concentrations were assessed by OD measurements at 260 nm NanoDrop (Nanodrop technologies, Wilmington, DE).

BOX, ERIC and RAPD amplifications

Molecular profiles of strains UAP-154 and CFN-535, as well as *A. brasilense* strains Cd, L4, NC9, NC16, PH1, R5(15), Sp245, Sp7, Wb1, Wb3, WN1, WS1 and ZN1 (Table 1) were generated using BOX-A1R, ERIC and RAPD primers (Table 2), as described respectively by Fancelli *et al.* 1998; Rademaker *et al.* 1998; Versalovic *et al.* 1998). For each type of PCR amplification, the profiles were compared using GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium) and subjected to clustering analysis, based on the presence/absence of bands (Jaccard similarity coefficient) and the Unweighted Pair Group Method with Arithmetic

(UPGMA) mean, using Primer v6 software (PRIMER-E, Plymouth, UK). Strain-specific bands (based on electrophoretic mobility) were identified.

Conversion of BOX, ERIC and RAPD fragments into SCAR markers

Strong strain-specific bands from BOX, ERIC and RAPD analyses that were at least 200 bp in length were excised from 1% agarose gels and purified using the Macherey & Nagel Nucleospin ExtractII kit, following the manufacturer's instructions. The purified DNA fragments were then ligated into pGEM®-T Easy vector (Promega, Madison, WI), according to manufacturer's instructions. *Escherichia coli* JM109 (Promega) was transformed with the resulting plasmids, as specified by the supplier, and grown overnight at 37°C on Luria-Bertani agar (Sambrook *et al.* 1989) supplemented with ampicillin (100 µg ml^{-1}), 0.5 mM IPTG and 80 µg X-Gal ml^{-1}. White colonies were selected for colony PCR in 50 µl of PCR mix for specific PCR amplification with universal primers M13f and M13r (Promega) to check the presence of plasmid insert. The clones selected for sequencing were grown overnight with shaking at 37°C in LB + ampicillin (100 µg ml^{-1}), and plasmids were purified using the Macherey & Nagel Plasmid kit. Plasmid inserts were then sequenced in both directions by Cogenics (Meylan, France) using M13 primers, Ampli*Taq* DNA polymerase, the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Waltham, Massachusetts, USA), and a Perkin-Elmer ABI377 sequencer FS. Sequences are available in GenBank (accessions GQ500126 and GQ500127).

Primer design

DNA sequences comparable to those of the SCAR markers were sought on the web using BLASTN at http://pbil.univ-lyon1.fr/BLAST/blast_nuc.php, with default parameters, as well as in preliminary genome sequences of *A. brasilense* Sp245. Putative strain-specific sequences, i.e. sequences without any match in sequence search were selected to design primer sets for *A. brasilense* UAP-154 and CFN-535. Primer design was done using FastPCR software (www.biocenter.helsinki.fi/bi/programs/fastpcr.htm) based on (i) an amplicon size inferior to 300 bp, and (ii) primers 18 to 22 bp in length. The Oligo 6.65 software (Molecular Biology Insights, West Cascade, CO) was then used to screen and select primer pairs for (i) high melting temperature of primers (Tm ~ 60°C, estimated using the nearest-neighbor thermodynamic method), (ii) low Tm difference between primers (ΔTm < 2°C), and (iii) lack of predicted hairpin loops, duplexes and primer-dimer formation.

Primer selection using strain collection

Primer selection was implemented based on (i) successful amplification of the target strain, and (ii) absence of cross-reaction with non-target strains. Three pools of bacterial genomic DNA were used as negative controls, i.e. an *A. brasilense* pool (13 non-target strains + UAP-154 or CFN-535 when testing respectively CFN-535 or UAP-154 primers), an *A. lipoferum* pool (11 strains), and a pool of other common rhizosphere bacteria (including the α-Proteobacteria *Rhizobium etli* and *Agrobacterium tumefaciens*, as well as Proteobacteria from other subdivision and belonging to *Pseudomonas* genus) (Table 1). A first step of primer selection was performed under qualitative PCR conditions, with about 30 ng of gDNA. A second step was performed under quantitative PCR conditions, using 30 pg of gDNA and primer concentrations ranging from 500 nM to 1 µM. To check primer specificity, the observation of melting curves (described below) was completed by agarose gel electrophoresis of real-time PCR products.

Real-time PCR conditions

Real-time PCR was done in 20 µl PCR volumes containing 10 µl LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science, Indianapolis, IN), 0.75 µM of each primer, 0.2 µl of T4 gene 32 protein (Roche Applied Science) and 2 µl of template DNA. White 96-well microplates and a LC-480 LightCycler were used (Roche Applied Science). The cycling program included a 10-min incubation at 95°C followed by 50 cycles consisting of 95°C for 30 s, 70°C for 30 s and 72°C for 30 s. Amplification specificity was studied by melting curve analysis of the PCR products performed by ramping the temperature to 95°C for 10 s and back to 65°C for 15 s followed by incremental increases of 0.1°C s^{-1} up to 95°C. Melting curve calculation and determination of Tm values were performed using the polynomial algorithm function of LightCycler Software v.1 (Roche Applied Science).

Generation of standard curves for genomic DNA

Genomic DNA from *A. brasilense* UAP-154 or CFN-535 was used to prepare ten-fold dilution series from 3×10^6 to 3×10^1 fg DNA μl^{-1} (in triplicate). Sterile water (2 µl) was used as negative control. The cycle threshold C_T, i.e. the number of PCR cycles necessary to reach the threshold fluorescence level, was automatically determined for each sample by the

LightCycler software v.1 (Roche Applied Science) based on the second derivative maximum method. A standard curve for each strain was generated by plotting the C_T number against the logarithm of bacterial DNA concentration for the three independent replicates, using LightCycler Software v.1 (Roche Applied Science). Amplification efficiency (E) was calculated from the slope of the standard curve using the formula $E = 10^{-1/slope} - 1$.

Generation of standard curves for real-time PCR quantification in maize rhizosphere

Each experiment involving real-time PCR quantification of *A. brasilense* inoculant UAP-154 or CFN-535 in the rhizosphere requires development of standard curves, which we tested using two contrasted soils. One was sampled from the loamy surface horizon of a French luvisol from a maize field at La Côte St André near Bourgoin (clay 16.2%, silt 44%, sand 40%, organic matter 2.1%, pH 7.0; El Zemrany *et al.* 2006), and the other from the sandy-clay-loam topsoil of a Mexican vertisol from a field at Zacatepec near Cuernavaca, Morelos (clay 30.9%, silt 7.3%, sand 61.8%, organic matter 1.9%, pH 7.6). To this end, Lysing-Matrix E tubes (BIO 101 Inc.) containing 250 mg lyophilized bulk soil were inoculated with one of the two *A. brasilense* strains to reach 10^3, 10^4, 10^5, 10^6, 10^7, or 10^8 CFU added per tube (three replicates per inoculation level per strain). No bacteria were added to the negative control. Colony counts were performed for each cell suspension used to generate standard curves. After 1 h of incubation at 4°C, DNA extraction was performed using the FastDNA® SPIN® kit (BIO 101 Inc.) as described above. Real-time PCR was done as described above. For each strain, a standard curve was generated for each replicate by plotting C_T number versus log CFU added per g of soil. Amplification efficiency was calculated as described above.

Standard curves were then used to estimate inoculant cell number in the rhizosphere of seed-inoculated maize plants. This was done by real-time analysis of the corresponding samples (obtained as described below) and the number of CFU in the rhizosphere was calculated from the C_T using the standard curve of the corresponding strain generated for the experiment.

Real-time PCR quantification of *Azospirillum* inoculants in the rhizosphere

Seeds of maize (*Zea mays*) hybrid PR37Y15 (Pioneer Hi-Bred International, Johnston, IA) were surface-sterilized by stirring in sodium dichloroisocyanurate-containing Bayrochlor Mini solution (Bayrol, Dardilly, France) for 15 min. Seeds were then washed 4-6 times with sterile water and germinated on water agar (8 g l^{-1}) for 2 d in the dark at 28°C. For each of the

two *A. brasilense* strains, cells from overnight NFb liquid cultures were collected by centrifugation at 4000 *g* for 10 min, gently washed and resuspended in 10 mM $MgSO_4$ solution to obtain 10^8 CFU per ml. Germinated maize seeds were inoculated by soaking for 1 h in one of the bacterial suspensions. Sterile water was used for the negative control. One maize seedling was placed in each pot, which contained 600 g sieved (at 5 mm) non-sterile soil from La Côte St André (4 pots per treatment) adjusted to 20% (w/w) water content. The pots were placed 10 d in a greenhouse with 18 h of light (under 400 W lamps; 22°C and 45-50% relative humidity) and 6 h of dark (18°C and 60-65% relative humidity), and fit with an automated irrigation system.

At sampling, each root system was dug up and shaken vigorously to discard soil loosely adhering to the roots. Roots and tightly-adhering soil were then transferred in a 1-liter bottle containing 300 ml of sterile distilled water, and the bottles were shaken for 15 min. The soil fraction was recovered by centrifugation for 30 min at 5,600 *g* and flash-freezed in liquid nitrogen. Samples were then lyophilized for 48 h in Falcon tubes and homogenized by crushing in the tubes using a spatula, and 250 mg were used for DNA extraction, as described above.

Assessment of real-time PCR data in comparison with colony counts

A microcosm experiment was performed under axenic conditions to compare real-time PCR data with colony counts. Maize seeds (cv. PR37Y15) were disinfected, pre-germinated and inoculated (or treated with water), as described above, using a cell suspension containing 10^8 CFU per ml of strains UAP-154 or CFN-535 (giving respectively about 10^4 and 10^6 CFU per seed, as indicated by colony counts). Two seedlings (one of the two was used for *Azospirillum* monitoring) were then added per microcosm, which consisted of 300 g of autoclaved La Côte St André soil placed in 150-cm^3 glass bottles and adjusted to 20% (w/w) water content. Each inoculated treatment and the negative control was studied using 12 microcosms, which were placed following a randomized block design (with four blocks) in a growth chamber at 75% relative humidity, with 16 h of light (30 W lamps) at 26°C and 8 h of dark at 18°C. At 1, 2 and 3 d after inoculation, root systems were sampled (as described above) and transferred each into a 15-ml Falcon tube containing 5 ml of 10 mM $MgSO_4$ solution. After high-speed vortexing (5 min), 1 ml from each of the 36 samples (3 treatments × 4 replicates × 3 samplings) was characterized by real-time PCR and colony counts, as described above.

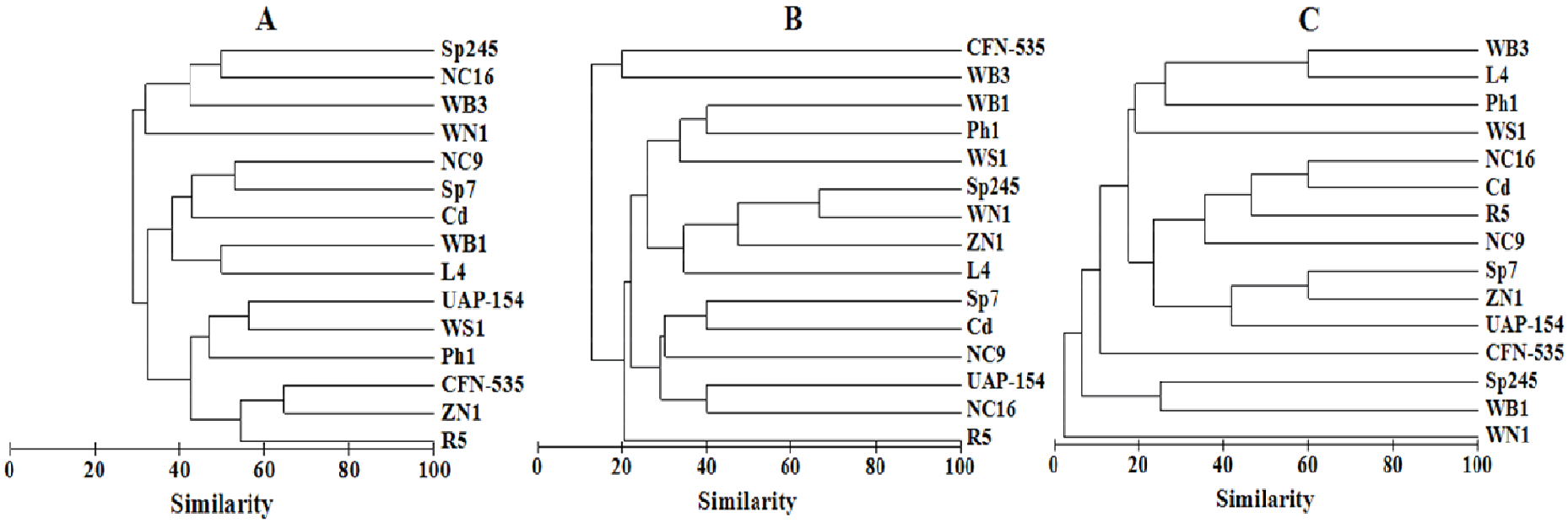

Figure 1 Genetic similarity of *A. brasilense* UAP-154 and CFN-535 and 13 other *A. brasilense* strains based on analysis of (A) BOX, (B) ERIC and (C) RAPD molecular profiles. The UPGMA clustering method was applied to a similarity matrix generated by GelCompar II software (Applied Maths) and calculated with the Jaccard coefficient.

Statistics

Greenhouse and growth chamber experiments followed a randomized block design. Colony counts were expressed as log CFU per root system or per g of dry root, and real-time quantification data were converted to log CFU equivalents per root system or per g of lyophilized soil. The relation between log CFU data and C_T values was assessed by (i) regression analysis when assessing standard curves, and (ii) correlation analysis for the comparison of real-time PCR data with colony counts. Statistical analyses were performed at $P < 0.05$, using S plus software (TIBCO Software Inc., Palo Alto, CA).

Table 3 Selection of SCAR markers for *A. brasilense* UAP-154 and CFN-535.

PCR type	Band number	Strain-specific bands*	Bands sequenced†	Bands kept after sequence analysis
Strain UAP-154				
BOX PCR	11	6	5	2
ERIC PCR	8	2	2	0
RAPD analysis	5	3	2	2
Strain CFN-535				
BOX PCR	11	6	4	2
ERIC PCR	7	4	2	2
RAPD analysis	4	1	0	0

*Strain-specific bands based on electrophoretic migration comparison in the collection of 15 *A. brasilense* strains.
†Strain-specific bands were sequenced provided they were (i) bright in agarose gel (facilitating purification) and (ii) at least 200 bp in length.

Results

Molecular comparison of *Azospirillum* strains and identification of SCAR markers

Molecular profiles of *A. brasilense* UAP-154 and CFN-535 generated 11 BOX PCR bands each, and respectively 8 and 7 ERIC PCR bands and 5 and 4 RAPD bands (Table 3). The profiles readily distinguished between the two strains, regardless of the method (Fig. 1). Based on size comparison with the entire collection of 26 *Azospirillum* strains (for each profiling method), only 11 of the 24 bands obtained for strain UAP-154 were specific of that strain, whereas another 11 bands out of the 22 CFN-535 bands were specific of strain CFN-535 (Table 3). A total of 9 (UAP-154) and 6 bands (CFN-535) were sequenced, but many of the SCAR markers thus obtained were discarded after *in silico* analysis due to their homology with DNA sequences recovered from Genbank database and Sp245 genome. Finally, four strain-specific SCAR markers were identified for each strain (Table 3).

Selection of SCAR-based primers

Screening of primer pairs derived from strain-specific SCAR markers based on Tm criteria and absence of PCR-impairing structure formation gave 28 primer sets for strain UAP-154 and 21 for strain CFN-535 (Table 4). However, most primer sets were then discarded because of cross-reaction with non-target strain(s), under qualitative or quantitative PCR conditions. Only one strain-specific primer set for strain UAP-154 and another for strain CFN-535 remained (Table 2), both designed from a BOX SCAR marker (Table 4).

Table 4 Strain-specific primers selection using three pools of genomic DNA

SCAR marker used (and length [bp])	Number primer sets*	Primer sets eliminated after qualitative PCR testing			Primer sets eliminated after quantitative PCR testing		
		Against other *A. brasilense* strains†	Against other bacteria††	Number primers remaining	Against other *A. brasilense* strains	Against other bacteria	Number primers remaining
Strain UAP-154							
BOX 2 (500)	13	10	3	0	0	0	0
BOX 3 (900)	6	3	1	2	1	0	1
RAPD 1 (500)	5	5	0	0	0	0	0
RAPD 2 (800)	4	4	0	0	0	0	0
Strain CFN-535							
BOX 4 (400)	4	4	0	0	0	0	0
BOX 5 (500)	3	3	0	0	0	0	0
BOX 6 (800)	12	8	2	2	1	0	1
ERIC 3 (400)	5	3	2	0	0	0	0

* Primer sets validated *in silico*. Suitable primers were not found for a 250-bp ERIC-based SCAR marker for strain UAP-154.
† The *A. brasilense* pool is composed of the genomic DNA from 13 non-target strains + the other target strain.
†† Tests were done separately for an *A. lipoferum* pool (11 strains) and a pool of non-*Azospirillum* rhizosphere bacteria (10 strains).

Table 5 Standard curve parameters and real-time PCR amplification efficiencies

Source of DNA	Strain	Detection limit	Quantification limit	Slope	Error	Amplification efficiency (%)
Bacterial culture	UAP-154	60 fg	60 fg	-3.571	0.0179	90.6
Bacterial culture	CFN-535	60 fg	60 fg	-3.580	0.0065	90.3
La Côte St André bulk soil	UAP-154	4×10^3 CFU g^{-1}	3×10^4 CFU g^{-1}	-3.365	0.0054	98.2
La Côte St André bulk soil	CFN-535	4×10^4 CFU g^{-1}	3×10^4 CFU g^{-1}	-3.415	0.0429	96.2
Zacatepec bulk soil	UAP-154	4×10^3 CFU g^{-1}	3×10^4 CFU g^{-1}	-3.283	0.0564	100
Zacatepec bulk soil	CFN-535	4×10^4 CFU g^{-1}	3×10^4 CFU g^{-1}	-3.709	0.0178	86.1

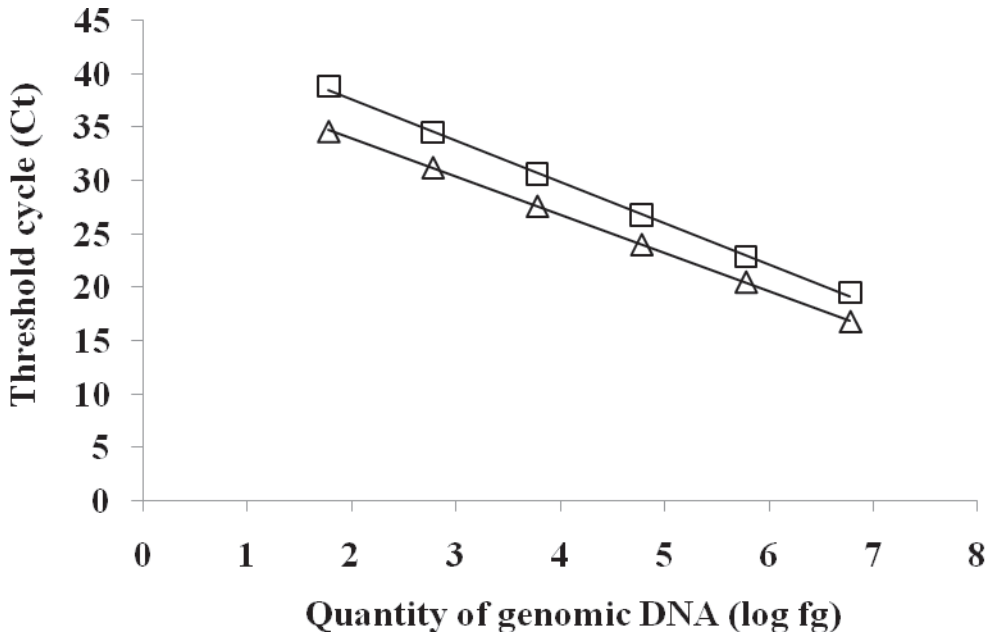

Figure 2 Real-time PCR standard curves for *A. brasilense* UAP-154 (□) and CFN-535 (Δ) *in vitro* generated by plotting C_T numbers against the quantity of genomic DNA added to the reaction mix. Means from three replicates are represented.

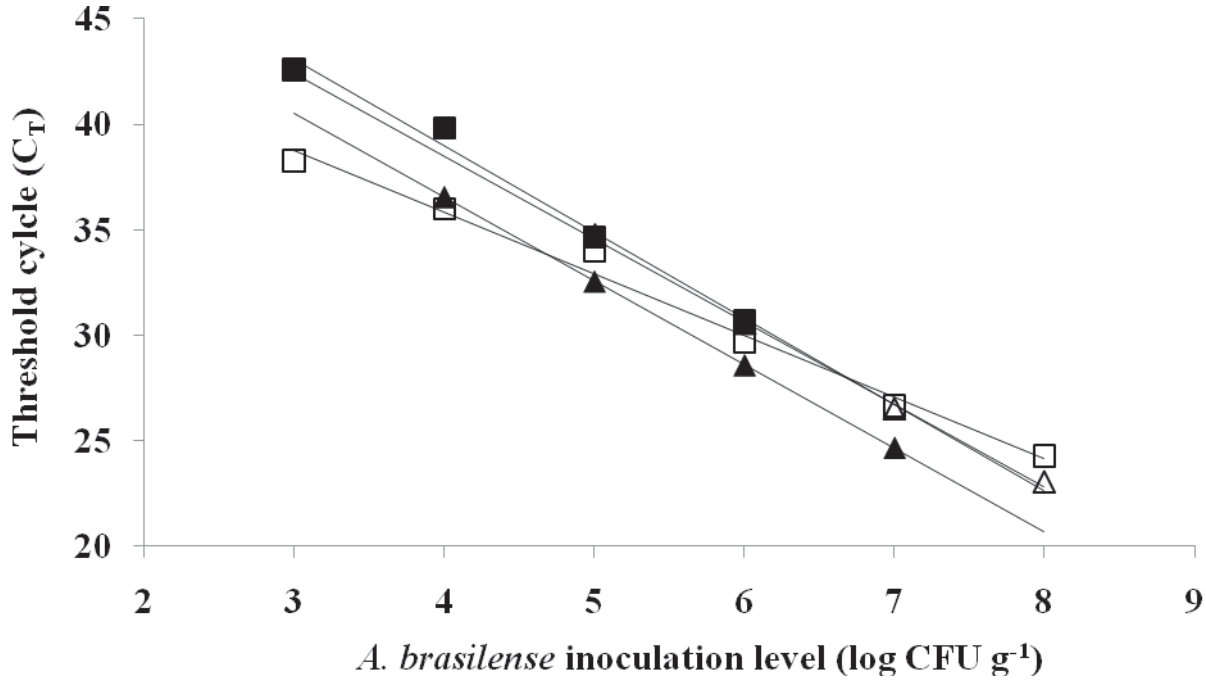

Figure 3 Real-time PCR standard curves for *A. brasilense* UAP-154 in non-sterile bulk soil from La Côte St André (■) and Zacatepec (□), and *A. brasilense* CFN-535 in non-sterile bulk soil from La Côte St André (▲) and Zacatepec (Δ). The standard curves were generated by plotting C_T numbers against the inoculation level of each *A. brasilense* strain. Means from three replicates are represented.

Real-time PCR quantification of *Azospirillum* strain in laboratory cultures

The usefulness of real-time PCR to quantify each of the two *A. brasilense* strains in laboratory cultures was assessed based on standard curves established after adding various amounts of purified genomic DNA to PCR mix. The limit of detection was 60 fg DNA for strains UAP-154 and CFN-535, corresponding respectively to 20 and 7 CFU. For each strain, R^2 values higher than 0.99 were found after regression analysis between DNA amount and C_T from real-time PCR over a range of 6 orders of magnitude (Fig. 2). Amplification efficiencies were about 90% for the two strains (Table 5).

Real-time PCR quantification of *Azospirillum* strains in the maize rhizosphere

Quantification of *A. brasilense* strains in maize rhizosphere was based on standard curves obtained with DNA extracted from bulk La Côte St André soil inoculated with 10^3 to 10^8 CFU of either strain. R^2 values higher than 0.98 were found after regression analysis between inoculation levels (as log CFU g^{-1}) and C_T from real-time PCR analysis of soil, over a log CFU range of at least 4 orders of magnitude (Fig. 3). Amplification efficiencies were above 96% (Table 5). In the maize rhizosphere, the detection limit was 4×10^3 (for UAP-154) and 4×10^4 CFU g^{-1} (for CFN-535), and the quantification limit was 4×10^3 CFU g^{-1} for both strains. Similar results were obtained when using Zacatepec soil, except that amplification efficiency with strain CFN-535 was only 86% (Table 5). For both strains, the highest amplification efficiencies were recorded when total DNA extract had been diluted 100 (La Côte St André soil) and 200 times (Zacatepec soil).

When UAP-154 and CFN- 535 strains were monitored in the rhizosphere of La Côte St André soil at 10 d, they were found at respectively 1.4 to 4.2×10^6 (mean 3.0×10^6) and 7.7 to 15×10^5 (mean 1.0×10^6) log CFU equivalents per root system, i.e. respectively 3.5 to 6.0×10^5 (mean value 4.9×10^5) and 1.3 to 2.9×10^5 (mean 1.9×10^5) log CFU equivalents per g of dry root.

Comparison of real-time PCR data with colony counts in axenic rhizosphere

The use of axenic rhizosphere microcosms enabled colony counts of the inoculants. *A. brasilense* UAP-154 and CFN-535, which were inoculated at respectively 10^6 and 10^4 CFU per seed, were recovered between 10^5 to 10^7 CFU per root system from days 1 to 3 after inoculation. When the same samples were studied by real-time PCR, a significant correlation

was obtained between log CFU and C_T for strains UAP-154 (r = 0.86, $P < 0.001$) and CFN-535 (r = 0.84, $P < 0.001$) (Fig. 4).

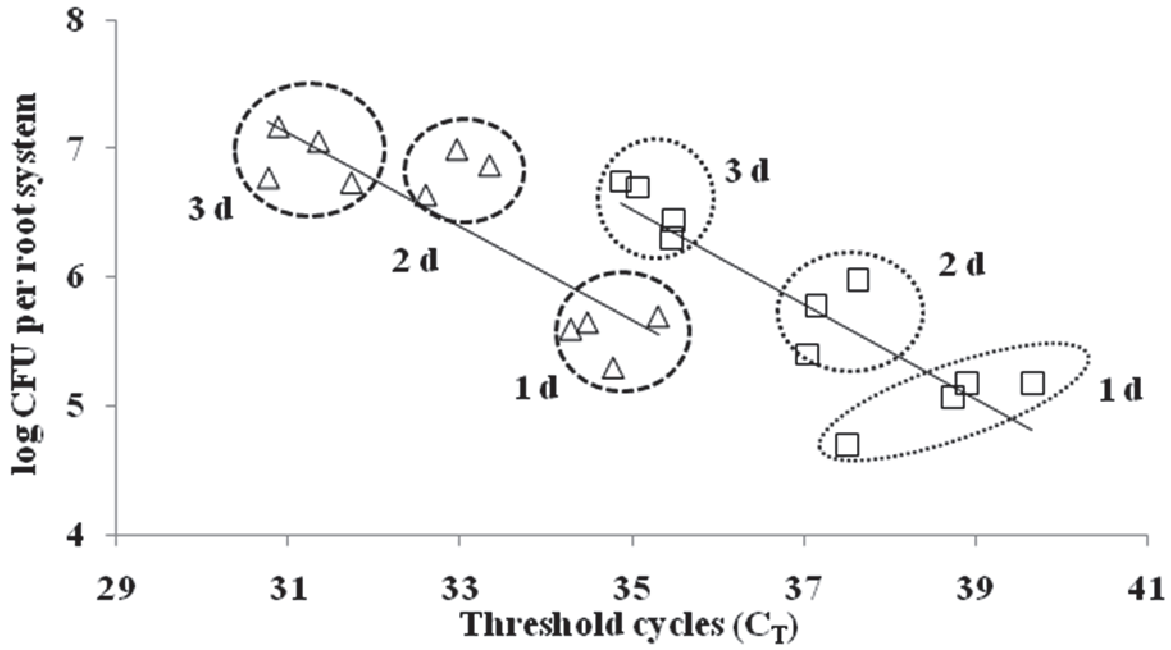

Figure 4 Relation between real-time PCR's C_T numbers and colony counts for *A. brasilense* strains UAP-154 (□) and CFN-535 (Δ) in axenic rhizosphere microcosms at 1, 2 and 3 d after inoculation. For visual clarity, data from a same sampling are gathered using dotted lines for each strain.

Discussion

A. brasilense PGPR strains have been extensively studied for phytostimulation of cereal crops (Okon et Labandera-Gonzalez 1994; Dobbelaere *et al.* 2001), and to a lesser extent for biological control of phytoparasitic plants (Miché *et al.* 2000) and microbes (Bashan et de-Bashan 2002), soil weathering (Puente *et al.* 2006), waste recycling (de-Bashan et Bashan 2004), and improving drought tolerance in plants (Rodriguez-Salazar *et al.* 2009). The use of *A. brasilense* inoculants in Latin American countries has constantly increased in recent years, reaching around 500,000 hectares of wheat and maize (Castro-Sowinski *et al.* 2007), but *Azospirillum* does not always survive well in soil or the rhizosphere (Bashan *et al.* 1995; Bashan 1999), which may affect inoculant performance (Dobbelaere *et al.* 2002). However, satisfactory methods for monitoring *A. brasilense* wild-type inoculants in the rhizosphere are lacking, which means cases of inoculation failure remain unexplained.

We describe here the development of a SYBR Green real-time PCR assay targeting SCAR markers for the maize inoculants *A. brasilense* UAP-154 and CFN-535. The use of SCAR markers is applicable to uncharacterized genomes, which was the case here. SCAR markers from BOX, ERIC and RAPD PCR amplicons were chosen because (i) BOX, ERIC and RAPD profiles can distinguish between *Azospirillum* strains including *A. brasilense* strains (Fani *et al.* 1993; Fancelli *et al.* 1998; Mirza *et al.* 2000; Vial *et al.* 2006b; Baudoin *et al.* 2009a), (ii) strain-specific RAPD markers can be used for detection of an *A. lipoferum* strain in soil (Fancelli *et al.* 1998; Jacoud *et al.* 1998), and (iii) they proved useful to develop strain-specific primers for real-time PCR quantification of a biocontrol strain of the bacterial genus *Pantoea* (Nunes *et al.* 2008). Indeed, we found that BOX, ERIC and RAPD PCR discriminated effectively between *A. brasilense* strains, but the number of total and especially of strain-specific bands was higher with BOX than with ERIC and RAPD PCR combined. However, it must be kept in mind that band yield of RAPD PCR might have been improved by changing primer sequence and/or concentration (Fani *et al.* 1993; Fancelli *et al.* 1998). Sequencing and BLASTN analyses resulted in the identification of four strain-specific SCAR markers for each strain, and since half of them were obtained by BOX PCR it makes BOX PCR the method of choice to obtain SCAR markers for *A. brasilense* strains.

Many primer sets were derived from the SCAR markers, but most of them were later discarded because cross-reaction was found with non-target strain(s), under qualitative or quantitative PCR conditions. It must be kept in mind that PCR was done with rather high quantity of genomic DNA of the 34 rhizosphere strains used as negative controls (respectively

30 ng and 30 pg in qualitative and quantitative PCR). Lower DNA concentrations would probably have resulted in the selection of additional primers, but with a higher risk of cross-reaction with indigenous bacteria in subsequent rhizosphere experiments and the need to optimize real-time PCR conditions.

The two real-time PCR assays were validated based on (i) PCR efficiency higher than 85% *in vitro* and in soil, (ii) ecologically-relevant detection and quantification limits, (iii) significant correlation with colony counts under axenic rhizosphere conditions, and (iv) the possibility to estimate population size of the inoculants (each found at about 10^6 log CFU equivalents per root system at 10 d) in the rhizosphere of maize grown in non-sterile La Côte St André soil. The detection limits of the two real-time PCR assays did not depend on the soil used, but differed according to the strain (4×10^3 and 4×10^4 CFU g^{-1} lyophilized soil for strains UAP-154 and CFN-535, respectively). The reason behind this difference is not known. Since *Azospirillum* inoculation requires at least 10^5 CFU $plant^{-1}$ for effective phytostimulation (Kapulnik *et al.* 1985; Arsac *et al.* 1990; Okon et Itzigsohn 1995; Benizri *et al.* 2001), these detection limits were satisfactory. Had it not been the case, the detection limit could have been lowered by developing an alternative real-time PCR method using TaqMan probes.

One main advantage of the standard curve approach is the integration of some of the bias linked to DNA extraction, as standard curves relate cell numbers to C_T values obtained with DNA from inoculated non-sterile bulk soil. Indeed, the latter involves DNA extracted with the same procedure subsequently used in quantification experiments. This standard curve approach proved effective for quantification of *Pseudomonas* groups in the wheat rhizosphere (Mavrodi *et al.* 2007), and here (as indicated by PCR efficiency results) to estimate the population densities of each *A. brasilense* strain.

In conclusion, SCAR markers were useful to design PCR primers for rhizosphere quantification of *A. brasilense* inoculants of maize, and this approach is advocated for other *Azospirillum* strains used on cereal crops. The two real-time PCR assays will be used in future work to assess the influence of ecological conditions on root colonization and maize phytostimulation by *A. brasilense* UAP-154 and CFN-535.

Acknowledgement

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II/ Development of a real-time PCR method to quantify the phytostimulatory PGPR inoculant *Azospirillum lipoferum* CRT1 on maize

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Abstract

A. lipoferum CRT1 is a promising phytostimulatory PGPR for maize, whose effect on the plant is cell density-dependent. A nested PCR method is available for detection of the strain but does not allow quantification. The objective was to develop a real-time PCR method for quantification of *A. lipoferum* CRT1 in the maize rhizosphere. Primers were designed based on a strain-specific RFLP marker, and their specificity was verified under qualitative and quantitative PCR conditions based on successful CRT1 amplification and absence of cross-reaction on genomic DNA from various rhizosphere strains. Real-time PCR conditions were then optimized using DNA from inoculated or non-inoculated maize rhizosphere samples. The detection limit was 60 fg DNA (corresponding to 19 CFU) with pure cultures and 4×10^3 CFU / g in the maize rhizosphere. Inoculant quantification was effective down to 10^4 CFU / g. This real-time PCR method will be available for direct rhizosphere monitoring of *A. lipoferum* CRT1 in greenhouse and field experiments.

Keywords: PGPR; *Azospirillum*; Root colonization; Inoculant quantification; Real-time PCR.

Introduction

Azospirillum is an α-proteobacterial genus that contains plant growth-promoting rhizobacteria (PGPR). These PGPR strains have been extensively used as seed inoculants for phytostimulation of cereal crops (Charyulu *et al.* 1985; Okon et Labandera-Gonzalez 1994; Dobbelaere *et al.* 2001; Pedraza *et al.* 2009). They promote plant growth, especially the root system, which is useful to enhance the uptake of water and nutrients by roots (Okon et Kapulnik 1986; Jacoud *et al.* 1999). In certain cases, *Azospirillum* inoculation was found to improve crop yield (Charyulu *et al.* 1985; Okon et Labandera-Gonzalez 1994; Dobbelaere *et al.* 2001; Pedraza *et al.* 2009). In areas of heavy mineral fertilisation, the goal may not be enhanced yield, but rather the possibility to reduce current doses of nitrogen fertilizers without affecting crop yield. This concern is becoming important when the focus is on lower-input farming (Fuentes-Ramirez et Caballero-Mellado 2006; El Zemrany *et al.* 2006).

Several modes of action are documented in *Azospirillum* PGPR, especially the production of phytohormones such as auxins, which is often proposed as the main phytobeneficial mechanism underpinning root system stimulation (Dobbelaere *et al.* 2003). Other significant traits include associative nitrogen fixation (James 2000), the synthesis of nitric oxide (Creus *et al.* 2005), and 1-aminocyclopropane-1-carboxylate deaminase activity (Prigent-Combaret *et al.* 2008).

In the case of maize, one of the main *Azospirillum* PGPR strains considered in Europe is *A. lipoferum* CRT1 (Fages et Mulard 1988). Phytostimulation by this strain is cell density-dependent (Jacoud *et al.* 1999), which means that it is important to monitor establishment of strain CRT1 in the maize rhizosphere (El Zemrany *et al.* 2006), especially shortly after germination (Jacoud *et al.* 1999). Two approaches are available to monitor *A. lipoferum* CRT1 in the maize rhizosphere. One is colony hybridization using a 16S rDNA-targeted probe (Jacoud *et al.* 1998; El Zemrany *et al.* 2006), but it is time-consuming and requires cultivation in semi-selective medium. The other is a nested PCR method, which targets the 16S-23S rDNA internal spacer region (Baudoin *et al.* 2009a). This PCR method is effective for identification of the strain, but does not allow inoculant quantification in the rhizosphere.

The objective of this work was to develop a quantitative PCR method for quantification of *A. lipoferum* CRT1 in the maize rhizosphere. Quantitative PCR of bacterial rhizosphere inoculants may be implemented by MPN-PCR (Rosado *et al.* 1996; Mirza *et al.* 2006), competitive PCR (Johansen *et al.* 2002; Mauchline *et al.* 2002; Rezzonico *et al.* 2005; Gobbin *et al.* 2007), and real-time quantitative PCR (Sørensen *et al.* 2009). During real-time

PCR, the amplicons generated are quantified in real time based on fluorescence level, which under certain conditions is proportional to the concentration of original template DNA. Here, a real-time PCR protocol was developed using a primer pair already available for strain CRT1 (Baudoin *et al.* 2003). Specificity of the primer pair was verified using various strains of *Azospirillum* spp. and other rhizosphere bacteria, and was further assessed for SYBR Green-based real-time PCR quantification in soil and maize rhizosphere.

Material and Methods

Bacterial strains

All *Azospirillum* strains (Table 1) were routinely grown at 27°C with shaking in N-free Nfb medium (Nelson et Knowles 1978) supplemented with 2.5% v/v LBm (i.e. Luria-Bertani medium containing only 5 g NaCl/l ; Pothier *et al.* 2007). *Pseudomonas* strains were grown in LBm, *Agrobacterium* in LPG (Roy *et al.* 1982) and *Rhizobium* in YEM (Vincent 1970). Colony counts of *A. lipoferum* CRT1 in media or gnotobiotic rhizosphere samples were performed after spreading dilutions on RC agar (Rodriguez Caceres 1982) and a 72-h incubation at 27°C.

DNA preparation

Three methods were used to obtain DNA. Genomic DNA from bacterial log cultures was extracted using Macherey & Nagel DNA Tissue kit (Düren, Germany) according to manufacturer's instructions. Rhizosphere DNA in the experiment where sterile soil was used was extracted by thermal shock (Baudoin *et al.* 2009a). The aliquots were heated for 10 min at 100°C and placed directly on ice for 5 min. DNA from the other rhizosphere samples and from bulk soil samples was extracted with the FastDNA® SPIN® kit (BIO 101 Inc., Carlsbad, CA). To this end, 250-300 mg samples (described below) were transferred in Lysing Matrix E tubes from the kit, and DNA was extracted and eluted in 50 µl of sterile ultra-pure water, according to the manufacturer's instructions. DNA concentrations were assessed by OD measurements at 260 nm with NanoDrop (Nanodrop technologies, Wilmington, DE).

Table 1 Strains used in this study

Species and strains	Host plant	Origin	Reference
Azospirillum brasilense			
UAP-154 ; CFN-535	Maize	Mexico	Dobbelaere *et al.* 2001
ZN1	Maize	Pakistan	Blaha *et al.* 2006
L4	Sorghum	France	Blaha *et al.* 2006
Sp245	Wheat	Brazil	Blaha *et al.* 2006
Wb1 ; Wb3; WS1 ; WN1	Wheat	Pakistan	Blaha *et al.* 2006
PH1	Rice	France	Blaha *et al.* 2006
R5(15)	Rice	Cuba	Blaha *et al.* 2006
Cd	*Cynodon dactilon*	USA	Blaha *et al.* 2006
Sp7	Digitaria	Brazil	Blaha *et al.* 2006
NC9	Soil	Mali	Blaha *et al.* 2006
NC16	Soil	Mali	Vial *et al.* 2006b
Azospirillum lipoferum			
CRT1	Maize	France	Blaha *et al.* 2006
Br17	Maize	Brazil	Vial *et al.* 2006b
B506 ; B510 ; B518	Rice	Japan	Blaha *et al.* 2006
RSWT1	Rice	Pakistan	Blaha *et al.* 2006
TVV3	Rice	Vietnam	Blaha *et al.* 2006
4B	Rice	France	Blaha *et al.* 2006
N4	Cotton	Pakistan	Blaha *et al.* 2006
Br10	Soil	Brazil	Vial *et al.* 2006b
NC4	Soil	Mali	Vial *et al.* 2006b
Pseudomonas fluorescens			
F113	Sugar beet	Ireland	Ramette *et al.* 2003
Pf-153	Tobacco	Switzerland	Gobbin *et al.* 2007
C10-186 ; S7-29	Tobacco	Switzerland	Ramette *et al.* 2003
Q37-87	Wheat	USA	Ramette *et al.* 2003
K94-41	Cucumber	Czech Republic	Wang *et al.* 2001
P97-1	Cucumber	Bhutan	Wang *et al.* 2001
Pseudomonas sp. CHA0	Tobacco	Switzerland	Ramette *et al.* 2003
Rhizobium etli CFN-42	Bean	Mexico	Romero *et al.* 1991
Agrobacterium tumefaciens C58	Prunus	USA	Blaha *et al.* 2006

Primer selection using strain collection

Primers CRT1-Q1 (ATCCCGGTGGACAAAGTGGA) and CRT1-Q2 (GGTGCTGAAGGTGGAGAACTG) were derived (Baudoin *et al.* 2003) from a 1.4-kb strain-specific genomic region obtained by genomic RFLP (U90627; Jacoud *et al.* 1998). Oligo 6.65 software (Molecular Biology Insights, West Cascade, CO) was then used to analyze primer characteristics such as (i) melting temperature (Tm) of each primer, using the nearest-neighbor thermodynamic method, and (ii) predicted hairpin loops, duplexes and primer-dimer formation. Primer selection was then implemented based on (i) successful amplification of strain CRT1 and (ii) absence of cross-reaction with non-target strains. Three pools of bacterial genomic DNA were used as negative controls, i.e. an *A. lipoferum* pool (10 strains), an *A. brasilense* pool (15 strains), and a pool of other common rhizosphere bacteria (including the α-Proteobacteria *Rhizobium etli* and *Agrobacterium tumefaciens*, as well as Proteobacteria from other subdivision and belonging to *Pseudomonas* genus) (Table 1). A first assessment of primer specificity was performed under qualitative PCR conditions with 4 different annealing temperatures (58 to 65°C), with about 30 ng of gDNA. A second assessment was performed under quantitative PCR conditions, using 30 pg of gDNA, primer concentrations ranging from 50 nM to 1 µM and 3 annealing temperatures (65, 68 and 70°C).

Rhizosphere experiments used for optimization of real-time PCR conditions

Maize (*Zea mays*) was grown in four different soils, which were sampled from the surface horizon of a French luvisol at La Côte Saint André near Bourgoin (loam: clay 16%, silt 44%, sand 40%, organic matter 2.1%, pH 7.0; El Zemrany *et al.* 2006), a French brunisol at Pouzol Etoile near Valence (clay loam: clay 32%, silt 29%, sand 21%, organic matter 2.1%, pH 8.1), a French brunisol at Marcellas Sud Etoile near Pouzol Etoile (loamy sand: clay 19%, silt 46%, sand 28%, organic matter 1.8%, pH 8.0), and a Swiss brunisol at Wangen bei Olten near Zurich. All fields were grown with maize except Pouzol Etoile (durum wheat).

Maize seeds of hybrid PR37Y15 (Pioneer Hi-Bred International, Johnston, IA) were surface-sterilized by stirring in sodium dichloroisocyanurate-containing Bayrochlor Mini solution (Bayrol, Dardilly, France) for 15 min. Seeds were then washed 4-6 times with sterile water and germinated on water agar (8 g l^{-1}) for 2 d in the dark at 28°C. Cells from overnight Nfb liquid cultures of strain CRT1 were collected by centrifugation at 4000 *g* for 10 min, gently washed and resuspended in 10 mM $MgSO_4$ solution to obtain 10^8 CFU per ml.

Germinated maize seeds were inoculated by soaking for 1 h in bacterial suspensions. Sterile water was used for the negative control. For each soil, one maize seedling was placed in each pot, which contained 600 g sieved (5 mm) non-sterile soil (4 pots per treatment) adjusted to 20% (w/w) water content. The pots were placed 10 d in a greenhouse with 18 h of light (under 400 W lamps; 22°C and 45-50% relative humidity) and 6 h of dark (18°C and 60-65% relative humidity), and fit with an automated irrigation system.

At sampling, the entire root system was dug up and shaken vigorously to discard soil loosely adhering to the roots. With the four soils tested, a first sampling procedure was applied to study rhizosphere soil, as follow. Roots and tightly-adhering soil were transferred in a 1-liter bottle containing 300 ml of sterile distilled water, and the bottles were shaken for 15 min. The soil fraction was recovered by centrifugation for 30 min at 5,600 *g* and flash-freezed in liquid nitrogen. With the Wangen soil, an additional sampling procedure was tested to include roots, by transferring roots and tightly-adhering soil in a 50-ml Falcon and flash-freezing in liquid nitrogen. With both methods, samples were then lyophilized for 48 h in Falcon tubes and homogenized by crushing in the tubes using a spatula, and 250-300 mg were used for DNA extraction, as described above.

Optimization of real-time PCR conditions in rhizosphere experiments

Optimized real-time PCR conditions were sought for maize rhizosphere samples originating from different types of soils. Rhizosphere DNA (described above) from inoculated and non-inoculated maize was used as positive and negative control, respectively. Optimized reaction mix composition was sought by comparing (i) 4 primer concentrations (0.05 to 1 µM), (ii) 3 volumes of template DNA (1 to 3 µl), and (iii) the effect of T4 gene 32 protein (50 or 100 ng per reaction mix; Roche Applied Science, Indianapolis, IN). In addition, optimized cycling conditions were sought by comparing (i) 4 primer melting temperatures (67°C to 70°C), (ii) 4 durations of the denaturation steps (15 to 30 s), (iii) 4 durations of the hybridization steps (15 to 30 s), and (iv) 5 durations of the elongation steps (10 to 30 s). To check primer specificity, the observation of melting curves (described below) was completed by agarose gel electrophoresis of real-time PCR products.

Optimized real-time PCR conditions

Optimized real-time PCR conditions were as follows. PCR was done in 20 µl PCR volumes containing 10 µl LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science),

0.50 µM primer, 50 ng of T4 gene 32 protein (Roche Applied Science) and 2 µl of template DNA. White 96-well microplates and a LC-480 LightCycler were used (Roche Applied Science). The cycling program included a 10-min incubation at 95°C followed by 50 cycles consisting of 95°C for 15 s, 68°C for 15 s and 72°C for 10 s. Amplification specificity was studied by melting curve analysis of the PCR products performed by ramping the temperature to 95°C for 10 s and back to 65°C for 15 s followed by incremental increases of 0.1°C/s up to 95°C. Melting curve calculation and determination of Tm values were performed using the polynomial algorithm function of LightCycler Software v.1 (Roche Applied Science).

Generation of standard curves for genomic DNA

Genomic DNA from *A. lipoferum* CRT1 was used to prepare ten-fold dilution series from 3×10^6 to 3×10^1 fg DNA μl^{-1} (in triplicate). Sterile water (2 µl) was used as negative control. Cycle threshold (C_T), i.e. the number of PCR cycles necessary to reach the threshold fluorescence level, was automatically determined for each sample by the LightCycler software v.1 (Roche Applied Science) based on the second derivative maximum method. A standard curve for each strain was generated by plotting the C_T number against the logarithm of bacterial DNA concentration for the three independent replicates, using LightCycler Software v.1 (Roche Applied Science). Amplification efficiency (E) was calculated from the slope of the standard curve using the formula $E = 10^{-1/slope} - 1$.

Generation of standard curves used for real-time PCR quantification in maize rhizosphere

Each experiment involving real-time PCR quantification of strain CRT1 in the rhizosphere requires development of standard curves, which can be performed either with rhizosphere soil or with bulk soil depending of the sampling procedure applied (described below). Standard curves using bulk soil were tested with the four different soils described above, and a comparison was made with standard curves obtained using one rhizosphere soil. To this end, Lysing-Matrix E tubes (BIO 101 Inc.) containing 250-300 mg lyophilized rhizosphere soil (obtained as described above) or lyophilized bulk soil were inoculated with strain CRT1 to reach 10^3, 10^4, 10^5, 10^6, 10^7, or 10^8 CFU added per tube (3 replicates per inoculation level). No bacteria were added to the negative control. After 1 h of incubation at 4°C, DNA extraction was performed using the FastDNA® SPIN® kit (BIO 101 Inc.) and real-time PCR was then performed, as described above. A standard curve was generated for each replicate by

plotting C_T number versus log CFU. Amplification efficiency was calculated as described above.

Standard curves were then used to estimate CRT1 cell number in the rhizosphere of seed-inoculated maize plants. This was done by real-time analysis of the corresponding samples (obtained as described above), and the number of CFU in the rhizosphere was calculated from the C_T using the standard curve generated for the experiment.

Assessment of real-time PCR data in comparison with colony counts

A microcosm experiment was performed under axenic conditions to compare real-time PCR data with colony counts. Maize seeds (cv. PR37Y15) were disinfected, pre-germinated and inoculated, as described above, using a cell suspension containing 10^8 CFU per ml of strain CRT1 (giving about 10^4 CFU per seed as indicated by colony counts). One seedling was added per microcosm, which consisted of 300 g of autoclaved La Côte Saint André soil placed in 150-cm^3 glass bottles and adjusted to 20% (w/w) water content. The inoculated treatment and the negative control were studied using 12 microcosms each, which were placed in a growth chamber at 75% relative humidity, with 16 h of light (30 W lamps) at 26°C and 8 h of dark at 18°C. Samples studied consisted of (i) the cell suspension used for seedling inoculation, (ii) seedling extracts obtained by high-speed vortexing (3 min) of 50-ml Falcon tubes containing 10 seedlings of each of the two treatments (immediately before sowing) in 10 mM $MgSO_4$ solution, and (iii) microcosm extracts obtained at each sampling (i.e. 1, 2 and 3 d after inoculation) by high-speed vortexing (5 min) of 15-ml Falcon tubes containing one root system in 5 ml of 10 mM $MgSO_4$ solution (4 replicates × 2 treatments at each sampling). Each of the 27 samples obtained was characterized by real-time PCR (using 1 ml of each) and colony counts, as described above.

Statistics

Greenhouse and growth chamber experiments followed a randomized block design. Colony counts were expressed as log CFU per root system or per g of dry root, and real-time quantification data were converted to log CFU equivalents per root system or per g of lyophilized soil. Treatment comparisons were performed by ANOVA and Fisher LSD tests ($P < 0.05$). The relation between log CFU data and C_T values was assessed by (i) regression analysis when assessing standard curves and (ii) correlation analysis for the comparison of

real-time PCR data with colony counts. Statistical analyses were performed at $P < 0.05$, using S plus software (TIBCO Software Inc., Palo Alto, CA).

Results

Primer characteristics and specificity in vitro

The analysis of primers Q1/Q2 by Oligo 6.65 software revealed primer melting temperatures only 2.4°C apart, and no formation of any PCR-impairing structure was predicted, which are characteristics needed for real-time PCR. Specificity testing against the three pools of genomic DNA, under qualitative PCR conditions, showed that cross-reaction occurred with the *A. lipoferum* DNA pool until raising annealing temperature to 65°C. At 65°C, amplification of strain CRT1 was effective and took place without any cross-reaction in any of the three DNA pools. When primers were then tested under quantitative conditions, at a concentration of 50 nM and at the same annealing temperature, no cross-reaction was detected but amplification was too late as indicated by high *Ct* (> 35). However, suitable real-time PCR conditions (i.e. effective CRT1 amplification without any cross-reaction) were identified with an annealing temperature of 70°C and a primer concentration of 1 μM. Thus, this primer set proved strain-specific *in vitro*, once real-time PCR conditions were adjusted.

Optimization of PCR conditions using bulk soil samples

During optimization of real-time PCR conditions, the use of T4 gene 32 protein had a positive impact on PCR efficiencies calculated with standard curves generated on the four different bulk soils tested, but it had a negative impact on PCR efficiencies with genomic DNA and, more importantly, it resulted in positive amplification with non-inoculated rhizosphere soils. This drawback was counteracted by decreasing (i) the quantity of T4 gene 32 protein added to the reaction mix from 100 to 50 ng, (ii) the concentration of CRT1 primers to 0.500 μM and (iii) denaturation and annealing to 15 s and elongation to 10 s, which in turn required Tm reduction to 68°C. These adjustments maintained PCR efficiency above 80% and gave satisfactory amplification specificity (i.e. no or only late amplifications with non-inoculated rhizosphere soils) for strain CRT1.

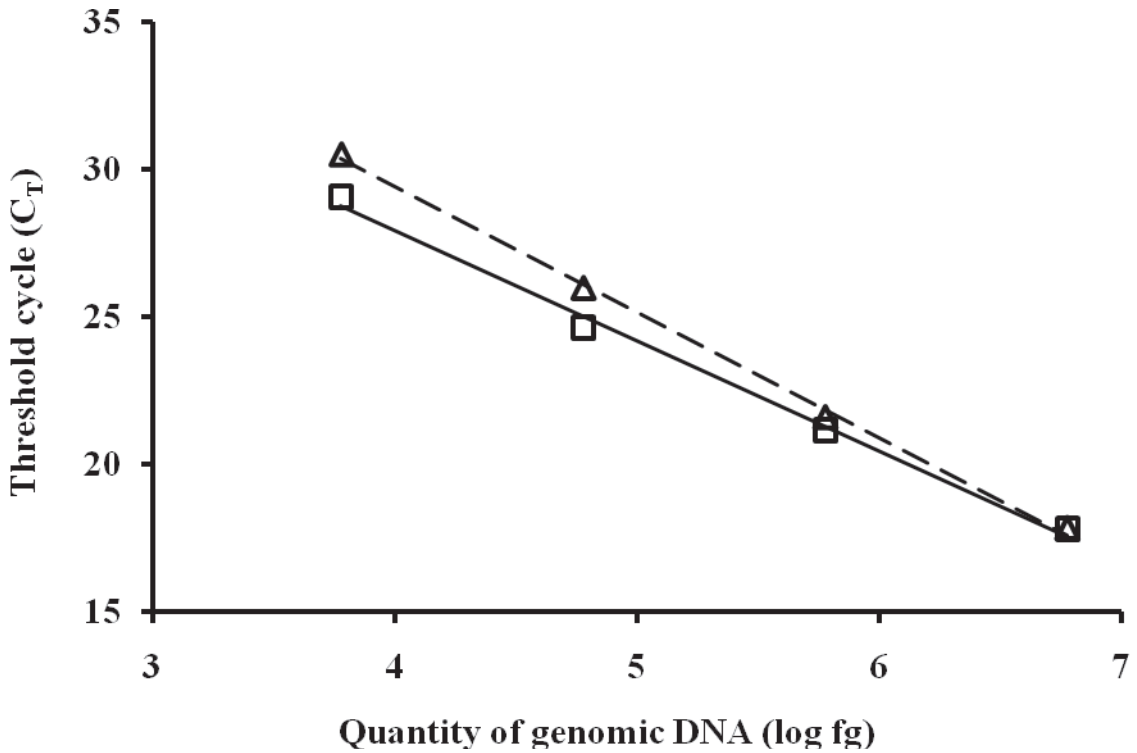
Evaluation *in vitro* of the real-time PCR assay developed

The evaluation of the real-time PCR assay was based on standard curves established after adding various amounts of purified CRT1 genomic DNA to the PCR mix. The limit of detection was found to be 60 fg DNA, corresponding to 19 CFU. A R^2 value higher than 0.99

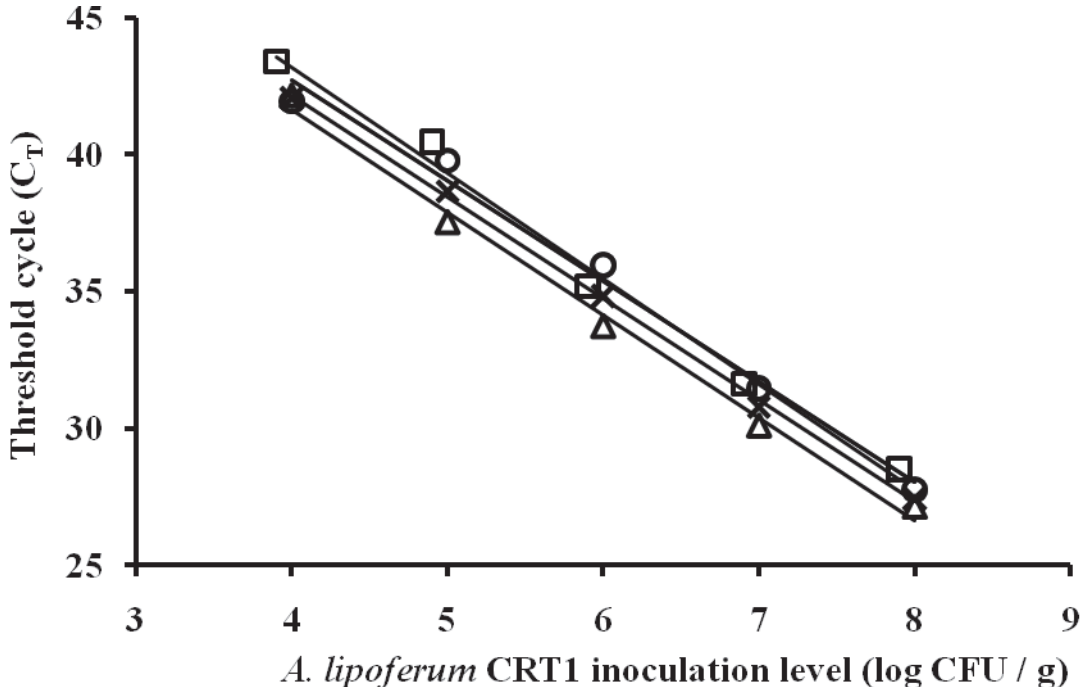
was obtained after regression analysis between DNA amount and C_T from real-time PCR over a range of 4 orders of magnitude (Figure 1A). Amplification efficiency decreased from 94% to 80% when adding T4 gene 32 protein in the reaction mix, as reflected by the variation of the slope of the standard curves (Figure 1A; Table 2).

Table 2 Standard curve parameters and real-time PCR amplification efficiencies used in this study.

Sample	Detection limit	R^2	Slope	Error	Amplification efficiency (%)
Bacterial culture (with T4 gene 32 protein)	60 fg	0.998	-3.904	0.045	80.4
Bacterial culture (without T4 gene 32 protein)	60 fg	0.995	-3.475	0.073	94.0
Inoculated bulk soil (La Côte St André soil)	3×10^3 CFU / g	0.991	-3.753	0.073	84.6
Inoculated bulk soil (Pouzol soil)	3×10^3 CFU / g	0.993	-3.706	0.029	86.1
Inoculated bulk soil (Marcellas soil)	3×10^3 CFU / g	0.999	-3.859	0.088	81.6
Inoculated bulk soil (Wangen soil)	3×10^3 CFU / g	0.988	-3.899	0.113	80.5
Inoculated rhizosphere soil (Wangen soil)	3×10^4 CFU / g	0.998	-3.349	0.124	98.9



A.



B.

Figure 1 Real-time PCR standard curves for *A. lipoferum* CRT1. **A,** Analysis of genomic DNA with (Δ) or without T4 gene 32 protein (□) in the reaction mix; C_T numbers were plotted against the quantity of genomic DNA added to the reaction mix, and means from three replicates are represented. **B,** Comparison of four non-sterile bulk soils from La Côte St André (□), Pouzol (Δ), Marcellas (×) and Wangen (○); C_T numbers were plotted against the inoculation level of strain CRT1, and means from three replicates are represented.

Real-time PCR quantification of *A. lipoferum* CRT1 in the maize rhizosphere in non-sterile soils

When standard curves were obtained using bulk soil, R^2 values higher than 0.98 were found with each of the four soils after regression analysis between log values of CFU / g of soil and C_T from real-time PCR over a range of 5 orders of magnitude (Figure 1B). All amplification efficiencies were above 80%, and the detection limit was 4×10^3 CFU / g (Table 2).

For the Wangen soil, the standard curve was also performed using rhizosphere soil, which gave a R^2 value of 0.99 obtained over a 4 log-CFU range. Amplification efficiency was 98%, with a detection limit of 4×10^4 CFU / g (Table 2). In addition, quantification was effective between 10^4 and 10^8 CFU g^{-1}, as when bulk soil was used. In summary, the same standard curve was obtained for the Wangen soil, regardless of whether bulk or rhizosphere soil was used.

When rhizosphere quantification of the seed-inoculant *A. lipoferum* CRT1 was performed for maize grown in the four non-sterile soils, based on real-time PCR and standard curves generated for each soil, levels ranging from 10^4 to 10^6 log CFU equivalents per root system were obtained at 10 d (Figure 2).

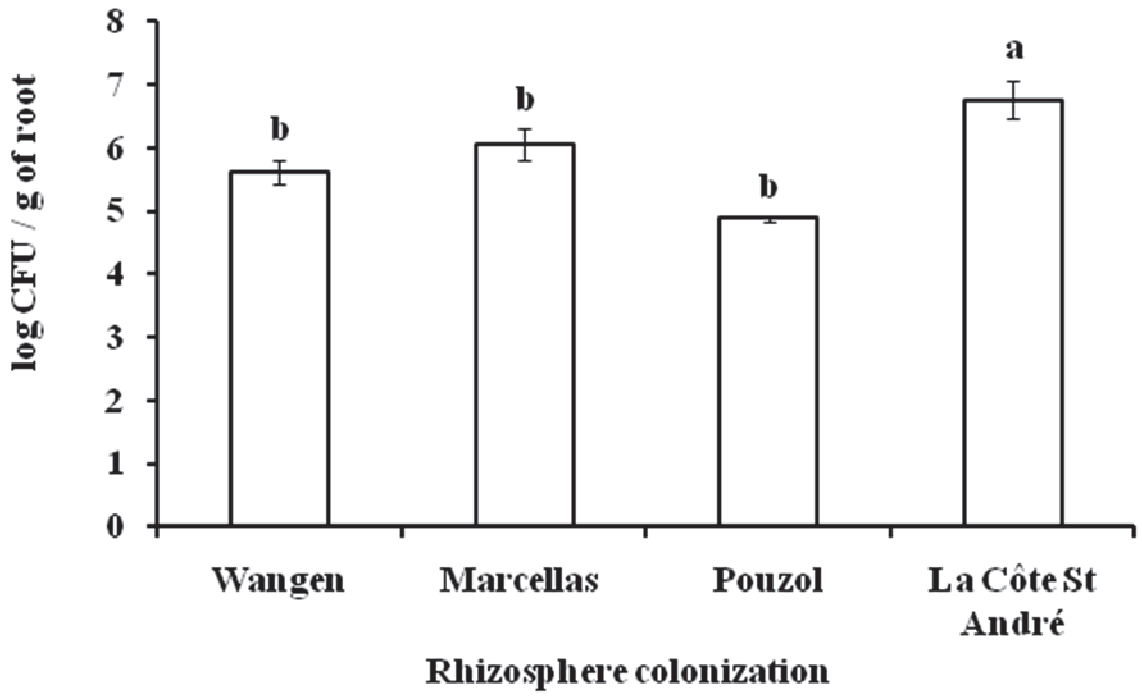


Figure 2 Quantification of the seed-inoculant *A. lipoferum* CRT1 at 10 d in the rhizosphere of maize grown in four non-sterile soils (mean log CFU equivalents ± SD; n = 4). Statistical differences between treatments are indicated with letters a-b (ANOVA and Fisher LSD tests; $P < 0.05$).

Comparison of real-time PCR data with colony counts in axenic rhizosphere

The use of axenic rhizosphere microcosms enabled implementation of colony counting technique, which gave *A. lipoferum* CRT1 at 10^4 CFU per maize seed and approximately 10^6 CFU per root system in rhizosphere samples taken at 1, 2 or 3 d after sowing. When the same samples were studied by real-time PCR, a statistically-significant correlation was obtained between log CFU and C_T (r = 0.71, $P < 0.005$) (Figure 3).

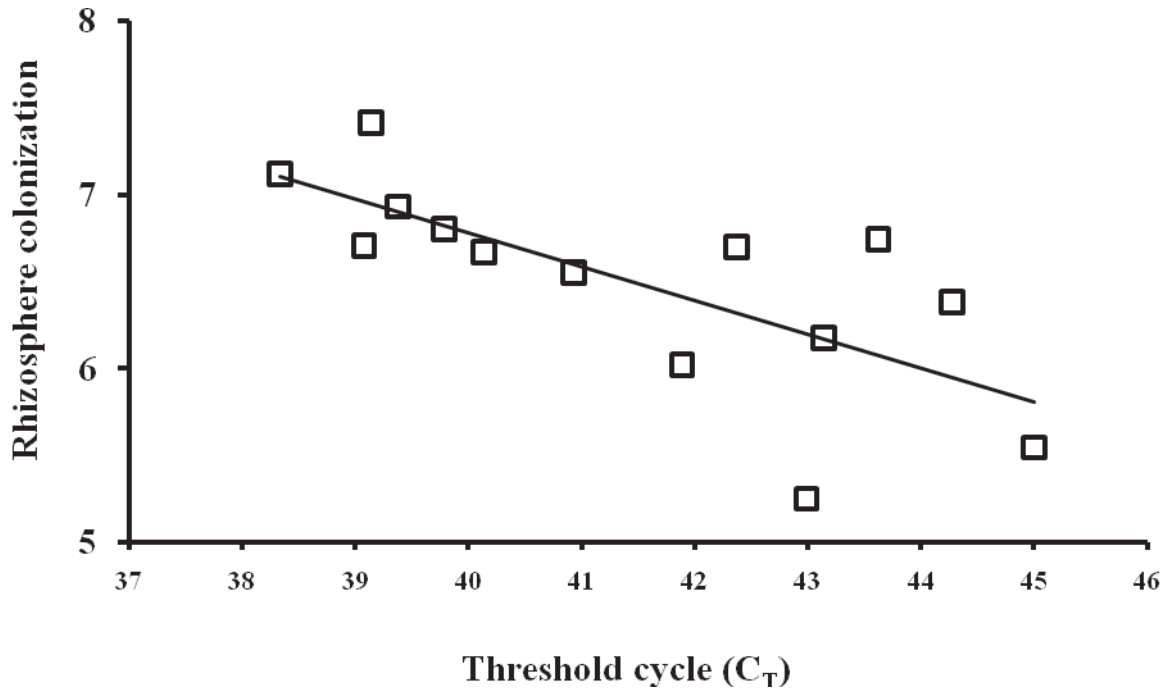


Figure 3 Relation between real-time PCR's C_T numbers and colony counts for *A. lipoferum* CRT1 (expressed as log CFU per root system) in axenic rhizosphere microcosms (autoclaved La Côte St André soil) at 1, 2 and 3 d after inoculation.

Discussion

A. lipoferum CRT1 is a prominent PGPR strain, which has been used for commercial inoculation of maize under agronomic conditions and included as reference strain in scientific studies (Jacoud *et al.* 1999; Revellin *et al.* 2001; El Zemrany *et al.* 2006; Baudoin *et al.* 2009b). However, effective methods are lacking to monitor this strain in the rhizosphere. Recently, a nested PCR method was proposed for rhizosphere detection of the strain (Baudoin *et al.* 2009a), but the primers (derived from the 16S-23S intergenic region) proved not specific enough to develop a real-time quantification method.

We describe here the development of a SYBR Green-based real-time PCR method for quantification of *A. lipoferum* CRT1 in maize rhizosphere, based on primers (Baudoin *et al.* 2003) designed from a PCR-RFLP Sequence Characterized Amplified Region (SCAR) marker identified by Jacoud *et al.* (1998). The method involves the use of standard curves, which are performed in each experiment (Gobbin *et al.* 2007; Mavrodi *et al.* 2007), thereby enabling direct integration of potential bias linked to DNA extraction efficiency and PCR amplification (Martin-Laurent *et al.* 2001; Braid *et al.* 2003; Sørensen *et al.* 2009).

When preliminary experiments were performed to select the DNA extraction procedure, it appeared that 250-mg sub-samples gave the same PCR results as larger 2-g samples of lyophilized rhizosphere soil + roots obtained from maize grown in non-sterile La Côte St André soil (data not shown). In addition, comparison of two soil DNA extraction kits (MOBio® Power Soil kit versus FastDNA® SPIN® kit) indicated that more DNA was recovered with the FastDNA® SPIN® kit, but more PCR inhibitors were co-extracted (data not shown). Two main differences can be highlighted between the two kits : (i) the mechanical lysis through bead beating in the FastDNA® SPIN® kit and (ii) the extensive purification steps in the MOBio® Power Soil kit (Schneegurt *et al.* 2003; Mumy et Findlay 2004; Cook et Britt 2007). Finally, the FastDNA® SPIN® kit was the more efficient with lyophilized rhizosphere samples, presumably because it includes a mechanical lysis procedure.

Development of the quantification tool required several optimizations, regarding to the specificity and the efficiency of our real-time PCR assay. Optimizations concerning PCR efficiency were mainly focused on the impact of potential PCR inhibitors, which were suspected to decrease PCR efficiency with soil samples. Crude DNA extracts obtained with the FastDNA® SPIN® kit were diluted in order to attenuate the inhibitory effects of humic acids (Schneegurt *et al.* 2003), which can be the main PCR inhibitors co-extracted with soil DNA (Tsai et Olson 1992; Bruce *et al.* 1999). Besides, the use of the T4 gene 32 protein

improved PCR efficiencies, presumable by relieving amplification inhibition (Kreader 1996; Wilson 1997) due to humic acids (Tebbe et Vahjen 1993). The addition of 50 ng of this protein per reaction mix combined with dilution of the crude DNA extract, both aimed at attenuating the inhibitory effects of humic acids, enabled to obtain satisfactory PCR efficiencies (i.e. above 80%; Mavrodi *et al.* 2002), which ranged from 81 to 99% depending on the soil and the presence of roots in the sample (Table 2). However, the use of this protein with genomic DNA had a negative effect on the slope of the standard curves (Figure 1A) and thus on real-time PCR efficiencies (Table 2), even though we added only 20-50% of the amount used in other studies (Henry *et al.* 2006; Dandie *et al.* 2007). This side effect may be explained by direct interactions of this protein with genomic DNA in absence of soil inhibitors (Jensen *et al.* 1976; Kelly *et al.* 1976). Thus, the evaluation of several parameters (such as amplicon length, primer composition, hairpin loops, duplexes, and primer-dimer formation) that can affect real-time PCR efficiency was performed without adding this protein.

The presence of roots in the samples had an impact on the detection limit of the real-time PCR assay, which was 3×10^3 CFU / g for lyophilized rhizosphere soil and 3×10^4 CFU / g for lyophilized rhizosphere soil + roots (Table 2). However, presence of roots did not affect the quantification range, which was 3×10^4 to 3×10^8 CFU / g of either rhizosphere soil or rhizosphere soil + roots. This means that including the roots for DNA extraction enabled successful integration of the background noise linked to the indigenous microbiota DNA without affecting the quantification range.

Applicability of the real-time PCR method for field and/or greenhouse CRT1-inoculation experiments analyses was validated by (i) significant correlation with colony counts under axenic rhizosphere conditions, and (ii) its ability to obtain population size estimates of the inoculant under natural rhizosphere conditions. First, a correlation of 0.71 was obtained with colony count data, which is significant in comparison to the one obtained by Mavrodi *et al.* 2007. Several factors can affect this correlation, noticeably the physiological state of the bacteria (Marsh *et al.* 1998; Rezzonico *et al.* 2003; Gedalanga et Olson 2008). Second, *A. lipoferum* CRT1 was estimated at 10^4 to 10^6 CFU equivalents per g of dry root by real-time PCR in a greenhouse experiment where inoculated maize plants were grown in four different non-sterile soils (Figure 2). As conclusion, a real-time PCR method is now available for CRT1 inoculant quantification in the maize rhizosphere.

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Partie Expérimentale 3
Compatibilité des PGPR au sein d'un consortium microbien inoculé sur du maïs cultivé en sol non stérile

Introduction

Dans cette partie, nous nous sommes intéressés à la compatibilité de PGPR *Azospirillum* et *Pseudomonas* lors de l'inoculation de maïs (cultivé en sol non stérile) par un consortium microbien comprenant également des champignons mycorhiziens à arbuscules. Les champignons mycorhiziens à arbuscules sont associés à la plupart des espèces cultivées, et ils peuvent avoir un impact important à la fois sur la croissance de la plante et sur la composition de la communauté microbienne rhizosphérique (Rillig 2004; Bonfante et Anca 2009). Dans un contexte d'inoculations multiples, ils correspondent donc à des microorganismes intéressants à prendre en compte. C'est pourquoi des champignons mycorhiziens à arbuscules ont été inclus dans les inoculations réalisées.

Cette expérience a été mise en place dans le cadre du projet MicroMaize, avec l'aide d'Augusto Ramírez-Trujillo, au sein de l'équipe de Jesus Caballero-Mellado du *Centro de Ciencias Genómicas* (CCG) de l'*Universidad Nacional Autónoma de México* (UNAM), à Cuernavaca au Mexique. Le suivi de l'expérience a concerné le végétal, les champignons mycorhiziens à arbuscules, et les bactéries inoculées. Les analyses ont été réparties entre différents partenaires du projet MicroMaize en fonction des méthodes impliquées. Le suivi de l'expression du gène *ipdC* chez *Azospirillum* a été effectué par Augusto Ramírez-Trujillo (UNAM, Mexique), la quantification par PCR en temps réel des taux de colonisation par différents champignons mycorhiziens à arbuscules (indigènes et inoculés) par Jan Jansa (*Institute of Plant Sciences*, ETH, Eschikon, Suisse), la quantification par PCR en temps réel de la colonisation racinaire par *P. fluorescens* F113 par Andreas von Felten (équipe Défago, *Institute of Integrative Biology*, ETH, Zürich, Suisse), l'analyse des profils métaboliques du maïs par Vincent Walker (équipe Rhizosphère). Pour ma part, j'ai (i) participé à la conception de l'expérience, (ii) contribué à sa réalisation lors d'un séjour à l'UNAM et (iii) assuré la quantification par PCR en temps réel de la colonisation racinaire par les souches d'*Azospirillum* CRT1, UAP-154 et CFN-535. Je me suis impliqué dans l'interprétation des résultats. Les résultats de l'ensemble de ces analyses sont présentés sous la forme d'un manuscrit, car il est prévu qu'ils soient soumis pour publication dans une revue scientifique.

Comparison of prominent *Azospirillum* strains in *Azospirillum-Pseudomonas-Glomus* consortia for promotion of maize growth

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Abstract

Azospirillum are prominent PGPR extensively used as crop-inoculants, but only few studies are dealing with *Azospirillum* mixed inocula involving more than two microorganisms. We describe here the comparison of three different prominent *Azospirillum* inoculants when inoculated in three-component consortia involving (i) the PGPR *Pseudomonas fluorescens* F113 and (ii) a mycorrhizal inoculant mix composed of three *Glomus* strains. The experiment performed on maize has shown that phytostimulation was comparable with all three-component consortia. This phytostimulation occurred despite (i) contrasted survival of *Azospirillum* strains, (ii) different F113 population levels depending on the *Azospirillum* strain, and (iii) different effects on maize secondary profiles. Unexpectedly, the presence of *Azospirillum* in the inoculum resulted in lower phytostimulation in comparison with the *Pseudomonas*-*Glomus* two-component consortium, whose molecular basis is unknown. However, this effect was transient. In conclusion, this study indicates that *Azospirillum*-*Pseudomonas*-*Glomus* three component consortia may be useful for maize growth promotion.

Introduction

Azospirillum are prominent PGPR used as inoculants for phytostimulation of several types of crops (mainly cereals) under different climatic conditions, and they may lead to improved crop yields (Charyulu *et al.* 1985; Okon et Labandera-Gonzalez 1994; Dobbelaere *et al.* 2001; Pedraza *et al.* 2009). Crop inoculation is also receiving attention as a mean to reduce nitrogen fertilizer without affecting crop yield, and can thus be evaluated as a component of integrated management strategies (El Zemrany *et al.* 2006; Fuentes-Ramirez et Caballero-Mellado 2006; Adesemoye *et al.* 2009). Several modes of action have been documented in *Azospirillum* PGPR (nitrogen fixation (James 2000), nitric oxide production (Creus *et al.* 2005), and 1-aminocyclopropane-1-decarboxylate deaminase activity (Prigent-Combaret *et al.* 2008), etc.), but production of phytohormones such as auxins is often proposed as the main phytobeneficial mechanism (Dobbelaere *et al.* 2003).

Interactions of *Azospirillum* PGPR inoculants with other rhizosphere microorganisms have been considered on one hand by studying the ecological impact of inoculation, and on the other hand, by studying mixed inocula involving *Azospirillum* strains. Studies focused on the ecological impact of *Azospirillum* inoculation did not evidenced any positive interactions with indigenous microorganisms (Basaglia *et al.* 2003; Russo *et al.* 2005; Herschkovitz *et al.* 2005a; Herschkovitz *et al.* 2005b; Lerner *et al.* 2006; Naiman *et al.* 2009; Baudoin *et al.* 2009b). On the other hand, interactions of *Azospirillum* inoculants with other rhizosphere microorganisms have been studied in order to evaluate the potential use of *Azospirillum* PGPR strains in mixed inocula (Bashan 1998a). By combining microorganisms with different metabolic capacities (N_2-fixation, P-mobilization, production of phytohormones and antibiotics, etc.), we can expect (i) an additive effect resulting from the combination of all phytobeneficial capacities, or (ii) a synergistic effect, which would surpass the effects of single inoculations.

Several studies have focused on *Azospirillum* dual-inoculation with (i) other *Azospirillum* (Han et New 1998; Bashan *et al.* 2000), (ii) *Bacillus* (El-Komy 2005), (iii) *Bradyrhizobium* (Steinberg *et al.* 1989; Cassan *et al.* 2009), (iv) phosphate-solubilizing bacteria (*Arthobacter* or *Agrobacterium*) (Belimov *et al.* 1995), (v) *Rhizobium* (Raverkar et Konde 1988; Remans *et al.* 2008), and (vi) *Glomus* (Mar Vázquez *et al.* 2000; Pulido *et al.* 2003), but only certain of them have shown enhanced plant growth stimulation compared to single inoculation (Belimov *et al.* 1995; El-Komy 2005; Remans *et al.* 2008). To our knowledge, two studies have focused on interactions established in complexed *Azospirillum*

mixed-inocula involving more than two microorganisms, such as (i) *Rhizobium* and AMF (Biró *et al.* 2000), and (ii) *Burkholderia*, *Gluconacetobacter*, and *Herbaspirillum* (Oliveira *et al.* 2009). Biro and associates (2000) have shown that co-inoculation of *Azospirillum* with *Glomus* reduced the phytostimulatory activity of the later, whereas synergistic effects were evidenced when those two microorganisms were co-inoculated with *Rhizobium*. Oliveirra and associates (2009) have shown that the competition occurring between *Burkholderia* and *Azospirillum* disappeared when the two were co-inoculated in a mixed-consortium involving three other rhizobacteria, leading to enhanced colonization rates. Thus, negative interactions observed in dual-inoculations were replaced by positive interactions in complexed inocula.

In this study, three different prominents *Azospirillum* inoculants were compared when inoculated in three-component consortia involving (i) another PGPR inoculant *Pseudomonas fluorescens* F113 and (ii) an AMF inoculant mix composed of three *Glomus* species. *P. fluorescens* F113 is another PGPR extensively studied as crop inoculant, and this strain has been shown to be a Mycorrhiza Helper Bacteria (MHB; Barea *et al.* 1998). In the case of maize, *A. lipoferum* CRT1 is one of the main PGPR strain used and commercialized in Europe (Jacoud *et al.* 1998; Lucy *et al.* 2004; El Zemrany *et al.* 2006), whereas *A. brasilense* UAP-154 and CFN-535 inoculants are extensively used in Mexico, which is one of the leading countries in practical field applications (Dobbelaere *et al.* 2001; Fuentes-Ramirez et Caballero-Mellado 2006). Phytostimulation by *Azospirillum* strains has been shown to be cell-density dependant (Jacoud *et al.* 1999), and effective root colonization is required for effective stimulation (Dobbelaere *et al.* 2002).

The objective of this study was to compare prominent *Azospirillum* strains in *Azospirillum-Pseudomonas-Glomus* consortia for promotion of maize growth. The experiment was performed to evaluate survival of the different microbial partners co-inoculated and to assess the impact on maize growth and secondary metabolism.

Table 1 Bacterial strains used in this study

Species	Strain	Host plant	Geographic Origin	Reference
Azospirillum brasilense	UAP-154	Maize	Mexico	Dobbelaere *et al.* 2001
	CFN-535	Maize	Mexico	Dobbelaere *et al.* 2001
Azospirillum lipoferum	CRT1	Maize	France	Fages et Mulard 1988
Pseudomonas fluorescens	F113	Sugar beet	Ireland	Fenton *et al.* 1992

Material and Methods

Microorganisms

All bacterial used in this study are listed in Table 1. To obtain *Azospirillum* inocula, strains were grown in NFb liquid medium (Döbereiner *et al.* 1976) supplemented with NH_4Cl (0.2 g l^{-1}) for 2 days at 30 °C with shaking at 200 rpm, giving 5×10^7 (for *A. lipoferum* CRT1), 2×10^9 (for *A. brasilense* UAP-154) and 7×10^9 (for *A. brasilense* CFN-535) CFU per ml. *P. fluorescens* F113 was grown in Luria Bertani medium supplemented with 0.25 g l^{-1} $MgSO_4 \cdot 7H_2O$ (LB-Mg ; Monika Maurhofer, ETH) for 8 h at 30 °C and 200 rpm. Cultures were adjusted with LB-Mg to an optical density (OD_{600}) of 0.2 to obtain 2×10^9 CFU per ml. Colony counts of inocula were obtained on RC agar (Rodriguez Caceres 1982) for *Azospirillum* strains and LB-Mg agar for *P. fluorescens* F113, after a 72-h incubation of plates at 30°C.

The mycorrhizal inoculum consisted of a mixture of strains *Glomus intraradices* JJ291, *G. claroideum* JJ360, and *G. mossae* JJ964. Each was prepared in plant cultures following commercial procedures by Symbio-M (Lanskroun, Czech Republic). Mycorrhized roots were chopped, mixed together and with zeolite carrier. The inoculum product contained 5.3×10^4 (*G. intraradices* JJ291), 2.9×10^4 (*G. claroideum* JJ360) and 2.5×10^3 (*G. mossae* JJ964) gene copies of the nuclear Large Ribosomal Sub-Unit (nLSU) per g.

Greenhouse experiment

A greenhouse experiment was performed with sieved (4 mm) non-sterile soil taken from the loamy-sandy surface horizon of a Mexican field at Zacatepec near Cuernavaca, Morelos (clay 4.8%, silt 7.9%, sand 87.3%, organic matter 4.3%, pH 7.5). Seeds of maize (*Zea mays*) var. Costeño Mejorado (PROSASOL, Huitchila Morelos, Mexico) were surface-sterilized by stirring in sodium dichloroisocyanurate-containing Bayrochlor Mini solution (Bayrol, Dardilly, France) for 15 min, and washed several times with sterile distilled water (Couillerot *et al.* submitted). The seeds were then germinated on water agar (8.5 g l^{-1}) for 24 h in the dark at 30°C.

Treatments included (i) a non-inoculated control, (ii) inoculation with a two-component consortium composed of *P. fluorescens* F113 and *Glomus* mix, and (iii) inoculation with a three-component consortium containing the two-component consortium and either *A. lipoferum* CRT1, *A. brasilense* UAP-154 or CFN-535. For each bacterial strain, inoculation was done by adding 1 ml of cell suspension (described above) to each germinated

seed. In addition, 65 g of zeolite-formulated *Glomus* inoculum was placed approximately 3 cm below each germinating seed. Sterile water (2 ml) and non-inoculated zeolite (65 g) were used in the non-inoculated control, and 1 ml sterile water in the *Pseudomonas*-*Glomus* treatment.

For the 10-d sampling, 4 maize plants were grown in 1-dm^3 pots containing 1.5 kg soil previously supplemented with 270 ml sterile nutrient solution (described in Rodriguez-Salazar *et al.* 2009). For the later samplings (i.e. at 21 and 35 d), 2 maize plants were grown in 2-dm^3 pots containing 2.3 kg soil previously supplemented with 340 ml of sterile nutrient solution. Five pots were used per treatment, and the 75 pots were placed in a greenhouse (randomized block design) with controlled temperature (26 ºC ± 4 ºC) and natural light and located at Cuernavaca (Mexico). Watering was done by adding 270 and 340 ml of nutrient solution each day in 1 and 2-dm^3 pots, respectively.

Sampling

Watering of the pots was reduced 48 h before the second and third samplings, and stopped 24 h before each sampling. At each sampling, all shoots were cut off and dried 2-4 d at 70°C for biomass determination, one root system per pot was used for *ipdC* RT PCR analysis, and another root system per pot for real-time PCR quantification of PGPR inoculants (and AMF genotypes at the 35-d sampling, after splitting the root system in two parts). In addition, two other root systems per pot were used for plant metabolomic analysis at the first sampling (10 d).

DNA preparation

For PGPR inoculant monitoring, each root system was shaken vigorously to discard soil loosely adhering to the roots. Roots and tightly-adhering soil were transferred in a 50-ml Falcon and flash-freezed in liquid nitrogen. Samples were then lyophilized for 48 h in Falcon tubes and homogenized by crushing in the tubes using a spatula. 250-300 mg of lyophilized sample (rhizosphere soil + roots) were transferred in Lysing Matrix E tubes from the FastDNA® SPIN® kit (BIO 101 Inc., Carlsbad, CA), and DNA was extracted and eluted in 50 µl of sterile ultra-pure water, according to the manufacturer's instructions. DNA concentrations were assessed by OD measurements at 260 nm using NanoDrop (Nanodrop technologies, Wilmington, DE).

For AMF monitoring, roots from the third sampling were cut in 5-cm pieces. They were washed in ice-cold tap water, flash-freezed in liquid nitrogen, and lyophilized for 48 h in Eppendorf tubes. Lyophilized roots samples (25-35 mg) were homogenized by dry bead-beating three times 45 s with glass balls (1 mm diameter) in Biospec Beadbeater-8. DNA was then extracted with Plant DNeasy kit (Qiagen, Courtaboeuf, France) following manufacturer's recommendations.

Real-time PCR assessment

Root colonization was assessed by real-time PCR, as described in Couillerot *et al.* (submitted; for *A. brasilense* inoculants), Couillerot *et al.* (in preparation; for *A. lipoferum* inoculant), von Felten *et al.* (submitted; for *P. fluorescens* inoculant), Thonar *et al*. (submitted) and Jansa *et al.* (submitted) for *Glomus*. Briefly, real-time PCR for *Azospirillum* strains was done using the FastStart DNA Master SYBR Green I kit and a LC-480 LightCycler (Roche Applied Science, Indianapolis, IN), and that for *P. fluorescens* F113 using the Fast SYBR Green kit and a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

The inoculant strain *G. intraradices* JJ291, as well as the species *G. intraradices*, *G. claroideum* and *G. mosseae* (to which the inoculants belonged) and the AMF genera *Gigaspora*, *Scutellospora* and *Diversispora* were assessed by targeting the nLSU or the mitochondrial Large ribosomal Sub-Unit (mtLSU). Real-time PCR was done using TapMan Master kit and a LightCycler 2.0 (Roche Applied Science). Melting curve calculation and determination of Tm values were performed using the polynomial algorithm function of LightCycler Software v.1 (Roche Applied Science) or of the Sequence detection Software v.1.4 (Applied Biosystems).

Data normalization

Plasmid APA9 (i.e. pUC19 with cassava virus insert; Genbank accession number AJ427910) was used as internal standard in order to normalize the C_T values, as described by Park et Crowley 2005). Normalization for *Azospirillum*, *Pseudomonas* and AMF was carried out as described in Couillerot *et al.* (submitted; for *A. brasilense* inoculants), Couillerot *et al.* (in preparation; for *A. lipoferum* inoculant), von Felten *et al.* (submitted; for *P. fluorescens* inoculant), Thonar *et al*. (submitted) and Jansa *et al.* (submitted) for AMF. Briefly, known quantities of purified plasmid APA9 were added at the first step of each DNA extraction protocol, and real-time PCR analyzes were then performed on each DNA extract. C_T values

thus obtained for the internal standard were used to normalize DNA extraction efficiency with the following formula:

$$\text{Normalized } C_T \text{ value of each sample} = \frac{C_T \text{ value of each sample}}{C_T \text{ value of each internal standard}} \times \text{Average } C_T \text{ value of internal standard}$$

Generation of standard curves for real-time PCR assessments

Real-time PCR quantification of PGPR inoculants in the rhizosphere required development of standard curves, as described in Couillerot *et al.* (submitted; for *A. brasilense* inoculants), Couillerot *et al.* (in preparation; for *A. lipoferum* inoculant), von Felten *et al.* (submitted; for *P. fluorescens* inoculant). Briefly, Lysing-Matrix E tubes (BIO 101 Inc.) containing 250-300 mg lyophilized sample (rhizosphere soil + roots) from the non-inoculated control (obtained as described above) were inoculated with one of the four PGPR strains. DNA extraction was performed using the FastDNA® SPIN® kit (BIO 101 Inc.) and real-time PCR was done as described above. A standard curve for each strain was generated by plotting the C_T number against the logarithm of CFU added per g of soil, for the three independent replicates. Amplification efficiency was calculated from the slope of the standard curve using the formula $E = 10^{-1/\text{slope}} - 1$ and standard curves were then used to estimate inoculant cell number in the rhizosphere of seed-inoculated maize plants. Real-time PCR quantification data were expressed as log CFU equivalents per g of dry root.

Real-time PCR assays of the different AMF phylotypes was calibrated by using serially-diluted cloned fragments (pGEM-T Easy vector, Promega) of the AMF large ribosomal subunit, as described by Thonar *et al*. (submitted) and Jansa *et al.* (submitted) for AMF. Real-time PCR quantification data were converted to log gene copies per g of dry root.

ipdC RT PCR analysis

Transcription of auxine synthesis gene *ipdC* by *Azospirillum* spp. was measured by reverse-transcriptase (RT) PCR. Each root system studied was placed (with adhering soil) in a 50 ml Falcon tube and flash freezed in liquid nitrogen. Samples were then washed with 35 ml solution of 100 mM $CaCl_2$ and 50 mM Tris-HCl pH 7.0 (prepared water treated with DEPC 0.5% v/v) supplemented with β-mercaptoethanol 5%. This solution was centrifuged for 5 min at 6 *g*. the pellet containing soil particles and root debris was discarded and the supernatant was centrifuged for 5 min at 2,250 *g*. The resulting pellet was resuspended in 1 ml of TRIZOL reagent (Invitrogene, Carlsbad, CA) according to manufacturer's instructions. DNAse

treatment was then performed and cDNA synthesis was done with RevertAid™ H minus cDNA synthesis kit (Fermentas, Ontario, CDN). cDNA was amplified with *Taq* DNA polymerase (Fermentas) and *Azospirillum* primers ipdcF3 (5′-CTTGCCCTTCTTCAAGGTGG-3′) and ipdCR3 (5′-GGGGGATTTCCAGATAGACC-3′) (unpublished).

Metabolomic analysis

At 10 days, the root systems from two plants per pot were washed with ice-cold distilled water and placed in aluminum envelops before being flash-freezing in liquid nitrogen. Samples were then lyophilized for 72 h and stored at -80°C until analysis. Freeze-dried roots were introduced in Eppendorf tubes, to which liquid nitrogen was added. Roots were crushed using a ball mill (TissueLyser II, Qiagen), and extraction was performed using 2 mL methanol for 10 mg of dry sample. Extraction was done twice and extracts were dried using Speedvac-assisted evaporation. Each sample was then resuspended in methanol to reach 10 mg dry extract/mL.

Chromatographic analysis of the extracts was achieved with an Agilent 1200 series HPLC equipped with a degasser (G132A), a quaternary pump module (G1311A), an automatic sampler (G1329A) and a Diode Array Detector (DAD G1315B). The separation was carried out at room temperature using a NUCLEODUR sphinx C18 column (250 × 4.6 mm; 5 µm-Macherey-Nagel®, Düren, Germany). For each sample, 20 µL of extract was injected and the column was eluted at 1 mL/min, with an optimized gradient established using solvents A (acetic acid 4 ‰ (v/v) in water) and B (acetic acid 4 ‰ (v/v) in acetonitrile) (Carloerba ® reagents, Val de Reuil, France). A step by step gradient was used with an increase of proportion of solvent B until 15% during 5 min, then an isocratic level from 30 min, with a flux of 1 mL/min. Chromatograms were recorded and processed at 254, 280, 310, and 366 nm. The Chemstation Agilent software was used for integration and comparison of chromatograms. Each chromatogram was integrated after standardization of integration parameters. Background peaks present on chromatograms were not integrated.

Statistics

Statistical analyses of real-time quantification data, *ipdC* expression data, and shoot biomass were performed at $P < 0.05$, using S plus software (TIBCO Software Inc., Palo Alto, CA). Chromatographic data obtained from root extracts, i.e. retention time and relative area of each

integrated peak, were compiled in a matrix for discriminant principal component analysis (PCA), as described by Walker *et al.* (submitted). Treatments were studied by ANOVA followed with Tukeys tests ($P < 0.05$).

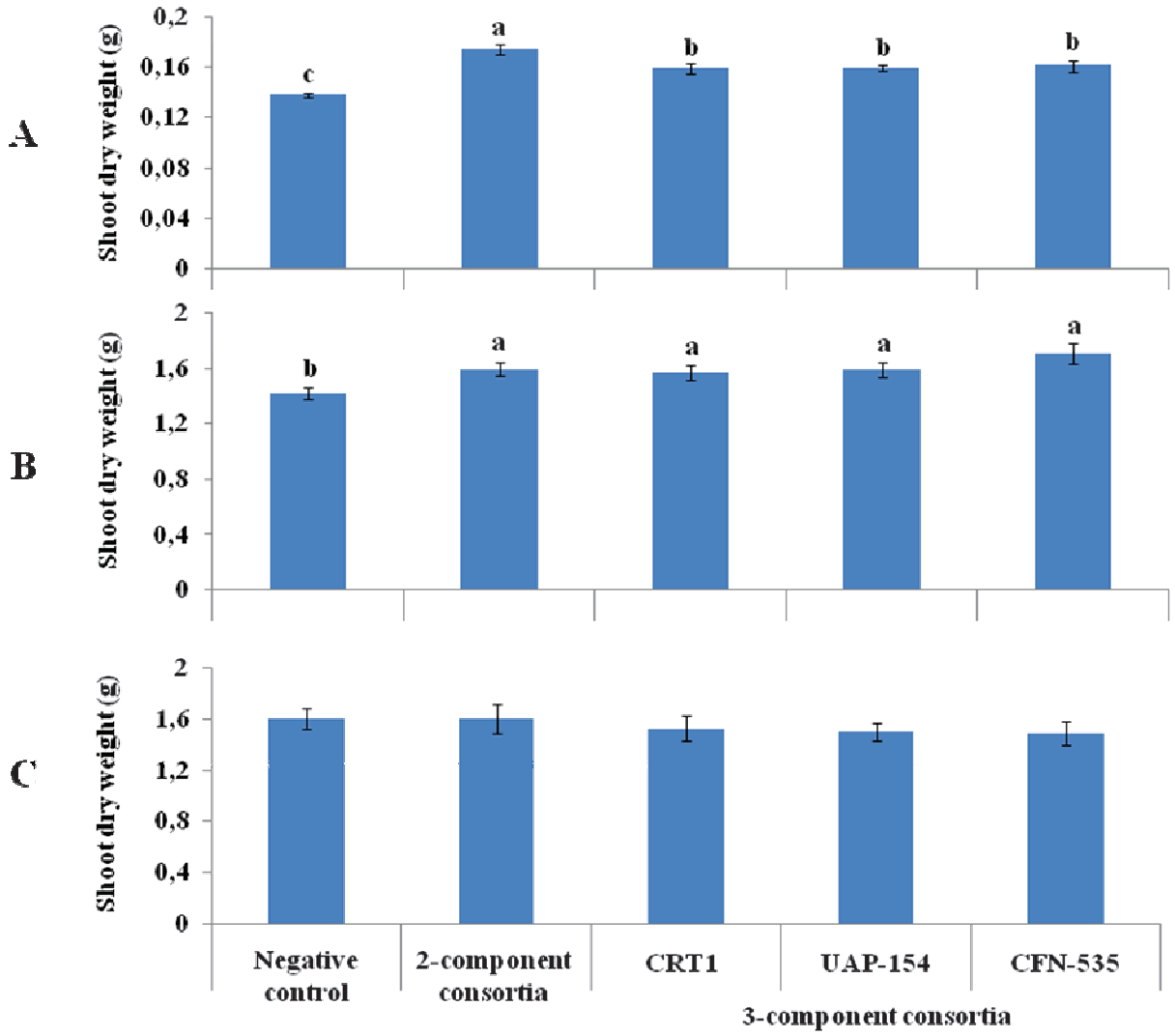

Figure 1. Shoot biomass of maize (g dry shoots per plant) at 10 (A), 21 (B), and 35 (C) days after inoculation with either the 2-component consortium or with 3-component consortia each differing by the *Azospirillum* strain involved (mean ± SD; n = 5). Statistical differences between treatments are indicated with letters a-b (ANOVA and Fisher LSD test ; $P < 0.05$).

Results

Effect of microbial consortia on maize growth

By comparison with the non-inoculated control, inoculation of maize with the *Pseudomonas-Glomus* two-component consortium resulted in higher shoot biomass at the first two samplings (Fig 1A-B). Shoot biomass was also higher in all three-component consortia than in the non-inoculated control at the first two samplings, but results were not influenced by the identity of the *Azospirillum* strain (Fig 1A-B). At the first sampling, however, shoot biomass with the two-component consortium was higher than those in the three-component consortia (Fig 1A). Shoot biomass was not influenced by inoculation at the third sampling (Fig 1C), but this result may be of limited significance since by then roots had extensively colonized the whole soil volume in the pots.

Inoculant colonization of maize roots

When the *Pseudomonas-Glomus* two-component consortium was used, *P. fluorescens* F113 was enumerated at about 10^8 CFU equivalents per g of rhizosphere at the first sampling and at 2 log units lower by the third sampling (Fig 2). The presence of an *Azospirillum* inoculant resulted in higher F113 population levels at two of the three samplings (with *A. lipoferum* CRT1 or *A. brasilense* UAP-154), or had no effect of the pseudomonad (with *A. brasilense* CFN-535). As expected, strain F113 was not found in the non-inoculated treatment.

The population size of *A. brasilense* CFN-535 dropped from 1.5×10^7 to 2.7×10^5 CFU equivalents per g of rhizosphere from the first to the third sampling (Fig 2). In comparison, *A. brasilense* UAP-154 and especially *A. lipoferum* CRT1 were recovered at lower levels, which fell below detection limit (4×10^3 CFU equivalents per g of rhizosphere) by the third (for strain UAP-154) or second sampling (for strain CRT1). None of the *Azospirillum* inoculants (i.e. even the two Mexican isolates) was not found in the non-inoculated treatment.

Among AMF inoculants, a quantification method was only available for *G. intraradices* JJ291. At the third sampling, this strain was not found in the non-inoculated treatment, but was detected in two of five replicate samples when the *Pseudomonas-Glomus* two-component consortium was used. With the three-component consortia, *G. intraradices* JJ291 was only found in one sample in the treatment where *A. brasilense* CFN-535 was included.

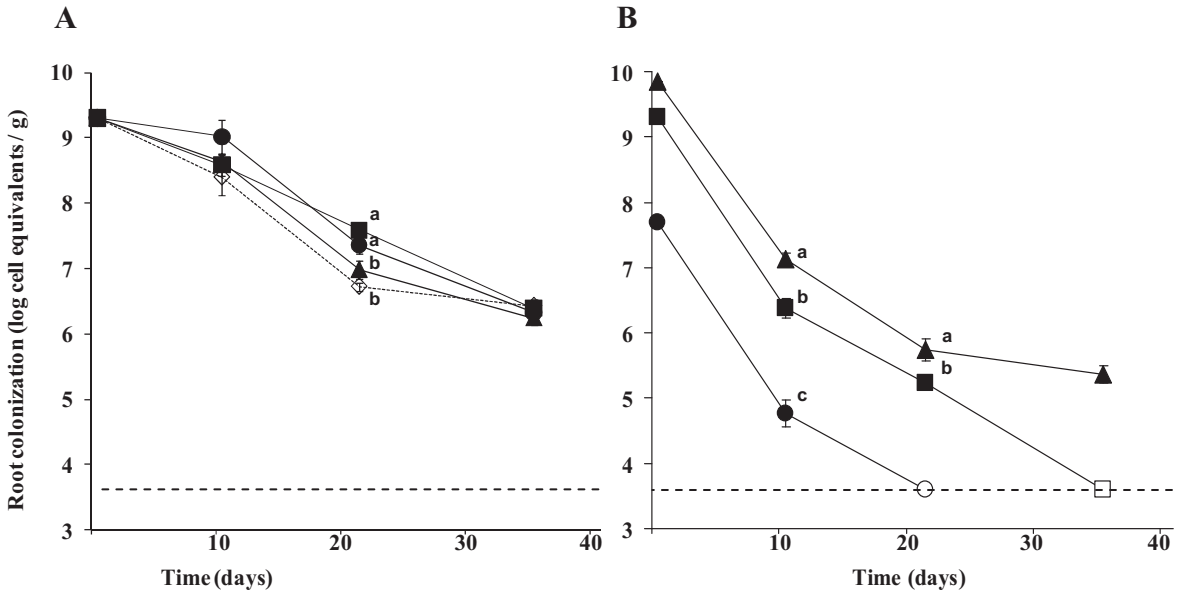

Figure 2. Root colonization of the PGPR strains *P. fluorescens* F113 (**A**) and *Azospirillum* (**B**) used as part of two-component (F113 + AMF mix; ◊) or three-component consortia (with *A. lipoferum* CRT1 (●), *A. brasilense* UAP-154 (■) or CFN-535 (▲)). Data represent means ± SD (n = 5) of log cell equivalents per g of rhizosphere. The detection limit (4×10^3 cell equivalents per g of rhizosphere) is shown by dotted lines and symbols appear in white for *Azospirillum* inoculants below detection limit. Statistical differences between treatments at each sampling time are indicated with letters (ANOVA and Fisher's LSD tests; $P < 0.05$).

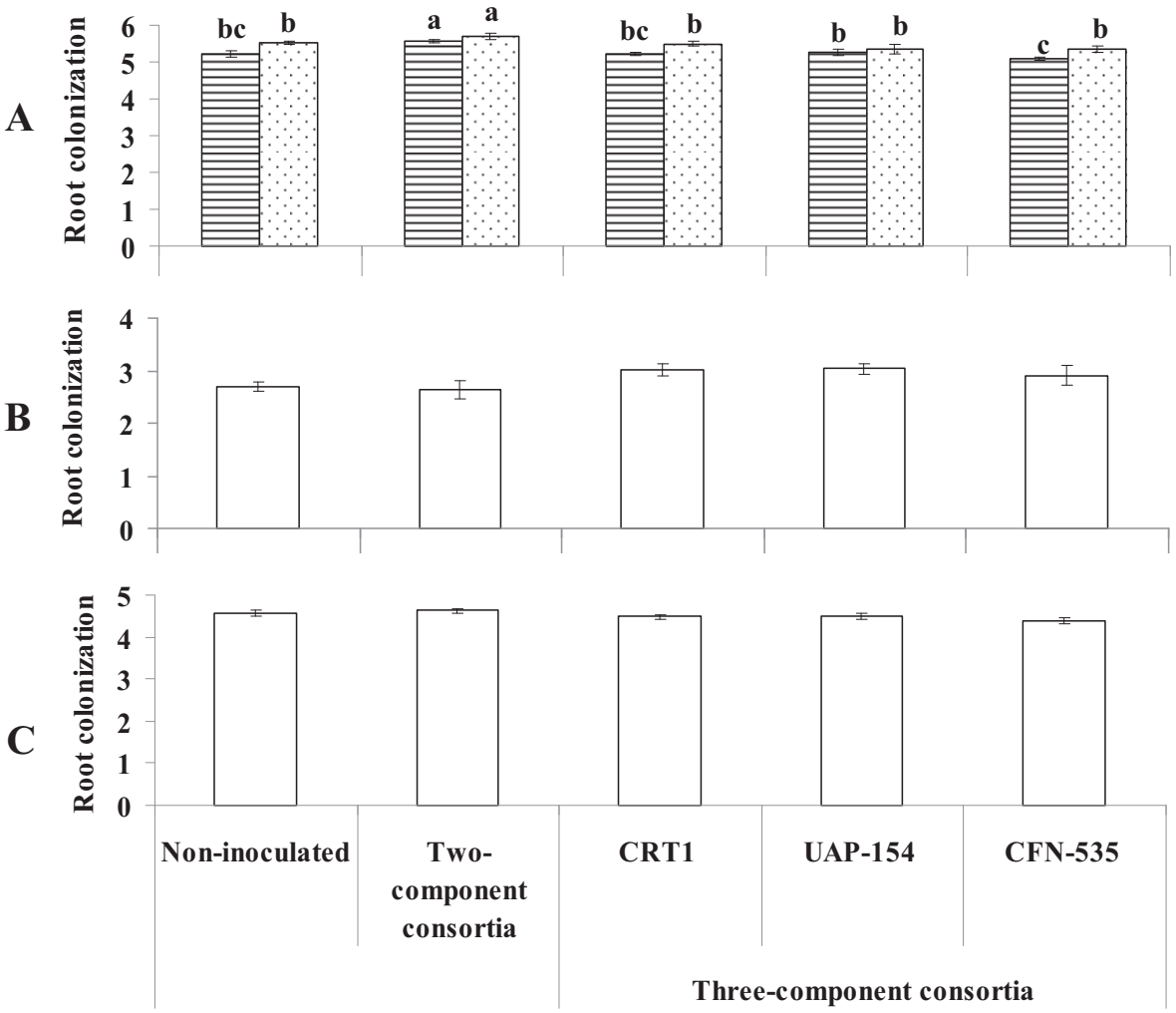

Figure 3. Effect of PGPR co-inoculation in two and three-component consortia on AMF root colonization of *G. intraradices* (nLSU and mtLSU methods; A), *Diversispora* (B) and *G. claroideum* (C) in the rhizosphere of maize grown in a Mexican non sterile soil, 5 weeks after inoculation (mean of log gene copies per g of dry root ± SD; n = 5). Statistical differences between treatments are indicated with letters (ANOVA and Fisher LSD tests; $P < 0.05$).

Table 2 RT-PCR detection of *Azospirillum ipdC* gene, as indicated by the number of plants (out of 5) for which amplification was successful

Treatments	1st sampling (10 days)	2nd sampling (21 days)	3rd sampling (35 days)
Non-inoculated control	1	2	0
Two-component control (F113 + AMF)	2	0	0
Three-component consortium (CRT1)	4	3	1
Three-component consortium (UAP-154)	3	4	2
Three-component consortium (CFN-535)	3	3	2

Effect of inoculation on root-associated AMF populations

In the non-inoculated treatment, at the third sampling, the *G. intraradices* species was enumerated at 1.8×10^5 (nLSU method) and 2.0×10^5 (mtLSU method) gene copies (Fig 3A), the *G. claroideum* species at 3.8×10^5 nLSU gene copies (Fig 3C), and the *Diversispora* genus at 5.4×10^2 nLSU gene copies (Fig 3B) per mg of dry root. When the two-component consortium was used, *G. intraradices* was recovered at higher level in comparison with the control, regardless of the method (Fig 3A). With the three-component consortia, the population size of *G. intraradices* was comparable to that in the non-inoculated control and lower to that detected when the two-component consortium was used. Inoculation had no effect on the size of the *G. claroideum* species (Fig 3C) or the *Diversispora* genus (Fig 3B). The *G. mosseae* species and the AMF genera *Scutellospora* and *Gigaspora* were not found in any of the treatments.

ipdC transcription in *Azospirillum*

Successful RT-PCR amplification of *Azospirillum*'s *ipdC* mRNAs was observed even in the absence of *Azospirillum* inoculation, i.e. in the non-inoculated control (at the first two samplings) and when the two-component consortium was used (at the first sampling) (Table 2). When three-component consortia were applied, transcription of *ipdC* gene in *Azospirillum* (i) was found in 3-4 of 5 replicates at the first two samplings (versus only 2 replicates or less in the other treatments) and (ii) was also detected at the third sampling.

Effect of inoculation on secondary metabolite profiles of maize roots

Chromatograms at 280 nm for root methanolic extract gave 18 major integrated peaks, 11 of them corresponding to benzoxazinoid derivatives based on UV spectra (Walker *et al.* submitted). Polar compounds (based on water elution) were cyclic hydroxamic acids, whereas two more apolar compounds were benzoxazinone derivatives.

Discriminant PCA indicated that all inoculation treatments resulted in changes in the secondary metabolite profile of maize (Fig 4). The inoculation impact varied according to the consortium, except that presence of *A. brasilense* UAP-154 within the *Pseudomonas*-*Glomus* two-component consortium had no effect. When assessing individual compounds responsible for treatment discrimination, it appeared that the prevalence of five PCA-discriminant secondary metabolites (including three benzoxazinoid derivatives and one

cinnamic acid) differed significantly between treatments based on ANOVA and Tukey's test (Fig 5).

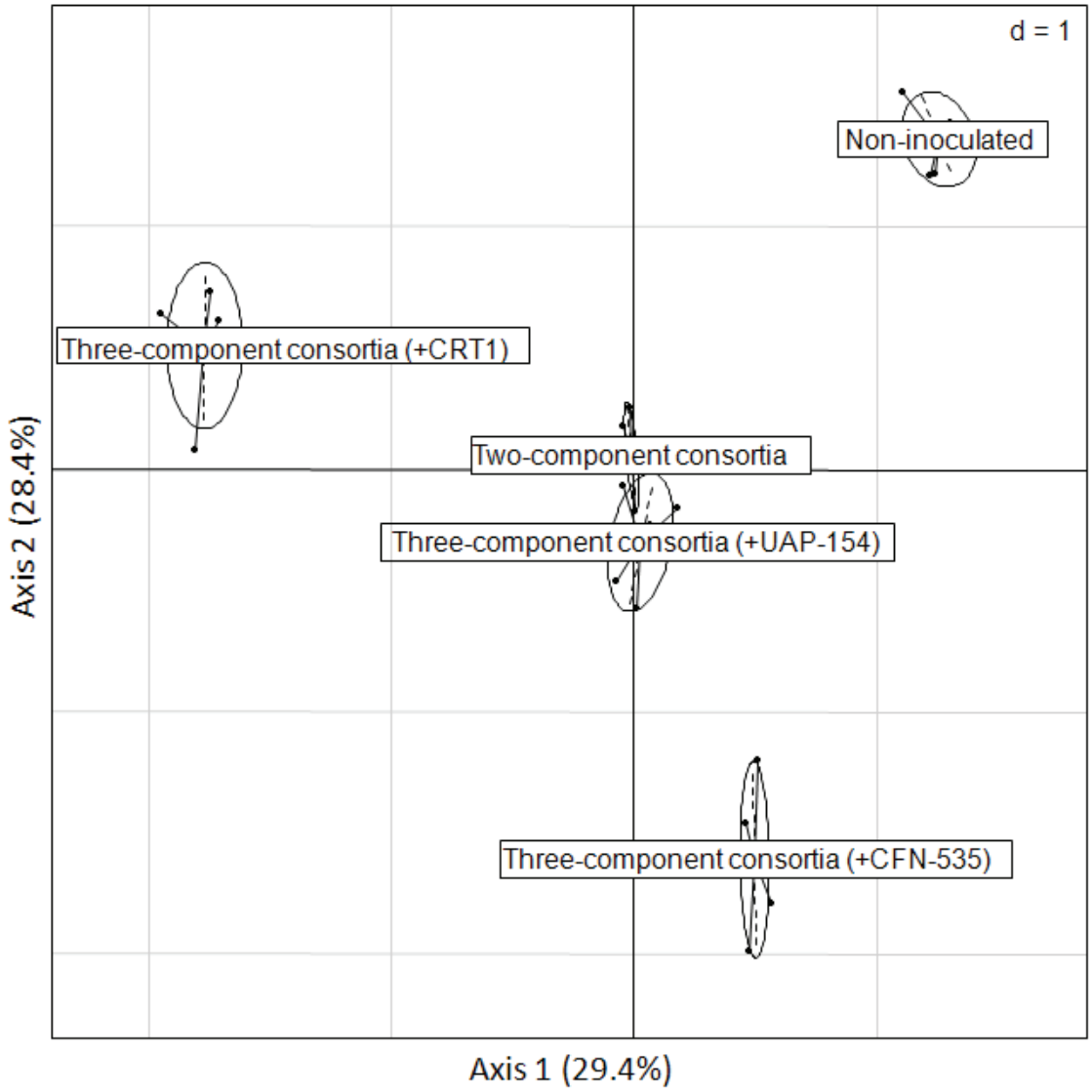

Figure 4. Discriminant PCA performed on chromatographic data obtained for each methanolic extract of maize. Analyses were based on peak areas and retention times. Each point represents two pooled extracts of the same treatment (2 plants).

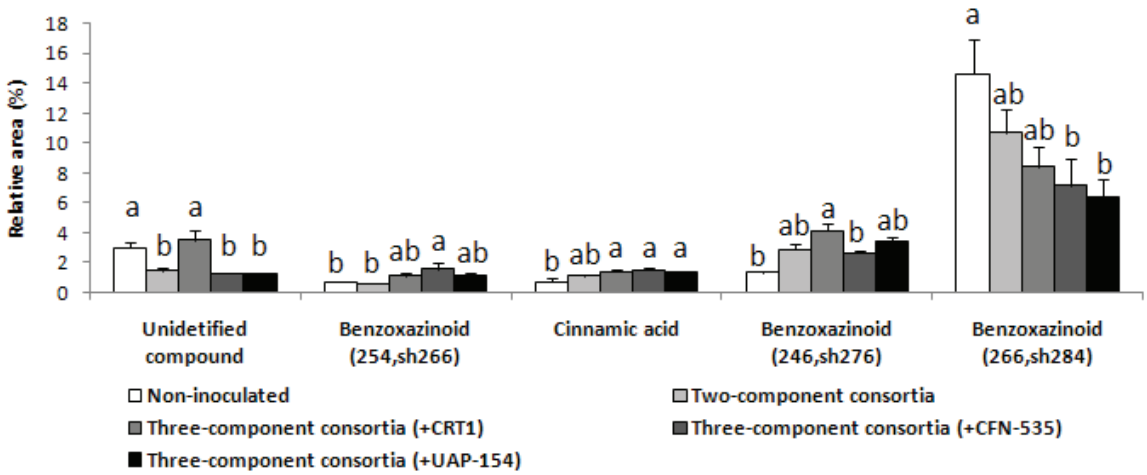

Figure 5. Effect of maize seed inoculation on root content in individual secondary metabolites that distinguished treatments in discriminant PCA. Statistical differences between treatments are indicated with letters (ANOVA and Tukey tests; $P < 0.05$).

Discussion

Azospirillum PGPR strains have been extensively studied as phytostimulatory inoculants of cereal crops (Okon et Labandera-Gonzalez 1994; Dobbelaere *et al.* 2001), and to a lesser extent in mixed inocula combining phytobeneficial microorganisms with different metabolic capacities (Bashan 1998a). Indeed, most studies on mixed inocula containing diazotrophic bacteria have been performed with bacteria other than *Azospirillum* (Biró *et al.* 2000; Remans *et al.* 2008; Cassan *et al.* 2009; Oliveira *et al.* 2009). To our knowledge, there is only one report in which *Azospirillum* was combined with a microorganism such as *Pseudomonas*, which functions also as a biocontrol agent (Corich *et al.* 1995). Combining *Pseudomonas* antagonistic biocontrol agents and *Azospirillum* requires special attention regarding potential inhibitory effects of *Pseudomonas* antimicrobial metabolites, such as 2,4-diacetylphloroglucinol (DAPG), against *Azospirillum*. We verified that the three *Azospirillum*

strains used in this study were rather resistant to DAPG, as growth inhibition required as much as 500 µM of synthetic DAPG.

The three *Azospirillum* strains showed very different root colonization abilities. Only the two *A. brasilense* strains managed to colonize roots significantly (i.e. at levels above 10^5 CFU equivalents per g of rhizosphere). *A. lipoferum* CRT1 declined rapidly, in contrast to results of maize experiments done in Europe (El Zemrany *et al.* 2006; Couillerot *et al.* in preparation). Perhaps this was due to the particular soil type occurring in the current Mexican experiment. In all inoculation treatments, *P. fluorescens* F113 colonized maize roots extensively. Unexpectedly, F113 population level was significantly enhanced when the pseudomonad was in presence of an *Azospirillum* inoculant, but the significance of this effect depended on the *Azospirillum* strain × sampling combination. The mechanism involved is unknown, but in future work it will be worth assessing potential syntrophic interactions between both taxa. Only one *Glomus* strain (i.e. *G. intraradices* JJ291) could be monitored by molecular means. Results indicated that this strain was not present in the non-inoculated control, but detection was poorly effective in the inoculation treatments, thereby limiting the usefulness of this assessment in the current experiment. Perhaps this resulted from strong competition with indigenous AMF (Biro 2000), a possibility raised by our molecular data on taxa of indigenous AMF, such as *Diversispora*.

Little is known about indigenous AMF taxa colonizing maize roots in Mexican soils. Here, AMF associated to roots were studied as a mean to explore potential effects of AMF inoculation. Results showed that several AMF taxa were well established, but they failed to evidence any effect of inoculation, except for a moderately higher prevalence of *G. intraradices* when the two-component consortium was used. It is tempting to speculate that this was due to the inoculation of *G. intraradices* JJ291, even though monitoring of the latter fell below expectations. It also appears that presence of DAPG-producing *P. fluorescens* F113 had no apparent deleterious impact on root-associated AMF, despite antifungal properties of DAPG (Barea *et al.* 1998; Mar Vázquez *et al.* 2000; Gaur *et al.* 2004). There was no sign either of negative effect of *Azospirillum* on AMF establishment, in accordance with previous mycorrhization assessments (Russo *et al.* 2005).

Stimulation of maize shoot growth was significant when seeds were inoculated with any three component-consortium, i.e. whatever the *Azospirillum* strain involved. It is interesting to note that this took place despite (i) contrasted survival dynamics for different *Azospirillum* inoculants, (ii) different F113 population levels depending on the *Azospirillum* strain, and (iii) maize secondary metabolite profiles that varied between most treatments.

Maize elaborated specific metabolic patterns according to the *Azospirillum* strain present, whose variation induced by microbial inoculation concerned several types of secondary compounds, including some already identified (Walker *et al.* submited). In addition, it was rather unexpected that presence of *Azospirillum* in the inoculum resulted in lower maize stimulation in comparison with the *Pseudomonas*-*Glomus* two-component consortium, but this effect was transient. Its molecular basis remains unknown. Finally, no maize shoot growth stimulation was observed at the last sampling, but this can be explained by the limitation of root growth by the size of the pots, and it correlated with lower detection of *Azospirillum ipdC* gene expression at this sampling time.

In conclusion, this study indicated that *Azospirillum*-*Pseudomonas*-*Glomus* three-component consortia may be useful for early stimulation of maize growth. Despite evidence for distinct interaction functioning according to the *Azospirillum* strain included, the identity of the *Azospirillum* strain was not a significant factor determining phytostimulation efficiency.

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Discussion générale

Au cours de cette thèse, nous nous sommes intéressés aux interactions qui peuvent s'établir entre les PGPR des genres *Azospirillum* et *Pseudomonas*, avec l'objectif de déterminer le niveau de compatibilité entre ces PGPR, qui appartiennent à des taxons très étudiés mais dont les interactions les unes avec les autres ont été jusqu'ici négligées. Les mécanismes d'interaction de ces deux genres de PGPR avec les plantes (et/ou les phytopathogènes) sont largement documentés dans la littérature scientifique (Steenhoudt et Vanderleyden 2000; Haas et Keel 2003; Bashan *et al.* 2004; Weller 2007; Couillerot *et al.* 2009). Ces PGPR font aussi l'objet de nombreuses recherches appliquées en vue de leur utilisation en tant qu'inocula dans les domaines de l'agronomie, l'horticulture, la bioremédiation ou le traitement d'effluents (Dobbelaere *et al.* 2001; Lucy *et al.* 2004; de-Bashan et Bashan 2005; Fuentes-Ramirez et Caballero-Mellado 2006). Lors de leur inoculation en sol non stérile, ces PGPR sont confrontées aux microorganismes indigènes (compétition, antagonisme, etc.) ainsi qu'à de nombreux facteurs environnementaux, qui peuvent influencer la survie et le fonctionnement de ces deux PGPR dans la rhizosphère (Bashan 1998a; Bashan 1999). L'ensemble de ces interactions biotiques et abiotiques établies par ces PGPR lors de leur inoculation va conditionner l'impact final des PGPR sur le fonctionnement de la plante ; et c'est dans ce contexte que l'étude de la compatibilité entre *Azospirillum* et *Pseudomonas* a été effectuée.

Inhibitions possibles entre PGPR : le cas du DAPG produit par certains PGPR *Pseudomonas*

Les bactéries appartenant au genre *Azospirillum* sont reconnues comme des PGPR phytostimulatrices, dont les principaux modes d'action reposent sur la synthèse d'enzymes et d'hormones modulant le développement des plantes (Steenhoudt et Vanderleyden 2000; Dobbelaere *et al.* 2003). Tandis que les bactéries appartenant au genre *Pseudomonas*, et plus spécialement les *Pseudomonas* fluorescents, sont reconnues comme des PGPR phytoprotectrices, dont l'un des principaux modes d'action est la production de métabolites antimicrobiens (Dwivedi et Johri 2003; Haas et Défago 2005; Weller 2007). Une des observations intéressantes effectuées par nos partenaires du projet MicroMaize, dans le cadre duquel cette thèse a été effectuée, est la capacité de certaines souches de *Pseudomonas* (étudiées habituellement comme phytoprotectrices) à stimuler la croissance du maïs, renforçant par la même l'intérêt de considérer leurs interactions avec des PGPR *Azospirillum* stimulatrices du maïs.

L'un des principaux métabolites antimicrobiens produits par les PGPR *Pseudomonas* est le DAPG (Haas et Keel 2003; Weller *et al.* 2007). Nous avons donc étudié dans un premier temps le rôle de ce composé dans les interactions entre *Azospirillum* et *Pseudomonas*. Afin de pouvoir clairement identifier l'impact du DAPG sur *Azospirillum*, des expériences préliminaires ont été réalisées avec du DAPG synthétique. Ces expériences ont permis de montrer que le DAPG avait un impact sur la physiologie des cellules d'*Azospirillum*, et notamment au niveau de l'accumulation de granules de PHB. L'accumulation de PHB chez *Azospirillum* pourrait jouer un rôle écologique important lors de la colonisation de la rhizosphère ainsi que dans la résistance à différentes conditions de stress (Okon et Itzigsohn 1992; Kadouri *et al.* 2003). Elle pourrait constituer un mécanisme de résistance au DAPG, même si nous n'avons pas obtenu de résultats confortant cette hypothèse. Ces expériences ont aussi permis de montrer que le DAPG inhibait la croissance d'*Azospirillum* de façon espèce-dépendante. Les concentrations inhibitrices s'étalent ainsi de 100 µM pour *A. lipoferum* 4B (une des souches les plus sensibles) à plus de 1000 µM pour *A. irakense* KBC1 (une des souches les plus résistantes). Quatre mécanismes de résistance au DAPG ont été identifiés à ce jour: (i) l'inhibition de la production de DAPG par *Fusarium* via la production d'acide fusarique (Notz *et al.* 2002), (ii) l'hydrolyse du DAPG en mono-acétylphloroglucinol, moins toxique (Bottiglieri et Keel 2006), (iii) l'implication d'une perméase (Abbas *et al.* 2004) et (iv) l'implication d'un système de pompe à efflux (Tian *et al.* 2009). L'ensemble de ces mécanismes pourrait être potentiellement impliqué dans la résistance de la souche *A. irakense* KBC1, mais aucune donnée n'est jusqu'ici disponible dans le cas d'*Azospirillum*. La répartition des souches d'*Azospirillum* le long d'un gradient de résistance au DAPG amène à penser que ces différents niveaux de résistance pourraient impliquer des barrières à la diffusion du DAPG. *Azospirillum* a en effet la capacité à produire différents exopolysaccharides (EPS ; Del Gallo *et al.* 1989; Katupitiya *et al.* 1995; Burdman *et al.* 1998; Pereg-Gerk *et al.* 1998), qui pourraient limiter la pénétration du DAPG dans la cellule.

Les concentrations en DAPG nécessaires pour inhiber *Azospirillum* sont nettement inférieures à celles requises *in vitro* pour l'inhibition totale de la croissance de certains phytopathogènes, mais supérieures à celles mesurées dans des sols rhizosphériques inoculés (Keel *et al.* 1992; Bonsall *et al.* 1997). Cela suggère qu'en conditions rhizosphèriques, (i) le DAPG ne pourrait avoir un effet inhibiteur qu'au niveau de microsites où *Azospirillum* et *Pseudomonas* seraient colocalisées et (ii) qu'*Azospirillum* ne serait sans doute pas inhibée par le DAPG. Pour tester ces deux hypothèses, nous avons pris en compte la grande variabilité dans les niveaux de sensibilité des souches d'*Azospirillum* spp. au DAPG en choisissant

quatre souches d'*Azospirillum* de sensibilité contrastée: *A. irakense* KBC1, *A. brasilense* Sp245 et Cd, et *A. lipoferum* 4B. Ces quatre souches ont ensuite été confrontées (i) à la souche DAPG$^+$ *P. fluorescens* F113, (ii) a un mutant DAPG$^-$, et (iii) un mutant complémenté DAPG$^+$, lors de leurs inoculations sur plante en systèmes gnotobiotiques. Ces expériences nous ont permis de prendre en compte la capacité de production du DAPG chez *Pseudomonas* et ainsi de montrer qu'elle était impliquée dans l'inhibition de la colonisation racinaire des souches d'*Azospirillum* sensibles au DAPG, en tout cas dans les conditions expérimentales choisies. La capacité de production de DAPG n'a cependant pas été directement impliquée dans l'impact de la souche F113 sur les effets phytostimulateurs d'*Azospirillum*. Or l'inhibition de la production de DAPG, chez le mutant DAPG$^-$, n'affecte pas ses capacités de colonisation racinaire (Carroll *et al.* 1995), et les *Pseudomonas* fluorescents synthétisent aussi d'autres composés antimicrobiens (Dwivedi et Johri 2003; Haas et Keel 2003). Ces résultats suggèrent donc que (i) la compétition spatiale au niveau de la colonisation racinaire suffirait à inhiber l'expression du potentiel phytostimulateur d'*Azospirillum* et (ii) d'autres métabolites synthétisés par la souche F113 (par exemple de l'acide cyanhydrique) pourraient avoir un impact sur *Azospirillum.*

Des expériences complémentaires ont été effectuées avec la souche *A. lipoferum* 4B, très sensible au DAPG, en prenant en compte sa capacité à générer des variants phénotypiques. Les résultats obtenus ont montré une plus forte résistance du variant $4V_I$, mais les mécanismes physiologiques impliqués dans cette résistance accrue restent à identifier. Nous avons néanmoins pu formuler deux hypothèses (non mutuellement exclusives) à l'issue de ces expériences : (i) le DAPG pourrait sélectionner la sous-population de cellules $4V_I$ au détriment de la sous-population de 4B, et (ii) le DAPG pourrait stimuler le processus de variation de phase chez 4B, et ainsi favoriser la mise en place de mécanismes de résistance chez cette bactérie.

La compatibilité entre *Azospirillum* et *Pseudomonas* : nécessité d'études en sol non stérile et de développer de nouveaux outils de suivi des souches inoculées

L'ensemble des mécanismes de régulation susceptibles de moduler la production de DAPG par *Pseudomonas* n'a cependant été que partiellement pris en compte au cours des expériences que nous avons réalisées en systèmes gnotobiotiques. Or la production de DAPG est régulée par de multiples facteurs biotiques et abiotiques, et cette régulation joue un rôle primordial dans l'expression du potentiel phytoprotecteur chez les *Pseudomonas* fluorescents (Schnider-Keel *et al.* 2000; Notz *et al.* 2001; Notz *et al.* 2002; Haas et Keel 2003; Maurhofer

et al. 2004; Baehler *et al.* 2005; de Werra *et al.* 2008; Jamali *et al.* 2008; Paulin *et al.* 2009). Afin d'intégrer certains de ces facteurs biotiques (présence d'une communauté microbienne indigène) et abiotiques (présence de sol) qui peuvent influencer la production de DAPG, nous avons souhaité poursuivre notre étude en sol non stérile. Il a alors été nécessaire de développer, au préalable, les outils nécessaires pour pouvoir caractériser ces PGPR.

Nous avons choisi de développer des protocoles de PCR quantitative en temps réel afin de palier au manque d'outils actuellement disponibles pour le suivi de souches inoculées en sol non stérile. De tels outils étant développés en parallèle sur *Pseudomonas*, par nos partenaires du projet MicroMaize, nous avons ainsi pu nous focaliser sur le développement de méthodes de PCR quantitative en temps réel pour le suivi des souches d'*Azospirillum* CRT1, UAP-154 et CFN-535 inoculées en sol non stérile. Deux approches différentes ont été suivies pour le développement de ces outils en fonction des souches concernées. Pour les souches *A. brasilense* UAP-154 et CFN-535, des amorces ont été conçues sur la base de marqueurs SCAR souche-spécifiques obtenus à partir de fragments générés aléatoirement par des PCR de type RAPD, BOX ou ERIC et ne présentant aucune homologie avec (i) les séquences de la base de donnée NCBI et (ii) le génome séquencé de la souche *A. brasilense* Sp245. Les SCAR-marqueurs (*Sequence Charaterized Amplified Region*) ainsi obtenus ont sans doute majoritairement concerné des régions inter-géniques. Des amorces souche-spécifiques ont ensuite été sélectionnées après un criblage en PCR qualitative avec des pools de souches rhizosphériques en tant que témoins négatifs, et une augmentation des températures de fusion de ces amorces a suffit à obtenir des méthodes de PCR quantitative suffisamment spécifiques en sol non stérile.

Tandis que pour la souche *A. lipoferum* CRT1, aucun jeu d'amorce suffisamment spécifique n'a pu être obtenu après le criblage en condition de PCR qualitative. Ce résultat peut s'expliquer par le manque de spécificité des SCAR-marqueurs à partir desquels les amorces ont été conçues. En effet, la recherche de séquences non homologues entre les souches CRT1 et Sp245 n'a sans doute pas été assez discriminante de part l'écart génétique entre les espèces *lipoferum* et *brasilense*. Les fortes différences mises en évidence entre les gènes de ménage de ces deux espèces (Blaha *et al.* 2005) ainsi que l'existence de plasmides spécifiques à chacune de ces deux espèces tend à soutenir cette hypothèse (Vande Broek et Vanderleyden 1995; Holguin *et al.* 1999). Des amorces ont donc été développées à partir d'un fragment spécifique préalablement identifié par Jacoud et collaborateurs en 1998, mais de nombreuses optimisations ont néanmoins été nécessaires pour obtenir une méthode souche-spécifique en sol non stérile.

Ces résultats indiquent que le développement d'outils de quantification en sol non stérile est possible pour des souches (i) pour lesquelles on ne dispose pas de SCAR-marqueurs préalablement identifiés mais dont au moins une souche de la même espèce a été séquencée ou (ii) dont la spécificité des amorces n'est pas totale en conditions de PCR qualitative. Il est aussi important de noter qu'au cours des deux approches abordées, nous avons procédé à de nombreux tests en conditions de PCR qualitative avec des pools de souches rhizosphériques en tant que témoins négatifs. Cette phase de test préliminaire, avant de passer en PCR quantitative, a sans doute été trop discriminante au vue de (i) du nombre de jeux d'amorces éliminés et (ii) des concentrations en ADN utilisées pour les témoins négatifs.

La compatibilité entre *Azospirillum* et *Pseudomonas* : analyse au sein de consortia utilisés sur maïs cultivé en sol non stérile

Les outils de PCR quantitative développés ont été utilisés pour caractériser la compatibilité entre *Azospirillum* et *Pseudomonas* au sein de consortia comprenant aussi des champignons mycorhiziens. Le choix de champignons mycorhiziens s'explique par (i) le rôle des PGPR en tant que bactéries auxiliaires de la mycorhization (Garbaye 1994; Barea *et al.* 1998; Frey-Klett *et al.* 2005), (ii) la constitution de niches spécifiques lors de la mycorhization (Bianciotto et Bonfante 2002), et (iii) la nécessité de prendre en compte les plantes, les champignons mycorhiziens et les bactéries en tant que partenaires d'un réseau d'interactions (Bonfante et Anca 2009). Sur cette base, un inoculum mixte de trois souches de *Glomus* a été co-inoculé avec la souche *P. fluorescens* F113, dont le rôle d'auxiliaire de la mycorhization a déjà été montré (Barea *et al.* 1998). Les trois souches d'*Azospirillum* CRT1, UAP-154 et CFN-535 ont été ensuite choisies pour être intégrée à notre dispositif expérimental, de par leur importance agronomique majeure (Dobbelaere *et al.* 2001; Lucy *et al.* 2004; El Zemrany *et al.* 2006; Fuentes-Ramirez et Caballero-Mellado 2006) et leur résistance relative au DAPG.

L'expérience avec *Azospirillum*, *Pseudomonas*, et des champignons mycorhiziens à arbuscules a été réalisée sur maïs cultivé en sol non stérile, sous serre. Elle a permis de montrer que malgré des niveaux de sensibilité au DAPG équivalents pour les trois souches d'*Azospirillum*, chacune d'elles a montré des niveaux de colonisation racinaires différents en co-inoculation avec *P. fluorescens* F113. Ces différences n'ont cependant pas eu d'impact sur le potentiel de phytostimulation de chaque consortium testé, ce qui peut amener à penser qu'*Azospirillum* ne joue qu'un rôle mineur dans la phytostimulation de la plante, lorsqu'il est co-inoculé avec *Pseudomonas* et des champignons mycorhiziens à arbuscules. Nous avons néanmoins pu montrer que la réponse des plantes à l'inoculation était spécifique de chaque

consortia testé, ce qui reflète l'impact d'*Azospirillum*, au niveau de la souche, dans l'interaction des consortia testés avec le maïs. Cette dernière observation est étayée par l'impact négatif d'*Azospirillum* sur la phytostimulation réalisée par le couple *Pseudomonas* / *Glomus*. Les interactions entre *Azospirillum* et *Pseudomonas* ont peut-être conduit à une perturbation de celles établies par *Pseudomonas* avec *Glomus*. La co-inoculation d'*Azospirillum* avec le couple *Pseudomonas* / *Glomus* a néanmoins conduit à une phytostimulation du maïs en comparaison avec le témoin non-inoculé. Par conséquent, ces expériences d'inoculations en sol non stérile suggèrent qu'*Azospirillum* et *Pseudomonas* peuvent être compatibles dans la rhizosphère, et que les interactions entre ces bactéries (i) sont souche-dépendantes, (ii) rendent possible l'utilisation de ces bactéries au sein de consortia tripartite avec des *Glomus*, mais (iii) sont susceptibles d'avoir un impact négatif sur l'efficacité phytostimulatrice du couple *Pseudomonas* / *Glomus*.

Perspectives

Ce travail de thèse ouvre de nouvelles perspectives, qu'il serait intéressant de considérer dans le futur. Premièrement, les mécanismes impliqués dans la résistance accrue du variant $4V_I$ par rapport à sa souche sauvage *A. lipoferum* 4B restent à déterminer. Or la variation de phase joue un rôle prépondérant dans la compétence rhizosphérique de certaines PGPR (van den Broek *et al.* 2005; Vial *et al.* 2006b). La relation qui pourrait être établie entre le processus de variation de phase et la résistance au DAPG pourrait ainsi (i) expliquer les niveaux de sensibilités très variables observés sur la collection de souches d'*Azospirilllum* spp. testées en condition *in vitro* et (ii) mettre en évidence un processus de communication entre *Azospirillum* et *Pseudomonas* avec l'implication du DAPG en tant que molécule signal. Il est important de rappeler que le DAPG est un signal pour la plante, en (i) stimulant et modifiant le processus d'exsudation racinaire et (ii) modifiant la morphologie des systèmes racinaires. Les *Pseudomonas* fluorescents producteurs de DAPG sont donc susceptibles de façonner leur habitat rhizosphérique, ce qui aura indirectement un impact sur la compatibilité de ces derniers avec les PGPR du genre *Azospirillum*.

Deuxièmement, l'ensemble des travaux présentés dans ce manuscrit de thèse repose sur des expériences de confrontation entre *Azospirillum* et *Pseudomonas* inoculés, alors que des populations indigènes d'*Azospirillum* et de *Pseudomonas* peuvent être mises en évidence dans une même rhizosphère (Kyselkovà *et al.* 2009). Cela pose la question de la compatibilité entre ces deux genres de PGPR au niveau des populations indigènes de la rhizosphère. De tels travaux pourraient être réalisés par une approche de puce à ADN ciblant plusieurs espèces de

ces deux genres. La puce 16S de Kyselkova *et al*. (2009) semble pertinente pour *Azospirillum* mais pas pour *Pseudomonas*, dont les espèces sont difficiles à distinguer sur la base du gène *rrs*. Pour *Pseudomonas*, il serait nécessaire de développer une puce de génotypage reposant sur un gène plus discriminant, comme par exemple *phlD* en ce qui concerne les *Pseudomonas* DAPG$^+$ ou *gacA* (Mavrodi *et al.* 2001; Picard et Bosco 2003; Rodrigo *et al.* 2007; Frapolli *et al.* 2008).

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ANNEXES

Applicability of the 16S-23S rDNA internal spacer for PCR detection of the phytostimulatory PGPR inoculant *Azospirillum lipoferum* CRT1 in field soil

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Abstract

Aims: To assess the applicability of the 16S-23S rDNA internal spacer regions (ISR) as targets for PCR detection of *Azospirillum* spp. and the phytostimulatory PGPR seed inoculant *A. lipoferum* CRT1 in soil.

Methods and Results: Primer sets were designed after sequence analysis of the ISR of *A. lipoferum* CRT1 and *A. brasilense* Sp245. The primers fAZO/rAZO targeting the *Azospirillum* genus successfully yielded PCR amplicons (400-550 bp) from *Azospirillum* strains but also from certain non-*Azospirillum* strains *in vitro*, therefore they were not appropriate to monitor indigenous *Azospirillum* soil populations. The primers fCRT1/rCRT1 targeting *A. lipoferum* CRT1 generated a single 249-bp PCR product but could also amplify other strains from the same species. However, with DNA extracts from the rhizosphere of field-grown maize, both fAZO/rAZO and fCRT1/rCRT1 primer sets could be used to evidence strain CRT1 in inoculated plants by nested PCR, after a first ISR amplification with universal ribosomal primers. In soil, a 7-log dynamic range of detection (10^2-10^8 CFU g^{-1} soil) was obtained.

Conclusions: The PCR primers targeting 16S-23S rDNA ISR sequences enabled detection of the inoculant *A. lipoferum* CRT1 in field soil.

Significance and Impact of the Study: Convenient methods to monitor *Azospirillum* phytostimulators in the soil are lacking. The PCR protocols designed based on ISR sequences will be useful for detection of the crop inoculant *A. lipoferum* CRT1 under field conditions.

Keywords*:* PGPR, *Azospirillum*, 16S-23S rDNA intergenic spacer, specific primers, soil detection

Introduction

Many strains of the genus *Azospirillum* (α-proteobacterial subclass) have received attention for their phytostimulatory effects on a wide range of plants, especially Gramineae (Baldani *et al.* 1986; Jacoud *et al.* 1999; Rothballer *et al*. 2003). These plant growth-promoting rhizobacteria (PGPR) have been extensively used over the past 30 years in field inoculation to improve crop yield and quality (Okon and Labandera-Gonzalez, 1994). Evaluating survival and fate of PGPR inoculants is part of this process (Tsushima *et al.* 1995; Mahaffee *et al.* 1997; El Zemrany *et al.* 2006).

Four main strategies are available to achieve this goal. The first relies on the introduction of one or several genetic markers (often conferring antibiotic resistance or encoding a fluorescent protein) in the bacterial strain prior to inoculation (Rothballer *et al*. 2003; Jäderlund *et al*. 2008). However, such genetically-modified strains are primarily designed for experimental purposes. Their release is regulated (Morrissey *et al.* 2002) and is seldom implemented in the field (Viebahn *et al.* 2003). This marker gene strategy presents other disadvantages, as (i) the exogenous genes can represent a metabolic burden and may affect ecological fitness and/or phytostimulatory properties, (ii) cell enumeration following selective plating or microscopic observations is cumbersome and (with selective plating) does not enable monitoring of viable non-culturable cells. The second strategy, which is based on the use of spontaneous antibiotic-resistant mutants (Moënne-Loccoz *et al.* 2001; Mascher *et al.* 2003), requires careful screening of candidate mutants (to ensure that other cell properties are not affected; Mahafee *et al*. 1997) and displays the drawbacks associated with colony counts. A third strategy based on natural antigenic properties of the cell may be followed if a specific antibody is available (Mascher *et al.* 2003). This strategy does not require any genetic modification but monitoring is tedious and the detection limit is rather high.

Research might also focus on a fourth strategy, which relies on the identification of strain-specific DNA sequences and the edition of probes/primers. This approach has been implemented on *Azospirillum* with randomly-selected sequences (Jacoud *et al.* 1998, Fancelli *et al.* 1998), but can also target selected genes. The ribosomal intergenic spacer region (ISR) located between the 16S rRNA and 23S rRNA genes shows a high degree of variation in length and sequence and holds potential for intraspecies discrimination (Gürtler and Stanisich, 1996; Buchan *et al.* 2001; Sadeghifard *et al*. 2006). Indeed, its sequence variability has been successfully exploited to edit probes and primers allowing species or subspecies

Table 1 Strains used in the study and PCR results using *Azospirillum* ISR-targeting primers fAZO/rAZO and fCRT1/rCRT1 with approximate amplicons size (bp) in parentheses.

Species	Strain	Reference	PCR band using fAZO/rAZO	PCR band using fCRT1/rCRT1
***Azospirillum* spp.**				
A. brasilense	Sp245	Baldani *et al.* 1986	+ (500)[a]	-
	PH1	Rinaudo, 1982	+ (480)	-
	Sp7	Tarrand *et al.* 1978	+ (500)	-
	L4	Kabir *et al.* 1996	+ (500)	-
	CFN-535	Dobbelaere *et al.* 2001	+ (520)	-
	UAP-154	Dobbelaere *et al.* 2001	+ (520)	-
A. irakense	KBC1	Khammas *et al.* 1989	+ (450)	-
A. lipoferum	CRT1	Fages and Mulard, 1988	+ (520)[b]	+ (250)[c]
	4B	Bally *et al.* 1983	+ (520)	-
	$4V_I$	Alexandre *et al.* 1999	+ (520)	-
	B506	Elbeltagy *et al.* 2001	+ (520)	+ (280)
	B510	Elbeltagy *et al.* 2001	+ (520)	+ (260)
	B518	Elbeltagy *et al.* 2001	+ (520)	+ (260)
	Br10	Tarrand *et al.* 1978	+ (520)	+ (280)
	Br17	Tarrand *et al.* 1978	+ (520)	+ (250)
	TVV3	Trân Van *et al.* 1997	+ (520)	+ (280)
Other α-Proteobacteria				
Agrobacterium tumefaciens	C58	van Larebeke *et al.* 1974	-	-
Nitrobacter hamburgensis	X14	Bock *et al.* 1983	-	-
N. winogradskyi (agilis)	AG	Degrange and Bardin, 1995	+ (1000)	-
Rhizobium etli	CFN42	Quinto *et al.* 1985	+ (500)	-
R. tropici	CFN299	Martínez-Romero *et al.* 1991	-	-
β-Proteobacteria				
Ralstonia solanacearum	GMI 1000	Boucher *et al.* 1985	+ (700)	-
Burkholderia vietnamensis	TVV75	Trân Van *et al.* 2000	-	-
Alcaligenes xylosoxidans	Cm4	Belimov *et al.* 2001	+ (500)	-
γ-Proteobacteria				
Enterobacter agglomerans	Cka5	This study	+ (500)	-
Stenotrophomonas maltophilia	Cy2	This study	+ (500)	-
Firmicutes				
Bacillus pumilus	Fp1	Belimov *et al.* 2001	+ (500)	-

Bacillus sp.	Cb17	This study	-	-
Microbacterium esteraromaticum	Cr59	This study	+ (600)	-
Rhodococcus sp.	Fp2	Belimov *et al.* 2001	-	-

[a] PCR product was made of 503 bp and 504 bp fragments as defined by sequencing

[b] PCR product was made of 517 bp and 529 bp fragments as defined by sequencing

[c] PCR product was made of 249 bp fragments as defined by sequencing

discrimination when applied on clinical or environmental isolates (Glennon *et al.* 1996, Rachman *et al*. 2004; Valcheva *et al*. 2007). To date, the usefulness of the ISR to design strain-specific PCR primers and develop tools for environmental detection of bacteria has been successfully applied only for rhizobia inoculants (Tan *et al.* 2001). This would be of particular interest in the case of PGPR inoculants, where strain monitoring after large-scale environmental release is an important issue because these bacteria interact in various ways with different microbial components of the soil/plant microbiota (Kabir *et al.* 1996; Moënne-Loccoz *et al*. 2001; Viebahn *et al*. 2003).

The aim of this study was to assess the applicability of 16S-23S ISR sequences to develop a PCR monitoring tool for field soil detection of the PGPR inoculant *Azospirillum lipoferum* CRT1, a commercial strain that has been used on crops worldwide (Okon and Labandera-Gonzalez, 1994). To reach this goal, the 16S-23S ISR from *A. lipoferum* CRT1 was sequenced and compared with that of *A. brasilense* Sp245. These sequences were used to design PCR primers targeting the *Azospirillum* genus and strain *A. lipoferum* CRT1, and their usefulness assessed to detect strain CRT1 in soil under field conditions.

Materials and methods

Bacterial strains

The bacterial strains used are listed in Table 1. They were grown overnight at 28°C with shaking in liquid Luria-Bertani (Sambrook *et al.* 1989) medium supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ (i.e. LB-$CaCl_2$-$MgSO_4$). Work with *Nitrobacter hamburgensis* X14 and *N. winogradskyi* AG was carried out using total DNA kindly provided by V. Degrange (Université Lyon 1).

Microcosm set-up and field inoculation

The soil used for the microcosms was collected at the experimental farm of La Côte Saint André (near Lyon, France). It was taken in the loamy surface horizon (clay 16%, silt 44%, sand 40%, organic matter 2.1%, pH (water) 7.0) of a luvisol (FAO), syn. alfisol (typic hapludalf; US Soil Taxonomy) cultivated with maize (El Zemrany *et al.* 2006).

Fresh soil was sieved (2 mm) and transferred into eight microcosms (14-cm diameter Petri dishes), each holding 103 g soil (equivalent to 90 g dry soil). Cells of *A. lipoferum* CRT1 in late log phase were collected from LB-$CaCl_2$-$MgSO_4$ plates and suspended in pure sterile

water prior to inoculation into soil at 10^1 to 10^8 culturable CRT1 cells per g of soil (eight inoculation levels), as follows. For each inoculation level, volume of the cell suspension was adjusted by reference to the calibration curve between OD at 580 nm and density of culturable CRT1 cells grown on modified Luria-Bertani plates. The suspension was centrifuged (5,000 × *g* for 15 min) and the pellet was resuspended in sterilised pure water (total volume 10.8 ml). Each inoculum was evenly spread onto the soil surface of a microcosm using a pipette and the soil was mixed thoroughly with a spatula. Soil was at water holding capacity (26% w/w as determined by gravimetry; Ranjard *et al*., 1997) after inoculation. The microcosms were incubated 24 h at 28°C and immediately processed for DNA extraction as described below.

For field inoculation in La Côte Saint André, *A. lipoferum* CRT1 was prepared commercially in a peat formulation (Azo-Green™; Lipha/Nitragin, Meyzieu, France) and used to inoculate seeds (3×10^7 CFU added per seed) of maize (cultivar PR38a24; Pioneer, Aussonne, France) immediately prior to sowing (80,000 seeds ha^{-1}), as described (El Zemrany *et al.* 2006). Current commercial farming practices were followed for chemical control of pests and weeds. The four non-inoculated and four inoculated plots (each 6 m wide × 15 m long) received 70 kg mineral N ha^{-1} (half nitrate and half ammonium). The two samplings (three plants per plot) were performed when maize reached 2-3 leaves (18 days after seed inoculation) and 9-10 leaves (57 days after seed inoculation). These samples were used for CRT1 enumeration based on colony hybridization (El Zemrany *et al*., 2006) and for the present study.

DNA extraction

Genomic DNA of bacterial cultures was extracted using Qiagen Genomic-tip, according to manufacturer instructions (Qiagen, Courtaboeuf, France) and DNA concentration was assessed by OD measurement at 260 nm (DU®-64 Spectrophotometer; Beckman, Roissy, France).

Environmental DNA was extracted in triplicate from 500 mg bulk soil (microcosm experiment) or crushed maize root system and adhering soil (field experiment; Baudoin *et al*., 2009) with the FastDNA® SPIN® Kit (For Soil) (BIO 101, Inc., Carlsbad, CA). DNA samples were resolved by electrophoresis in a 0.8 % agarose gel, stained with ethidium bromide and photographed using a Gel Doc 1000 camera (Bio-Rad, Ivry sur Seine, France). Dilutions of calf thymus DNA (Boehringer Mannheim, Meylan, France) were included in each gel and a

standard curve of DNA concentration (25, 50, 100, 200, 400 and 800 ng) versus integrated pixel (R^2 = 0.99) was used to estimate the final DNA concentration in the extracts. The ethidium bromide staining intensities were analysed using Molecular Analyst software (Bio-Rad).

PCR analyses

The 16S-23S ISR was amplified by PCR using 10 ng of genomic DNA and the eubacterial universal primers FGPS1490-72 and FGPL132-38 (Table 2). Amplicons were resolved in a 5% polyacrylamide gel and their length compared, as described (Ranjard *et al.* 2000).

Primers fAZO and rAZO were designed to amplify target sequences of the genus *Azospirillum*, and primers fCRT1 and rCRT1 for detection of strain CRT1 (Table 2). These primers were used in a nested configuration: a PCR reaction was performed first on strain genomic DNA or environmental DNA (10 ng) using the universal primers FGPS1490-72/FGPL132-38. Then, 0.5 µl of these PCR products was used as target DNA in a second PCR round, using either fAZO/rAZO or fCRT1/rCRT1. In the case of environmental DNA, the intensities of the banding patterns produced in a 2% agarose electrophoretic gel by the first-round PCR products were quantified by image analysis and amplicon concentrations were adjusted to a same level (when necessary) to perform the second PCR round on equivalent amounts of target DNA. This second PCR was performed in a 50-µl volume containing 5 µl of 10× dilution buffer (supplemented with 1.5 mM $MgCl_2$), 200 µM of each dNTP, 0.5 µM of each primer, 1.5 µg of T4 gene 32 protein (Roche, Meylan, France), 2.5 units of Expand® High Fidelity *Taq* polymerase (Boehringer Mannheim) and 0.5 µl of ISR amplicon. Amplifications were done using a GeneAmp PCR System 2400 (Perkin-Elmer, Courtaboeuf, France), with an initial denaturation for 3 min at 94°C, 25 PCR cycles (1 min at 94°C, 1 min at 59°C for fAZO/rAZO or 58°C for fCRT1/rCRT1, 1 min at 72°C), a final elongation for 5 min at 72°C, followed with a cooling step at 5°C. Nested PCR amplicons were resolved in 2% agarose gels with Smart ladder (Eurogentec, Seraing, Belgium), stained with ethidium bromide and photographed using Gel Doc 1000 (Bio-Rad).

Selected *A. lipoferum* strains were compared based on BOX-A1R PCR (Versalovic *et al.* 1998), ERIC PCR as described by Rademaker *et al.* (1998), and RAPD analysis using primer 1253 (5'-GTTTCCGCCC-3'; Fancelli *et al.* 1998), as described in Vial *et al.* (2006). Electrophoretic profiles were compared using GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium) and were combined for the three methods. Similarity analysis of the

Table 2 Primers used in the study

Primers	Sequence	Reference
Eubacterial 16S-23S ISR		
FGPS1490-72	5'-TGCGGCTGGATCCCCTCCTT-3'	Ranjard *et al.* 2000
FGPL132-38	5'-CCGGGTTTCCCCATTCGG-3'	Ranjard *et al.* 2000
Genus *Azospirillum*		
fAZO	5'-GGCGCATCCCTTCTCACGG-3'	This work
rAZO	5'-GCTTGCGCCACGCGCAGG-3'	This work
Strain CRT1		
fCRT1	5'-CGCCCGATTACGAGGACC-3'	This work
rCRT1	5'-CCACGCGCAGGAACAAGC-3'	This work

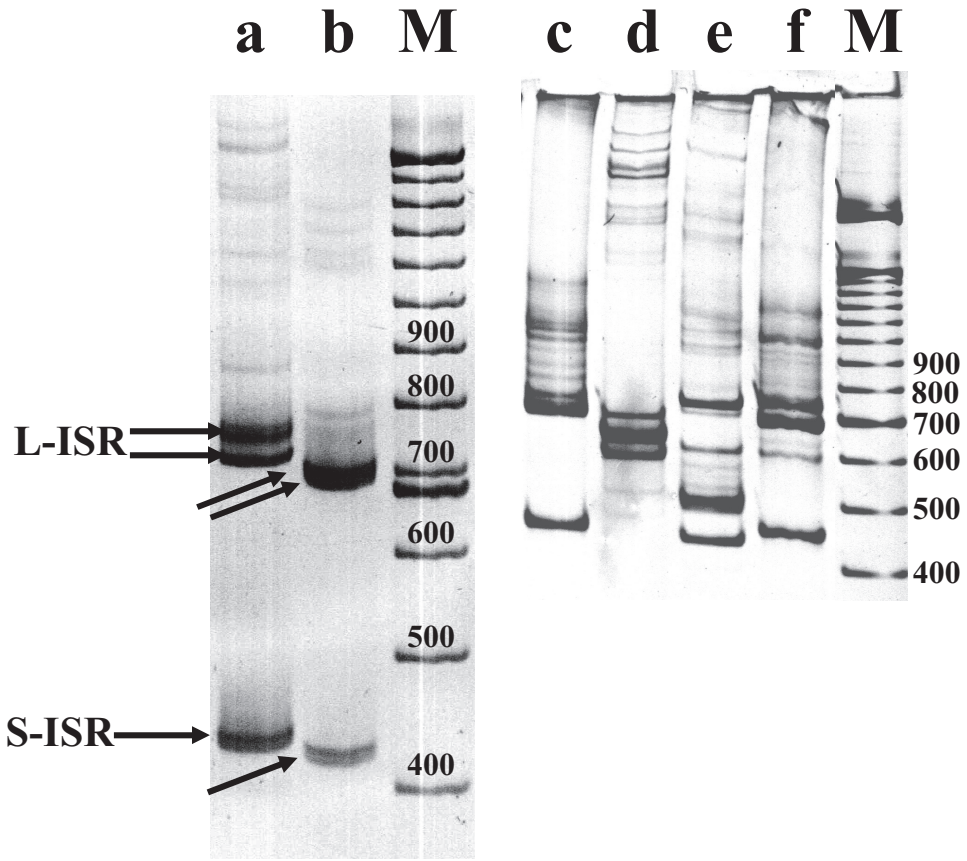

Figure 1 16S-23S rDNA intergenic spacer fingerprints of *A. lipoferum* CRT1 (a), *A. brasilense* Sp245 (b), *A. lipoferum* $4V_I$ (c), *A. brasilense* Sp7 (d), *A. brasilense* PH1 (e) and *A. brasilense* L4 (f) on a 5%-polyacrylamide gel. M: 100-bp ladder. Three main bands were

obtained for each strain; in lane b, band visualisation is hampered by the proximity between two of the main bands. Arrows indicate large and small ISR bands that were excised from agarose gels for sequencing.

resulting composite profiles was done based on presence/absence of bands (Jaccard coefficient) and clustering using the Unweighted Pair Group Method with Arithmetic means (UPGMA).

16S-23S rDNA ISR cloning and sequencing

Clone libraries of the PCR-amplified rDNA of *A. lipoferum* CRT1 and *A. brasilense* Sp245 obtained with primers FGPS1490-72/FGPL132-38 were constructed using the pGEM®–T Easy Vector (Promega, Charbonnières, France) and chemically-competent *Escherichia coli* DH5α™ cells (Invitrogen, Cergy Pontoise, France). Briefly, for each strain the two main bands, as identified on agarose gel, were excised and purified with Minelute (Qiagen). In both cases, the agarose band of the highest length harbored indeed two sequences of similar sizes as revealed by an acrylamide gel migration (see Fig. 1). The PCR products were then ligated with pGEM®–T Easy Vector (Promega), according to manufacturer instructions. *E. coli* DH5α™ cells (Invitrogen) were transformed with the constructs, as specified by the supplier, and grown overnight at 37°C on LB-$CaCl_2$-$MgSO_4$ medium supplemented with 100 µg ml^{-1} ampicillin, 0.5 mM IPTG and 80 µg ml^{-1} X-Gal. From 20 to 100 colonies were selected for each type of 16S-23S ISR identified in *Azospirillum* strains. Cloned DNA was extracted by boiling in 100 µl ultrapure water for 15 min. The bacterial lysates were centrifuged (10,000 × *g*, 10 min) and 2 µl supernatant was used as template DNA in subsequent PCR steps. Plasmid inserts from the positive clones were amplified with primers M13f and M13r (Promega) and digested with *Alu*I and *Hae*III (Boehringer Mannheim) for *A. lipoferum* CRT1 and *Alu*I for *A. brasilense* Sp245. Individual clones were placed into restriction groups based on a 100% identity threshold of the restriction patterns. A total of 16 and 7 clones were sequenced from the two libraries of *A. lipoferum* CRT1, respectively, and 10 and 5 clones from those of *A. brasilense* Sp245. The clones selected for sequencing were amplified with primers M13f/M13r. Purified PCR products were then sequenced in both directions with primers FGPS1490-72/ FGPL132-38. Sequencing was performed by Genome Express (Meylan, France) on an ABI377 sequencer FS (Perkin-Elmer) using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with Ampli*Taq* DNA polymerase (Perkin-Elmer).

Sequence analysis

The sequences of 16S-23S ISR DNA were compared with GenBank sequences (database nr/nt) using BLASTN 2.2.18+ (Altschul *et al.* 1997) at http://blast.ncbi.nlm.nih.gov/Blast.cgi, as well as with preliminary data from the Sp245 sequencing project (courtesy of I.B. Zhulin, The University of Tennessee and Oak Ridge National Laboratory). Sequences from clones were aligned using CLUSTAL W (Thompson *et al.* 1994) with manual refinements. Localisation of the 3' end of 16S rDNA and the 5' end of 23S rDNA genes was done based on *E. coli* sequences (GenBank accession no. J01695). Identification of tRNA sequences was performed with tRNAscan-SE search server (http://lowelab.ucsc.edu/tRNAscan-SE/).

Nucleotide sequence accession numbers

The sequences of the small and large 16S-23S ISR DNA of *A. lipoferum* CRT1 and *A. brasilense* Sp245 were deposited in the GenBank nucleotide database under the numbers AY685928, AY685927, AY685926, AY685925, AY685924 and AY685923.

(A)

16S rDNA

```
CRT1-G1     TGCGGCTGGATCCCCTCCTTTCTAAGGAAGC-CGACCCTGGCTGGTCCGGCACCTTCAAA
CRT1-G2     TGCGGCTGGATCCCCTCCTTTCTAAGGAAGC-CGACCCTGGTTGGTCCGGCACCTTCAAA
Sp245-G1    TGCGGCTGGATCCCCTCCTTTCTAAGGAAAAGCCGGCCCGTCCGATCGGGCCGCGCGCCG
Sp245-G2    TGCGGCTGGATCCCCTCCTTTCTAAGGAAAAACCGGCCCGTCCAATCGGGCCGCGCGCCG
            ****************************   *   ** *     ** ***  *

                                        fAZO
CRT1-G1     GCCCAGATGGCGCGTCTCTGCCGCCGCCGGCGCATCCCTTCTCACGGTTCTCGACGTGCT
CRT1-G2     GCCCAGATGGCGCATCTCTGCCGCCGCCGGCGCATCCCTTCTCACGGTTCTCGACGTGCT
Sp245-G1    ACCAAGAA-----------GCCGCCGCCGGCGCATCCCTTCTCACGGATCTCATCGTTGT
Sp245-G2    ACCAAGAA-----------GCCGCCGCCGGCGCATCCCTTCTCACGGATCTCATCGTTGT
            ** ***            ***************************** ****  ***  *

                                        tDNA^Ile
CRT1-G1     CCTCAG----------TGGGGCACGGCCGGGCTAGTAGCTCAGTTGGTTAGAGCGCGCGC
CRT1-G2     CCTCTG----------TGGGGCACGGCCGGGCTAGTAGCTCAGTTGGTTAGAGCGCGCGC
Sp245-G1    CAACCAAGTGATGAGCTTGGACAGCGAGGGGCTAGTAGCTCAGTTGGTTAGAGCGCGCGC
Sp245-G2    CAACCAAGTGATGAGCTTGGACAGCGAGGGGCTAGTAGCTCAGTTGGTTAGAGCGCGCGC
            *  *            * ** **  *  *******************************

CRT1-G1     TTGATAAGCGTGAGGTCGGAGGTTCAAATCCTCCCTGGCCCACCAT--GTTTAGCGATC
CRT1-G2     TTGATAAGCGTGAGGTCGGAGGTTCAAATCCTCCCTGGCCCACCAT--GTTTAGCGATC
Sp245-G1    TTGATAAGCGTGAGGTCGGAGGTTCAAATCCTCCCTGGCCCACCACCCATCAGGCGACA
Sp245-G2    TTGATAAGCGTGAGGTCGGAGGTTCAAATCCTCCCTGGCCCACCACCCATCAGGCGACA
            *********************************************    *   ****

                                  tDNA^Ala
CRT1-G1     GTGCGT-TTGCCGAT-CGGGGGCATAGCTCAGTTGGGAGAGCGCCTGCTTTGCAAGCAG
CRT1-G2     GTGCGT-TTGCCGAT-CGGGGGCATAGCTCAGTTGGGAGAGCGCCTGCTTTGCAAGCAG
Sp245-G1    CCGCGTTACACCGACATGGGGGCATAGCTCAGTTGGGAGAGCGCCTGCTTTGCAAGCAG
Sp245-G2    CCGCAC-GCACCGACATGGGGGCATAGCTCAGTTGGGAGAGCGCCTGCTTTGCAAGCAG
            **      ****   ****************************************

CRT1-G1     GAGGTCGTCGGTTCGATCCCGTCTGCCTCCACCAGTTTTT--------------CTGGTG
CRT1-G2     GAGGTCGTCGGTTCGATCCCGTCTGCCTCCACCAGTTTCCGAGACGGAC----GCTGGTG
Sp245-G1    GAGGTCGTCGGTTCGATCCCGTCTGCCTCCACCAGTCTT---------------CTGGTG
Sp245-G2    GAGGTCGTCGGTTCGATCCCGTCTGCCTCCACCAGGAACCTCACTCTGGAGGGGCTGGTG
            ***********************************                   ******
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```
                                          fCRT1
CRT1-G1     TCGAGCGTGGATGAT--------CGGCCGCCCGATTACGAGGACCGTTGGAAGGAACCACA
CRT1-G2     TCGATGGTGGAGGC---------GAGCCGCTCAGCTTCGAGGACCGTTGGAAGGAACCACA
Sp245-G1    TCGAGGCTGCAGGGTTGGGACCG-GATGTTCCGGCA--GAGATCCGTCAGAAGGAAACGCA
Sp245-G2    TCGAGGCGA--------------TGGTGGTCTCCCT--GGGATCCGTCAGAAGGAAACGCA
            **** *                               * *  ****  ******* * **

CRT1-G1     ACACGGCAACGTGAACAGCAACGAGCGCGCAGCGCTCGTTGCTGTGTCCCTGAC-------
CRT1-G2     ACACGGCAACGTGAACAATAACGAGCGCTCCGCGCTCGTTATTGTGTCCCAAACCACATTG
Sp245-G1    ACACGGAAACGTGA------------GCTTCGGGCTCCTCATCGCTGAGGGGACT------
Sp245-G2    ACACGGAAACGTGA------------GCTTCGGGCTCCTCATCGCTGAGGGGACT------
            ****** *******            **   * **** *    *        **


CRT1-G1     --------GGGACGGGATCAT GGACAA-GTGAAGATGAAGTGCAAGTGACCGAGGACGCT
CRT1-G2     GTGGTGCAGGGACGGGATCATGGACAA-GTGAAGATGAAGTGCAAGTGACCGAGGACGCTC
Sp245-G1    --------GGAGCGGGATCAT GGACAGTGTGAAGACGATTGTTAAGTGACCGAGGACGGA
Sp245-G2    --------GGAGCGGGATCAT GGACAGTGTGAAGACGATTGTTAAGTGACCGAGGACGGA
                    **  ********* *   *     *     *     *     *   *

CRT1-G1     CCTCGGCCGGCCCAGACCCACAAGGTCAAAGCTGGCTGGGAGTAGCATCGAACGGCGGAAA
CRT1-G2     CTCGGCCGGG---------AGAATACC----CTGGCTGGGAGTAGCATCGAACGGCGGAAA
Sp245-G1    CCTCGGGCCGG------C--------T----CTGAAGAAGGGTTGGTTCGA-TGGTCAATG
Sp245-G2    CCTCGGGCCGG------C--------T----CTGAAGAAGGGTTGGTTCGA-TGGTCAATG
            *   *    *                     ***     * ** *  ****  **   *

                                                rCRT1  rAZO
CRT1-G1     CGACCAGCCCTGTCGGTTGGTTCGCGAGCAGGCTTGTTCCTGCGCGTGGCGCAAGCGTTTT
CRT1-G2     CAGTCGGTCCTGTCGACCGGCTCGCGAGCAGGCTTGTTCCTGCGCGTGGCGCAAGCGTTTT
Sp245-G1    CATCTTGCGGCGTTGTGCG---TGCGTCTGGGCTTGCCCCTGCGCGTGGCGCAA------C
Sp245-G2    CATCTTGCGGCGTTGTGCG---TGCGTCTGGGCTTGCCCCTGCGCGTGGCGCAA------C
            *     *    ** *   *    ***    ******  ****************

CRT1-G1     CGTTGGAGTTGAGATCAAGCGTCTGAAGGGCATCCGGTGGATGCCTTGGCA
CRT1-G2     CGTTGGAGTTGAGATCAAGCGTCTGAAGGGCATCTGGTGGATGCCTTGGCA
Sp245-G1    CGCTGAGTTTAGGATCAAGCGTCTGAAGGGCATCTGGTGGATGCCTTGGCA
Sp245-G2    CGCTGAGTTTAGGATCAAGCGTCTGAAGAGCATCTGGTGGATGCCTTGGCA
            ** **   **  **************** ***** ****************
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23S rDNA

16S rDNA

(B)

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CRT1        TGCGGCTGGATCCCCTCCTTTCTAAGGAAGCCGACCCTGGTGGGTCCGGCACCTTCAAGT
Sp245       TGCGGCTGGATCCCCTCCTTTCTAAGGAA---AAGCC-GGCCCGTCCGA-----TCGGGC
            ****************************    * ** **   *****      **  *

CRT1        CCAGATGGCGCATCTCTGCCGCCGCCGGCGCATCCCTTCTCGACGATCCGG--AACACCC
Sp245       C--GCGGACACGACGAAGCCGCCGCCGGCGCATCCCTTCTCGACAGCCAATCCAAGATGA
            *  *  * * *  *   **************************   *     ** *

CRT1        GCTGACAGTGCATCCGCACTGTCTCGGCCGGATTGACGAGAAGCGCTTGGCGTTTTGAAA
Sp245       ACCGGGCATTTCAAAGTGCCCGTTCATGATGGCTGCCGGGAAGTGGTAAG-GCGTTGCTT
             * *    *      *  *    **     *  ** ** **** * *  * *  ***

CRT1        TTTTCAAAACGCGGGATCTTTCAAATCGTGAATAAAGTCGAGTTTTAAGTGACCGAGGAT
Sp245       TCGAAGCGACGCGGATCTTTGAAAATCGTGAATAAAGTCGAGAAT--AGTGACCGAGGAT
            *       ******    **  ********************  *  *************

CRT1        GCATCTTCATGAGCGTCCACGAGGCAGGAGCGGTTTGCCCCTGTGCGTGACGTTCGGAAT
Sp245       GCA                 CCGGGCACAAGGGGCGGTTTGTCCCTGTGCGCGACGTTCGCGAG
            ***                 **  *    *** ******** ********* ********  *

CRT1        AGAGATCAAGCGTCTGAAGGGCATCTGGTGGATGCCTTGGCA
Sp245       AAGGATCAAGCGTCTGAAGGGCATCTGGTGGATGCCTTGGCA
            *  ************************************
```

23S rDNA

Figure 2 Multiple sequence alignment from representative sequences of the two L-ISR groups (A) and the S-ISR group (B) for *A. lipoferum* CRT1 and *A. brasilense* Sp245. In A, the L-ISR are termed CRT1-G1 and CRT1-G2 (for CRT1), and Sp245-G1 and Sp245-G2 (for Sp245). tRNA sequences are shaded in grey. Primers fAZO/rAZO are indicated in bold and fCRT1/rCRT1 are shaded in grey. rAZO and rCRT1 have a 11 bp overlap. Sequence matching is indicated by an asterisk.

Results

Size and sequence of the 16S-23S ISR

PCR amplification using the universal primers FGPS1490-72/FGPL132-38 was performed with genomic DNA of eight strains of various *Azospirillum* species. Extensive length polymorphism was evidenced for the 16S-23S ISR, as illustrated in Figure 1. It appeared that there was no species-specific pattern, as clearly exemplified with *A. brasilense* strains. A majority of bands ranged from 400 to 900 bp, which is the true size of the spacer plus 16S (20 bp) and 23S (130 bp) rDNA tails. In all cases, strains exhibited at least three major bands. *A. lipoferum* CRT1 was characterised by two large major bands (L-ISR) of approximately 750 bp and a small major band (S-ISR) in the vicinity of 430 bp. The profile of *A. brasilense* Sp245 was very similar in terms of number and length of bands.

Restriction analysis of the L-ISR revealed two restriction groups within each strain. Alignment of L-ISR sequences obtained for *A. lipoferum* CRT1 yielded a 613-bp sequence (60.8% GC) and a 601-bp sequence (61.6% GC), which corresponded to the two restriction groups (Fig. 2A). These two sequences showed 87.8 % identity with one another. Similarly, a 572-bp sequence (61.1% GC) and a 571-bp sequence (61.4% GC) identical at 92% were obtained for *A. brasilense* Sp245. For each strain, alignments of the S-ISR sequences depicted a single sequence (Fig. 2B) of 281 bp for *A. lipoferum* CRT1 (56.6% GC) and 269 bp for *A. brasilense* Sp245 (58.7% GC). Each of the two L-ISR and the S-ISR of *A. lipoferum* CRT1 shared respectively 46% and 62% homology with those of *A. brasilense* Sp245. Multiple alignments of L-ISR and S-ISR sequences representative of the different restriction groups also highlighted several domains identical in strains CRT1 and Sp245 (Fig. 2). The two largest domains conserved (76-77 bp), which were located in tandem within the L-ISR sequences, were identified as the genes encoding $tRNA^{Ile}$ and $tRNA^{Ala}$. BLASTN analysis revealed that the latter genes were highly conserved when considering the Proteobacteria (more than 96% homology among the first 100 sequences producing significant alignments). The S-ISR elements were lacking tRNA genes. The other domains identical in CRT1 and Sp245 were much smaller (at the most 28 bp and 31 bp in L-ISR and S-ISR, respectively).

BLASTN of the L-ISR and S-ISR of *A. brasilense* Sp245 with preliminary data from the Sp245 sequencing project gave 96% and 91% homology, respectively. The observed partial homologies could then be related to the presence of several copies of the *rrn* operons

in the genome of *A. brasilense* Sp245 (Martin-Didonet *et al.* 2000) as well as the incomplete sequencing of the genome and partial screening of ISR regions in our study.

Design of genus- and strain-specific PCR primers

For the design of primers targeting the *Azospirillum* genus (i.e. fAZO/rAZO), only highly-specific domains identical in all *Azospirillum* species can be exploited, which implied that tRNA sequences had to be discarded. Such relevant domains, large enough for primer edition, were identified at both extremities of all L-ISR copies. The primers fAZO/ rAZO amplified a 529 bp and a 517 bp product from the two L-ISR of *A. lipoferum* CRT1. Amplicon sizes were 504 bp and 503 bp for the two L-ISR of *A. brasilense* Sp245.

For the definition of primers targeting *A. lipoferum* CRT1 (i.e. fCRT1/rCRT1), only *Azospirillum*-relevant domains differing from the corresponding ones in *A. brasilense* Sp245 were explored. The primers were chosen based on one of the L-ISR sequences (Fig. 3). The primers fCRT1/ rCRT1 amplified a 249-bp fragment in strain CRT1 and nothing in strain Sp245. The sequence amplified (Fig. 2A) did not yield any BLASTN result matching a rDNA sequence.

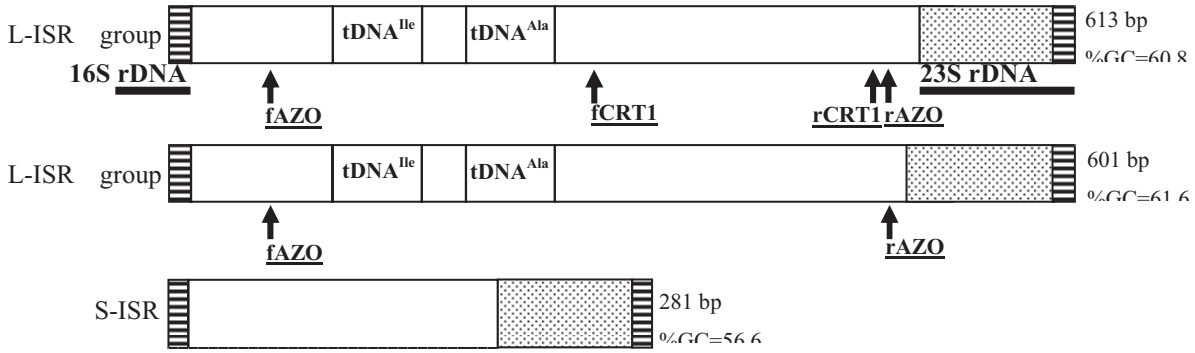

Figure 3 16S-23S rDNA intergenic spacer structure for the two L-ISR groups and the single S-ISR group of *A. lipoferum* CRT1. Length and %GC are given for ISR sequences (without the 16S and 23S rDNA tails). Locations of primers fCRT1/rCRT1 and fAZO/rAZO are indicated. Primer rCRT1 has a 11 bp overlap with primer rAZO. (▒ 23S rDNA tail; (▤) ribosomal universal primers used for IGS amplification and (↑) approximate location of annealing sites for the designed primers.

Specificity of primers targeting the *Azospirillum* genus

The primers fAZO/rAZO, which target the genus *Azospirillum*, were first used on bacterial DNA extracted from *Azospirillum* and non-*Azospirillum* strains. All 16 *Azospirillum* strains were successfully amplified, yielding mainly one strong band (approximately 450 bp to 520 bp; Table 1). In addition, 8 of 14 non-*Azospirillum* strains also yielded a unique band (Table 1), but of lower intensity, and whose size (500 bp to 1000 bp) was in some cases similar to that of the dual band visible for *A. lipoferum* CRT1 (at about 520 bp). Among them, the phylogenetically-closest bacteria were the α-Proteobacteria *Rhizobium etli* CFN42 and *N. winogradskyi* AG. Increasing the annealing temperature from 59°C to 60°C increased specificity, but when tested on seven representative *Azospirillum* strains the amplification was only successful with three of them (i.e. CRT1, Sp245 and PH1; not shown). Nevertheless, these primers were applied in the next evaluation steps (at 59°C) to assess their usefulness with complex environmental DNA extracts.

No amplification signal could be detected by direct PCR when using primers fAZO/rAZO on soil DNA extracts, regardless of whether CRT1 inoculation was performed. With a nested PCR approach (i.e. after PCR with the universal primers FGPS1490-72/FGPL132-38), however, strong PCR signals consisting of a single band migrating on agarose gel at a similar position to that of strain CRT1 (i.e. two L-ISR bands within an acrylamide gel) were obtained when studying DNA extracts from soil microcosms inoculated with strain CRT1. No signal was detected when strain CRT1 had been added below 10^3 CFU g^{-1} soil (data not shown). When applied on maize field DNA extracts, primers yielded a signal for all CRT1-inoculated plots and PCR signals were strongest with samples from the first sampling stage (Fig. 4). Here again, the PCR bands were similar in size to that produced by strain CRT1. Faint signals were visible for at least certain non-inoculated plots at the first sampling stage and for one of these plots at the second sampling.

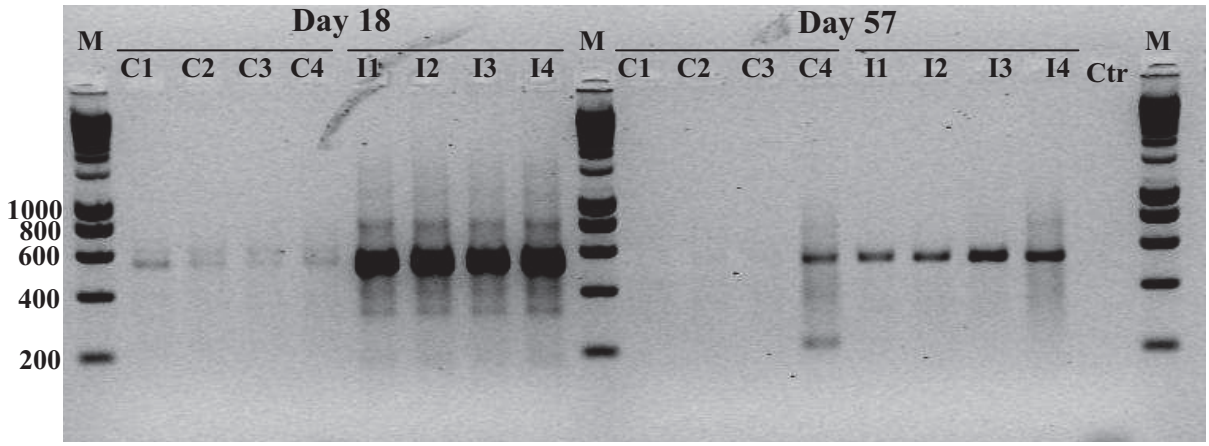

Figure 4 2% agarose migration profiles of amplicons obtained by nested PCR with universal primers FGPS1490-72/FGPL132-38 and then primers fAZO/rAZO on DNA extracts from field maize rhizosphere, at 18 and 57 days after sowing. C1 to C4 : non-inoculated control (one sample per plot), I1 to I4 : seed inoculation with *A. lipoferum* CRT1 (one sample per plot). M: molecular weight marker (bp). Ctrl: negative control.

Specificity and detection level of primers targeting *A. lipoferum* strain CRT1

Primers fCRT1/ rCRT1, which amplify a 249-bp fragment in *A. lipoferum* CRT1, were tested against the bacterial DNA collection. Amplification was not successful, except for six of the eight other *A. lipoferum* strains (Table 1). For the latter, the band produced (i) was either similar or somewhat different in size, and (ii) displayed different restriction properties (digestion done separately with *Alu*I, *Taq*I and *Hae*III) in comparison with the band from strain CRT1. Combined analysis of BOX-A1R, ERIC and RAPD markers indicated that a positive response of *A. lipoferum* strains to fCRT1/rCRT1 amplification was not associated with a higher genetic similarity with strain CRT1 (Fig. 5).

To assess whether these primers could detect *A. lipoferum* CRT1 in environmental samples, they were directly applied in a PCR assay using total soil DNA extracted from microcosms inoculated with various levels of CRT1 cells. Strain CRT1 could be detected after refinement of initial PCR conditions (i.e. primer concentration doubled and annealing

temperature raised from 55 to 58°C), provided it was at least at 10^6 CFU g^{-1} soil (not shown). However, the detection level was increased 10^4 fold when using a nested PCR protocol starting with the universal primers FGPS1490-72/FGPL132-38 (Fig. 6A). Signal intensity decreased progressively (yet in a non-linear way) between 10^8 and 10^3 CFU g^{-1} soil, and dropped abruptly between 10^3 and 10^2 CFU g^{-1} soil.

With field DNA extracts, a band whose size (about 250 bp) was identical to that of the band produced by *A. lipoferum* CRT1 was efficiently detected by nested PCR in all inoculated plots at the first sampling (Fig. 6B). At the second sampling, the band was still detected in all inoculated plots but was not as strong. In the non-inoculated control, no PCR band was obtained at the first sampling whereas a very faint 250-bp band was detected in some of the plots at the second sampling.

To verify the specificity of PCR bands, nested PCR products obtained from DNA originating from *A. lipoferum* strain CRT1 or soil taken from (i) microcosms inoculated with CRT1 at 10^4 CFU g^{-1} soil and (ii) one inoculated field plot (at both samplings) were digested separately with *Alu*I, *Taq*I and *Hae*III. For each enzyme, the restriction profiles were identical for all soil samples and they matched both the theoretical (digestions *in silico*) and experimental profiles for CRT1 amplicons (data not shown).

These primers were also tested using a variety of soil DNA extracts obtained from fields (Ranjard *et al.* 2001) located in France, Senegal and French Guyana. No signal was seen for any of the soils whatever the PCR conditions used (data not shown).

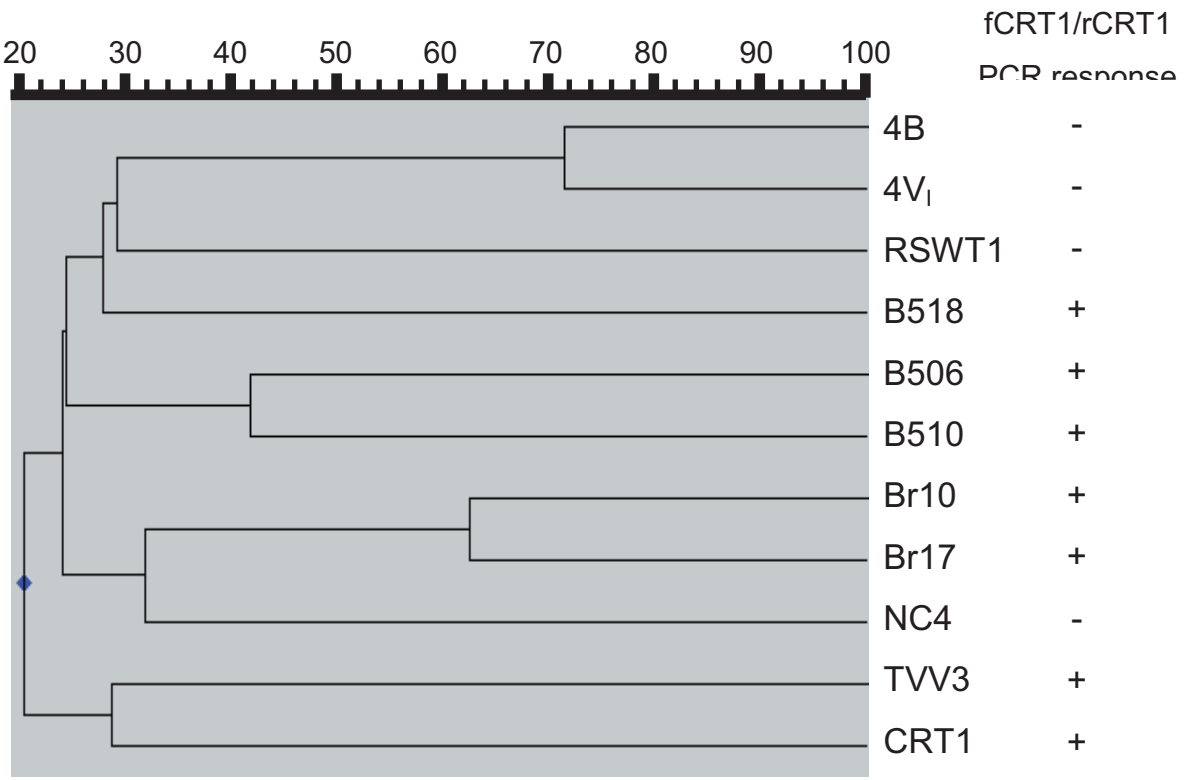

Figure 5 Genetic similarity, based on combined analysis of BOX-A1R, ERIC and RAPD markers, of *A. lipoferum* strains responding or not to amplification with primers fCRT1/rCRT1. The UPGMA clustering method was applied to a similarity matrix calculated with the Jaccard coefficient.

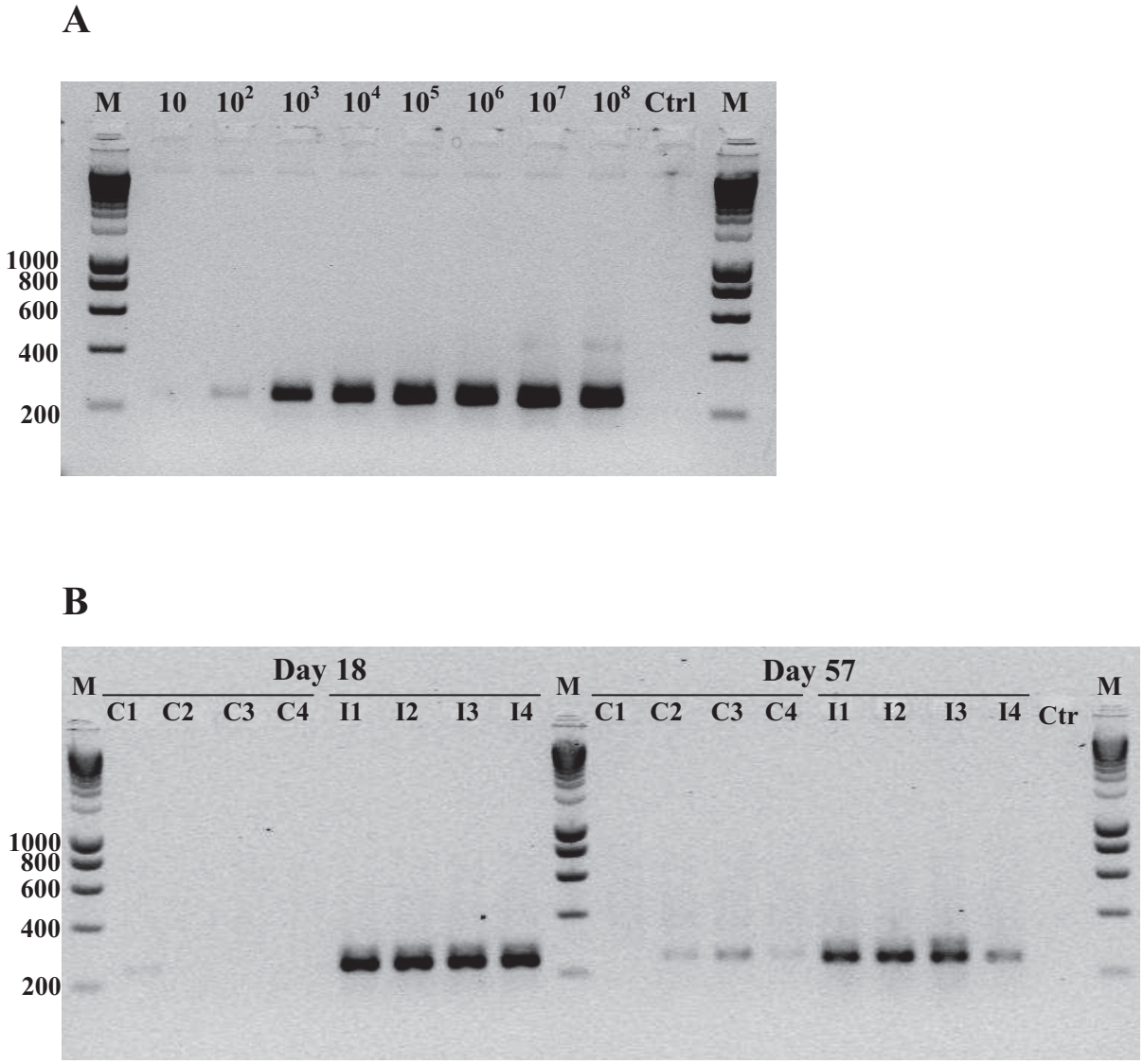

Figure 6 2% agarose migration profiles of amplicons obtained by nested PCR with universal primers FGPS1490-72/FGPL132-38 and then primers fCRT1/rCRT1 on DNA extracts from microcosm soil (A) or on DNA extracts from field maize rhizosphere, at 18 and 57 days after sowing (B). Top lane numbers on (A) indicate inoculum size of *A. lipoferum* CRT1 (CFU g^{-1} soil). M: molecular weight marker (bp). Ctrl: negative control. C1 to C4: non-inoculated control (one sample per plot), I1 to I4 : seed inoculation with *A. lipoferum* CRT1 (one sample per plot).

Discussion

Despite the use of *A. lipoferum* CRT1 as phytostimulatory inoculant of crops in different countries (Okon and Labandera-Gonzalez, 1994), no rapid method was available to monitor the fate of the strain once released into the field. So far, detection of CRT1 in soil is based on the use of a semi-selective medium followed by colony hybridisation to a DNA probe obtained randomly from the CRT1 genome (Jacoud *et al.* 1998; El Zemrany *et al.* 2006). However, this approach is time consuming since it relies on two cultivation steps (5 days incubation on a semi-selective medium followed by two days of growth on a generalist medium) and hybridization procedure. In this study, the sequences of the 16S-23S ISR of two strains from different *Azospirillum* species were determined to assess their applicability as priming sites for a PCR detection tool targeting the *Azospirillum* genus and the crop inoculant *A. lipoferum* CRT1 in environmental samples.

As a preliminary step, comparison of the 16S-23S ISR profiles across various *Azospirillum* species evidenced variability between and within species, and the presence of several major bands. This points to the presence of several, variable copies of the ribosomal operon in *Azospirillum* species, but their precise number has not been determined. This would require Southern hybridisation (Klappenbach *et al.* 2000), quantitative PCR (Candela *et al.* 2004) targeting the ISR region, or genome sequencing. In both *A lipoferum* CRT1 and *A. brasilense* Sp245 strains, sequencing of the large and the small 16S-23S ISR showed that the former differed by the presence of the genes coding for $tRNA^{Ile}$ and $tRNA^{Ala}$ (Fig. 3). The presence of tandem $tRNA^{Ile}$ and $tRNA^{Ala}$ genes is a common feature among Proteobacteria, including α-Proteobacteria such as *Agrobacterium*, *Rhizobium* and *Bradyrhizobium* (Tan *et al.* 2001; Stewart and Cavanaugh, 2007). To our knowledge, this is the first report dealing with *Azospirillum* species.

ISR polymorphism has been widely used to identify or differentiate isolates belonging to various clinical and environmental genera, such as *Legionella* (Riffard *et al.* 1998), *Escherichia* (Buchan *et al.* 2001), *Pseudomonas* (Moënne-Loccoz *et al.* 2001), or *Streptococcus* (Chen *et al.* 2005), either by edition of probes or comparison of RFLP profiles. Even though the ribosomal spacer element is thought to be highly variable among eubacteria (Gürtler and Stanisich, 1996), the level of this heterogeneity in a given bacterial species is not always so high, and the usefulness of this element in defining species- or subspecies-specific primers cannot be taken for granted. Successful primer design was reported for screening isolates at the species level, such as in several *Lactobacillus* (Berthier and Ehrlich, 1998), and

Carnobacterium (Rachman *et al*. 2004) species, *Mycoplasma pulmonis* (Takahashi-Omoe *et al.* 2004) or *Lactobacillus sanfranciscensis* (Valcheva *et al*. 2007). In contrast, the minor differences in ISR sequences from *Bacillus thuringiensis* strains were insufficient to design species-specific probes (Bourque *et al.* 1995). In our study, we observed sufficient sequence variability to suggest that the ISR could be useful to design PCR-based detection protocols. Thus, two couples of primers, one targeting the genus *Azospirillum* and the other the strain *A. lipoferum* CRT1, were designed from the L-ISR sequences.

All *Azospirillum* strains from the three species studied positively responded to primers fAZO/rAZO, even though they were designed on the basis of sequences related to two species only. Thus, the targeted sequences are probably highly conserved across *Azospirillum* taxa. PCR products were also obtained for some non *Azospirillum* strains (i.e. *R. etli*, *N. winogradsky* or *B. pumilus*). However, the size and the restriction profiles of the bands were different from those produced by *Azospirillum* strains including CRT1 (data not shown). Furthermore, the tests performed on pure culture DNA extracts from non-*Azospirillum* strains always led to signal intensities lower than those produced with *Azospirillum* strains. These observations suggested that amplification using soil DNA would preferentially amplify *Azospirillum*-originating fragments. Here, PCR bands of the size and sequence, as confirmed by restriction, of those produced by CRT1 were evidenced when studying inoculated maize in the field suggesting that non-CRT1 strains possibly responsive to these primers were not present or that *Azospirillum* CRT1 was a dominant population. Only barely visible PCR signals were obtained for non-inoculated control plots. It could be that effective amplification required high cell numbers, which was the case for strain CRT1 in the rhizosphere of inoculated plants (> 10^7 CFU g^{-1} root; El Zemrany *et al*. 2006) and perhaps not for the indigenous *Azospirillum* spp. colonizing maize (Sanguin *et al*. 2006). Overall, it means that primers fAZO/rAZO may be useful to detect the inoculant in a complex background of bacterial DNA.

Nevertheless, a strategy directly focused on *A. lipoferum* CRT1 was also followed. The primers fCRT1/rCRT1 designed to target strain CRT1 were not strain-specific since PCR products of comparable size were obtained with various *A. lipoferum* strains. However, the restriction profiles of the bands were different from those produced by *A. lipoferum* CRT1. With field DNA extracts strong PCR bands were obtained only from plots in which seeds inoculated with strain CRT1 had been used. Indeed, the PCR amplicons obtained from soil taken in inoculated microcosms or field plots gave the same restriction profiles as those given by strain CRT1, pointing to detection of the CRT1 inoculant in soil.

Detection limit was 10^3 and 10^2 CFU of the inoculant with the primers targeting the *Azospirillum* genus and strain CRT1, respectively. However, it must be kept in mind that this sensitivity could not be reached without a nested PCR, using first universal primers FGPS1490-72/FGPL132-38. Some authors succeeded in applying group- (Smart *et al.* 1996), species- (Grote *et al.* 2002; Wang *et al.* 2003) and even strain-specific primers (Tan *et al.* 2001) to environmental DNA extracts. Detection limits of the assay were not always reported, but it appears that with such a complex DNA mix, the nested PCR approach gave the best detection limit, enhanced by several 10 folds by comparison with direct PCR. For instance, as little as 60 fg of *Phytophthora nicotianae* DNA was detected in DNA extracts obtained from artificially-inoculated healthy roots, which was 1000 times more sensitive than conventional PCR (Grote *et al.* 2002). Likewise, our data indicate a 10^4-fold lowering of the detection limit. Authors working with other target genes also improved their threshold detection in soil with a nested protocol (Tsushima *et al.* 1995; Rosado *et al.* 1998), but here this was perhaps facilitated by the fact that rDNA sequences are present in several copies in bacterial genomes, which is likely to have a positive effect on PCR efficiency. A comparison of the detection sensitivity of our approach to the published methods based on culture and colony hybridization (Jacoud *et al*., 1998; El Zemrany *et al*., 2006) showed that our method is very efficient. Detection limit was 10^2 CFU per gram soil using fCRT1/rCRT1 primers in a culture-independent approach whereas other methods could not detect *A. lipoferum* CRT1 below 10^3 CFU, which corresponded to the culture detection limit. Furthermore, our approach is less time consuming allowing the treatment of a large number of samples. The use of fAZO/rAZO or fCRT1/rCRT1 primers for monitoring of *A. lipoferum* CRT1 in field plots where inoculated seeds were used gave a stronger signal at the first sampling (18 days after inoculation) than at the second one. This could suggest a decline of the population size of strain CRT1 in maize rhizosphere between the two samplings, as often found with bacterial inoculants in field release studies (Tsushima *et al.* 1995; Moënne-Loccoz *et al.* 1998), but here such a decline was not observed when monitoring the inoculant by colony counts (El Zemrany *et al.* 2006). This discrepancy might result from a decrease in the efficacy of the current protocol between the two samplings, as (i) the efficiency of DNA extraction from rhizosphere samples decreases as plant ages (our unpublished data), (ii) plant metabolites and/or rhizodeposits inhibiting PCR are perhaps more prevalent in rhizosphere extracts from older plants, and (iii) exposure to environmental stress reduces the effectiveness of PCR protocols in bacteria (Rezzonico *et al.* 2003). Putative CRT1 cells were also detected in certain control plots, perhaps as a result of transport of soil and crop residues across

neighbouring plots during tillage. However, it cannot be totally discounted that CRT1 or related *A. lipoferum* strains are naturally present in low numbers in this soil and managed to colonize the rhizosphere of certain maize plants, as the results of El Zemrany *et al.* (2006) suggest.

In conclusion, ISR analysis revealed a marked length and sequence polymorphism among *Azospirillum* strains. This variability enabled the design of a primer set primarily dedicated to the PCR detection of *Azospirillum* strains in soil whose specificity has to be improved since PCR amplicons might be contaminated with unspecific bands originating from non-*Azospirillum* strains. The second primers set allowed for a rapid nested PCR detection of inoculated CRT1 cells in soil with a low and improved sensitivity threshold (i.e. 10^2 cells per gram soil) as compared to the current colony isolation procedure. Combined to the more powerful real-time PCR technique, this will be useful in future work for monitoring of the crop inoculant *A. lipoferum* CRT1 under field conditions.

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Composition des milieux de culture

Milieu LBm

Bacto-tryptone (Difco)	10 g
Extrait de levure (Difco)	5 g
NaCl	5 g
Eau déminéralisée qsp	1 L
→ Pour milieu solide Agar granulé (Difco)	15 g

Milieu NFB

- Solution majeure (à autoclaver) :

$C_4H_4O_5Na_2$	25 g
Eau déminéralisée qsp	933 ml

Ajuster à pH 7
Pour milieu solide Agar granulé (Difco) 15g

- Solution de sels (à autoclaver) :

$MgSO_4 : 7H_2O$	0,8 g
$CaCl_2 : 2H_2O$	0,104 g
NaCl	0,4g
$Na_2MoO_4 : 2H_2O$	0,008 g
$MnCl_2 : 4H_2O$	0,028 g
Eau déminéralisée qsp	400ml

- Solution de phosphates (à autoclaver) :

KH_2PO_4	44g
K_2HPO_4	46g
Eau déminéralisée qsp	100ml

- Solution de fer-EDTA (à autoclaver)

$FeSO_4 : 7H_2O$	0,631 g
EDTA	0,592 g
Eau déminéralisée qsp	50ml

- Solution de biotine (à filtrer sur 0,22 µm, Millipore)

Biotine	0,05 g
Ethanol absolu qsp	50 ml

→ Pour l'utilisation, ajouter à 933 ml de solution majeure :
- 50 ml de solution de sels
- 15 ml de solution de phosphates
- 1 ml de solution de fer-EDTA
- 1 ml de solution de biotine

Agar B de KING (base) (Merck, Ref :10989)

- Composition type de la poudre (en g / L)

peptone de caséine	10
peptone de viande	10
$MgSO_4$	1,5
hydrogénophosphate dipotassique	1,5
agar-agar	12

- Préparation

poudre	35 g
Glycérol 95%	10 ml
Eau déminéralisée qsp	1 L

Milieu RC

DL-Malic acid	5,00 g
K_2HPO_4	0,50 g
$MgSO_4$, $7H_2O$	0,20 g
NaCl	0,10 g
Yeast Extract	0,50 g
$FeCl_3$, $6H_2O$	0,015 g
KOH	4,8 g
Solution aqueuse Congo Red 1 :400	15 ml
Agar granulé	18,0 g
H_2O_γ qsp	1000 ml

Ajuster à pH 6,8 avec KOH 0,1N