# Sélection des rhizobactéries phytostimulatrices par la plante : impact sur la distribution des propriétés phytobénéfiques chez les Pseudomonas fluorescents

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# Sélection des rhizobactéries phytostimulatrices par la plante : Impact sur la distribution des propriétés phytobénéfiques chez les *Pseudomonas* fluorescents

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# Sélection des rhizobactéries phytostimulatrices par la plante : Impact sur la distribution des propriétés phytobénéfiques chez les *Pseudomonas* fluorescents


## Résumé

Les plantes interagissent en permanence avec une grande diversité de microorganismes qu'elles sélectionnent entre autre au niveau de leurs racines. Certaines bactéries, qualifiées de PGPR (*Plant Growth-Promoting Rhizobacteria*), sont capables de stimuler la croissance et la santé de la plante, grâce à l'expression d'une large panoplie de propriétés phytobénéfiques. L'hypothèse actuelle serait que les PGPR possédant un nombre maximal de ces fonctions auraient un plus fort impact bénéfique sur le végétal. Toutefois, l'occurrence de ces PGPR multifonctions dans la rhizosphère n'est pas connue. De plus, les possibles interactions entre propriétés co-occurrentes au sein d'une même PGPR et la résultante de ces interactions sur la plante sont relativement peu documentées. Dans ce contexte, ce projet de thèse a eu comme objectif général de mieux comprendre la distribution des propriétés phytobénéfiques chez un groupe bactérien possédant un large éventail de ses propriétés, celui des *Pseudomonas* fluorescents et d'évaluer chez une PGPR modèle multifonction, *Pseudomonas fluorescens* F113, si des interactions fonctionnelles existent entre ces propriétés et déterminer leurs contributions respectives à l'effet phytostimulateur. L'étude d'environ 700 isolats de *Pseudomonas* provenant de la rhizosphère de deux cultivars de maïs et de sol non-rhizosphérique, a mis en évidence que ces cultivars sélectionnent majoritairement des *Pseudomonas* fluorescents (i) arborant un nombre réduit de propriétés phytobénéfiques (1 à 5 propriétés) et (ii) appartenant à des sous-groupes taxonomiques particuliers. Par ailleurs, l'étude de génomique comparative que nous avons menée au sein du groupe des *Pseudomonas* fluorescents souligne le lien entre phylogénie et les profils de propriétés phytobénéfiques possédées par les *Pseudomonas*. Enfin, chez *P. fluorescens* F113, les propriétés co-occurrentes ne contribuent pas de façon égale à l'effet observé sur la plante. L'existence d'interactions entre ces propriétés co-occurrentes a également été mise en évidence. De ce réseau d'interactions, résultera un effet bénéfique observé sur la plante particulier. Ces résultats sont importants pour mieux comprendre la place et le rôle de ces PGPR multifonctions dans la rhizosphère. Ils permettent d'approfondir nos connaissances à propos du fonctionnement écologique des *Pseudomonas* fluorescents au sein du rhizomicrobiote.



**Mots clés :** *Pseudomonas*, Multifonction, Propriétés phytobénéfiques, Biocontrôle, Rhizosphère, Maïs.

# Plant-beneficial rhizobacteria selection by plant: Impact on plant-beneficial property distribution in fluorescent *Pseudomonas*


## Abstract

Plants are constantly interacting with a huge diversity of microorganisms, especially in the rhizosphere, where plant roots (through rhizodeposition) will select particular bacterial populations. Some bacteria, called PGPR (Plant Growth-Promoting Rhizobacteria), are able, in turn, to improve plant growth and health, through the expression of a wide range of plant-beneficial properties. The current hypothesis is that PGPR harboring a maximum number of these plant-beneficial properties would provide a better effect on plant. However, the occurrence of these multi-trait PGPR in the rhizosphere is unknown. In addition, the interaction between co-occurring plant-beneficial properties within the same PGPR and their resulting effects on plant are poorly documented. In this context, the aims of this thesis were to (i) determine the distribution of plant-beneficial properties in the fluorescent *Pseudomonas* bacterial group, known to harbor a wide range of these properties, and (ii) to evaluate in the PGPR model strain *Pseudomonas fluorescens* F113, if a crosstalk between plant-beneficial properties occurs and to determine the relative contribution of each co-occurring plant-beneficial properties. The study of 700 *Pseudomonas* isolates from the rhizosphere of two maize cultivars and from non-rhizosphere soils shows that cultivars mostly select fluorescent *Pseudomonas* (i) displaying few plant-beneficial properties (up to 5 properties) and (ii) belonging to particular taxonomic subgroups. Furthermore, the comparative genomic study we conducted within the fluorescent *Pseudomonas* group emphasizes the link between phylogeny and plant-beneficial profiles owned by *Pseudomonas*. Finally, in *P. fluorescens* F113, co-occurring plant-beneficial properties do not contribute equally to the observed effects on plant. The existence of crosstalks between these co-occurring plant-beneficial properties was also highlighted. This network of functional interactions may lead to specific effects on plant. These results are important for understanding the place and role of multi-trait PGPR in the rhizosphere. They help to deepen our understanding of the ecological functioning of fluorescent *Pseudomonas* among the rhizomicrobiote.



**Key words:** Fluorescent *Pseudomonas*, Multi-trait PGPR, plant-beneficial properties, Biocontrol, Rhizosphere, Maize.

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écologie microbienne lyon

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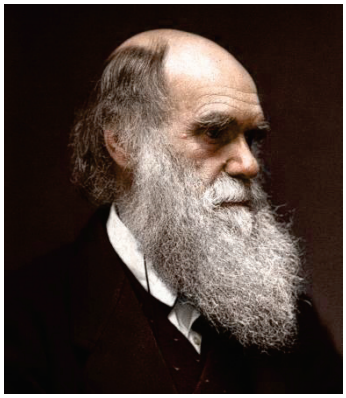
# INTRODUCTION GENERALE

*"Les espèces qui survivent ne sont pas les espèces les plus fortes, ni les plus intelligentes, mais celles qui s'adaptent le mieux aux changements."*

Charles DARWIN (1809-1882)



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L'évolution de la vie sur Terre a été entraînée par un petit nombre de grands changements, aussi appelés « transitions évolutives » (West *et al.* 2015). L'une des grandes transitions évolutives est attribuée à la sortie des eaux des organismes qui a eu lieu du Silurien au Dévonien, il y a entre 443 et 358 millions d'années. La capacité des organismes à interagir entre eux est un moteur de leur évolution, pouvant ainsi permettre la réalisation de telles transitions. D'après des études de fossiles (Taylor *et al.* 1995) et des analyses moléculaires (Wang *et al.* 2010), le succès de la colonisation terrestre par les plantes aurait été possible grâce à l'établissement d'une interaction coopérative avec un champignon (Heckman *et al.* 2001). Cette interaction n'était probablement pas unique, puisque les bactéries avaient déjà colonisé la Terre (Watanabe *et al.* 2000). Par ailleurs, il est proposé que la naissance du genre bactérien *Azospirillum* (décrit comme comportant des bactéries en interaction avec différentes plantes) coïnciderait avec l'émergence des plantes vasculaires sur Terre (Wisniewski-Dyé *et al.* 2011). De nombreux microorganismes devaient être en interaction avec les plantes lors de cette transition évolutive, constituant pour la plante un microbiote primitif. Dès lors, les plantes se sont diversifiées comptant en 2010 plus de 330 000 espèces végétales (Source : Union Internationale pour la Conservation de la Nature – IUCN) dont certaines ont même colonisé des environnements extrêmes sur Terre, comme des milieux extrêmement chauds et arides (Ali *et al.* 2000) ou polaires (Berrios *et al.* 2012). La diversification et l'adaptation des plantes à leurs milieux pourraient avoir été favorisées par leur co-évolution avec leurs microbiotes associés. Ainsi, le terme d'« holobionte » a été proposé pour désigner la plante et ses microbiotes associés comme une seule et même entité soumise à des processus évolutifs (**Figure 1**) (Vandenkoornhuyse *et al.* 2015).

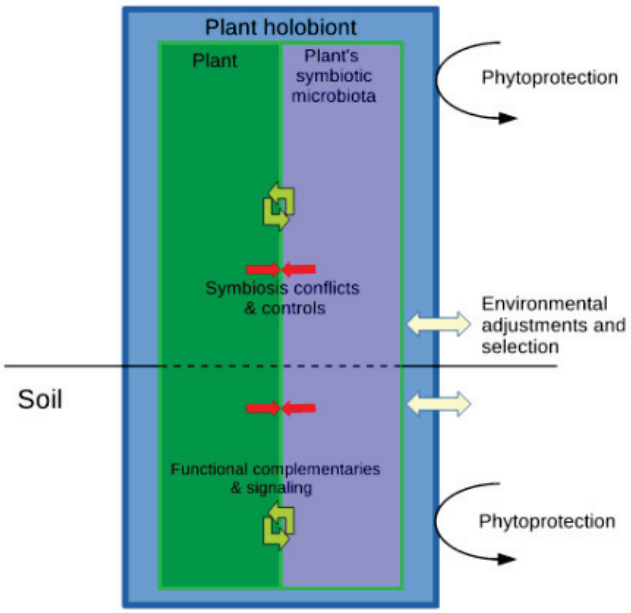


**Figure 1** : Schéma de l'holobionte végétal et des relations existant entre la plante et ses microbiotes (d'après Vandenkoornhuyse *et al.* 2015).

## ***Le microbiote rhizosphérique***

Différents microbiotes sont décrits chez les plantes et classés selon les différents organes qu'ils occupent. On différentie les microbiotes associés aux feuilles (de la phyllosphère), à la graine (de la spermosphère), mais l'un des microbiotes de plantes les plus étudié correspond à celui qui est présent dans la zone de sol sous l'influence des racines (rhizosphère - Hiltner *et al.* 1904), le rhizomicrobiote. Le rhizomicrobiote est constitué de très nombreux microorganismes dont l'abondance et la diversité diffèrent d'un groupe taxonomique à l'autre (**Figure 2**).

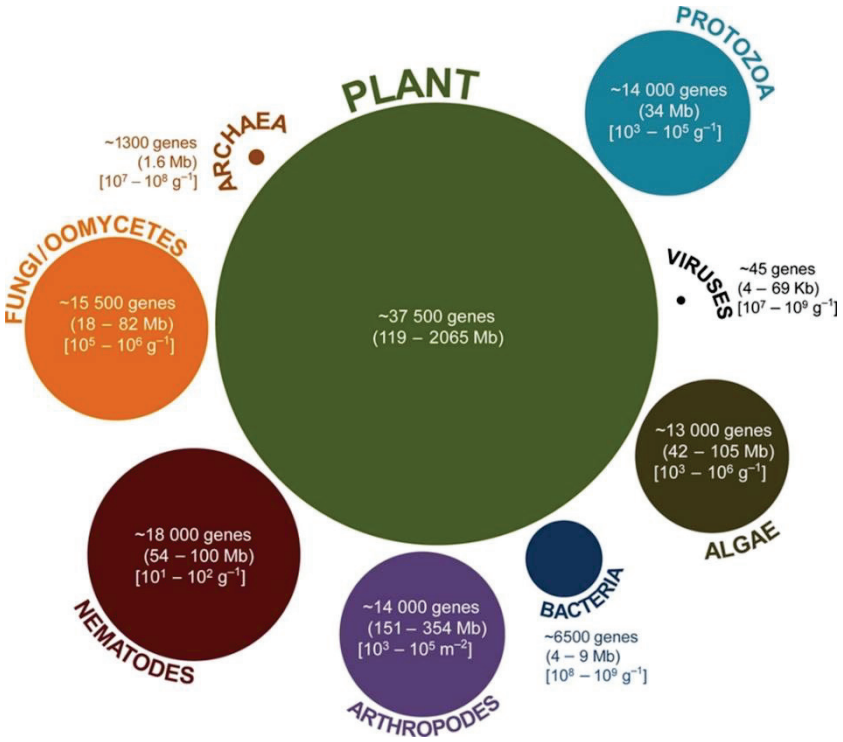


**Figure 2 : Représentation de l'ensemble des microorganismes présents dans la rhizosphère**
La taille des cercles est proportionnelle au nombre moyen de gènes présents dans les génomes d'espèces représentatives de chaque groupe d'organismes. La taille (ou gamme de tailles) de leurs génomes respectifs est indiquée entre parenthèses. L'abondance de chacun des microorganismes est indiquée entre crochets (tiré de Mendes *et al.* 2013).

La composition du rhizomicrobiote peut varier selon différents facteurs biotiques et abiotiques. Le sol est souvent assimilé à un « réservoir » contenant de nombreuses populations microbiennes diversifiées et susceptibles d'interagir avec la plante (Alabouvette et Steinberg 2006). Le type de sol - autrement dit le type de « réservoir » - est un déterminant majeur modulant la composition du rhizomicrobiote (Lundberg *et al.* 2012, Bulgarelli *et al.* 2012, Schlaeppi *et al.* 2013). Le partenaire végétal module également son rhizomicrobiote *via* les exsudats qu'il relargue (Haichar *et al.* 2008). Les profils d'exsudation variant entre deux plantes de famille, genre, espèce ou variété différentes (Lesuffleur *et al.* 2007), les rhizomicrobiotes associés auront ainsi une composition taxonomique différente (Grayston *et al.* 1998 ; Berendsen *et al.* 2012). Le rhizomicrobiote est un système dynamique qui change également au cours du temps, notamment en fonction du stade de développement de la plante (Mougel *et al.* 2006, Li

*et al.* 2014). Enfin, des études récentes ont montré que l'histoire évolutive des plantes y compris suite à leur domestication est un facteur qui a influencé la composition du rhizomicrobiote (Bouffaud *et al.* 2012, Bouffaud *et al.* 2014, Perez-Jamarillo *et al.* 2015).

Ainsi, l'interaction entre la plante et son environnement constitue sans doute le facteur prépondérant influençant l'assemblage du rhizomicrobiote (Peiffer *et al.* 2013).

## ***Interactions biologiques dans la rhizosphère : cas de la coopération***

Différents types d'interactions biologiques sont partagées par la plante avec les microorganismes dans la rhizosphère. Ces interactions peuvent être néfastes pour la plante lorsqu'elles concernent des microorganismes phytopathogènes. D'autres en revanche peuvent être bénéfiques pour le végétal. Ces interactions font intervenir des microorganismes coopérant de façon « libre » avec la plante (cas des symbioses associatives ou coopérations) impliquant des bactéries pouvant stimuler la santé et la croissance du végétal, les « *Plant Growth-Promoting Rhizobacteria* » (PGPR) ou en mettant en place un organe dédié spécifiquement à cette association (cas des symbioses dites mutualistes, selon la définition de Ramade 1984), comme la nodosité dans le cas des symbioses *Rhizobium-Fabaceae*. Ces interactions bénéfiques à la fois pour le partenaire végétal et pour le partenaire microbien peuvent être schématisées sous la forme d'une boucle de rétroaction s'établissant entre les 2 partenaires (**Figure 3**).

Werner *et al.* (2014) ont suggéré l'existence au sein du rhizomicrobiote de deux types de partenaires bactériens bénéfiques pour la plante. Le premier est un partenaire proposant des services multiples et diversifiés plutôt qu'un seul – analogue à une PGPR pouvant posséder plusieurs fonctions bénéfiques pour la plante. Le second partenaire est un partenaire spécialisé dans un nombre restreint de services proposés à la plante – analogue à une PGPR possédant peu de fonctions bénéfiques pour la plante mais fournissant un bénéfice important à cette dernière. Cependant, savoir lequel des deux partenaires bactériens pourrait être préférentiellement sélectionné par la plante demeure une question sans réponse.

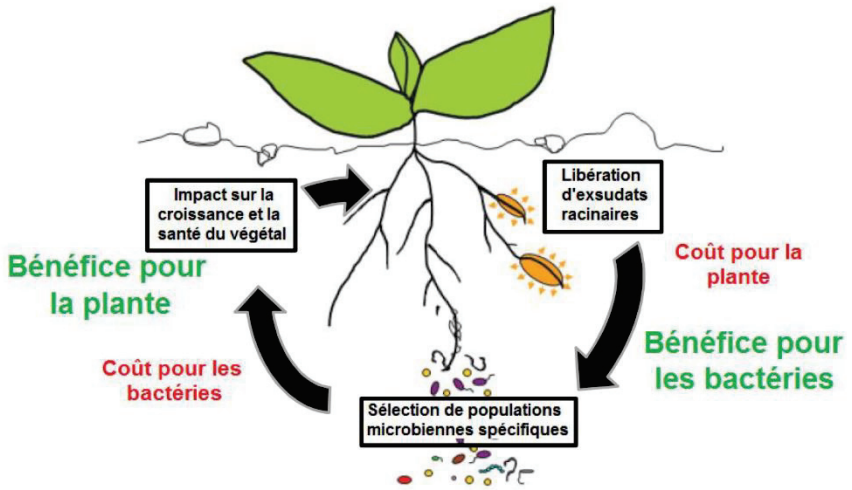


**Figure 3 : Boucle de rétroaction représentant l'interaction bénéfique plante-rhizobactéries.**
La libération des exsudats représente un coût métabolique pour la plante qui va être investi dans la sélection de populations microbiennes particulières. En retour, certaines populations sélectionnées vont avoir un impact positif sur la croissance et la santé de la plante en exprimant des fonctions bénéfiques pour la plante (modifié de Lemanceau *et al.* 2014).

## ***Les Pseudomonas fluorescents de la rhizosphère : des partenaires importants dans la coopération***

Parmi les partenaires connus pour être en coopération avec la plante figurent les bactéries appartenant au genre *Pseudomonas* et plus particulièrement au groupe *Pseudomonas fluorescens* (**Figure 4**).

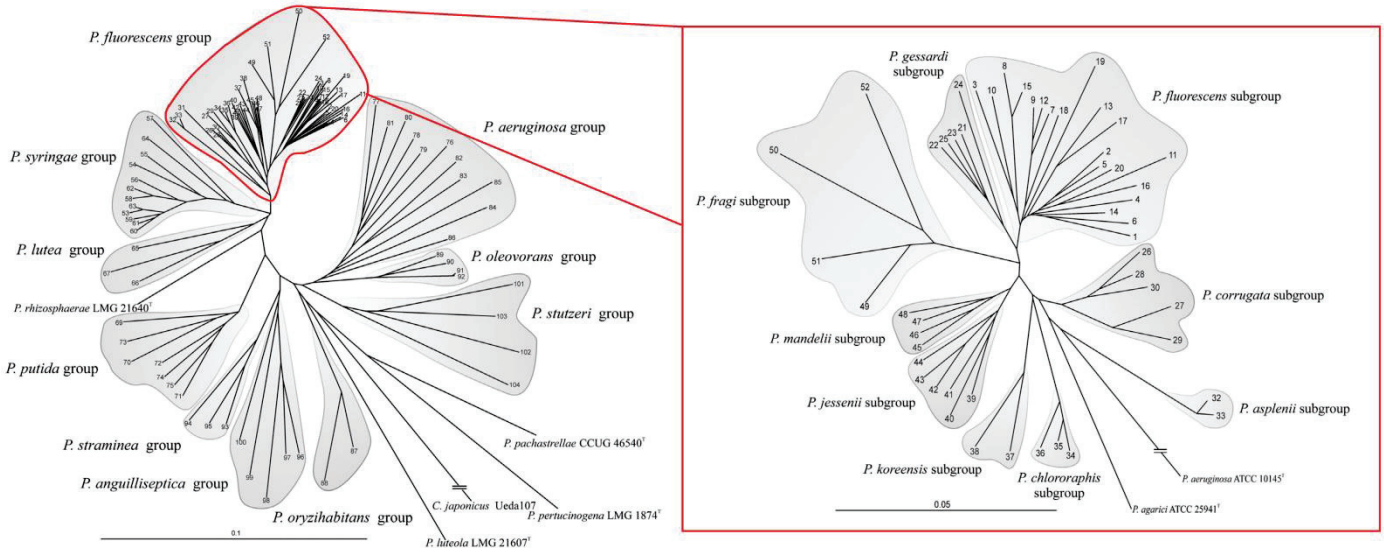


**Figure 4 : Arbre phylogénétique des bactéries du genre *Pseudomonas***
Arbre phylogénétique réalisé à partir de la concaténation de 4 gènes de ménage *rrs*, *rpoD*, *gyrB* et *rpoB* (d'après Mulet *et al.* 2010).

Les bactéries du groupe des *P. fluorescens* sont retrouvées abondantes dans la rhizosphère de nombreuses plantes (Couillerot *et al.* 2009, Almario *et al.* 2014, Haney *et al.* 2015) et possèdent une large gamme de fonctions phytobénéfiques avec différents modes d'action sur le végétal (Naik *et al.* 2008, Ahmad *et al.* 2008, Loper *et al.* 2012). Ces rhizobactéries peuvent agir (i) sur le développement de la plante –ils modifient l'architecture racinaire en produisant des hormones végétales ou en modulant la production d'hormones dans la plante (Garcia de Salamone *et al.* 2001, Picard et Bosco 2005, Shaharoona *et al.* 2006), (ii) sur sa nutrition – ils rendent biodisponibles pour la plante certains minéraux essentiels comme le phosphore (Meyer *et al.* 2010) et (iii) sur sa santé – ils produisent des métabolites secondaires antimicrobiens pouvant nuire au développement de phytopathogènes et/ou activer les mécanismes de défenses de la plante (Bakker *et al.* 2007, Gross et Loper 2009). Par ailleurs, la production de métabolites secondaires chez ces rhizobactéries peut également réguler l'expression de gènes phytobénéfiques chez d'autres PGPR parmi le rhizomicrobiote (Combes-Meynet *et al.* 2011).

Du fait de ce large éventail de fonctions phytobénéfiques possédées, ces rhizobactéries ont un rôle écologique primordial dans la rhizosphère. C'est en particulier le cas dans les sols qualifiés de « sols résistants » où l'implication de ces bactéries dans la résistance naturelle de certains sols à des maladies et dans la protection de la plante a ainsi été démontrée (Mazzola *et al.* 2002, Ramette *et al.* 2003).

La diversité des modes d'action a fait des rhizobactéries appartenant au groupe des *Pseudomonas fluorescens* des candidats de 1er ordre pour la lutte biologique depuis les années 1970 (Weller 2007).

## ***Hypothèses de travail et objectifs de la thèse***

La recherche de nouvelles souches bactériennes d'intérêt agronomique s'oriente vers des candidats arborant un maximum de fonctions parce qu'ils pourraient apporter des bénéfices plus importants à la plante en terme d'acclimatation à son milieu, de lutte envers les nuisibles (micro ou macroorganismes), d'amélioration de son développement et de sa croissance. De ce fait, l'hypothèse générale de ce travail est que les plantes – en particulier le maïs – sélectionneraient préférentiellement au niveau de leur rhizosphère des *Pseudomonas* fluorescents possédant un maximum de fonctions phytobénéfiques du fait de la diversité des bénéfices qu'elles peuvent potentiellement apporter à la plante.

Trois principaux objectifs ont été poursuivis. *Le premier* a été d'évaluer si les *Pseudomonas* fluorescents présentant plusieurs fonctions phytobénéfiques co-occurrentes, qualifiés de multi-fonctions, sont préférentiellement sélectionnés dans la rhizosphère du maïs et d'identifier les critères de sélection des *Pseudomonas* fluorescents par les plantes (taxonomie, nombre et type de fonctions phytobénéfiques). *Le second* a été d'étudier la distribution et la co-occurrence des fonctions phytobénéfiques au sein de la lignée des *P. fluorescens* au travers d'une étude de génomique comparative, en incluant 9 souches issues des travaux précédents. *Le troisième* et dernier objectif a eu pour but de déterminer, chez la PGPR modèle multi-fonction *P. fluorescens* F113, les interactions qu'il peut exister entre les fonctions phytobénéfiques qu'elle possède et la résultante de ces interactions potentielles sur l'effet observé sur la plante.

## ***Démarche scientifique***

La démarche employée pour répondre au premier objectif a été d'isoler des bactéries appartenant au groupe des *P. fluorescens* (défini taxonomiquement d'après Mulet *et al.* 2010), taxon comportant des PGPR, à partir des rhizosphères de deux cultivars de maïs, cultivés sur 4 sols d'origines géomorphologiques et possédant des caractéristiques physico-chimiques différentes. Une collection de 698 isolats a été construite et criblée vis-à-vis (i) de la présence/absence de gènes codant des propriétés phytostimulatrices de premier plan et (ii) de tests biochimiques d'activités phytostimulatrices. La caractérisation fonctionnelle mais également taxonomique des isolats nous a permis d'évaluer l'occurrence des bactéries multifonctions du groupe des *P. fluorescens* dans la rhizosphère du maïs et de révéler que la plante sélectionne des individus de ce groupe bactérien possédant préférentiellement entre 1 à 5 fonctions ce qui pourrait être lié à leur colonisation plus efficace de la rhizosphère.

Le second objectif a été de déterminer, par une étude de génomique comparative, s'il existait un lien entre la taxonomie des *Pseudomonas* et les propriétés phytobénéfiques qu'ils possèdent. Dans ce but, nous avons construit une banque de 114 génomes de *Pseudomonas* comportant 9 génomes séquencés provenant de souches isolées au cours de travaux du 1er axe.

Les analyses de reconstruction phylogénétique et de distribution des propriétés phytobénéfiques dans les génomes nous ont permis de proposer un scénario retraçant les histoires évolutives de l'acquisition des propriétés impliquées dans la production de métabolites secondaires antimicrobiens chez les *Pseudomonas*. De plus, la caractérisation détaillée de ces 9 souches a été réalisée afin de visualiser si un lien existe entre les combinaisons des propriétés phytobénéfiques des souches et leur aptitude à stimuler la plante et à inhiber la croissance de microorganismes phytopathogènes.

Pour le troisième objectif, nous avons opté pour une approche de mutagenèse afin de supprimer chacune des 4 fonctions phytobénéfiques majeures de la PGPR modèle, *P. fluorescens* F113, dans le but de déterminer si certaines fonctions phytobénéfiques ont un impact sur les autres fonctions présentes dans cette même souche. Pour cela, nous avons mesuré chaque fonction phytobénéfique étudiée chez chacun des mutants obtenus. L'importance de chacune des fonctions phytobénéfiques vis-à-vis de la fitness de l'individu a également été mesurée. Enfin, nous avons cherché à savoir si chacune des fonctions phytobénéfiques possédées contribuaient de façon égale à l'effet stimulateur induit par F113 sur la plante. De ce fait, nous avons procédé à des tests d'inoculation des souches mutées et sauvage, en condition *in vitro* sur la plante modèle *Arabidopsis thaliana*, puis sur du maïs planté dans du sol non stérile en serre, et mesuré des paramètres de croissance et de développement des plantes.

En parallèle à ces objectifs, une démarche de sélection de souches pour une potentielle valorisation agronomique et des essais préliminaires de phytoprotection ont été engagés.

## ***Plan du manuscrit de thèse***

Le manuscrit se compose d'un premier chapitre correspondant à une synthèse bibliographique, suivi de trois chapitres décrivant les résultats des travaux de recherche et pour terminer, d'un chapitre de discussion générale et perspectives.

### ***1er chapitre***

Ce chapitre comprend une première partie correspondant à une revue bibliographique publiée en 2013 dans le journal *Frontiers in Plant Science* intitulée « *Plant growth-promoting rhizobacteria and root system functioning* » qui fait un état des lieux des modes d'actions des PGPR sur le végétal (en termes de physiologie et de morphologie) et leur investissement dans le fonctionnement microbien de la rhizosphère. La seconde partie intitulée « Les fonctions phytobénéfiques co-occurrentes » traite de (i) la distribution des propriétés phytobénéfiques co-occurrentes parmi les rhizobactéries, de (ii) leur régulation et de (iii) leurs rôles écologiques.

### ***2ème chapitre***

Ce chapitre, intitulé « *Occurrence des rhizobactéries multi-fonctions dans la rhizosphère : cas des Pseudomonas fluorescents* » est présenté sous la forme d'un article rédigé en anglais qui a été soumis à *Environmental Microbiology*. Dans ce chapitre, l'occurrence des *Pseudomonas* fluorescents dans la rhizosphère du maïs selon (i) le nombre et le type de fonctions phytobénéfiques qu'ils possèdent et (ii) leur taxonomie a été déterminée.

### ***3ème chapitre***

Ce chapitre, intitulé « *Distribution des propriétés phytobénéfiques parmi la lignée des Pseudomonas fluorescens*» a été rédigé sous la forme d'un article scientifique en français et porte sur l'analyse de la distribution des propriétés phytobénéfiques parmi des génomes de *Pseudomonas* fluorescents afin d'évaluer si il existe des liens entre la taxonomie des *Pseudomonas* et les propriétés phytobénéfiques qu'ils possèdent.

### ***4ème chapitre***

Ce chapitre, intitulé « *Interactions fonctionnelles et contribution relative des fonctions phytobénéfiques à l'effet phytostimulateur : cas de la PGPR multi-fonction modèle Pseudomonas fluorescens F113* » contient deux parties expérimentales.

- ∴ La partie I, intitulée « *Functional interactions and relative contribution to phytostimulation between plant-beneficial properties in Pseudomonas fluorescens F113* » (article en préparation) concerne l'obtention de mutants inactivés dans la production de 4 fonctions phytobénéfiques majeures de la PGPR modèle *P. fluorescens* F113 dans le but d'évaluer les interactions fonctionnelles potentielles existant entre ces propriétés phytobénéfiques co-occurentes et la contribution de chacune de ces fonctions phytobénéfiques à l'effet de modulation de l'architecture racinaire d'*A. thaliana* en condition *in vitro*.
- ∴ La partie II, intitulée « *Root colonization and maize growth were differently affected by the inoculation of the wild-type PGPR Pseudomonas fluorescens F113 compared to mutants impaired in the biosynthesis of plant-beneficial functions* » a été rédigée comme une note scientifique (en préparation) et analyse cette fois-ci l'importance des fonctions phytobénéfiques de *P. fluorescens* F113 dans sa capacité à coloniser la rhizosphère du maïs en condition *in situ* et de visualiser son impact sur la physiologie et la morphologie du maïs.

### ***5ème chapitre***

Dans la discussion générale, la place et le rôle des *Pseudomonas* fluorescents dans le fonctionnement de la rhizosphère sont discutés en regard des résultats obtenus. Les limites du travail sont également discutées et des perspectives ainsi que de potentielles pistes de recherches sont suggérées.

### ***Annexes***

En Annexe, est présentée une partie de la production scientifique en lien avec le projet de thèse.

- ∴ Une étude rédigée sous la forme d'un article scientifique décrivant la sélection de deux souches de *Pseudomonas* candidates pour lutter contre le champignon phytopathogène *Thielaviopsis basicola* ainsi que les essais préliminaires réalisés en Loire Atlantique pour lutter contre ce champignon dans le cadre de la culture de la mâche (*Valerianella locusta*). Ce travail a été réalisé en collaboration avec le CTIFL (Centre Technique Interprofessionnel des Fruits et Légumes).

∴ Un article de congrès publié en 2015 dans le cadre du congrès « *Natural products and Biocontrol 2012* » et intitulé « *Plant growth-promoting properties of Pseudomonas biocontrol agent producing 2,4-diacetylphloroglucinol* ».

∴ Un chapitre de livre publié en 2015 dans le livre « *Handbook for Azospirillum* » et intitulé « Alleviat*ion of abiotic and biotic stresses in plants by Azospirillum* ».

# CHAPITRE 1

# SYNTHESE BIBLIOGRAPHIQUE

# ***Introduction à la synthèse bibliographique***

La rhizosphère, définie comme la zone de sol sous l'influence des racines par L. Hiltner en 1904, est une zone de transition (écotone) entre le sol et la racine. Cette zone est soumise à des conditions physico-chimiques particulières dues, d'une part, à la composition physico-chimique du sol d'origine et, d'autre part, aux molécules sécrétées (sucres, composés phénoliques etc.) par la plante (Haichar *et al.* 2008 ; Hinsinger *et al.* 2009). Les racines vont interagir avec la communauté microbienne du sol entrainant, au travers des exsudats libérés, une sélection préférentielle de certains microorganismes au niveau de la rhizosphère, constituant alors le rhizomicrobiote (Hartman *et al.* 2009). Comme dans tout système biologique, les individus présents dans le rhizomicrobiote vont accomplir un certain nombre de fonctions permettant un développement optimal de la plante en réponse aux contraintes environnementales. Certaines de ces fonctions pourront être assurées par plusieurs microorganismes d'espèces, genres, *phyla* différents formant ce que l'on appelle des groupes fonctionnels (définition donnée initialement par B. H. Walker en 1992). La diversité taxonomique des groupes fonctionnels est synonyme d'une redondance fonctionnelle qui va favoriser le maintien d'un équilibre des fonctions assurées par ce rhizomicrobiote au sein de la rhizosphère. Ainsi dans le rhizomicrobiote, certains microorganismes vont posséder des fonctions pouvant être bénéfiques pour la plante (propriétés phytobénéfiques). Ces microorganismes possédant de telles propriétés représentent un potentiel agronomique important pour une agriculture durable et résiliente. Certains d'entre eux ont été caractérisés, leurs effets bénéfiques sur la plante validés et ont été appelés pour la première fois « PGPR » pour « *Plant Growth-Promoting Rhizobacteria* » en 1979 par J. W. Kloepper et M. N. Schroth.

Depuis le nombre de souches décrites, possédant de tels effets, ne cesse d'augmenter et la description de leurs modes d'action envers la plante également. Par ailleurs, de plus en plus d'études s'intéressent à la distribution de ces propriétés phytobénéfiques parmi les individus du rhizomicrobiote et mettent en évidence la possibilité pour une même rhizobactérie de posséder plusieurs propriétés phytobénéfiques (rhizobactérie multifonction).

La première partie de ce chapitre bibliographique décrit l'état des connaissances concernant les différents modes d'actions des PGPR envers la plante à une échelle phénotypique, physiologique et moléculaire. Cette partie s'intéresse également à l'effet de la plante sur les PGPR et leurs propriétés phytobénéfiques. Enfin, une vision globale du fonctionnement de la rhizosphère est abordée en se plaçant non-plus à l'échelle de l'individu mais au niveau de l'ensemble des populations de PGPR et des groupes fonctionnels. Cette revue a été publiée dans *Frontiers in Plant Science*.

La seconde partie de la synthèse bibliographique dresse un état des lieux de la distribution des propriétés phytobénéfiques et s'intéresse plus précisément aux propriétés présentes chez un même individu (qualifiées de propriétés phytobénéfiques co-occurrentes). Cette partie traite également des mécanismes intracellulaires de régulation de ces propriétés phytobénéfiques co-occurrentes ainsi que de la contribution de chacune de ces propriétés co-occurrentes à l'effet phytobénéfique observé.

# PARTIE A

## *LES PGPR ET LE FONCTIONNEMENT DE LA RHIZOSPHERE*

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### Plant growth-promoting rhizobacteria and root system functioning

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## ABSTRACT

The rhizosphere supports the development and activity of a huge and diversified microbial community, including microorganisms capable to promote plant growth. Among the latter,plant growth-promoting rhizobacteria (PGPR) colonize roots of monocots and dicots, and enhance plant growth by direct and indirect mechanisms. Modification of root system architecture by PGPR implicates the production of phytohormones and other signals that lead, mostly, to enhanced lateral root branching and development of root hairs. PGPR also modify root functioning, improve plant nutrition and influence the physiology of the whole plant. Recent results provided first clues as to how PGPR signals could trigger these plant responses. Whether local and/or systemic, the plant molecular pathways involved remain often unknown. From an ecological point of view, it emerged that PGPR form coherent functional groups, whose rhizosphere ecology is influenced by a myriad of abiotic and biotic factors in natural and agricultural soils, and these factors can in turn modulate PGPR effects on roots. In this paper, we address novel knowledge and gaps on PGPR modes of action and signals, and highlight recent progress on the links between plant morphological and physiological effects induced by PGPR. We also show the importance of taking into account the size, diversity, and gene expression patterns of PGPR assemblages in the rhizosphere to better understand their impact on plant growth and functioning. Integrating mechanistic and ecological knowledge on PGPR populations in soil will be a prerequisite to develop novel management strategies for sustainable agriculture.


**Keywords: plant-PGPR cooperation, rhizo-microbiome, rhizosphere, phytohormone, plant nutrition, ISR, functional group**

# INTRODUCTION

Photosynthetic terrestrial plants play key roles as ecosystem engineers (Wright and Jones, 2006; Hartmann *et al.*, 2009). They contribute, for instance, to the establishment of specific microbial ecological niches in plant-based systems. This is particularly the case in the rhizosphere, i.e., the soil in contact with plant roots. Besides its role in plant anchorage in soil, absorption of water and ions, nutrient storage, and plant vegetative growth, the root system is in close contact with a wide range of soil microbial populations (Berg and Smalla, 2009).

Despite their interactions with the biotic environment, the root system and its rhizosphere have received much less attention by plant physiologists than the rest of the plant. Plant roots exude a huge diversity of organic nutrients (organic acids, phytosiderophores, sugars, vitamins, amino acids, nucleosides, mucilage) and signals that attract microbial populations, especially those able to metabolize plant-exuded compounds and proliferate in this microbial habitat (Bais *et al.*, 2006; Pothier *et al.*, 2007; Badri *et al.*, 2009; Shukla *et al.*, 2011; Drogue *et al.*, 2013). Root exudates being the largest source of carbon supply within soil, the rhizosphere compartment houses a rich microbial community, comprising up to $10^{10}$ bacteria per gram of soil (Gans *et al.*, 2005; Roesch *et al.*, 2007) and encompassing a large diversity of taxa (Kyselková *et al.*, 2009; Gomes *et al.*, 2010). The corresponding microbial community associated to plant roots can be referred to as the rhizomicrobiome (Chaparro *et al.*, 2013). Its composition is distinct from that of the microbial community of the surrounding soil, a direct consequence of bacterial competition for nutrients liberated in the vicinity of plant roots (Raynaud *et al.*, 2008; Bulgarelli *et al.*, 2013; Chaparro *et al.*, 2013). Since root exudate composition changes along the root system, according to stages of plant development and to plant genotypes, the rhizomicrobiome composition differs accordingly (Berg and Smalla, 2009; Aira *et al.*, 2010; Bouffaud *et al.*, 2012; Bulgarelli *et al.*, 2013; Chaparro *et al.*, 2013). Plant-driven selection of bacteria is an important issue recently discussed in several reviews (Hartmann *et al.*, 2009; Doornbos *et al.*, 2012; Drogue *et al.*, 2012; Bulgarelli *et al.*, 2013).

Within the rhizomicrobiome, some microorganisms can promote plant growth and provide better plant health through several indirect or direct mechanisms (Couillerot *et al.*, 2009; Richardson *et al.*, 2009). Beneficial plant-microbe interactions are symbiotic interactions in which costs and benefits are shared by the plants and the microorganisms (Odum and Barrett, 2005; Bulgarelli *et al.*, 2013) and can be categorized into two main types of interactions (Drogue *et al.*, 2012). First, mutualistic interactions correspond to intimate and mostly obligate interactions between microbes and a restricted range of compatible host plants. They generally lead to the formation of a structure specifically dedicated to the interaction (e.g., nodules during the symbiosis between nodulating rhizobia and Fabaceae, arbuscules in the endomycorrhizal symbiosis; Parniske, 2008; Masson-Boivin *et al.*, 2009). Second, cooperations (also called associative symbioses) correspond to less obligate and specific interactions (Barea *et al.*, 2005; Drogue *et al.*, 2012). They involve soil bacteria able to colonize the surface of the root system (and sometimes root inner tissues) and to stimulate the growth and health of the plant, and are referred to as plant growth-promoting rhizobacteria (PGPR; Barea *et al.*, 2005). Colonization of plant host roots by PGPR is heterogeneous along the root system; their competitiveness regarding this process is a *sine qua non* for plant growth promotion (discussed in Benizri *et al.*, 2001; Compant *et al.*, 2010; Dutta and Podile, 2010;

Drogue *et al.*, 2012). In comparison to mutualistic symbionts, PGPR are thought to interact with a large range of host plant species and to encompass a huge taxonomic diversity, especially within the Firmicutes and Proteobacteria phyla (Lugtenberg and Kamilova, 2009; Drogue *et al.*, 2012). PGPR can enhance plant nutrition *via* associative nitrogen fixation, phosphate solubilization, or phytosiderophore production (Richardson *et al.*, 2009). They can improve root development and growth through the production of phytohormones or enzymatic activities, as well as favor the establishment of rhizobial or mycorrhizal symbioses. Others can protect the plant through inhibition of phytoparasites, based on antagonism or competition mechanisms, and/or by eliciting plant defenses such as induced systemic resistance (ISR; Couillerot *et al.*, 2009; Lugtenberg and Kamilova, 2009). Some PGPR can also help plants withstand abiotic stresses including contamination by heavy metals or other pollutants; certain are even able to increase the capacity of plants to sequester heavy metals (Jing *et al.*, 2007; Saharan and Nehra, 2011; Tak *et al.*, 2013). Therefore, utilizing PGPR is a new and promising approach for improving the success of phytoremediation of contaminated soils (for recent reviews see Zhuang *et al.*, 2007; Shukla *et al.*, 2011; Tak *et al.*, 2013).

Understanding and quantifying the impact of PGPR on roots and the whole plant remain challenging. One strategy is to inoculate roots with a PGPR *in vitro* and monitor the resulting effects on plant. This showed that many PGPR may reduce the growth rate of the primary root (Dobbelaere *et al.*, 1999), increase the number and/or length of lateral roots (Combes-Meynet *et al.*, 2011; Chamam *et al.*, 2013), and stimulate root hair elongation *in vitro* (Dobbelaere *et al.*, 1999; Contesto *et al.*, 2008). Consequently, the uptake of minerals and water, and thus the growth of the whole plant, can be increased. Some of these effects, including increased root and shoot biomass, are also documented for PGPR-inoculated plants growing in soil (El Zemrany *et al.*, 2006; Minorsky, 2008; Veresoglou and Menexes, 2010; Walker *et al.*, 2012).

The focus of this paper is to review the main modes of action of PGPR strains, the functioning of PGPR populations, and their ecology in the rhizosphere. Description of plant-beneficial properties of PGPR has been the focus of several reviews (e.g., Vessey, 2003; Richardson *et al.*, 2009; Bashan and de-Bashan, 2010), but without integrating actual PGPR gene expression on roots, the interactions between different PGPR populations in the rhizosphere, or the resulting plant-beneficial effects. This paper is organized into four sections. In the first section, we present the molecular mechanisms through which PGPR may affect the architecture of the root system and interfere with the plant hormonal pathways, and review our current understanding of their impact on the structural properties of the roots. In the second section, recent findings related to the impact of PGPR on the physiology of the whole plant are presented, with a focus on plant nutrient acquisition, plant transcriptome and plant metabolome. The third section shows how expression of plant-beneficial properties can be affected within the rhizosphere by molecules emitted by other microbial populations or by the plant. As PGPR strains are not acting individually in the rhizosphere, the ecology of PGPR populations and notably the complexity of the interactions taking place between PGPR populations is discussed in the fourth section. Finally, we conclude on the importance of integrating molecular investigations on the modes of action and ecology of PGPR strains with high-throughput analyses on the abundance, taxonomic/functional diversity and activity of rhizosphere microbial communities, and with the monitoring of plant molecular responses.

## IMPACT OF PGPR ON ROOT SYSTEM ARCHITECTURE AND ROOT STRUCTURE

Most terrestrial plants develop their root system to explore soil and find nutrients to sustain growth. Root is a complex organ made of distinct regions such as the root tip, root meristem, differentiation and elongation zones, and emerging lateral roots (Scheres *et al.*, 2002). These regions have distinct roles. For instance, root hairs are differentiated epidermal cells important for plant mineral nutrition, as inferred from gene expression studies (Lauter *et al.*, 1996; von Wiren *et al.*, 2000) and nutrient accumulation measurements (Ahn *et al.*, 2004). Root functional specificity is also reflected at the level of plant-microbe interactions. In *Fabaceae* for example, the root tip is the most important region to initiate the rhizobial colonization process leading eventually to the formation of a root nodule (Desbrosses and Stougaard, 2011). In *Poaceae*, root hairs and lateral roots are preferentially colonized by PGPR, where they may express their plant beneficial properties (Pothier *et al.*, 2007; Combes-Meynet *et al.*, 2011). Root system architecture (RSA) integrates root system topology, the spatial distribution of primary and lateral roots, and the number and length of various types of roots. Several abiotic and biotic factors can influence RSA, including PGPR strains. PGPR modify RSA and the structure of root tissues mainly through their ability to interfere with the plant hormonal balance(**Figure 1**).

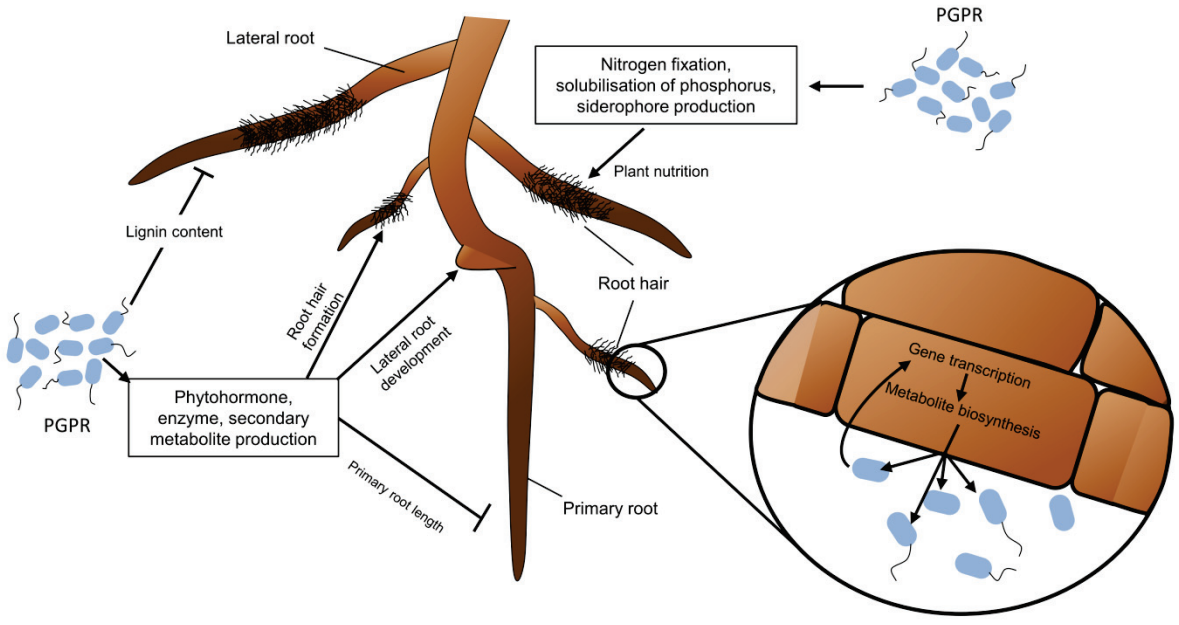


**Figure 1: Impact of phytostimulating PGPR on RSA, nutrient acquisition and root functioning**. PGPR can modulate root development and growth through the production of phytohormones, secondary metabolites and enzymes. The most commonly observed effects are areduction of the growth rate of primary root, and an increase of the number and length of lateral roots and root hairs. PGPR also influence plant nutrition *via* nitrogen fixation, solubilization of phosphorus, or siderophore production, and modify root physiology by changing gene transcription and metabolite biosynthesis in plant cells.

## PGPR effects on RSA *via* modulation of host hormonal balance

Changes in RSA may result from interferences of PGPR with the main hormonal pathways involved in regulating plant root development: auxin, cytokinin, ethylene, and to a lesser extend gibberellin, and abscisic acid (ABA) (Moubayidin *et al.*, 2009; Stepanova and Alonso, 2009; Dodd *et al.*, 2010; Overvoorde *et al.*, 2011). The balance between auxin and cytokinin is a key regulator of plant organogenesis, and shapes root architecture (Aloni *et al.*, 2006). The auxin to cytokinin ratio can be affected by PGPR because they are able to produce a wide range of phytohormones, including auxins and/or cytokinins, and secondary metabolites that can interfere with these hormonal pathways.

Many PGPR are able to produce phytohormones and secondary metabolites interfering with the plant auxin pathway, such as auxins, 2,4-diacetylphloroglucinol (DAPG), and nitric oxide (NO). Indole-3-acetic acid (IAA) is the best-characterized auxin produced by many plant-associated bacteria, including PGPR (Spaepen *et al.*, 2007a). Exogenous IAA controls a wide variety of processes in plant development and plant growth: low concentrations of IAA can stimulate primary root elongation, whereas high IAA levels stimulate the formation of lateral roots, decrease primary root length and increase root hair formation (**Figure 1**; Dobbelaere *et al.*, 1999; Patten and Glick, 2002; Perrig *et al.*, 2007; Spaepen *et al.*, 2007b; Remans *et al.*, 2008). IAA is usually synthesized by rhizobacteria from tryptophan, which is found at different concentrations in root exudates according to plant genotype (Kamilova *et al.*, 2006). In PGPR strains, several IAA biosynthetic pathways have been described depending on the metabolic intermediates (Spaepen *et al.*, 2007a). The indole-3-pyruvate decarboxylase (encoded by the *ipdC/ppdC* bacterial gene) is a key enzyme involved in the indolepyruvic acid pathway. Effects of *ipdC* mutants on plant root morphology are often altered in comparison to those of wild-type strains (Brandl and Lindow, 1998; Dobbelaere *et al.*, 1999; Patten and Glick, 2002; Suzuki *et al.*, 2003; Malhotra and Srivastava, 2008).

Plant growth promotion by PGPR can also result from indirect stimulation of the plant auxin pathway. For example, several PGPR strains like *Azospirillum brasilense* have a nitrite reductase activity and consequently are able to produce NO during root colonization (Creus *et al.*, 2005; Pothier *et al.*, 2007; Molina-Favero *et al.*, 2008). NO is involved in the auxin signaling pathway controlling lateral root formation (Creus *et al.*, 2005; Lanteri *et al.*, 2006, 2008; Molina-Favero *et al.*, 2008). DAPG is a well-known antimicrobial compound produced by biocontrol fluorescent pseudomonads(Couillerot *et al.*, 2009). At lower concentrations, DAPG can also be a signal molecule for plants, inducing systemic resistance (Iavicoli *et al.*, 2003; Bakker *et al.*, 2007), stimulating root exudation (Phillips *et al.*, 2004), and enhancing root branching (Brazelton *et al.*, 2008; Couillerot *et al.*, 2011; Walker *et al.*, 2011). DAPG can interfere with an auxin-dependent signaling pathway and thus modify RSA (Brazelton *et al.*, 2008). Indeed, applications of exogenous DAPG, at a concentration around 10 µM, inhibited primary root growth and stimulated lateral root production in tomato seedlings. Furthermore, roots of an auxin-resistant *diageotropica* mutant of tomato displayed reduced DAPG sensitivity (Brazelton *et al.*, 2008).

The growth-promotion effect of auxin or auxin-like compounds by PGPR may require functional signaling pathways in the host plant. To test that hypothesis, one could use a host

plant defective at a particular step of the hormone-signaling pathway and assess whether PGPR inoculation complements or not the effect of the mutation. This strategy requires the use of model plant such as *Arabidopsis*, the only biological system that provides to date enough documented mutant plants (Dubrovsky *et al.*, 1994; Alonso *et al.*, 2003). Consistent with that, some *Arabidopsis* auxin-signaling mutants failed to show the typical root architecture changes in response to the beneficial rhizobacterium *Phyllobacterium brassicacearum* STM196 (Contesto *et al.*, 2010). However, auxin content was not increased in roots upon inoculation with *Phyllobacterium brassicacearum* STM196, ruling out the potential implication of auxin of bacterial origin (Contesto *et al.*, 2010). Nevertheless, the use of *Arabidopsis DR5*::*GUS* reporter line, whose expression is restricted to the root meristem where the auxin maximum is located (Ulmasov *et al.*, 1997; Casimiro *et al.*, 2001), showed a change of expression pattern in response to STM196 inoculation (**Figure 2**). GUS staining appeared more intense on a wider region of the root tip as well as in the vasculature, suggesting that there was a change of auxin distribution in the root in response to STM196 inoculation, even though this strain is a low auxin producer (Contesto *et al.*, 2010). Interestingly, a similar observation was made when *Arabidopsis* plantlets were inoculated with the PGPR *Bacillus subtilis* GB03 (Zhang *et al.*, 2007), which emits volatile organic compounds (VOCs), or with *Pseudomonas fluorescens* WCS417 (Zamioudis *et al.*, 2013).

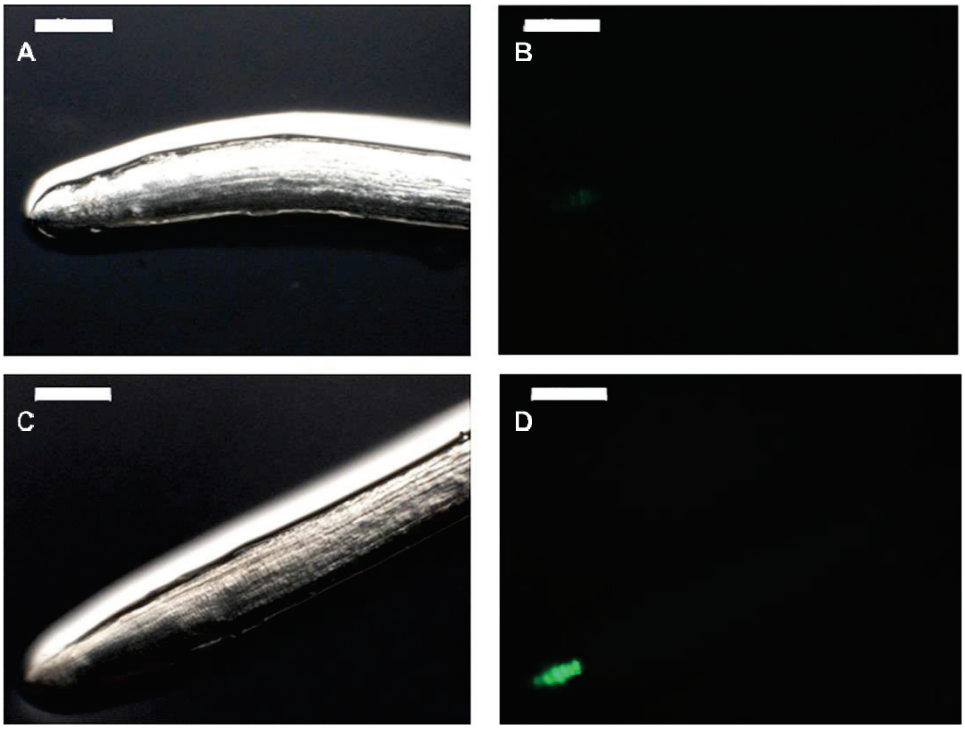


**Figure 2: PGPR-mediated changes in RSA may relate to modifications of auxin content in roots**. Six-day-old *Arabidopsis* plantlets expressing the *GFP* gene under the control of the auxin-sensitive *DR5* artificial promoter were inoculated **(C, D)** or not **(A, B)** with the PGPR *Phyllobacterium brassicacearum* STM196. Six days later, root tips were observed under normal light **(A, C)** or UV light **(B, D)** with a microscope (Z16APO, Leica, Bensheim, Germany). Scale bars represent 200 µm. Inoculation by STM196 modified root traits such as root hair elongation and primary root growth, which coincided with an increase in GFP signal in the root tip in inoculated **(D)** compared with control plants **(B)**. These observations confirm previous results with a different *Arabidopsis DR5* reporter line (Contesto *et al.*, 2010).

Cytokinin production (especially zeatin) has been documented in various PGPR like *Arthrobacter giacomelloi*, *Azospirillum brasilense, Bradyrhizobium japonicum, bacillus licheniformis, Pseudomonas fluorescens*, and *Paenibacillus polymyxa* (Cacciari *et al.*, 1989; Timmusk *et al.*, 1999; de García Salamone *et al.*, 2001; Perrig *et al.*, 2007; Cassán *et al.*, 2009; Hussain and Hasnain, 2009). Cytokinins stimulate plant cell division, control root meristem differentiation, induce proliferation of root hairs, but inhibit lateral root formation and primary root elongation (Silverman*et al.*, 1998; Riefler *et al.*, 2006). Inoculation of plants with bacteria producing cytokinin has been shown to stimulate shoot growth and reduce the root to shoot ratio (Arkhipova *et al.*, 2007). Bacterial genes involved in cytokinin biosynthetic pathways have been identified *in silico* but their role has not yet been validated through functional analyses (Frébort *et al.*, 2011). Consequently, the contribution of cytokinin production by PGPR toRSA modifications remains speculative.

Ethylene is another key phytohormone, which inhibits root elongation and auxin transport, promotes senescence and abscission of various organs, and leads to fruit ripening (Bleecker and Kende, 2000; Glick *et al.*, 2007). Ethylene is also involved in plant defense pathways (Glick *et al.*, 2007). This phytohormone can be produced in small amounts from the precursor methionine bysome PGPR, like *Azospirillum brasilense* (Thuler *et al.*, 2003; Perrig *et al.*, 2007). The ability of *Azospirillum brasilense* to produce ethylene presumably promotes root hair development in tomato plants. Indeed, exogenous ethylene supply to the plant mimicked the effect of *Azospirillum brasilense* inoculation, while the addition of an ethylene biosynthesis inhibitor blocked this effect (Ribaudo *et al.*, 2006). Actually, PGPR are more widely able to lower plant ethylene levels through deamination of 1-aminocyclopropane-1-carboxylic acid (ACC). Many genomes of PGPR do contain a gene (*acdS*) coding for an ACC deaminase, which degrades ACC into ammonium and α-ketobutyrate (Blaha *et al.*, 2006; Contesto *et al.*, 2008; Prigent-Combaret *et al.*, 2008). By lowering the abundance of the ethylene precursor ACC, the PGPR AcdS activity is thought to decrease root ethylene production, which can in turnalleviate the repressing effect of ethylene on root growth (Glick, 2005). Despite being widely accepted and supported by experimental data (Penrose *et al.*, 2001; Contesto *et al.*, 2008), the model raises issues that have not been well addressed yet. The first one deals with ethylene production within roots. Light is promoting ethylene biosynthesis, providing there is a sufficient $CO_2$ supply for shoots (Yang and Hoffman, 1984). Exposition of roots to light was shown to trigger an increase in ethylene production (Lee and Larue, 1992). In soil however, roots are sheltered from light, suggesting that this organ might not be able to synthesize large amounts of ethylene. In agreement with that, Fabaceae roots did produce ethylene in response to rhizobial colonization in presence of light, but less when they were in the dark (Lee and Larue, 1992). Secondly, there is a regulation of ethylene synthesis by a feedback loop (Yang and Hoffman, 1984). This loop should stimulate ethylene biosynthesis when the level of ACC is low. Unless PGPR disconnect that feedback loop, lowering ACC content would eventually result in stimulation of ethylene production. There is no indication yet how the feedback loop would work in presence of a PGPR. Last but not least, if ethylene plays a key role during the plant-PGPR interaction, one would expect that eitherplant ethylene mutants or impaired AcdS activity in the bacteria would result in clear disturbance of the plant responses to bacteria. However, minor effects on RSA were observed when plants were inoculated with an *acdS* bacterial mutant, or when plants affected in

their ethylene signaling pathway were inoculated with wild-type PGPR (Contesto *et al.*, 2008; Galland *et al.*, 2012; Zamioudis *et al.*, 2013). It suggests that ethylene participates to the root architecture response but is not a key player. Taken together, the functional importance of the bacterial ACC deaminase function needs further clarification. One hypothesis could be that AcdS contributes to the fine-tuning of ethylene biosynthesis during the plant-PGPR cooperation.

Several reports have revealed that PGPR are able to produce ABA or gibberellic acid, or to control the level of these hormones in plants (Richardson *et al.*, 2009; Dodd *et al.*, 2010). The first one, ABA, is well known for its involvement in drought stress. During water stress, increase in ABA levels causes closing of stomata, thereby limiting water loss (Bauer *et al.*, 2013). However, this hormone also plays different roles during lateral root development (De Smet *et al.*, 2006; Dodd *et al.*, 2010). Inoculation with *Azospirillum brasilense* Sp245 led to an increase of ABA content in *Arabidopsis*, especially when grown under osmotic stress (Cohen *et al.*, 2008). Gibberellins promote primary root elongation and lateral root extension (Yaxley *et al.*, 2001). Production of gibberellins has been documented in several PGPR belonging to *Achromobacter xylosoxidans, Acinetobacter calcoaceticus, Azospirillum* spp., *Azotobacter* spp., *Bacillus* spp., *Herbaspirillum seropedicae, Gluconobacter diazotrophicus* and rhizobia (Gutiérrez-Mañero *et al.*, 2001; Bottini *et al.*, 2004; Dodd *et al.*, 2010). Application of gibberellic acid on maize, at a concentration similar to that produced by *Azospirillum*, promotes root growth; furthermore, gibberellin content increases in maize root inoculated with *Azospirillum* (Fulchieri *et al.*, 1993). In addition to playing a role in plant RSA, these two hormones are involved in plant defense mechanisms. Thus, PGPR producing these hormones may modulate the hormonal balance involved in plant defense, including the jasmonate and salicylic acid pathways (for a review see Pieterse *et al.*, 2009).

Although the production of hormones by PGPR has been well described, the genetic determinants involved in their biosynthesis remain largely unknown and bacterial mutants affected in hormone biosynthesis are mostly lacking. Consequently, the involvement of hormones of bacterial origin in the modulation of plant hormonal balance has not been fully demonstrated.

## Modification of root cell wall and root tissue structural properties by PGPR

Many PGPR can lead to modifications of the chemical composition and therefore structural properties of root cell walls (**Figure 1**; El Zemrany *et al.*, 2007; Zhang *et al.*, 2007). For example, the biocontrol agent *Bacillus pumilus* INR-7 is able to enhance lignin deposition in pearl millet epidermal tissues, and this plant defense response appears much more rapidly in PGPR-primed plants infected by the pathogen *Sclerospora graminicola* compared to nonprimed plants (Niranjan Raj *et al.*, 2012). The sole inoculation of INR-7 led to callose apposition. Although the precise location of these deposited polymers was not investigated, it is possible that their enhanced accumulation may participate to pathogen inhibition and disease suppression. A similar response was also triggered by *Bacillus pumilus* SE34 and *Bacillus subtilis* UMAF6639 when inoculated to respectively pea and melon roots. In both cases, it led to enhanced fungal pathogen tolerance (García-Gutiérrez *et al.*, 2013). Inoculation of *Pseudomonas fluorescens* 63-28R to pea roots induced accumulation of lignin in root cells and

inhibited colonization by the oomycete *Pythium ultimum* (Benhamou *et al.*, 1996). The same result was observed with a *Pseudomonas putida* strain inoculated on bean roots (Anderson and Guerra, 1985). These cell wall modifications have been reported in the case of PGPR that protect plants against phytopathogens by activating ISR plant defense responses (Iavicoli *et al.*, 2003; Desoignies *et al.*, 2012; Weller *et al.*, 2012; García-Gutiérrez *et al.*, 2013). One of the consequences of ISR is thus the reinforcement of the cell wall through enhanced lignin synthesis and callose apposition (Kovats *et al.*, 1991; Strömberg and Brishammar, 1993), which restricts the progression of phytopathogens through plant tissues (García-Gutiérrez *et al.*, 2013).

Modifications of the chemical composition of root cell walls are also triggered by PGPR that directly promote plant growth (**Figure 1**). Through the analysis of the infrared spectral characteristics of crude cell wall preparations of maize roots, El Zemrany *et al.* (2007) concluded that roots inoculated with *Azospirillum lipoferum* CRT1 had lower lignin content than uninoculated ones. This result contrasts with those aforementioned for biocontrol agents. Nevertheless, lower lignin content may facilitate cell elongation, and therefore overall root elongation. Similarly, *Azospirillum irakense* produces pectate lyases that are capable of degrading the pectate content of root cell wall and might allow its progression between root cortex cells and its functioning as an endophyte (Bekri *et al.*, 1999). Up to now, the impact on plant lignin content of PGPR that are both inducing ISR and promoting root growth has not been clarified.

Modifications of root cell wall ultrastructure are thought to result mainly from PGPR-triggered changes in plant gene expression. Indeed, *Bacillus subtilis* GB03 promotes *Arabidopsis* growth by producing VOCs that were shown to modulate the expression of 38 genes with known functions associated with cell wall structure (Ryu *et al.*, 2003; Zhang *et al.*, 2007). Among them, 30 were implicated in cell wall expansion or loosening. The endophytic PGPR *Azospirillum irakense* was also shown to stimulate the expression of polygalacturonase genes in inoculated rice roots (Sekar *et al.*, 2000).

Chemical mediators involved in the effects of PGPR on root cell walls have received little attention. A single report has indicated that the exogenous addition of auxins enhanced the induced polygalacturonase activities observed in *Azospirillum irakense* inoculated rice roots (Sekar *et al.*, 2000).

## SYSTEMIC EFFECTS OF PGPR ON WHOLE PLANT PHYSIOLOGY AND FUNCTIONING

In addition to their effects on root tissues, PGPR can modify the physiology and functioning of plant tissues located at a substantial distance from the colonized sites, such as shoots. Two types of mechanisms are involved. On the one hand, some PGPR can enhance nutrient availability/uptake for plant roots. Stimulation of plant nutrition will lead to modifications in primary metabolism and consequently will contribute to enhance growth. On the other hand, certain PGPR trigger specific systemic responses, mostly by unknown signaling mechanisms. High-throughput analyses of plant transcriptomic and metabolomic responses have evidenced the effects of PGPR on plant gene expression and metabolite accumulation,

respectively. These results highlight the extensive effect of PGPR on whole plant physiology and functioning (**Figure 1**), and provide clues to understand the systemic effect of PGPR.

## Impact of PGPR on plant nutrition

The impact of PGPR on plant nutrition may result from effects on plant nutrient uptake and/or on plant growth rate (Mantelin and Touraine, 2004). It is indeed commonly hypothesized that nutrient uptake is increased as a consequence of increased root surface area triggered by PGPR. However, root ion transporters are under the control of regulatory processes that adjust their activity to the plant nutritional demand (Imsande and Touraine, 1994; Lappartient and Touraine, 1996; Lappartient *et al.*, 1999; Nazoa *et al.*, 2003), so that regulations of root development and ion transporter activities are antagonistically coordinated to maintain steady nutrient acquisition rate (Touraine, 2004). Hence, PGPR must interfere with pathways coordinating plant development and plant nutrition to elicit both increased nutrient acquisition rate and plant growth promotion (**Figure 1**).

Plant growth-promoting rhizobacteria can directly increase nutrient supply in the rhizosphere and/or stimulate ion transport systems in root. With regards to increased nutrient supply, two main types of bacterial activities can be considered. Firstly, phosphate solubilization is one key effect of PGPR on plant nutrition. Soils generally contain a large amount of phosphorus, which accumulates in the wake of regular fertilizer applications, but only a small proportion of the latter is available for plants. Plants are able to absorb on their own mono and dibasic phosphate; organic or insoluble forms of phosphate need to be mineralized or solubilized by microorganisms, respectively (Richardson *et al.*, 2009; Ramaekers *et al.*, 2010). Many PGPR – such as *Pseudomonas*, *Bacillus*, *Rhizobium* – are able to dissolve insoluble forms of phosphate (for a review see Richardson *et al.*, 2009). Two main processes exist: acidification of the external medium through the release of low molecular weight organic acids (such as gluconic acid) that chelate the cations bound to phosphate (Miller *et al.*, 2009), and production of phosphatases/phytases that hydrolyse organic forms of phosphate compounds. Secondly, many associated bacteria can fix $N_2$ so that they could provide nitrogen to the plant. Evidence in favor of the participation of PGPR to the plant N budget has been reported for several plants, especially sugar cane (Boddey *et al.*, 2003). However, the impact of $N_2$-fixation by PGPR is still debated and is rarely credited for the stimulation of plant growth (for review see Dobbelaere *et al.*, 2003). In addition, non-fixing rhizobacteria can promote plant growth, showing that N provision is not obligatory for plant growth promotion. For instance, *Phyllobacterium brassicacearum* STM196 is unlikely to fix $N_2$ while it promotes the growth of canola and *Arabidopsis* (Bertrand *et al.*, 2000, 2001; Mantelin *et al.*, 2006).

With regards to the impact of PGPR on nutrient uptake systems, only very few studies have been published so far. Inoculation of canola with *Achromobacter* sp. strain U80417 resulted in an increase of both $NO_3^-$ and $K^+$ net influx rates per root surface area unit (Bertrand *et al.*, 2000). In this study, the net $H^+$ efflux was also enhanced, so that increased $NO_3^-$ and $K^+$ uptake rates may be part of a general mechanism leading to increased ion uptake rate, similar to energization of nutrient transport by enhanced proton pump activity (Sondergaard *et al.*, 2004). In favor of this hypothesis, acidification of the rhizosphere has also been reported with

*Arabidopsis* exposed to the VOC-emitting *Bacillus subtilis* strain GB03 (Zhang *et al.*, 2009).

In *Arabidopsis*, $NO_3^-$ uptake measurement in response to PGPR, over time, can lead to contradictory results: $NO_3^-$ influx was increased in seedlings, upon 24 h-inoculation with *Phyllobacterium brassicacearum* STM196, while it was reduced 7 days later (Mantelin *et al.*, 2006). However, it is hard to draw a firm conclusion as the net $NO_3^-$ uptake rate remained unknown since ion efflux was not measured in these experiments. Except for the *NRT2.5* and *NRT2.6* genes, the accumulation of transcripts of nitrate and ammonium transporters was very slightly or not significantly changed upon *Phyllobacterium brassicacearum* STM196 inoculation (Mantelin *et al.*, 2006). Interestingly, these two genes have recently been shown to be required in *Arabidopsis* growth promotion by this PGPR (Kechid *et al.*, 2013). Since these two genes code for two plasma membrane-localized $NO_3^-$ transporters (Kotur *et al.*, 2012), this discovery raises the question of the interactions between N nutrition and plant development in PGPR-inoculated plants. The *NRT2.5* and *NRT2.6* genes are predominantly expressed in shoots (Mantelin *et al.*, 2006). Their role in *Phyllobacterium brassicacearum* STM196 plant growth promotion and/or root architecture modification are not linked to changes in $NO_3^-$ uptake rate or $NO_3^-$ distribution between roots and shoots (Kechid *et al.*, 2013), suggesting an involvement in N-signaling rather than a direct role in N-metabolism.

Evidence in favor of a regulation of ion transporters at a transcriptional level by PGPR has been obtained in studies with Bacillus subtilis GB03. This strain induces concomitant down and up-regulation of HKT1 expression in roots and shoots of Arabidopsis seedlings, respectively (Zhang *et al.*, 2008). In the shoots, HKT1 functions in phloem tissues to retrieve $Na^+$ from the xylem (Berthomieu *et al.*, 2003) and in the roots it is involved in $Na^+$ uptake (Rus *et al.*, 2001). The differential regulation of HKT1 expression in roots and shoots resulted in reduced accumulation of $Na^+$ and increased accumulation of $K^+$ in both organs of GB03-inoculated seedlings under salt-stress conditions (Zhang *et al.*, 2008). Consistent with the effect of GB03 on HKT1, GB03 failed to rescue salt-stressed hkt1 mutant seedlings from elevated $Na^+$ accumulation.

Volatile organic compounds emitted by GB03 also activate the plant's iron acquisition machinery leading to increased iron assimilation (Zhang *et al.*, 2009). Firstly, this PGPR leads to acidification of the rhizosphere, both directly due to chemical effects of some unidentified VOCs and indirectly through increased root proton efflux. Secondly, GB03 up-regulates the expression levels of FRO2 and IRT1 genes, coding respectively for a $Fe^{3+}$ chelate reductase and a $Fe^{2+}$ transporter. As a result, GB03-exposed Arabidopsis has enhanced ferric chelate reductase activity and increased iron content. Finally, it has been shown that this PGPR induces the expression of the FIT1 transcription factor that regulates positively FRO2 and IRT1 expressions (Zhang *et al.*, 2009). The fact that GB03 fails to increase root ferric reductase activity and plant iron content in Arabidopsis fit1 mutants shows that PGPR can modify indirectly ion uptake by interfering with plant regulatory processes that control ion transporter expressions and/or activities (Zhang *et al.*, 2009).

## Impact of PGPR on plant transcriptome

Targeted or genome-wide analyses of plant gene expression following root inoculation by PGPR were reported with various bacterial models: phytostimulating PGPR, endophytes and PGPR exerting a biocontrol activity. Inoculation of the phytostimulator Pseudomonas putida MTCC5279 triggered overexpression of 520 genes and repression of 364 genes (threefold changes) in leaves of Arabidopsis; upregulated genes were involved in maintenance of genome integrity, growth hormone and amino acid syntheses, ABA signaling and ethylene suppression, $Ca^{2+}$ dependent signaling and induction of ISR (Srivastava *et al.*, 2012). On rice, a recent study performed with *Azospirillum* points towards association specificity (Vargas *et al.*, 2012). The targeted expression of ethylene receptors was followed after inoculation of *Azospirillum brasilense* Sp245 on two rice cultivars of contrasted ability to gain nitrogen from biological nitrogen fixation. Seedlings of cultivar IR42, which enabled higher nitrogen fixation, also displayed higher expression of ethylene receptors compared to cultivar IAC 4440 (Vargas *et al.*, 2012). The transcript accumulation of all ethylene receptors might be necessary for the establishment of a beneficial association between the plant and the bacteria.

As for endophytes, differential colonization of rice roots was observed with an *Azoarcus* PGPR. In a less compatible interaction, a slight defense response occurred and was accompanied by the induction of pathogenesis-related proteins and proteins sharing domains with receptor-like kinases induced by pathogens; those proteins were also induced by a jasmonate treatment (Miché *et al.*, 2006). Inoculation of rice roots with the endophytic PGPR *Herbaspirillum seropedicae* triggered the expression of genes responsive to auxin and ethylene and the repression of the defense-related proteins PBZ1 and thionins (Brusamarello-Santos *et al.*, 2012). These studies suggest that endophytes modulate plant defense responses during colonization. Plants treated with biocontrol PGPR, usually belonging to the *Pseudomonas* genus, are more resistant to subsequent infections by bacterial or fungal pathogens. In *Arabidopsis*, this rhizobacteria-mediated ISR requires sensitivity to jasmonate and ethylene, and the regulators MYC2 (Pieterse *et al.*, 1996, 2000; Pozo *et al.*, 2008), NPR1 (Pieterse *et al.*, 1998), and MYB72 (Van der Ent *et al.*, 2008) played a central role in this signaling. One of the earliest transcriptomic study performed with *Pseudomonas fluorescens* WCS417r indicated that bacteria elicited a substantial change in the expression of 97 genes in roots whereas none of the approximately 8,000 genes tested showed a consistent change in expression in the leaves (Verhagen *et al.*, 2004). Subsequent studies on *Arabidopsis* reported an increase of defense-related transcripts, including PR-related proteins, in shoots of bacterized plants compared to untreated shoots (Cartieaux *et al.*, 2003; Wang *et al.*, 2005; van de Mortel *et al.*, 2012). Interestingly, the ISR induced by *Pseudomonas fluorescens* SS101 was recently reported to be dependent on salicylic acid signaling and not on jasmonic acid and ethylene signaling (van de Mortel *et al.*, 2012); moreover, a prominent role of camalexin and glucosinolates in the ISR was proposed. In wheat, bacterization with *Pseudomonas fluorescens* Q8r1-96 also triggered the accumulation of defense-related transcripts (Okubara *et al.*, 2010; Maketon *et al.*, 2012) and neither DAPG nor the type three secretion system were key single factors in the expression of these genes (Maketon *et al.*, 2012). The establishment of beneficial associations requires mutual recognition and substantial coordination of plant and microbial responses and consequently beneficial microbes modulate plant immunity.

## Impact of PGPR on plant metabolome

Several studies have addressed the metabolomic changes triggered by PGPR inoculation, by analyzing metabolite contents of root exudates, root tissues and shoots under normal or stressful conditions (**Figure 1**). Some studies have shown that PGPR can elicit changes in the activities of root enzymes involved in the production of metabolites, especially flavonoids, leading to changes in the pattern of root exudation (Lavania *et al.*, 2006; Shaw *et al.*, 2006). Some *Azospirillum* PGPR stimulated by up to one-third the level of carbon compounds exuded from roots (Heulin *et al.*, 1987). Moreover, compounds of microbial origin, such as phenazines and DAPG, could enhance total net efflux of amino acids in plant species (Phillips *et al.*, 2004). On soybean roots, the PGPR *Chryseobacterium balustinum* Aur9 influences flavonoids exudation (Dardanelli *et al.*, 2010). PGPR strains from *Chryseobacterium* (Dardanelli *et al.*, 2010) or *Azospirillum* (Burdman *et al.*, 1996) may influence flavonoid exudation by Fabacea roots. This property can be important for the design of mixed inoculants that will include a PGPR strain promoting flavonoid exudation together with rhizobia that will respond to plant flavonoids (Burdman *et al.*, 1996).

In addition to effects on root exudates, PGPR can trigger modifications of metabolite composition of the whole plant. For instance, rice plants inoculated with *Herbaspirillum seropedicae* showed higher shoot contents in malate and in key amino acids than those of control plants (Curzi *et al.*, 2008). Many more studies focused on modifications of secondary metabolites. Elicitation of isoflavone accumulation was observed on soybean inoculated with various PGPR, either by increasing the total isoflavone content in seedlings or by causing an asymmetric distribution of isoflavones throughout the plant (Ramos-Solano *et al.*, 2010). Increase in the content of several alkaloid and terpenoid compounds of pharmaceutical relevance was demonstrated in medicinal plants following PGPR inoculation (Manero *et al.*, 2003; Jaleel *et al.*, 2007; Bharti *et al.*, 2013). Recent studies investigated the early impact of several *Azospirillum* strains on root and shoot secondary metabolite profiles of maize and rice; analysis of secondary metabolites of two maize cultivars, inoculated by three different *Azospirillum* strains under greenhouse conditions, revealed major qualitative and quantitative modifications of the contents of secondary metabolites, especially benzoxazinoids (Walker *et al.*, 2011). In the same way, a metabolic profiling approach of two rice cultivars inoculated with two different *Azospirillum* strains under gnotobiotic conditions, showed that profiles of secondary metabolites were modified with phenolic compounds such as flavonoids and hydroxycinnamic derivatives being the main metabolites affected (Chamam *et al.*, 2013). Both studies evidenced a specific response, as plant metabolic changes differed according to the *Azospirillum* strain-cultivar combination. Moreover, PGPR applied to the roots can affect the composition of secondary metabolites in shoots, pointing towards systemic effects (Chamam *et al.*, 2013).

Accumulation of secondary compounds was also modified in several plants inoculated with consortia containing arbuscular mycorrhizal fungus and PGPR. Blumenin accumulation triggered by *Rhizophagus irregularis* (formerly *Glomus intraradices*) in barley and wheat roots was increased when a rhizosphere bacterium was applied with the fungus (Fester *et al.*, 1999). Leaf secondary metabolites (total phenols and ortho dihydroxy phenols), as well as leaf mineral content (phosphorus, potassium, zinc, copper, and iron) were maximal when *Begonia*

*malabarica* or *Solanum viarum* were inoculated with consortia containing two fungi and a *Bacillus coagulans* strain (Selvaraj *et al.*, 2008; Hemashenpagam and Selvaraj, 2011). Field-inoculation of maize with selected strains of *Pseudomonas*, *Azospirillum* or *Rhizophagus*/*Glomus*, or with these strains combined two by two or all three together, led to qualitative and quantitative modifications of root secondary metabolites, particularly benzoxazinoids and diethylphthalate (Walker *et al.*, 2012). These modifications depended on fertilization level and on the type of microorganisms inoculated. The three selected strains gave distinct results when used alone, but unexpectedly all microbial consortia gave somewhat similar metabolic responses.

Plant growth-promoting rhizobacteria can help plants to withstand saline stress, a feature that may be linked to accumulation of specific metabolites. A higher level of proline content was reported in inoculated *Bacopa monnieri* (Bharti *et al.*, 2013), as well as higher accumulation of glycine betaine-like quaternary compounds in rice inoculated with *Pseudomonas pseudoalcaligenes* (Jha *et al.*, 2011). Similarly, Arabidopsis inoculation with the VOC-emitting strain Bacillus subtilis GB03 induced strong plant accumulation of glycine betaine and its precursor choline, and GB03-induced drought tolerance was lost in the *xipotl* mutant of Arabidopsis with reduced choline production (Zhang *et al.*, 2010). Alleviation of cold stress was demonstrated for *Burkholderia phytofirmans* PsJN on grapevine; this endophytic strain promotes plant post-chilling recovery by improving acclimation to cold (Ait Barka *et al.*, 2006). This is accompanied by accumulation of stress-related metabolites such as proline, malondialdehyde and aldehydes (known as lipid peroxidation markers), hydrogen peroxide, and by higher expression of defense and cold-related genes (Theocharis *et al.*, 2012). Bacterization resulted in a 1.2-fold increase in starch content and in a two-fold increase in total soluble sugars, with sugars known to be involved in low-temperature tolerance (glucose, sucrose, and raffinose) displaying higher concentrations in treated plantlets (Fernandez *et al.*, 2012). Independently of temperature, inoculation also enhanced phenolic content (Ait Barka *et al.*, 2006).

## EXPRESSION OF PLANT-BENEFICIAL FUNCTIONS OF PGPR IN THE RHIZOSPHERE

One PGPR strain can harbor several plant-beneficial properties, which may be co-regulated or not. Within the rhizosphere, the expression of PGPR's plant-beneficial properties is affected by both abiotic factors (like pH, oxygen, clay mineralogy, heavy metals, etc.) and biotic factors (i.e., compounds produced by plants or the rhizo-microbiome) that can lead to distinct expression patterns in space and time, possibly with different effects on host plant (Piccoli and Bottini, 1994; Pothier *et al.*, 2008; Prigent-Combaret *et al.*, 2008; Dutta and Podile, 2010; Almario *et al.*, 2013b; Drogue *et al.*, 2013). In this section, a focus is put on the regulation of the expression of PGPR plant-beneficial properties by biotic factors occurring in the rhizosphere.

## Regulation of PGPR functions by root exudates

Through the release of root exudates, plants can impact bacterial gene expression, especially genes encoding plant-beneficial traits. Composition of root exudates is dependent upon intra and inter-specific genetic variability (Bertin *et al.*, 2003; Czarnota *et al.*, 2003; Phillips *et al.*, 2004), plant developmental stage (Lynch and Whipps, 1990; Bacilio-Jiménez *et al.*, 2003) and soil abiotic factors (Lipton *et al.*, 1987). One of the major studies aiming at analyzing the impact of root exudates variability on bacterial gene expression was carried out on *phlA*, involved in DAPG biosynthesis, in *Pseudomonas protegens* (formely *Pseudomonas fluorescens*) CHA0 (Notz *et al.*, 2001). The expression of *phlA* was increased four-fold in the rhizosphere of monocots (maize and wheat) compared to the rhizosphere of dicots (bean and cucumber). The analysis of six maize cultivars also revealed that *phl* expression and hence biocontrol activity could be affected by plant genotype (Notz *et al.*, 2001). Specific components of root exudates, notably sugars, were shown to affect the production of antimicrobial compounds, such as DAPG, pyoluteorin and pyrrolnitrin by fluorescent pseudomonads, with some strain-dependent effects (Duffy and Défago, 1999). Among 63 plant compounds related to defense or development, or involved in plant-microbe interactions (flavonoids, phenolic acids, phytohormones, etc.), many could modulate the expression of *phlA* and *pltA* in *Pseudomonas protegens* CHA0 (de Werra *et al.*, 2011). No specific chemical structures were identified that generally induced or repressed *phlA* or *pltA* expression (de Werra *et al.*, 2011). Umbelliferone led to the strongest inhibition of *phlA*; salicylate, jasmonate, and methyl jasmonate, all slightly reduced *phlA* expression, whereas the plant hormone IAA induced *phlA* expression. None of these compounds had an effect on *pltA* expression (de Werra *et al.*, 2011) whereas a previous study reported repression of both DAPG and pyoluteorin biosynthesis genes by salicylate (Baehler *et al.*, 2005).

1-Aminocyclopropane-1-carboxylic acid deamination (encoded by *acdS*) is another bacterial function that may be differentially expressed according to plant genotypes. Indeed, *in vitro* experiments demonstrated that some compounds present in root exudates tightly control *acdS* expression. First, ACC, the precursor of ethylene that is metabolized by AcdS, can positively regulate *acdS* expression (Hontzeas *et al.*, 2004; Prigent-Combaret *et al.*, 2008). Second, leucine, by inhibiting oligomerization of the Lrp-type regulator AcdR, prevents transcription of *acdS* leading to a decrease of ACC deaminase activity in *Pseudomonas putida* UW4 (Li and Glick, 2001) and in *Azospirillum lipoferum* 4B (Prigent-Combaret *et al.*, 2008). Finally, carbon sources can also influence *acdS* transcription (Prigent-Combaret *et al.*, 2008).

As presented above, bacterial IAA biosynthesis mostly depends on tryptophan-related pathways (Spaepen *et al.*, 2007a). The main source of tryptophan for PGPR is root exudates. Measurement of tryptophan bioavailability from graminaceous roots (*Avena barbata*) indicated that this amino acid is abundant at the emergence of secondary roots (Jaeger *et al.*, 1999). In the absence of exogenous tryptophan supply, bacterial IAA biosynthesis is insignificant (Ona *et al.*, 2005; Malhotra and Srivastava, 2006). Next to being an IAA precursor, tryptophan also plays an important role in regulating positively the *ipdC/ppdC* gene (Ona *et al.*, 2005). Other root-exuded amino acids like tyrosine and phenylalanine can also induce *ipdC/ppdC* expression (Rothballer *et al.*, 2005). Besides amino acids, plant roots release compounds like vitamins (e.g., pyridoxine and nicotinic acid) and organic acids (e.g.,

phenylacetic acid and prephenic acid; Shukla *et al.*, 2011). All these compounds increase significantly IAA production in *Azospirillum brasilense* Sp245 (Zakharova *et al.*, 2000; Somers *et al.*, 2005).

Metabolites present in root exudates can thus specifically modulate the expression of key genes involved in plant-beneficial functions. Consequently, specific physiological responses of the plant are dependent on the PGPR strain/plant cultivar combination (Drogue *et al.*, 2012).

## Regulation of PGPR functions by microbial signals

Plant growth-promoting rhizobacteria exchange several types of cell-to-cell communication signals between each other and with other rhizosphere-inhabiting bacteria and fungi, i.e., quorum-sensing (QS) signals that allow bacteria to monitor their density and to coordinate gene expression only when a quorum of cells is achieved (Fuqua *et al.*, 1994) and other bacterial signals that regulate gene expression independently of the cell density.

Quorum-sensing relies on the synthesis and perception of small diffusible molecules, such as *N*-acyl-homoserine lactones (AHLs). In fluorescent pseudomonads, colonization properties and biosynthesis of antimicrobial metabolites, such as phenazines, is often subjected to an AHL-based QS regulation (Pierson *et al.*, 1994; Chin-A-Woeng *et al.*, 2001; De Maeyer *et al.*, 2011). Production of pyrrolnitrin in *Serratia plymuthica* HRO-C48, a strain isolated from the rhizosphere of oilseed rape and able to protect crops against *Verticillium* wilt, is also under QS regulation (Liu *et al.*, 2007). In *S. plymuthica* G3, an endophytic strain, QS positively regulates antifungal activity, production of exoenzymes, but negatively regulates IAA production (Liu *et al.*, 2011). Among the genus *Azospirillum*, only a few strains belonging to the *lipoferum* species and isolated from rice, display the ability to produce AHL signals (*Via*l *et al.*, 2006). In the rice endophyte *Azospirillum lipoferum* B518, AHL inactivation abolishes pectinase activity, increases siderophore synthesis and reduces IAA production (in stationary phase) but no effect is observed on cellulase activity and on the phytostimulatory effect (Boyer *et al.*, 2008). Moreover, a proteomic approach indicates that AHL-based QS regulation in *Azospirillum* is rather dedicated to control functions linked to rhizosphere competence and adaptation to plant roots (Boyer *et al.*, 2008).

Interestingly, several studies have shown that bacterial communication of a specific bacterial population could be jammed by other microbes; indeed, some soil bacteria can inactivate AHL (notably members of the genus *Bacillus*), whereas others can intercept AHL or can act as a physical barrier preventing their diffusion (Boyer and Wisniewski-Dyé, 2009). Consequently, other members of the bacterial rhizosphere community can compromise expression of biocontrol traits in PGPR. Conversely, cross-talk between species using the same AHL signal or a structurally-related AHL can occur in natural habitats and was evidenced in the rhizosphere of wheat and tomato (Pierson *et al.*, 1998; Steidle *et al.*, 2001). Finally, plant compounds designated as AHL-mimics can also interfere with bacterial QS and may influence the expression of plant-beneficial functions (Teplitski *et al.*, 2000; Vandeputte *et al.*, 2010). Some *Pseudomonas fluorescens* strains unable to synthetize AHLs but possessing the cognate receptor may even recognize a plant compound to trigger expression of genes involved in biocontrol properties (Subramoni *et al.*, 2011). Exometabolites produced by

microbial populations including pathogenic fungal strains can also affect PGPR plant-beneficial properties. For instance, fusaric acid produced by *Fusarium oxysporum* represses the production of DAPG in the biocontrol strain *Pseudomonas protegens* CHA0 (Notz *et al.*, 2002). Next to their antifungal effect, some *Pseudomonas*-produced compounds can influence gene expression of biocontrol traits in pseudomonads. Indeed, in *Pseudomonas protegens* strains CHA0 and Pf-5, DAPG and pyoluteorin productions are influenced by positive autoregulation; moreover, DAPG and pyoluteorin mutually inhibit one another's production (Brodhagen *et al.*, 2004; Baehler *et al.*, 2005). In order to determine if DAPG could act as a signal on other PGPR strains than those of the fluorescent *Pseudomonas* group, a differential fluorescence induction promoter-trapping approach based on flow cytometry was developed on *Azospirillum*. Using this approach DAPG was shown to enhance expression of a wide range of *Azospirillum brasilense* genes, including genes involved in phytostimulation. Four of them (i.e., *ppdC*, *flgE*, *nirK*, and *nifX-nifB*) were upregulated on roots in the presence of *Pseudomonas fluorescens* F113 compared with its DAPG-negative mutant (Combes-Meynet *et al.*, 2011). Accordingly, *Pseudomonas fluorescens* F113 but not its DAPG-negative mutant enhanced the phytostimulatory effect of *Azospirillum brasilense* Sp245 on wheat. Thus, DAPG can act as a signal by which some beneficial pseudomonads may stimulate plant-beneficial activities of *Azospirillum* PGPR (Combes-Meynet *et al.*, 2011). This finding is also relevant in the context of inoculation with microbial consortia, in which different types of PGPR may be combined. The number of studies investigating the efficacy of such combined inoculations is growing, with variations in the number of microorganisms and the nature of the combinations (PGPR strains only, PGPR and fungi, etc.; Cassán *et al.*, 2009; Couillerot *et al.*, 2012; Kumar *et al.*, 2012; Walker *et al.*, 2012). Field inoculation of sorghum with fluorescent *Pseudomonas* strains alone or in combination with arbuscular mycorrhizal fungi showed a better effect when in presence of the latter (Kumar *et al.*, 2012). Field inoculation of maize with a consortium consisting of two PGPR (*Azospirillum lipoferum* CRT1 and *Pseudomonas fluorescens* F113) and one mycorrhizal strain (*Rhizophagus irregularis*/*Glomus intraradices* JJ291) showed an increase of root surface, root volume and number of roots, although data were not statistically significant compared to the single *Rhizophagus* inoculation (Walker *et al.*, 2012). Modification of one member of this consortium (three different *Azospirillum* strains were tested) could lead to significant modification of maize growth (Couillerot *et al.*, 2012). Further studies are needed to describe the synergistic effects between beneficial microorganisms at a molecular scale and to analyse the expression of plant-beneficial functions when consortia are used.

## ECOLOGY OF PGPR POPULATIONS AND IMPACT ON ROOT SYSTEM FUNCTIONING

Many studies have deciphered the mechanisms of action of PGPR using one individual strain and one host plant. But in reality, as described above, PGPR strains are not acting individually in the rhizosphere but rather as part of bacterial communities, in which cell communication signals may coordinate the activities of all individual strains. Indeed, a vast array of PGPR populations displaying co-occurring plant-beneficial activities and that may share between each other antagonistic or synergistic effects are interacting with a same host plant (**Figure 3**). When analysing plant growth-promoting effects, it is thus important to integrate

the complexity of the interactions between PGPR populations within the rhizo-microbiome. To do so, functional ecology approaches are needed, in which the relations between the size, diversity and activities of PGPR assemblages in the rhizosphere are taken into account. This is of particular importance when assessing the effect of various environmental factors, including that of plant genotype.

## PGPR ecology in the rhizosphere: from individual strains to functional groups

Plant growth-promoting rhizobacteria strains occur in various taxonomic groups, and these different taxonomic groups may be present simultaneously in a given soil (Kyselková *et al.*, 2009; Almario *et al.*, 2013a). This suggests that taxonomically-contrasted PGPR strains may coexist in soil and colonize a same rhizosphere, along with all non-PGPR members of the bacterial community. This possibility has been documented repeatedly, especially when characterizing the taxonomic status of bacterial isolates selected based on their positive effect on plant growth or health, their ability to inhibit phytopathogens, or the occurrence of a particular gene or property of relevance for PGPR effect (Bertrand *et al.*, 2001; Barriuso *et al.*, 2005; Upadhyay *et al.*, 2009). In fact, this possibility seems to be the rule rather than the exception. PGPR populations contributing to a same type of function (i.e., ISR, nitrogen fixation, nutrient solubilization, plant development enhancement, etc.) belong to a same functional group. Functional group approaches can be implemented when specific genes are documented. For instance, nitrogen fixers can be assessed using the *nifH* gene, which encodes the dinitrogenase subunit of the nitrogenase. Its sequence is well conserved within the functional group and it is commonly used as marker to monitor the size and diversity of the diazotrophic community (Poly *et al.*, 2001; Dixon and Kahn, 2004). Some of these PGPR functional groups are taxonomically narrow, such as the *Pseudomonas* DAPG producers (Frapolli *et al.*, 2012). In contrast, others are much more diversified, and certain bacterial functional groups may also comprise both PGPR and non-PGPR strains. For instance, nitrogen fixers include PGPR as well as mutualistic symbionts and even a few pathogens (Herridge *et al.*, 2008).

When considering PGPR-plant relationship in fields, the co-occurrence of genetically contrasted PGPR strains from a same functional group in the rhizosphere has two consequences. First, the activity of a given PGPR functional group corresponds to the resulting contributions of all active individual cells from each type of bacterium within the functional group. If synergistic effects occur between the PGPR populations, the expected performance level for the PGPR function might be higher than if only one type of strain was involved. On this basis, knowing the size of the functional group will help understand the potential importance of the corresponding function. Indeed, for functions leading to enhanced nutrient availability to the plant, such as nitrogen fixation or phosphorus solubilization, the higher the better. For others where optimality matters, such as the production of auxinic signals (Dobbelaere *et al.*, 1999; Spaepen *et al.*, 2007b), the performance level of the functional group will need fine-tuning to avoid production levels too small or too great. How this is ecologically regulated at the scale of the corresponding functional group is unknown, but it raises the

possibility of co-evolutionary patterns. To bridge the gap between the potential of a plant-beneficial PGPR function and its actual implementation by PGPR strains, the regulatory effects need also to be taken into consideration. Some of these regulatory effects will be common to all members of the functional group (Prigent-Combaret *et al.*, 2008). However, other regulatory effects may be relevant for a subset only of the functional group. For instance, zinc sulfate stimulates DAPG production in certain but not all genetic groups of *Pseudomonas* PGPR strains (Duffy and Défago, 1999).

Second, the relationships amongst the different PGPR strains co-occurring in a same rhizosphere are important. Interactions will take place within a functional group, as illustrated above with QS regulation of phenazine production in fluorescent *Pseudomonas* PGPR (Pierson *et al.*, 1994). Interactions may also take place between different PGPR functional groups (**Figure 3**), integrating competitive and inhibitory effects (Couillerot *et al.*, 2011), signal jamming (Boyer and Wisniewski-Dyé, 2009) and positive signaling (Combes-Meynet *et al.*, 2011), as well as more indirect processes such as root exudation modifications (Phillips *et al.*, 2004; Dardanelli *et al.*, 2010). These interactions have the potential to modulate spatial colonization patterns of PGPR on roots (Couillerot *et al.*, 2011) and to affect PGPR performance (Pierson *et al.*, 1998). This also suggests that members of different PGPR functional groups can function together, as consortia, with the possibility of synergistic effects or, contrariwise, antagonistic effects. These positive effects may be sought by implementing inoculation procedures in which different types of plant-beneficial microorganisms are used in combination, as highlighted above. Even in this context, interactions between the different microbial strains that are inoculated and indigenous microorganisms (including PGPR) probably matter.

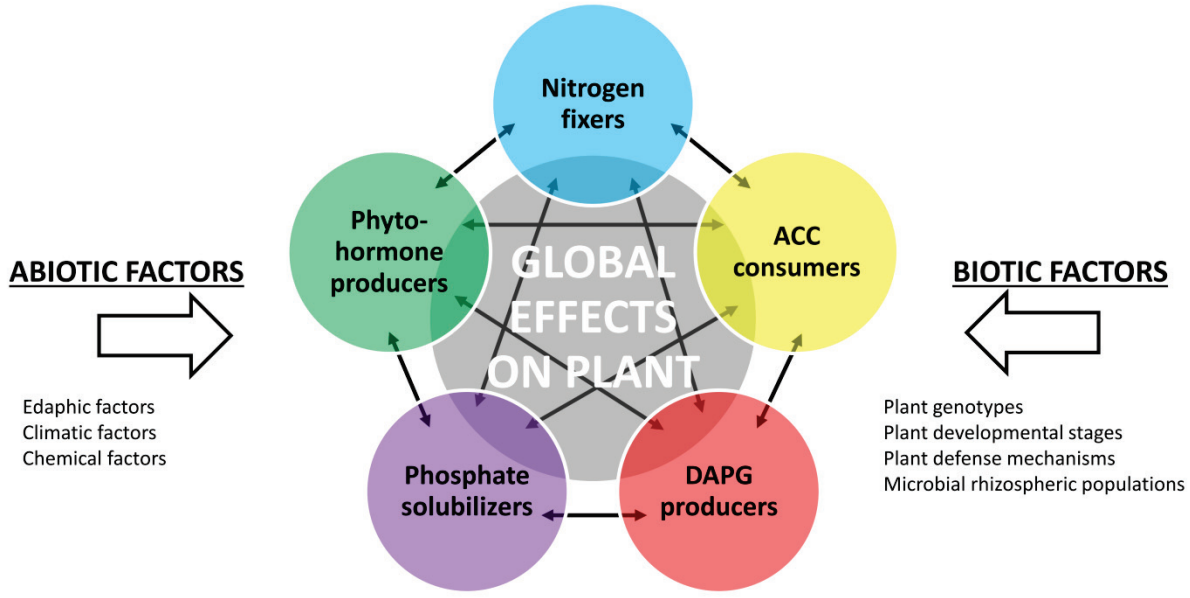


**Figure 3: Implementation of plant-growth promoting traits in PGPR functional groups**. Selected PGPR functional groups are represented by different colored circles. The resulting effect of all PGPR functional groups on the plant is symbolized by the gray circle. Abiotic and biotic factors may influence the activity of each functional group. Solid arrows represent potential interactions (inhibition, signaling, etc.) between members of the functional groups, which may impact on the size, diversity and activity of these groups.

## Impact of plant genotypes on PGPR functional groups

Plants at species, sub-species and variety levels exhibit substantial genetic and phenotypic diversity (Salamini *et al.*, 2002; Vaughan *et al.*, 2008). In the rhizosphere, different plant genotypes will have a different impact on the number, diversity and activity of microorganisms (Bais *et al.*, 2006; Micallef *et al.*, 2009). This has been shown when comparing different plant species (Grayston *et al.*, 1998; Costa *et al.*, 2006; Berg and Smalla, 2009) or varieties within species (Germida and Siciliano, 2001; van Overbeek and van Elsas, 2008; I˙nceog˘lu *et al.*, 2010; Bouffaud *et al.*, 2012). It entails differences noticeably in root system structure, root exudation profile, and nutrient acquisition (Czarnota *et al.*, 2003; Comas and Eissenstat, 2009). These effects have also been evidenced when considering microbial functional groups of PGPR or where PGPR predominate.

Nitrogen-fixing bacteria are particularly important for plant nitrogen nutrition (Hsu and Buckley, 2009; Turk *et al.*, 2011). The analysis of functional groups indicated that the size and/or composition of nitrogen-fixing bacteria is influenced by host plant features (**Figure 3**), both at plant species (Perin *et al.*, 2006) and variety levels (Coelho *et al.*, 2009; Wu *et al.*, 2009). Analysis of *nifH* gene transcripts extracted from the rhizosphere showed that only a fraction of the community expresses *nifH*, and that the corresponding bacterial species differed according to the plant variety, pointing to an influence of plant genotype on the functioning of nitrogen-fixing bacteria (Knauth *et al.*, 2005; Mårtensson *et al.*, 2009; Orr *et al.*, 2011). Similar findings were made with the functional group of phosphate solubilizers (Richardson and Simpson, 2011). Their selection by roots varies according to host plant species (Kaeppler *et al.*, 2000; Chen *et al.*, 2002; Ramaekers *et al.*, 2010).

Other functional groups, such as those involved in plant protection from parasites, act mainly by competition or antagonism, even though direct ISR effects might also take place (Weller *et al.*, 2012). For these microorganisms as well, plant genotype can have a major effect on microbial selection processes, as shown with fluorescent pseudomonads producing DAPG (Picard *et al.*, 2004; Bergsma-Vlami *et al.*, 2005; Picard and Bosco, 2006; Frapolli *et al.*, 2010) or hydrogen cyanide (Jamali *et al.*, 2009; Rochat *et al.*, 2010). Plant-genotype differences in rhizosphere ecology may also matter in terms of plant protection efficiency (Smith and Goodman, 1999; Mazzola and Gu, 2002; Mazzola *et al.*, 2004; Ryan *et al.* 2004).

# CONCLUSION

Plants have evolved different types of biotic interactions with soil microbial populations, ranging from commensalism to mutualism. Within this continuum of interactions, the plant-PGPR cooperation plays a major role by enhancing growth and health of widely diverse plants. Recent progress has helped to understand key features regarding the modes of action and ecology of plant-PGPR interactions, but major knowledge gaps remain. In terms of molecular signaling and functioning, whether PGPR fine-tune plant hormonal pathways similar to those induced bypathogens and symbionts and/or trigger yet-unknown specific pathways requires clarification.

Plant growth-promoting rhizobacteria are able to modulate RSA and *in fine* the vegetative growth and physiology of the whole plant. RSA effects have long been associated

with the production of IAA by PGPR. Surprisingly, bacterial modulation of plant auxin distribution and IAA signal transduction pathways, independently of IAA production by PGPR, has also been revealed. It is obviously a step forward in our understanding of plant-PGPR cooperation but it does not fully clarify the bacterial functions and plant hormonal networks involved. Plant hormones regulate genes for the biosynthesis of other hormones or components of hormonal pathways. Consequently, it is possible that PGPR can affect these cross-talks too. It would explain why PGPR can have such pleiotropic effects on plants. One of the major current scientific challenges lying ahead is to understand how these different signaling pathways are integrated to coordinate plant growth and development, and how PGPR influence the plant hormonal network.

Distinct PGPR populations present in a same soil can express plant-beneficial properties in concert. As aforementioned, the relationships between plants and their rhizo-microbiome are complex and vary both according to plant genotypes and soil inhabiting populations (and thus local soil properties, more generally speaking). Next-generation sequencing technologies have started to reveal their taxonomic and functional diversity. They have begun to bring new knowledge on the ecology of PGPR functional groups. In the near future, it is expected that metatranscriptomics and metaproteomics will develop drastically, and will allow further progress on the understanding of the activity and ecological behavior of natural PGPR populations within the rhizosphere. However, given the heterogeneity in space and time of the rhizosphere habitat, samplings at different times and locations within the plant rhizosphere and within fields will be essential to better understand the ecology and performance of PGPR at plant and field plot scales. Nevertheless, despite being very reductionist, mechanistic functional studies using one PGPR and one plant are still useful to investigate the ways PGPR exert beneficial effects on plants. We think that the most important advances on plant-PGPR cooperation will be brought in the future by combining both ecology and functional biology approaches.

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# PARTIE B

## *LES PROPRIETES PHYTOBENEFIQUES CO-OCCURRENTES*

# LA MULTIFONCTIONNALITE CHEZ LES RHIZOBACTERIES BENEFIQUES

Les fonctions ou propriétés phytobénéfiques énoncées dans la partie précédente ne sont pas distribuées de manière aléatoire chez les rhizobactéries et confèrent aux bactéries les possédant un avantage sélectif potentiel pour leur sélection dans le rhizomicrobiote.

## Etat des lieux

La sélection de nouvelles souches exerçant une action bénéfique sur les plantes (qualifiées de PGPR) cible généralement des bactéries possédant un large éventail de propriétés phytobénéfiques différentes. Le criblage de ces propriétés phytobénéfiques peut être réalisé par des approches (i) génétiques en ciblant un (des) gène(s) clé(s) impliqué(s) dans la(les) propriété(s) phytobénéfique(s) considérée(s), ou (ii) biochimiques *via* des mesures d'activités. En effet, l'étude des PGPR a montré que la plupart des souches possèdent plusieurs propriétés phytobénéfiques co-occurrentes (Picard et Bosco 2005 ; Ahmad *et al.* 2008 ; Kang *et al.* 2010). Avec l'avènement du séquençage, l'analyse des génomes de PGPR a confirmé la présence de plusieurs gènes codant des propriétés phytobénéfiques simultanément au sein d'un même génome (Ma *et al.* 2011, Duan *et al.* 2013, Redondo-Nieto *et al.* 2013, Jousset *et al.* 2014) mais a également permis la recherche de nouvelles fonctions phytobénéfiques par des méthodes de prédiction aussi appelées « Genome-mining » permettant par exemple la détection de potentiels métabolites secondaires anti-microbiens *via* l'utilisation de plateformes en ligne comme AntiSMASH ou encore BAGEL3 (Webber *et al.* 2015 ; van Heel *et al.* 2013).

## Distribution des propriétés phytobénéfiques

L'association préférentielle de certaines propriétés phytobénéfiques entre-elles est souvent en lien avec les relations phylogénétiques existant entre les bactéries qui les possèdent, du fait que deux bactéries taxonomiquement très proches vont avoir un contenu de gènes codant de propriétés phytobénéfiques très similaire, c'est ce que l'on appelle le signal phylogénétique (Bruto *et al.* 2014) (**Figure 1**). L'écologie des souches bactériennes influence également le contenu génique des bactéries (Lassalle *et al.* 2012, 2015), dont certainement des gènes impliqués dans des propriétés phytobénéfiques lorsque la bactérie est en interaction avec la plante.

L'acquisition de nouvelles propriétés phytobénéfiques peut se faire par des échanges horizontaux de gènes entre microorganismes (Wisniewski *et al.* 2012), et conférer un avantage adaptatif/compétitif aux souches réceptrices. Ces transferts seraient responsables de l'acquisition des gènes *nif* chez la PGPR *Pseudomonas stutzeri* A1501. Ces gènes sont situés au sein d'un îlot génomique d'environ 50 kb possédant un pourcentage GC significativement plus élevé que le reste du génome (66,8% *vs*. 63,8%) (Yan *et al.* 2008). Cet îlot génomique se serait inséré entre les gènes qui codent la glutathion péroxydase et les gènes impliqués dans la biosynthèse de cobalamine ; gènes qui présentent une synténie conservée chez la majorité des espèces de *Pseudomonas*. Parmi l'ensemble des gènes présents dans l'îlot génomique, les gènes *nif* sont orthologues à ceux retrouvés chez *Azotobacter vinelandii*, ce qui suggère que ces gènes auraient été transférés horizontalement (Yan *et al.* 2008). D'autres études montrent que les gènes peuvent être acquis par transferts de plasmides, comme c'est le cas des gènes *nif* de *Burkholderia phymatum* (Zehr *et al.* 2003).

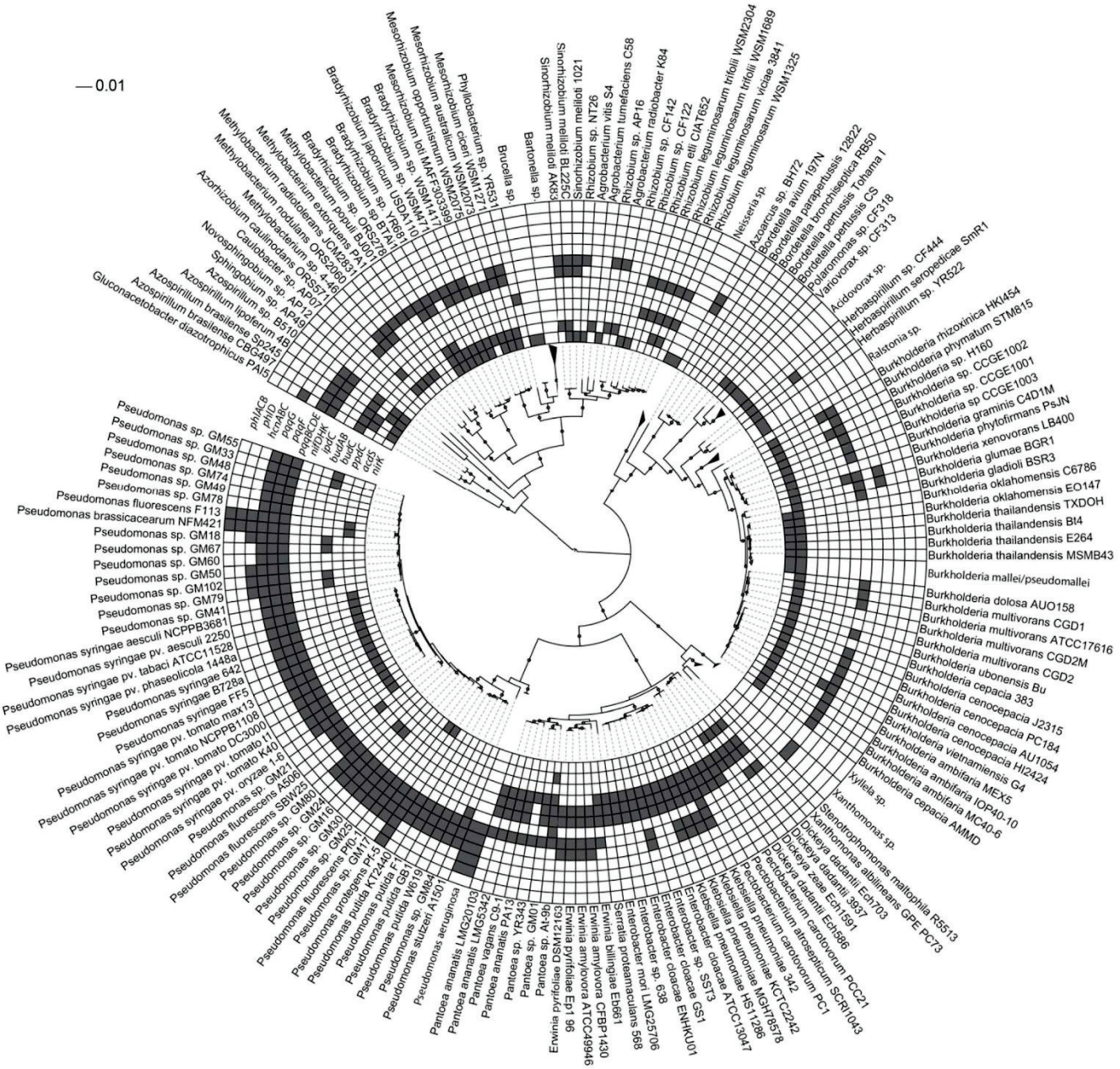


**Figure 1 : Distribution phylogénétique de propriétés phytobénéfiques parmi les Protéobactéries (tiré de Bruto et al. 2014).** Cercle interne : la présence du gène est indiquée par un carré gris, l'absence du gène par un carré blanc.

*Cooccurrence entre la production de DAPG et l'ACC désaminase*

La cooccurrence entre l'opéron *phl* (codant la voie de biosynthèse du métabolite 2,4 diacétylphloroglucinol ou DAPG) et du gène *acdS* codant la 1-AminoCyclopropane-1-Carboxylate (ACC) désaminase a été montrée chez certaines souches de *Pseudomonas* fluorescents. En effet, sur 50 souches de *Pseudomonas fluorescens* isolées de sols rhizosphériques en Suisse, 42 possèdent les deux fonctions, de même en République Tchèque (8 souches sur 10), ou encore en Italie (4 souches sur 7) (Wang *et al.* 2001). Chez la PGPR

modèle isolée en Irlande, *P. fluorescens* F113, la cooccurrence de ces deux fonctions a également été décrite (Redonto-Nieto *et al.* 2013). Cette co-occurrence est également retrouvée chez des *Pseudomonas* phylogénétiquement proches de *P. fluorescens* F113 (Almario *et al.* 2014), appartenant au même sous-groupe des *Pseudomonas corrugata* (Mulet *et al.* 2010). En revanche, si cette cooccurrence a été clairement établie chez de nombreux *Pseudomonas* fluorescents, aucune étude ne fait part des interactions potentielles de ces deux fonctions entre elles.

*Cooccurrence entre la production de DAPG et la production d'auxine*

Picard et Bosco ont isolé des *Pseudomonas* de la rhizosphère de trois variétés de maïs différentes, deux lignées parentales et leur hybride (Picard et Basco 2005). La variété hybride est plus performante que les variétés parentales; ce gain de performance correspond à ce que l'on nomme l'hétérosis. Les auteurs ont mis en évidence que les *Pseudomonas* isolés de la rhizosphère de la variété hybride présentaient deux fonctions phytobénéfiques cooccurrentes, la production d'auxine et de DAPG. Ces *Pseudomonas* n'étaient que très peu présents dans la rhizosphère des variétés parentales (Picard et Basco 2005). De ce fait, l'hétérosis favoriserait la sélection des populations spécifiques de *Pseudomonas* fluorescents producteurs d'auxine et de DAPG. Même si le DAPG est particulièrement connu pour être impliqué dans des interactions d'antagonisme envers des microorganismes phytopathogènes (Cronin *et al.* 1997 ; Weller *et al.* 2007), il a également un effet concentration-dépendante sur le végétal en modulant son architecture racinaire *via* l'utilisation de voies de transduction du signal auxine-dépendantes (Brazelton *et al.* 2008). Ainsi, ces deux fonctions empruntent des voies de signalisations similaires chez le végétal, pouvant permettre une régulation fine de la balance hormonale de la plante. Elles peuvent être toutefois complémentaires du fait que le DAPG induit également chez la plante la mise en place d'une réponse systémique induite (ISR – Iavicoli *et al.* 2003).

*Cooccurrence entre la fixation de l'azote et la production d'auxine*

La fixation de l'azote atmosphérique par des bactéries en interaction non-mutualiste avec des plantes est décrite comme une fonction phytobénéfique. La fixation « libre » du $N_2$ est une fonction caractéristique du genre *Azospirillum*. Plusieurs espèces du genre *Azospirillum*, comme *A. lipoferum*, *A. amazonense*, possèdent en plus de leur capacité à fixer le $N_2$, des gènes codant la voie de production d'auxines tel le gène *ipdC* codant une indole-3-pyruvate décarboxylase (Malhotra et Srivastava 2008) ou le gène *ppdC* codant une phénylpyruvate décarboxylase (Spaepen *et al.* 2007). Cette association est également trouvée chez des gamma-protéobactéries comme *Klebsiella pneumoniae* 342, *Pantoea sp.* At-9b et *P. stutzeri* A1501 (Bruto *et al.* 2014). Certains auteurs focalisent leur attention sur l'isolement de bactéries rhizosphériques arborant ces deux fonctions pour de futures valorisations agronomiques (Roesch *et al.* 2007), du fait que ces deux propriétés n'ont pas le même rôle écologique, la fixation d'azote participant à l'amélioration de la nutrition végétale, la production d'auxine interférant avec le développement et la croissance de la plante.

*Coocurence entre une plus large diversité de propriétés phytobénéfiques et apport de la génomique comparative à l'étude de leurs associations préférentielles*

Avec l'augmentation du nombre de génomes de PGPR séquencés, les analyses de génomiques comparatives ont simplifié les études de description d'associations préférentielles

de propriétés phytobénéfiques. Ainsi, en recherchant les déterminants génétiques impliqués dans ces propriétés phytobénéfiques, il est possible de dresser un profil fonctionnel putatif d'une souche à partir de son génome. Loper *et al.* (2012) ont dressé le profil fonctionnel de 10 souches appartenant aux groupes des *P. fluorescens* dont le génome est disponible et les ont relié aux relations phylogénétiques existant entre ces souches (**Figure 2**).

Des associations préférentielles sont retrouvées parmi ces 10 *Pseudomonas* comme notamment la production de DAPG et d'HCN préalablement décrit par Ramette *et al.* 2003. D'autres études de génomique étudiant la distribution des propriétés phytobénéfiques ont été réalisées à différentes échelles taxonomiques comme celle des protéobactéries (Bruto *et al.* 2014), à l'échelle d'un genre (cas des *Azospirillum* – Wisniewski-Dyé *et al.* 2012), ou encore à l'échelle d'une espèce (cas des *Paenibacillus polymyxa* – Eastman *et al.* 2014).

Ainsi, il est possible de retrouver des associations préférentielles de propriétés phytobénéfiques impliquées ou non dans un même rôle écologique. C'est par exemple le cas des propriétés antimicrobiennes diverses possédées par certaines bactéries du groupe des *Pseudomonas fluorescens* comme la souche *P. protegens* CHA0 (i.e. DAPG, HCN, pyolutéorine, pyrrolnitrine) ou comme les *P. chlororaphis,* (i.e. phénazine, 2-hexyl 5-propyl résorcinol (HPR), pyrrolnitrine, HCN) (Romanowski *et al.* 2011, Haas et Keel 2001 ; Loper *et al.* 2012 ; Caldéron *et al.* 2013 ; Chen *et al.* 2015). Cette forme de redondance fonctionnelle n'est pourtant pas absolue du fait que certaines propriétés phytobénéfiques peuvent avoir des rôles pléïotropes dans l'interaction avec la plante hôte (exemple du DAPG comme discuté précédemment – Cronin *et al.* 1997 ; Brazelton *et al.* 2008 ; Philipps *et al.* 2004 ; Weller *et al.* 2007) ou peuvent également être impliquées dans des processus cellulaires (comme par exemple la solubilisation du phosphate impliquant la production d'acide gluconique, cette molécule étant également une molécule carbonée impliquée dans le métabolisme primaire des bactéries – de Werra *et al.* 2008 ; Miller *et al.* 2010). La présence de co-occurrences entre les propriétés phytobénéfiques est de plus en plus recherchée et étudiée afin de mesurer la complémentarité de leurs modes d'action (Liu these 2015), mais également afin d'obtenir des bactéries phytobénéfiques efficientes, que certains auteurs qualifient des PGPR « d'élite » (Kumar *et al.* 2011). Toutes ces études se focalisent sur le type de propriétés possédées, en revanche leur distribution selon leur nombre et leurs interactions au sein des bactéries phytobénéfiques restent mal-étudiées.

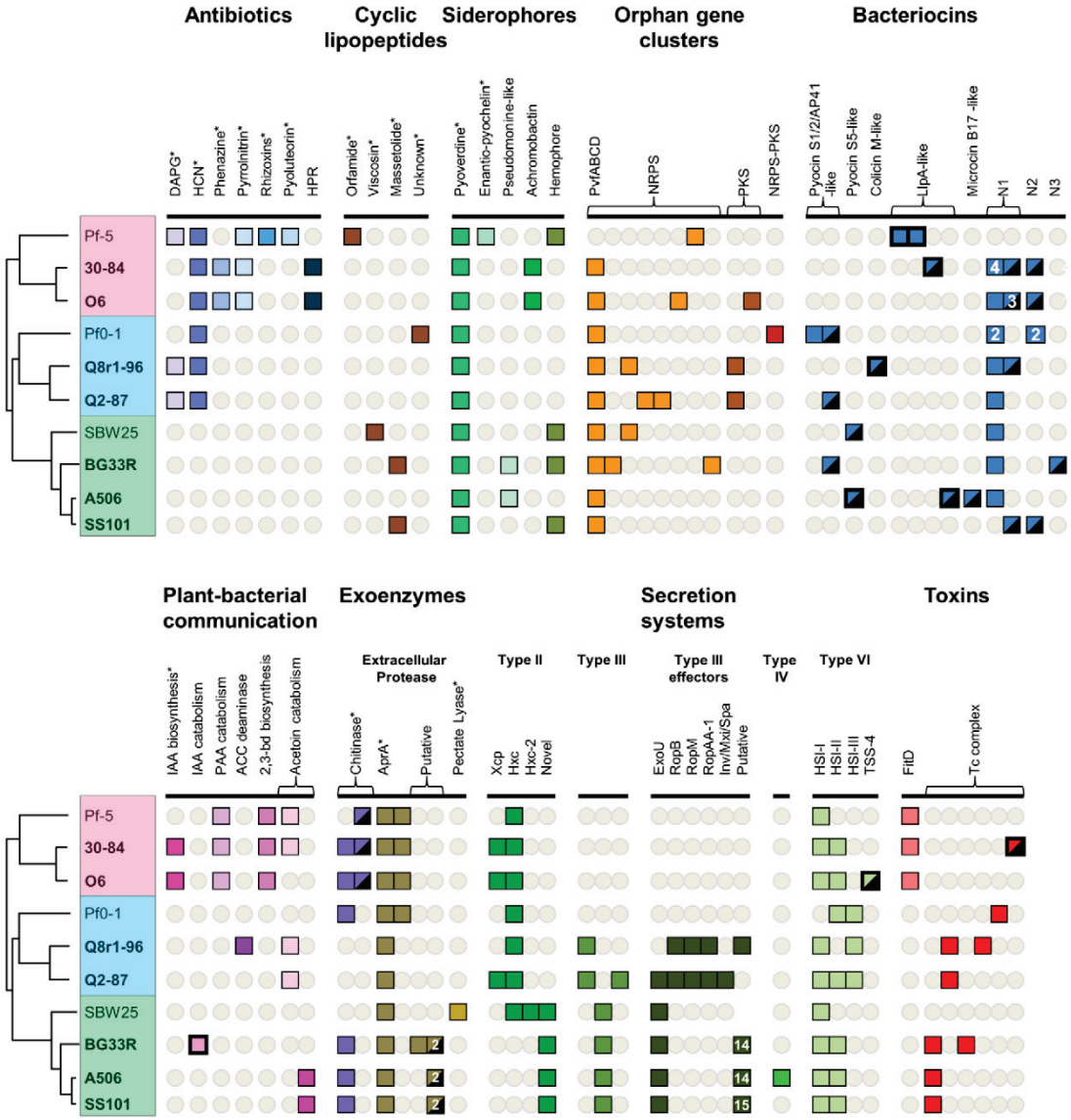


**Figure 2 : Distribution des propriétés phytobénéfiques au sein des souches séquencées du groupe des *Pseudomonas fluorescens* (Tiré de Loper et al. 2012)** Les cases colorées représentent la présence d'un groupe de gènes ou d'un gène dans un génome, alors que l'absence d'un groupe est représentée par un cercle gris ; les numéros dans une boîte représentent le nombre de copies d'un gène ou d'un cluster dans un génome. Les gènes au sein d'un élément génétique mobile sont présentés sous la forme d'une boîte dont le contour est épaissi; les gènes au sein de régions atypiques en contenu trinucléotidique sont indiqués par des boîtes à moitié colorées en noir. Les groupes de gènes impliqués dans la communication plantes-bactéries sont composées de: *iaaMH* (IAA biosynthèse); *iacR*, un transporteur ABC, et *iacHABICDEFG* (catabolisme des auxines en particulier de l'acide indole-3-acétique); *paaCYBDFGHIJKWLN* (catabolisme du PAA); *acdS* (ACC désaminase); *budC* / *ydjL* + *ilvBN* (biosynthèse de 2,3-butanediol); *acoRABC* + *acox* + *bdh* (en rose clair, catabolisme de l'acétoïne); *acoRABC* + *budC* (rose foncé, catabolisme de l'acétoïne). Les abré*via*tions sont : 2,4-diacétylphloroglucinol (DAPG); le cyanure d'hydrogène (HCN); des dérivés de rhizoxine (Rhizoxins); 2-hexyl-5-propyl-alkylrésorcinol (HPR); peptide non-ribosomique synthétase (PNR); polycétide synthétase (PKS); nouveaux groupes, respectivement, des carocine, pyocine et bactériocines et que l'on trouve dans ces souches (N1, N2, N3); acide indole-3-acétique (IAA); phénylacétique (PAA); aminocyclopropane-1-carboxylique (ACC); systèmes de sécrétion de type VI trouvés, homologues des loci de virulence HSI-I, HSI-II, et HSI-III de *P. aeruginosa* (HSI-I, II, II); TSS-4 de *Burkholderia pseudomallei* (TSS-4). Les astérisques indiquent que le phénotype attendu a été détecté chez les souches ayant les gènes ou les groupements de gènes indiqués.

**Nombre de propriétés phytobénéfiques possédé : Le plus = le mieux ?**

Bashan et collègues ont formulé l'hypothèse d'additivité de ces fonctions phytobénéfiques. Cette théorie considère que l'ensemble des fonctions co-occurrentes dans un génome de PGPR participe, de façon simultanée ou non, à l'amélioration de la plante (**Figure 3**) (Bashan *et al.* 2004). L'amélioration de la croissance et/ou de la santé du végétal résulterait de plus de la somme des contributions relatives des fonctions phytobénéfiques d'une PGPR (Bashan et de Bashan 2010).

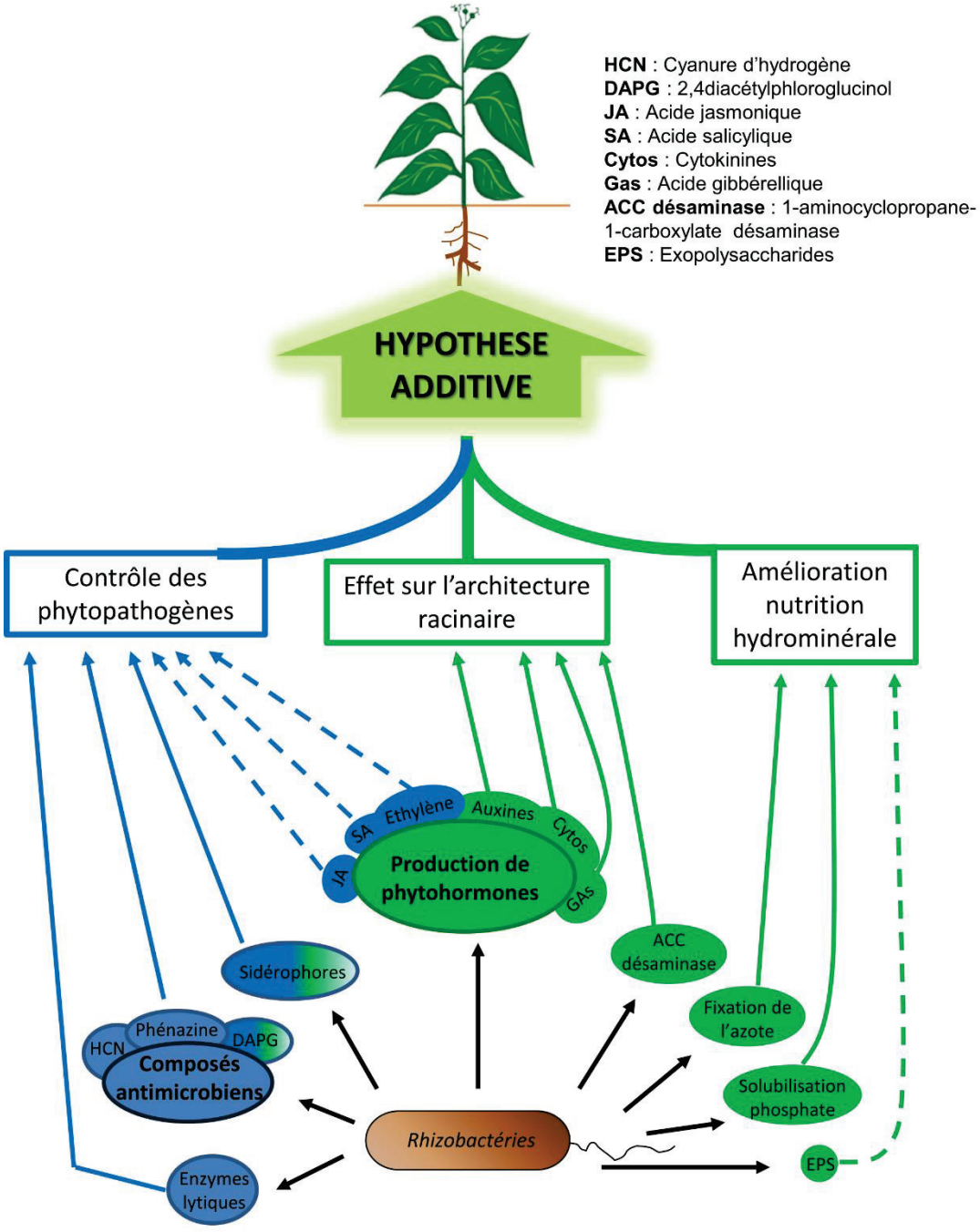


**Figure 3 : Hypothèse d'additivité des principales activités phytobénéfiques.** La liste des fonctions présentes sur cette figure n'est pas exhaustive. La couleur bleue correspond aux activités phytoprotectrices et la couleur verte aux activités phytostimulatrices. Les traits pleins indiquent un effet direct et les traits en pointillés caractérisent un effet indirect. (Adapté de Bashan et de Bashan 2010).

Toutefois, cette théorie n'est pas basée sur des observations qui mettent en évidence qu'une bactérie « élite » serait davantage sélectionnée/enrichie par les plantes. Werner *et al.* (2014) ont récemment proposé qu'afin d'optimiser leur succès de sélection par la plante au sein du rhizomicrobiote, les rhizobactéries phytobénéfiques auraient deux stratégies possibles: la première consisterait à proposer une large gamme de propriétés phytobénéfiques dans le but de répondre de façon optimale aux besoins du végétal; la seconde stratégie consisterait, au contraire, à n'assurer qu'une fonction écologique spécialisée mais avec une très forte efficacité et donc à posséder donc un nombre restreint de propriété phytobénéfiques. Il est proposé que ces deux stratégies soient présentes dans le rhizomicrobiote (Werner *et al.* 2014). Toutefois, la proportion des individus optant pour l'une ou l'autre de ces deux stratégies n'a jusqu'à présent pas été étudiée.

# REGULATIONS COMMUNES DES PROPRIETES PHYTO-BENEFIQUES CO-OCCURRENTES

L'association de fonctions potentiellement impliquées dans l'écologie de la bactérie qui les possèdent implique des systèmes de régulation permettant l'expression simultanée ou successive des gènes codant ces fonctions en réponse aux conditions environnementales.

## Régulation des propriétés phytobénéfiques co-occurrentes par *quorum sensing* (QS) et par des systèmes à deux composants

Il est établi que les bactéries sont capables de communiquer entre elles afin de coordonner certaines activités. De ce fait, les bactéries ont développé un système complexe de communication pour contrôler et synchroniser dans une population l'expression de certaines fonctions. La communication bactérienne par *quorum sensing* (QS) est basée sur la production de molécules signales appelées autoinducteurs dont la concentration est proportionnelle à la densité bactérienne (pour revue voir Grandclément *et al.* 2015). Ainsi, la reconnaissance des autoinducteurs entraine une modulation de l'expression génique (induction ou répression) de différents gènes impliqués dans des fonctions variées. La régulation de 5 à 10% des gènes de *Pseudomonas aeruginosa* et *Burkholderia cepacia* est réalisées *via* le QS (Riedel *et al.* 2003, Shuster *et al.* 2003). Parmi les gènes régulés par QS, certains sont impliqués dans des propriétés phytobénéfiques chez les rhizobactéries phytobénéfiques. Une approche de *quorum-quenching* visant à introduire sur un plasmide un gène codant une lactonase (enzyme dégradant des auto-inducteurs possédant un noyau lactone) a permis d'identifier les gènes régulés par QS chez deux souches *d'Azospirillum lipoferum* (Boyer *et al.* 2009). Il a été montré que le QS régule la production d'auxine et de sidérophore chez la souche *Azospirillum* B518 tandis qu'aucune de ces deux fonctions ne semble être régulée par QS chez la souche *Azospirillum* TVV-3 (Boyer *et al.* 2009). Par ailleurs, il a été découvert un système de régulation globale – le système Gac (*Global antibiotic and cyanide control*) contrôlant la production de métabolites secondaires antimicrobiens comme le DAPG, l'HCN et la pyolutéorine chez *P. protegens* CHA0 (Laville *et al.* 1995). Le système Gac est constitué d'une protéine kinase senseur transmembranaire (GacS) percevant les signaux de l'environnement et phosphorylant, lorsqu'elle est activée, le régulateur GacA. Ce régulateur va à son tour activer la transcription de petits ARN régulateurs (*rsmX*, *rsmY* et *rsmZ*) (Valverde *et al.* 2003 ; Kay *et al.* 2005) qui peuvent séquestrer la protéine RsmA, levant alors l'inhibition de l'expression des gènes impliqués dans la production de

métabolites secondaires (Blumer *et al.* 1999 ; Valverde *et al.* 2003). Si ce système de régulation a été bien décrit chez le pathogène opportuniste humain *P. aeruginosa* (Nadal-Jimenez *et al.* 2012), cela reste un système de régulation transposable à de nombreux *Pseudomonas,* quel que soit leur écologie (**Figure 4**). De plus, le système de régulation par QS et le système à deux composantes GacA/GacS ne sont pas indépendants, car ce dernier module également l'expression des gènes impliqués dans le QS (Venturi *et al.* 2005). Chez la souche *P. chlororaphis* PA23, la production d'HCN et d'autres composés antimicrobiens est également régulée par un autre système QS, PhzI/PhzR (Nandi *et al.* 2015).

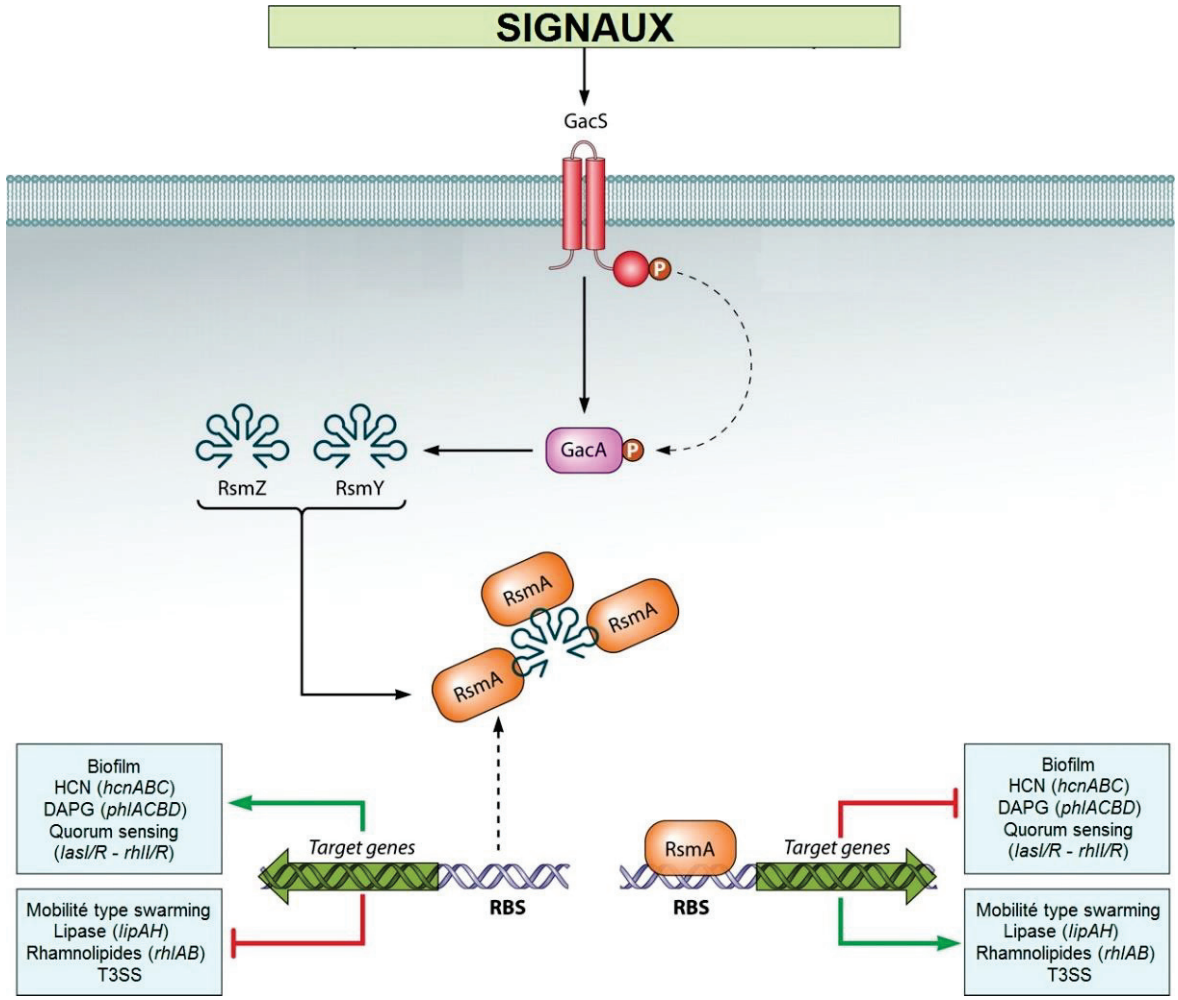


**Figure 4 : Schéma simplifié de la régulation par le système GacA/GacS chez les *Pseudomonas*** (adapté de Nadal Jimenez *et al.* 2012). La protéine régulatrice RsmA se fixe sur la région promotrice de gènes multiples dont elle induit (flèche verte) ou réprime la transcription (flèche rouge). La phosphorylation de GacA *via* GacS stimule la production des petits ARNs RsmY et RsmZ qui se fixent sur la protéine régulatrice RsmA, levant la répression de l'expression des gènes que cette protéine inhibe.

## Régulation des propriétés phytoprotectrices co-occurrentes par la réponse stringente et par les facteurs de transcription

La réponse stringente est un mécanisme de réponse cellulaire mis en œuvre par les bactéries lorsque leurs conditions de croissance deviennent défavorables, conditions au cours

desquelles la concentration en certains acides aminés ne permet par exemple plus une synthèse protéique « normale ». Il résulte de ce mécanisme une augmentation intracellulaire de la concentration en (p)ppGpp, une alarmone pouvant activer l'expression de facteurs de transcription alternatifs comme RpoS (σ38 ou Sigma S) qui vont alors réguler l'expression de gènes codant pour la production de métabolites secondaires antimicrobiens. Ainsi, l'absence du gène *rpoS* chez un mutant de *P. chlororaphis* PA23 augmente l'expression de l'opéron *prn* (impliqué dans la production de la pyrrolnitrine) et diminue l'expression du gène *phzA* (impliqué dans la production de phénazines) conduisant alors une régulation inverse de l'expression, en conditions stringentes, de ces deux propriétés co-occurrentes (Manuel *et al.* 2012). De plus, la régulation de ces deux métabolites antimicrobiens par RpoS est également sujette à une régulation *via* PhzI/PhzR (Selin *et al.* 2012), et *via* le système GacA/GacS chez *P. chlororaphis* PA23 (Selin *et al.* 2014). RpoS est également impliqué dans la régulation de la production du DAPG et de la pyolutéorine chez la souche *P. protegens* Pf-5 (Sarniguet *et al.* 1995) mais également le facteur de transcription RpoN (σ54 ou sigma N) chez *P. protegens* CHA0 (Péchy-Tarr *et al.* 2005).

**Autres types de régulations des propriétés phytobénéfiques co-occurrentes**

Si le QS et les systèmes à deux composants jouent un rôle prépondérant dans la régulation de l'expression des propriétés phytobénéfiques (en particulier l'expression des propriétés affiliées à de la phytoprotection), certaines peuvent moduler directement l'expression ou l'activité d'autres propriétés phytobénéfiques. C'est notamment le cas chez *P. protegens* CHA0, où la production de pyolutéorine et de DAPG s'inhibent mutuellement à un niveau transcriptionnel (Baehler *et al.* 2005). La même régulation est observée chez *P. protegens* Pf-5, où il a même été démontré que le phloroglucinol (composé intermédiaire de la synthèse du DAPG) inhibait la production de pyolutéorine à forte concentration et l'active à des concentrations nanomolaires (Kidarsa *et al.* 2011, Clifford *et al.* 2015). D'autres composés d'origines microbiennes et végétales influencent également l'expression de ces deux gènes (Schnider-Keel *et al.* 2000, de Werra *et al.* 2011).

Si les interactions entre fonctions *via* des réseaux de régulation communs sont bien décrites dans la littérature pour les propriétés impliquées dans la production de molécules antimicrobiennes chez les *Pseudomonas* fluorescents, les interactions entre propriétés de phytostimulation ne sont que pauvrement décrites. Toutefois, certains liens fonctionnels ont été établis entre fonctions « phytostimulatrices » et « phytoprotectrices » comme par exemple le lien entre la solubilisation du phosphate et de la production de DAPG chez *P. protegens* CHA0 (de Werra *et al.* 2009). La production de DAPG se trouve augmentée lorsque le gène *gcd* codant une glucose deshydrogénase, impliquée dans la production d'acide gluconique, est mutée. Le système mettant en œuvre la répression de la production de DAPG par la voie de l'acide gluconique n'a jusqu'à l'heure pas encore été élucidé (de Werra *et al.* 2009). Enfin, certaines propriétés phytobénéfiques peuvent être liées l'une à l'autre du fait qu'elles partagent un précurseur commun dans leur voie biosynthèse, comme par exemple le tryptophane précurseur de la synthèse d'auxine (Spaepen *et al.* 2007), mais également de la pyrrolnitrine (Hamill *et al.* 1967). Cependant, à notre connaissance, aucune étude ne s'est encore intéressée aux interactions potentielles entre ces deux propriétés phytobénéfiques.

# ROLE ECOLOGIQUE DES FONCTIONS PHYTOBENEFIQUES: « QUI CONTRIBUENT A QUOI ? »

Pour comprendre le rôle écologique d'une fonction phytobénéfique chez les bactéries, deux approches peuvent être envisagées. Ces approches consistent à déléter la fonction phytobénéfique chez une bactérie qui la possède (cas de l'approche « perte de fonction ») ou à rajouter la fonction phytobénéfique à une bactérie qui ne l'a possède pas (cas de l'approche « gain de fonction »). La contribution de la propriété peut alors se mesurer en quantifiant un ou plusieurs traits impliqués dans l'interaction bactérie-plante (comme la colonisation racinaire ou des paramètres végétaux indicateurs de la santé et de la croissance du végétal).

Ces approches ont souvent été documentées en considérant l'implication de ces fonctions phytobénéfiques d'une façon individuelle.

## Contribution des propriétés phytobénéfiques : Approche perte de fonction

Pour comprendre la contribution d'une propriété à un effet bénéfique sur la plante, il est possible d'opter pour une approche « perte de fonction » en mutant la propriété phytobénéfique d'intérêt. Ainsi, la mutation de cette propriété renseigne sur sa contribution à l'effet observé en termes d'effet antagoniste vis-à-vis d'un phytopathogène ou de stimulation de la croissance du végétal. L'approche par mutagenèse permet en particulier de savoir si la propriété mutée est à elle seule responsable de cet effet, ou si d'autres facteurs ou propriétés sont impliqués. De cette façon, de nombreux mutants de propriétés phytobénéfiques impliquées dans le biocontrôle ont été étudiés afin de voir l'impact de la fonction mutée sur la capacité de cette souche à exercer son antagonisme envers des microorganismes phytopathogènes. Ainsi, un mutant *hcn*, ne produisant plus d'acide cyanhydrique (HCN) protège de façon moindre le tabac contre le champignon phytopathogène *Thielaviopsis basicola* comparé à l'inoculation de la souche sauvage (Voisard *et al.* 1989). Pour autant, la production d'HCN ne contribue pas à elle seule à expliquer l'effet obtenu par l'inoculation de la souche sauvage. D'autres études ont montré que la production de DAPG par *P. fluorescens* F113 contribue pleinement à la réduction de la mobilité du nématode phytopathogène *Globodera rostochiensis* (Cronin *et al.* 1997a), à l'inhibition de la croissance d'*Erwinia carotovora* subsp. a*troseptica* (Cronin *et al.* 1997b), de *Pythium ultimum* (Fenton *et al.* 1992). Des études impliquant la perte de plusieurs fonctions au sein d'une même bactérie ont également été menées. L'une d'entre elles utilise une souche mutée dans le régulateur GacA de *P. protegens* CHA0 ayant comme phénotype l'absence de production de tous les composés antimicrobiens régulés par ce système, ainsi que des mutants simples de ces fonctions. Chacun des composés microbiens produit par CHA0 ne contribue pas de façon égale à l'inhibition de la sporulation de *Peronospora parasitica*, le DAPG étant le composé contribuant majoritairement à l'inhibition de la sporulation de ce phytopathogène (Iavicoli *et al.* 2003). Une autre étude a analysé la contribution de 3 composés antimicrobiens produits par *P. chlororaphis* PCL1606 (la pyrolnitrine, l'HCN et le 2-hexyl 5-propyl résorcinol (HPR)) en réalisant des mutants simples, doubles et triple. Il apparait que la production d'HPR est majoritairement responsable de l'inhibition de *Rosellinia necatrix* et de *Fusarium oxysporum* (Calderón *et al.* 2013).

La contribution de la production d'auxine à la phytostimulation a également été analysée chez *Azospirillum brasilense* SM en mutant le gène *ipdC* (Malhotra *et al.* 2008). Les effets du mutant sur l'architecture racinaire, notamment le nombre de racines latérales ainsi que la

longueur racinaire, sont affectées lors de l'inoculation du mutant comparativement aux effets observés avec la souche sauvage. En revanche, les paramètres racinaires restent toutefois plus élevés quand le mutant est inoculé que dans la condition non-inoculée. La production d'auxine n'est cependant pas totalement abolie chez le mutant, ce qui conduit aux effets observés sur le végétal (Malhotra *et al.* 2008). Une autre étude similaire, réalisée avec la souche *A. brasilense* Sp245 sauvage et son mutant *ipdC*/*ppdC,* montre la contribution majoritaire de la production d'auxine dans l'effet racinaire observé (Spaepen *et al.* 2007).

**Contribution des propriétés phytobénéfiques : Approche gain de fonction**

L'ajout de fonction peut être effectué par insertion chromosomique *via* l'utilisation d'un transposon comme cela a été réalisé chez *P. fluorescens* SBW25 avec l'ajout de l'opéron *phz* impliqué dans la production de phénazine (Timms-Wilson et al 2000). Ce gain de fonction confère une meilleure capacité de biocontrôle contre le champignon phytopathogène *Pythium ultimum* que ce soit *in vitro* ou lors d'essai d'infection sur le pois. Ce même opéron a été introduit par insertion d'un transposon au niveau du chromosome de *P. putida* WCS358r, résultant en la souche WCS358r::*phz*. De la même façon, l'opéron *phl* a été ajouté à la même souche sauvage, donnant lieu à la souche WCS358r::*phl* (Glandorf *et al.* 2001). Ces souches ont présenté une augmentation de leur activité biocontrôle vis-à-vis de *Gaeumannomyces graminis* var. *tritici* (Glandorf *et al.* 2001). Leurs impacts sur les communautés fongiques et bactériennes du sol, dans des essais au champ ont également été analysés en vue d'une potentielle commercialisation de ces souches génétiquement modifiées (Glandorf *et al.* 2001 ; Viebahn *et al.* 2003 ; Bakker *et al.* 2005).

D'autres études ont intégré le gène *acdS* codant l'ACC désaminase en aval d'un promoteur constitutif chez *Azospirillum brasilense* Cd (Holguin et Glick 2001), une souche connue pour stimuler la croissance des plantes *via* sa capacité à produire des auxines (Omay *et al.* 1993 ; Zaadi *et al.* 1993). Comme montré dans de nombreux exemples au cours des paragraphes précédents, la PGPR modèle *P. protegens* CHA0 est connue pour avoir un fort pouvoir biocontrôle du fait de la capacité de cette souche à produire une large gamme de métabolites antimicrobiens. De ce fait, l'incorporation dans cette souche de propriétés phytobénéfiques impliquées dans la stimulation de la croissance de la plante comme la production d'une activité ACC désaminase ou encore de la production d'auxine a été réalisée. L'introduction de l'activité ACC désaminase chez CHA0 lui permet de stimuler la rhizogenèse du canola (Wang *et al.* 2000) alors que la souche sauvage ne produit aucun effet sur cette plante. De plus, l'introduction de cette fonction a également amélioré les capacités de biocontrôle de cette souche vis-à-vis de *Pythium ultimum* (Wang *et al.* 2000). Par ailleurs, CHA0 est capable de produire de l'auxine (sous la forme d'acide indole-3-acétique) par deux voies différentes, la voie impliquant la « *tryptophan side chain oxidase* » (TSO) et celle impliquant la tryptophane transaminase (Oberhänsli *et al.* 1991). La production d'auxine par ces deux voies de biosynthèse reste faible. Ainsi, l'incorporation de gènes impliqués dans la production d'auxine *via* la voie de la tryptophane monooxygénase (gènes *iaaM*, *iaaH*) a été réalisée et montre une augmentation significative la production d'auxine *in vitro*, résultant lorsque cette souche est inoculée à du concombre en une augmentation de la biomasse racinaire (Beleyer *et al.* 1999). Les études de contribution par gain de fonction sont souvent réalisées en ciblant une seule fonction. A notre connaissance, aucune étude ne s'est intéressée à l'ajout de plusieurs propriétés

phytobénéfiques simultanément dans un même individu dans le but de caractériser la contribution de chacune de ces propriétés.

# CONCLUSION

Cette étude bibliographique n'est pas exhaustive et montre l'importance de considérer l'ensemble des propriétés phytobénéfiques possédées par une bactérie afin de mieux comprendre pour chacune d'entre elle leur importance dans l'interaction avec la plante hôte et l'effet observé sur celle-ci. En effet, la contribution relative des fonctions phytobénéfiques peut être différente selon la fonction considérée, mais également d'une bactérie à une autre. Ces mêmes propriétés pourront être régulées par des systèmes différents selon la bactérie qui les possède.

# CHAPITRE 2

## OCCURRENCE DES RHIZOBACTERIES MULTI-FONCTIONS DANS LA RHIZOSPHERE : CAS DES *PSEUDOMONAS* FLUORESCENTS

# ***Préambule chapitre 2***

Le chapitre bibliographique précédent souligne l'importante diversité des modes d'action des bactéries phytobénéfiques sur la plante, à savoir (i) l'amélioration de sa nutrition, (ii) la modulation de sa balance phytohormonale et (iii) sa protection envers des microorganismes phytopathogènes. Cette étude bibliographique a également mise en lumière la nécessité de prendre en compte l'ensemble des propriétés phytobénéfiques co-occurrentes possédées par une bactérie phytobénéfique pour mieux comprendre leurs rôles écologiques.

Au sein du rhizomicrobiote, les rhizobactéries du groupe des *P. fluorescens* sont retrouvées abondantes dans la rhizosphère de nombreuses plantes (Couillerot *et al.* 2009, Almario *et al.* 2014, Haney *et al.* 2015) et possèdent un large éventail de fonctions phytobénéfiques ayant des modes d'action différents sur le végétal (Naik *et al.* 2008, Ahmad *et al.* 2008, Loper *et al.* 2012). De fait, ces rhizobactéries ont un rôle écologique primordial dans la rhizosphère. Nous avons donc focalisé notre attention sur ce groupe bactérien. Les études de la distribution des propriétés phytobénéfiques au sein de ce groupe s'intéressent rarement au nombre de propriétés co-occurrente possédées par les individus. Pour autant, la recherche de nouvelles souches bactériennes d'intérêt agronomique s'oriente vers des candidats arborant un maximum de fonctions parce qu'ils pourraient apporter des bénéfices plus importants à la plante en terme d'acclimatation à son milieu, de sa résistance envers les nuisibles (micro ou macroorganismes), d'amélioration de son développement et de sa croissance.

L'hypothèse de ce chapitre est que des individus appartenant au groupe des *Pseudomonas* fluorescents possédant un grand nombre de propriétés phytobénéfiques sont préférentiellement sélectionnés par la plante au sein de son rhizomicrobiote.

Pour répondre à cette hypothèse, la co-occurrence de 18 propriétés impliquées dans l'amélioration de la nutrition, la modulation phytohormonale et la protection de la plante a été analysée par des méthodes moléculaires et biochimiques, dans une collection d'environ 700 isolats bactériens appartenant au groupe des *Pseudomonas* fluorescents et provenant de la rhizosphère de maïs ainsi que de sols non-plantés. Contrairement à notre hypothèse de départ, le maïs sélectionne dans son rhizomicrobiote des *Pseudomonas* ne possédant qu'un faible nombre de propriétés phytobénéfiques (jusqu'à cinq). Cette sélection, qui n'a pas d'influence sur l'abondance relative de *Pseudomonas* sp. dans le rhizomicrobiote, n'est pas due à la prédominance d'isolats possédant des associations de propriétés phytobénéfiques particulières. Un nombre optimal de ces propriétés par individu pourrait favoriser leur capacité de colonisation des racines et d'établissement dans la rhizosphère. Ces résultats sont présentés sous la forme d'une publication intitulée *'Fluorescent Pseudomonas strains with only few plant-beneficial properties are favored in the maize rhizosphere'*.

Ces travaux ont été soumis à la revue *Environmental Microbiology*.

# Fluorescent *Pseudomonas* strains with only few plant-beneficial properties are favored in the maize rhizosphere

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**Running title:** Distribution of plant beneficial properties in pseudomonads

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## SUMMARY

Plant Growth-Promoting Rhizobacteria (PGPR) enhance plant health and growth using a variety of traits. Effective PGPR strains typically exhibit multiple plant-beneficial properties, but whether they are better adapted to the rhizosphere than PGPR strains with fewer plant-beneficial properties is unknown. Here, we tested the hypothesis that strains with higher numbers of plant-beneficial properties would be preferentially selected by plant roots. To this end, the co-occurrence of 18 properties involved in enhanced plant nutrition, plant hormone modulation, or pathogen inhibition was analyzed by molecular and biochemical methods in a collection of maize rhizosphere and bulk soil isolates of fluorescent *Pseudomonas*. Twelve plant-beneficial properties were found among the 698 isolates. Contrarily to expectation, maize preferentially selected pseudomonads with low numbers of plant-beneficial properties (up to five), but strains with higher numbers of properties still reached high population densities due to overall rhizosphere enrichment. This selection, which did not influence the relative amount of *Pseudomonas* spp. in the community, was not due to the predominance of strains with specific assortments of these properties. Since over-representation of pseudomonads harboring few plant-beneficial properties in the rhizosphere was not linked to their taxonomic status, an optimum number of these properties seems to favor root colonization ability.

**Key-words:** Fluorescent *Pseudomonas*; PGPR; maize; functional groups; plant-beneficial properties

# INTRODUCTION

Plant roots interact with huge amounts of soil bacteria, including Plant Growth-Promoting Rhizobacteria (PGPR), which may have beneficial effects on plant growth, development and/or health (Richardson *et al.*, 2009; Vacheron *et al.*, 2013). This entails various modes of action, related to improved nutrient supply (associative nitrogen fixation, phosphate solubilization, etc.), modulation of plant hormonal balance (*via* production of auxins, cytokinins, nitric oxide etc., or deamination of 1-aminocyclopropane-1-carboxylic acid (ACC)), and enhanced plant defense against parasites (through induction of systemic resistance pathways, or production of antimicrobial secondary metabolites, extracellular lytic enzymes, or surfactants) (Spaepen *et al.*, 2009; Vacheron *et al.*, 2015).

It is not unusual that a given PGPR strain displays several different plant-beneficial properties, which can lead to higher positive effects on the plant (Bashan and de-Bashan, 2010). The latter is expected to take place because (i) the effects of different modes of action may add-up quantitatively, or (ii) it could ensure that at least one mode of action is expressed in particular environmental conditions. Indeed, the most effective PGPR are typically multi-function strains (Rana *et al.*, 2011; Almario, *et al.*, 2014). In line with this, the loss of one mode of action often reduces plant-beneficial effects (Thomashow and Weller, 1988; Keel *et al.*, 1990) while the gain of one may enhance plant-beneficial effects (Wang *et al.*, 2000; Bakker *et al.*, 2002; Holguin and Glick, 2003; Baudoin *et al.*, 2010), and the combined use of PGPR strains with different modes of action can also improve the effects on the plant (Dunne *et al.*, 1998; Combes-Meynet *et al.*, 2011; Walker *et al.*, 2012). Comparative genomics and network analysis evidenced that certain plant-beneficial properties tend to occur jointly in bacteria (Loper *et al.*, 2012; Bruto *et al.*, 2014). This situation is reminiscent of the accumulation of virulence factors in pathogens (Friesen *et al.*, 2006).

As coevolution between PGPR and plants is thought to occur (Lambers *et al.*, 2009; Combes-Meynet *et al.*, 2011), it should favor strains with multiple plant-beneficial properties in the rhizosphere since they can provide higher benefits to the host. This means that such PGPR strains should be selected preferably by plant roots, at the expense of related PGPR strains displaying a smaller number of plant-beneficial properties. However, this hypothesis remains to be tested. Therefore, the objective of the present study was to assess whether the rhizosphere preferentially selects bacteria with higher numbers of plant-beneficial properties.

To this end, we compared in various soils the effect of maize on the diversity of fluorescent *Pseudomonas* spp., a taxonomic group that displays a wide range of plant-beneficial properties and contains PGPR strains capable of phytostimulation and phytoprotection (Ahmad *et al.*, 2008; Naik *et al.*, 2008; Loper *et al.*, 2012; Sarma *et al.*, 2014; Yadav *et al.*, 2014). Plant-beneficial properties documented in these bacteria include associative nitrogen fixation (genes *nif* ; Mirza *et al.*, 2006), synthesis of antimicrobial compound and root-branching signal 2,4-diacetylphloroglucinol (genes *phl*; Keel *et al.*, 1990; De Leij *et al.*, 2002; Brazelton *et al.*, 2008), phosphate solubilization (Meyer *et al.*, 2010), synthesis of the auxinic phytohormone indole-3-acetic acid (Oberhänsli *et al.*, 1991) and the cytokinins isopentenyl adenosine (IPA) and trans-zeatin ribose (ZR) (García de Salamone *et al.*, 2001), synthesis of the root-branching signal nitric oxide (nitrite reductase gene *nirS*; Arese *et al.*, 2003), deamination of the plant's ethylene

precursor 1-aminocyclopropane-1-carboxylate (ACC; Glick *et al.*, 1998; Prigent-Combaret *et al.*, 2008), hydrogen cyanide synthesis (Frapolli *et al.*, 2012), pyrrolnitrin and pyoluteorin synthesis (Souza and Raaijmakers, 2003), phenazine production (Mavrodi *et al.*, 2006) and the production of extracellular lytic enzymes such as chitinase and protease (Sarma *et al.*, 2014). A collection of fluorescent *Pseudomonas* isolates was thus established and screened for the presence of 18 plant-beneficial properties, using established and novel methods targeting relevant genes, enzymatic activities and/or compounds. This isolate library was used to address the question whether fluorescent pseudomonads harboring a high number of co-occurring plant-beneficial properties are selected preferably in maize rhizosphere.

## RESULTS

### Maize preferentially selects fluorescent *Pseudomonas* harboring one to five plant-beneficial properties

To assess the hypothesis that plant preferentially selects fluorescent *Pseudomonas* isolates harboring a high number of co-occurring plant-beneficial properties, two maize cultivars, PR37Y15 and DK315, were grown 21 days in soils MS8, Ysa5, YSa8 and Bmo1 (Table S1), and a collection of 698 *Pseudomonas* isolates was obtained on selective $KB^{+++}$ agar (210 isolates from PR37Y15, 254 from DK315 and 234 from bulk soil). These *Pseudomonas* isolates possessed from 0 (11 isolates) to 9 (1 isolate) of 18 plant-beneficial properties studied, with a mean of 3.6 properties per isolate. When data from all soils were pooled together, most rhizosphere isolates (433 of 464, i.e. 93%) displayed one to five plant-beneficial properties, whereas as many as 81 of 234 bulk soil isolates (i.e. 35%) displayed six to nine of them (**Fig. 1-A**). Therefore, the hypothesis that plant preferentially selects fluorescent *Pseudomonas* isolates harboring a high number of co-occurring plant-beneficial properties was not valid in this maize experiment.

### Maize preferential selection for *Pseudomonas* with one to five plant-beneficial properties is counterbalanced by rhizosphere enrichment effect

Since pseudomonads may undergo rhizosphere enrichment, the numbers of fluorescent *Pseudomonas* were compared in bulk soil and maize rhizospheres according to the prevalence of plant-beneficial properties. Culturable fluorescent *Pseudomonas* populations did not differ between maize cultivars, but were significantly more abundant in rhizosphere soils of maize PR37Y15 ($7.09 \pm 0.26$ log cells.$g^{-1}$) and DK315 ($6.99 \pm 0.22$ log cells.$g^{-1}$) than in bulk soils ($4.96 \pm 0.16$ log cells.$g^{-1}$). When the amounts of plant-beneficial properties were taken into consideration, it appeared that the numbers of culturable fluorescent *Pseudomonas* with one, two, three, four or five plant-beneficial properties reached about 6 log cells/g in the rhizosphere of PR37Y15 and DK315 ($P>0.001$), but only 4 log cells.$g^{-1}$ in bulk soil (**Fig. 1-B**). Pseudomonads with six to nine plant-beneficial properties were found in the rhizosphere in substantial numbers ($5.51 \pm 0.45$ log cells.$g^{-1}$ for PR37Y15 based on the three soils MS8, Ysa8 and Bmo1 where they were detected, and $5.69 \pm 0.37$ log cells.$g^{-1}$ for DK315) compared with bulk soil ($4.32 \pm 0.39$ log cells.$g^{-1}$). Thus, stimulation of *Pseudomonas* growth on maize roots enhanced the rhizosphere effect on pseudomonads with one to five plant-beneficial properties

(density higher by two log compared with bulk soil), but it also counterbalanced the low prevalence of pseudomonads with six to nine plant-beneficial properties (whose overall density was not lower than in the bulk soil).

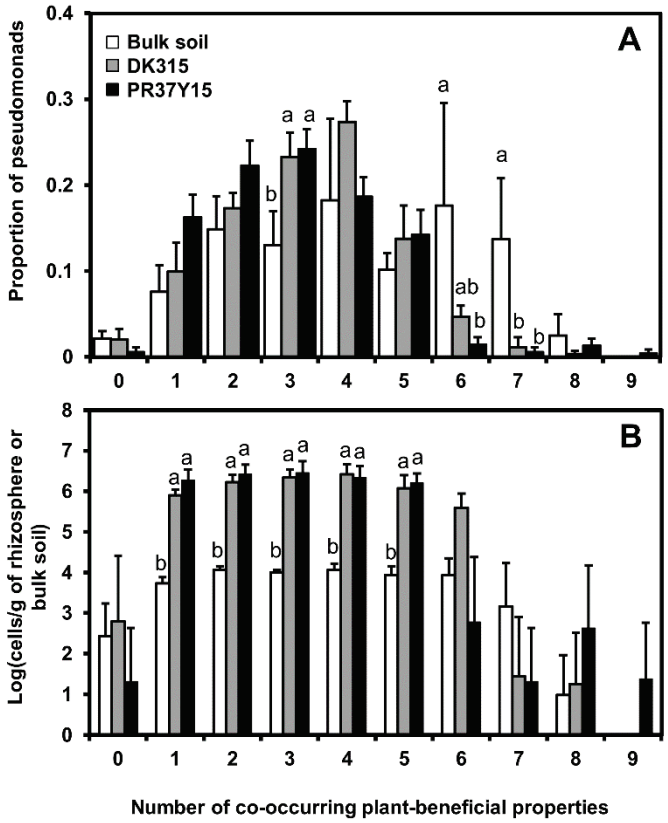


**Fig. 1: Proportion (A) and number (B) of pseudomonads according to the number of plant-beneficial properties harbored (from 0 to 9), in bulk soil and in the rhizosphere of maize cultivars DK315 and PR37Y15.** Data from soils Bmo1, Ysa5, MS8 and Ysa8 were combined and error bars (standard errors) reflect the variability among the four soils. Statistical differences between conditions (bulk soil/PR37Y15/DK315) are indicated with letter a-b. (ANOVA, Fisher's LSD test, $P < 0.05$).

## Maize preferential selection among *Pseudomonas* took place without higher proportions of strains with specific individual properties

Whereas nitrogen fixation gene *nifH* or the ability to synthesize the auxin indole-butyric acid or the cytokinins trans-zeatin, trans-zeatin riboside isopentenyl adenosine or 6-benzylaminopurine were not found in any of the *Pseudomonas* isolates, the 12 other plant-beneficial properties studied were found in 2% (chitinase activity) to 96% (production of indole-3-acetic acid) of the 698 pseudomonads (**Fig. 2-A**). Differences were not significant when comparing ($\chi^2$ tests) bulk soil and PR37Y15 and DK315 rhizospheres based on the proportion of strains displaying a given plant-beneficial property, regardless of the property tested. Essentially the same findings were made when comparisons were restricted to the subset of 502 pseudomonads with one to five plant-beneficial properties (except that extracellular protease activity was in a higher proportion in rhizosphere strains), or to the subset of 106 pseudomonads with six to nine plant-beneficial properties (not shown). When treatments were

analyzed separately, the distribution of these properties in pseudomonads differed according to soil type and maize cultivar (**Fig. S1**).

However, when considering the numbers (rather than relative proportions) of fluorescent *Pseudomonas* with a particular plant-beneficial properties, we found that the ACC deaminase gene *acdS*, the NO gene *nirS*, indole-3-acetic acid production, extracellular protease activity, the phenazine gene *phzCD*, phosphate solubilization activity or HCN production were each present in a significantly higher number of strains in the rhizosphere (for both cultivars) than in bulk soil, by approximately two log values (**Fig. 2-B**). In addition, a higher number of *phlD*$^+$ isolates (DAPG) was obtained in the rhizosphere for the four soils (for DK315) and three of four soils (for PR37Y15). For *prnD*$^+$ isolates (pyrrolnitrin) and *pltC*$^+$ isolates (pyoluteorin), the higher rhizosphere numbers were retrieved in the four soils (for PR37Y15) and three of four soils (for DK315) (not shown). Numbers of kinetin producers and of pseudomonads with chitinase activity were below 2 log cells.g$^{-1}$ in all three treatments.

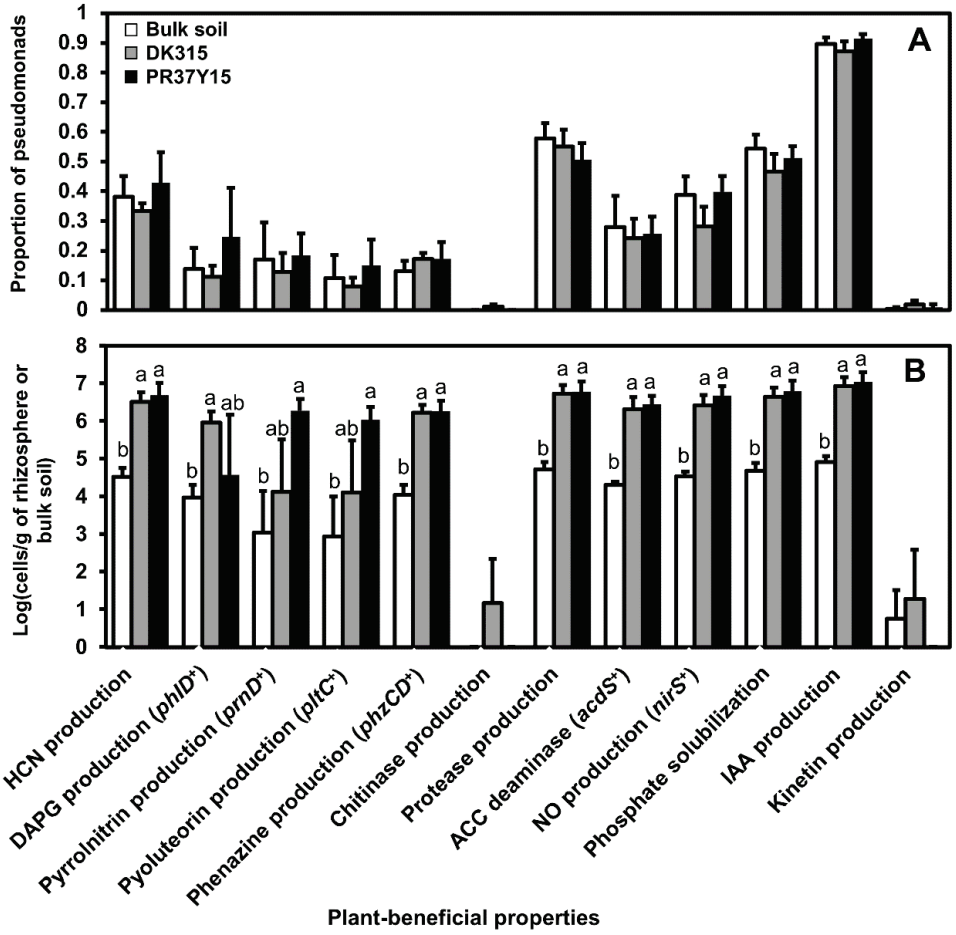


**Fig. 2: Proportion (A) and number (B) of pseudomonads according to plant-beneficial properties harbored, in bulk soil and in the rhizosphere of maize cultivars DK315 and PR37Y15.** Data from soils Bmo1, Ysa5, MS8 and Ysa8 were combined and error bars (standard errors) reflect the variability among the four soils. HCN: Cyanhydric acid; DAPG: 2,4- diacetylphloroglucinol; ACC: 1-amino-cyclopropane carboxylic acid; NO: Oxide nitric. Error bars correspond to standard error. Statistical differences between conditions (bulk soil/PR37Y15/DK315) are indicated with letter a-b (ANOVA, Fisher's LSD test, $P < 0.05$).

Therefore, even though preferential selection by maize of *Pseudomonas* with one to five plant-beneficial properties had no effect on the relative proportions of strains displaying one of

them, in all soils it took place in parallel to higher population densities in the rhizosphere for pseudomonads with a particular plant-beneficial property in the case of 7 plant-beneficial properties.

**Maize preferential selection among *Pseudomonas* did not result in the predominance of strains with specific assortments of properties**

The co-occurrence of different plant-beneficial properties within strains was not random, as genes for synthesis of organic antimicrobial compounds (i.e. DAPG, pyrrolnitrin, pyoluteorin and phenazines) clustered together following hierarchical clustering analysis (Fig. S1) and significantly correlated with one another (except for phenazines with pyrrolnitrin or with pyoluteorin) (**Fig. S2**). Likewise, properties involved in modulation of plant hormonal balance (i.e. ACC deaminase activity gene *acdS* and NO production gene *nirS*, which correlated positively; $r = 0.86$, $P < 0.010$), phosphate solubilization, protease activity and HCN production grouped together (**Figs S1** and **S2**).

Considering all the 12 displayed plant-beneficial properties together, a total of 175 different combinations (i.e. functional profiles) were found for the 698 isolates. The ratio of the number of functional profiles to the number of isolates was significantly higher ($P = 0.03$) in PR37Y15 ($0.56 \pm 0.02$) compared to DK315 ($0.45 \pm 0.03$) and bulk soil ($0.45 \pm 0.05$). This difference was due to the contribution of pseudomonads harboring one to five plant-beneficial properties (**Fig. S3**). However, correspondence analysis indicated that bulk soil and rhizospheres overlapped completely when assessing the 698 isolate profiles (**Fig. S4**). Results thus point to a lack of selection of pseudomonads with specific assortments of plant-beneficial properties in the rhizosphere (**Fig. S4**).

**Maize preferential selection among *Pseudomonas* also impacted on distribution of *Pseudomonas* clades**

*rpoD* sequencing was successful for 498 isolates (60% of PR37Y15 isolates, 82% for DK315 isolates and 81% of bulk soil isolates ; i.e. 70% overall) and confirmed their fluorescent *Pseudomonas* status. *rpoD* phylogeny (**Fig. S5**) also enabled affiliation to the *Pseudomonas* subgroups proposed by Mulet *et al.* (2010). Thus, the *rpoD*-sequenced isolates were distributed in 7 of the 10 subgroups. *rrs* sequencing was carried out on the 210 remaining isolates to confirm them as *Pseudomonas* spp., but did not enable affiliation to the *Pseudomonas* subgroups (i.e. they were thus referred as unclassified pseudomonads). Strains from the *P. fluorescens*/*P. mandelii*/*P. jessenii*/*P. koreensis* subgroups (hereafter referred to as clade FMJK) or the *P. corrugata*/*P. protegens*/*P. chlororaphis* subgroups (clade CPC) were present in same relative proportions (40%) in bulk soil, but in the rhizosphere, clade FMJK was retrieved at 60% whereas clade CPC was below 10% (**Fig. 3-A**). Accordingly, the numbers of fluorescent *Pseudomonas* (below 5 log cells/g in bulk soil) reached in the DK315 and PR37Y15 rhizospheres almost 7 log cells/g for clade FMJK versus a significantly lower level of 6 log cells.$g^{-1}$ or less (i.e. significantly lower) for clade CPC (**Fig. 3-B**).

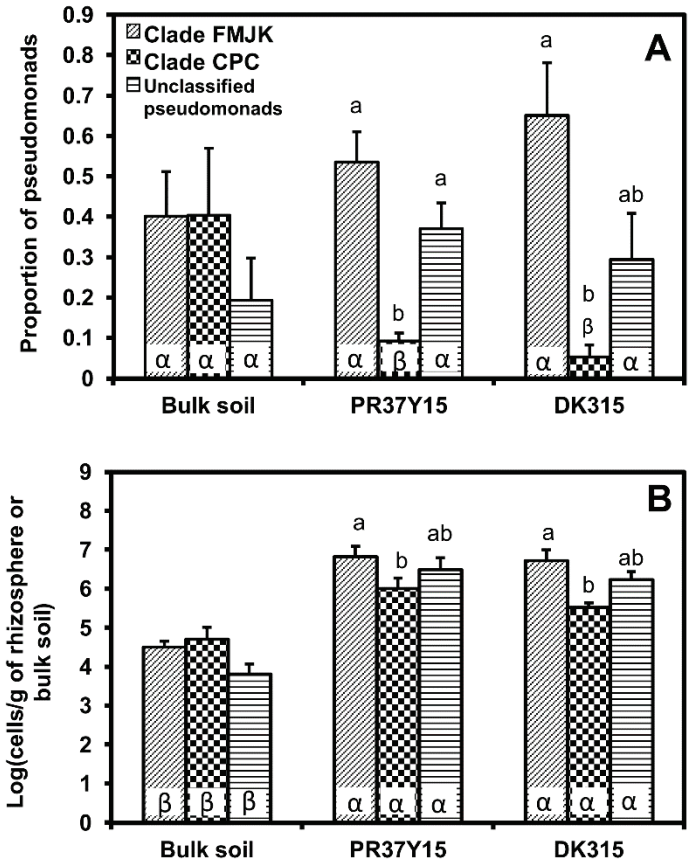


**Fig. 3: Comparison of the proportion (A) and number (B) of pseudomonads belonging to clades FMJK, CPC and unclassified 16S pseudomonads in bulk soil, and in the PR37Y15 and DK315 rhizospheres.** Error bars correspond to standard error. Statistical differences between clades are indicated with letter a-b and statistical differences between conditions (bulk soil/PR37Y15/DK315) are indicated with letter α-β (ANOVA, Fisher's LSD test, $P < 0.05$).

The number of plant-beneficial properties found per strain was significantly lower ($P < 0.001$) in clade FMJK (3.36 ± 0.07 properties; 346 of 367 isolates [i.e. 94%] with one to five plant-beneficial properties) than in clade CPC (5.76 ± 0.13 properties; 45 of 131 isolates [i.e. 34%] with one to five plant-beneficial properties). The proportions of each biocontrol property (i.e. genes for organic antimicrobial compounds DAPG, pyrrolnitrin, pyoluteorin and phenazine, and HCN production) in the pseudomonads were higher ($P < 0.001$ each) in clade CPC than clade FMJK, which shared more diversified patterns of plant-beneficial properties (**Fig. 4**). Contrariwise, properties related to plant hormonal balance modulation (i.e. ACC deaminase and NO genes, and kinetin and indole-3-acetic acid productions) were found in similar proportions in the two clades. Moreover, plant-beneficial properties harbored by clade CPC correspond mainly to biocontrol properties whereas clade FMJK shared a more diversified pattern of plant-beneficial properties. In addition, the proportion of strains with at least one individual biocontrol property or at least one plant hormonal modulation property were negatively correlated, and clades CPC and FMJK could be distinguished (r = -0.93, $P = 0.001$; **Fig. S6**).

In summary, pseudomonads of clade FMJK were more abundant in both DK315 and PR37Y15 rhizospheres and displayed a lower number of co-occurring plant-beneficial properties in comparison with pseudomonads of clade CPC.

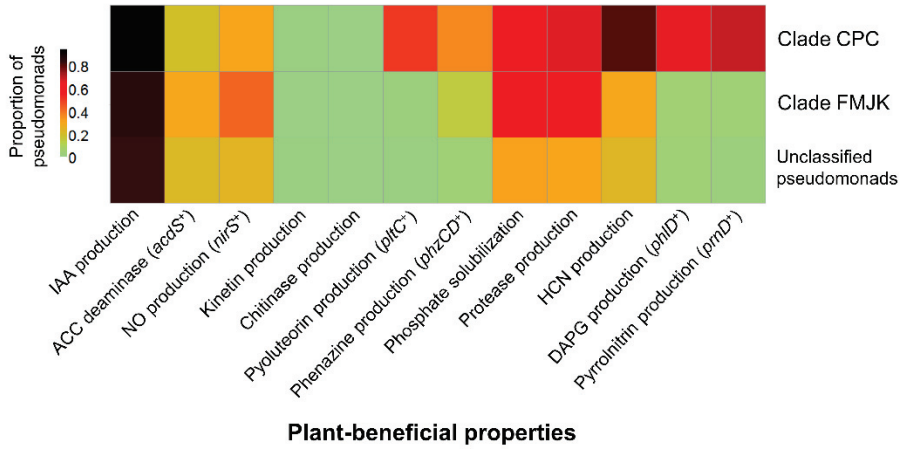


**Fig. 4: Repartition of plant-beneficial properties among pseudomonads clades FMJK, CPC and unclassified 16S pseudomonads.** The heatmap represents the proportion of pseudomonads harboring plant-beneficial properties harbored according to the clade that they belong to.

## Maize preferential selection among *Pseudomonas* was little influenced by strain taxonomy

Analysis of strain proportions in bulk soil and the rhizospheres indicated that clade FMJK pseudomonads effectively colonized the rhizosphere, but clade FMJK isolates with one to five plant-beneficial properties were more selected than clade FMJK strains with six to nine plant-beneficial properties ($P < 0.001$) (**Fig. 5A**). This was also the case when considering population densities (**Fig. 5B**). Indeed, numbers of *Pseudomonas* from clade FMJK harboring one to five plant-beneficial properties gained more than 2 log units ($P < 0.001$) in the rhizospheres of both maize cultivar compared to bulk soil, whereas for clade FMJK pseudomonads harboring six to nine plant-beneficial properties the difference between rhizosphere and bulk soil (only 1 to 1.5 log units) was not significant ($P = 0.89$).

In parallel, proportions of isolates belonging to clade CPC with six to nine plant-beneficial properties were smaller in the rhizospheres of both cultivars ($P = 0.002$) than in bulk soil, whereas those with one to five plant-beneficial properties were found in similar proportions in bulk and rhizosphere soils ($P = 0.28$) (**Fig. 5C**). When considering population densities (**Fig. 5D**), numbers of the clade CPC isolates with six to nine plant-beneficial properties were similar in bulk soil and the rhizospheres ($P = 0.55$), whereas numbers of the clade CPC strains with one to five plant-beneficial properties were similar in the DK315 rhizospheres ($P = 0.87$) but significantly higher (by more than 2 log units) in the PR37Y15 rhizospheres ($P = 0.040$) compared with bulk soil.

In conclusion, the selection of *Pseudomonas* strains in the maize rhizosphere depended more on the number of plant-beneficial properties harbored than the clade they belonged to.

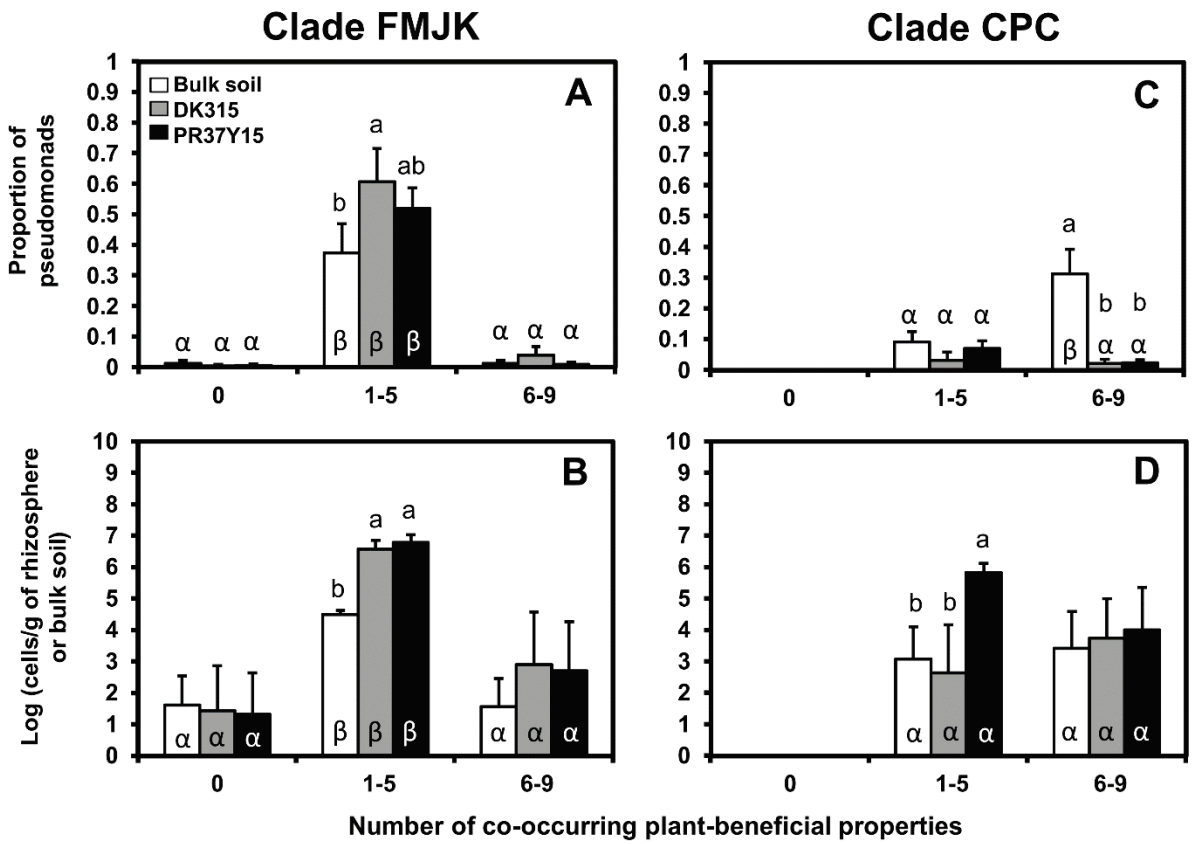


**Fig. 5: Comparison of the proportion (A, C) and number (B, D) of pseudomonads belonging to clades FMJK (A, B) and CPC (C, D) in bulk soil, and in the PR37Y15 and DK315 rhizospheres according to the number of co-occurring plant-beneficial properties they harbored.** Error bars correspond to standard error. Statistical differences between plant-beneficial property classes according to conditions (bulk soil/PR37Y15/DK315) are indicated with letter α-β (ANOVA, Fisher's LSD test, $P<0.05$). Statistical differences between conditions within plant-beneficial property class are indicated with letter a-b.

## Maize rhizosphere effect had no effect on the relative amount of *Pseudomonas* spp. in the community

Euclidean cluster analysis of V4 bacterial *rrs* sequences obtained from bulk soil and maize rhizospheres at 21 days after sowing evidenced Proteobacteria as the most abundant phylum (41-55%) in all four soils, regardless of whether bulk or rhizosphere soil was considered, followed by Bacteroidetes (11-16%), Actinobacteria (7-12%) and Verrucomicrobia (6-14%) (**Fig. S7**). As expected, the bacterial community differed when comparing rhizosphere vs bulk soil, or the two cultivars. The relative abundance of *Pseudomonas* spp. did not differ between treatments, as *Pseudomonas* OTUs amounted to 1.11 ± 0.45 % in bulk soil, 1.35 ± 0.39 % in PR37Y15 rhizosphere, and 1.09 ± 0.29 % in DK315 rhizosphere. Therefore, maize preferential selection for *Pseudomonas* with one to five plant-beneficial properties took place while the relative abundance of the genus remained unchanged within the whole bacterial community.

## DISCUSSION

The fluorescent *Pseudomonas* are key models to assess beneficial plant-bacteria interactions, because they display a wide range of plant-beneficial properties and play an important role in the rhizosphere, including in disease-suppressive soils (Weller and Cook, 1983; Lemanceau and Alabouvette, 1991; O'Sullivan and O'Gara, 1992; Bakker *et al.*, 2007; Barret *et al.*, 2009; Almario, Muller, *et al.*, 2014). A given *Pseudomonas* PGPR strain generally displays many different modes of action on the plant, which is thought important to maximize plant benefits and has resulted in PGPR screening programs where effective strains with several plant-beneficial modes of action were being sought for. However, contrarily to expectations, we found that *Pseudomonas* rhizobacteria with high numbers (> 5) of plant-beneficial properties were not favored for colonization of the maize rhizosphere, as they were outcompeted by counterparts with lower numbers (1 to 5) of these properties. Therefore, this questions the current selection of *Pseudomonas* inoculants with high numbers (> 5) of plant-beneficial properties, as their high effectiveness in short term greenhouse trials may be counterbalanced by insufficient rhizosphere survival under field conditions, an issue long identified (Weller, 1988).

The current finding is hard to track back to literature data, as direct comparisons of *Pseudomonas* strains with 1-5 vs 6-9 plant-beneficial properties are not available. A trend for high-property-number strain CHA0 not colonizing maize as well as strain F113 that possesses lower property number upon seed inoculation was found by quantitative PCR, but it was not significant in short-term experiments (Von Felten *et al.*, 2010). *P. protegens* CHA0 survived poorly in the rhizosphere of well-established maize and wheat (Troxler *et al.*, 1997) and was seldom evidenced by *phlD* PCR-DGGE even in its habitat of origin, i.e. roots of tobacco grown in Morens suppressive soils (Frapolli *et al.*, 2010). In contrast, pseudomonads closely related to strain F113 were prominent in the strawberry rhizosphere based on *rrs* PCR-DGGE (Costa *et al.*, 2006) and *gacA* PCR-DGGE (Costa *et al.*, 2007), whereas pseudomonads related to *P. protegens* CHA0 were not found.

Eighteen contrasted plant-beneficial properties were screened in the *Pseudomonas* collection and 12 were evidenced. Auxin production is widespread in fluorescent *Pseudomonas* (Picard *et al.*, 2004; Picard and Bosco, 2005), as was confirmed here, but (i) it is often deduced from the Salkowski colorimetric assay, which responds also to other indolic compounds (Glickmann and Dessaux, 1995; Szkop and Bielawski, 2012), and (ii) different auxins may play different roles in plant (Simon and Petrášek, 2011). Therefore, a HPLC approach coupled with a diode array detector was implemented to detect the types of auxins (and cytokinins), which showed that only indole-3-acetic acid (and the cytokinin kinetin) was produced by pseudomonads. The gene *nifH*, which occurs in certain *Pseudomonas* strains (Mirza *et al.*, 2006), was not evidenced here.

The distribution of plant-beneficial properties in pseudomonads was not random, as co-occurrence patterns were found. This concerned *acdS* (ACC deaminase) and *nirS* (nitric oxide), both involved in the modulation of plant hormonal balance and plant defenses (Wendehenne *et al.*, 2004; Glick, 2005), which suggests possible fine tuning of plant hormonal conditions by rhizosphere *Pseudomonas* populations. It was also the case for *acdS* and indole-3-acetic acid, in line with the proposition that auxins stimulate ACC synthase activity in roots (Glick *et al.*,

1998). Finally, the co-occurrence of genes for production of organic antimicrobial compounds, which is well documented in model strains (Ramette *et al.*, 2003; Haas and Défago, 2005; Raaijmakers *et al.*, 2006; Mazurier *et al.*, 2009), raises the possibility of additive or even synergistic effects in phytoprotection (Clifford *et al.*, 2015).

A broader study analyzed the distribution of plant-beneficial properties among a total of 304 genomes of Proteobacteria with different ecologies (Bruto *et al.*, 2014). Among the 25 PGPR sequenced genomes, the co-occurrence between *phl* and *hcn* genes was found. In contrast, no relation was described between the *acdS* gene and *nirK* gene involved in NO production, regardless bacterial ecology (Bruto *et al.*, 2014). This can be explained by the fact that our study targeted a lower taxonomic level (i.e. *Pseudomonas fluorescens* group) and the identification of specific co-occurrence between plant-beneficial properties may be possible in a bacterial group due to phylogenetic signal phenomenon (Blomberg *et al.*, 2001; Bruto *et al.*, 2014).

In this work, differences of plant-beneficial property distributions were found between (i) the four soils (**Fig. S1**), (ii) bulk soil and rhizosphere, and (iii) the two maize cultivars, as could be expected in relation to documented rhizosphere effects (Picard & Bosco, 2005; İnceoğlu *et al.*, 2012; Marschner *et al.*, 2001; Berg & Smalla, 2009). The cultivar comparison is of particular interest since DK315 responds poorly to inoculation with plant-beneficial bacteria and fungi whereas PR37Y15 is well responsive (Walker *et al.*, 2011). The plant-beneficial profiles harbored by pseudomonads in DK315 rhizosphere were less diversified than those found in PR37Y15 rhizosphere (**Fig. S3**). Moreover, the pseudomonads proportions of *phlD*$^+$, *prnD*$^+$, *pltC*$^+$ and *phzCD*$^+$, were higher in PR37Y15 rhizosphere than in DK315 rhizosphere.

Among the 7 subgroups (Mulet *et al.*, 2010) evidenced here for the *P. fluorescens* group, the four subgroups forming clade FMJK included pseudomonads with a lower number of co-occurring plant-beneficial properties compared with the three subgroups forming clade CPC, in which biocontrol properties were more prevalent. It is interesting to note that the lower rhizosphere competitiveness of CPC pseudomonads affected particularly strains with 6-9 plant-beneficial properties in comparison with CPC pseudomonads with 1-5 of these properties. Results suggest that there is a trade-off between rhizosphere colonization and the ability to maintain a large number of plant-beneficial properties in the rhizosphere environment, where competition for rhizodeposits and other root exudates is high (Bais *et al.*, 2006). This may have functional implications, as the two *Pseudomonas* clades tend to implement different arrays of plant-beneficial effects (phytostimulation vs. biocontrol) in the rhizosphere, and it is tempting to speculate that their joint contributions may be useful to optimize symbiotic benefits for root system functioning. This is in line with co-inoculation experiment of different bacterial and/or fungal strains which offer a functional complementarity between plant-beneficial actions they display (Srivastava *et al.*, 2010; Combes-Meynet *et al.*, 2011; Couillerot *et al.*, 2012). Indeed, the inoculation of consortia may also favor other symbioses in the rhizosphere, as the inoculation of two pseudomonads increased the number of rhizobial root nodules and improved symbiotic performance (Egamberdieva *et al.*, 2010).

This report suggests that plants shape the composition of *Pseudomonas* populations by preferentially selecting pseudomonads harboring one to five plant-beneficial properties. This is

the first study that characterizes fluorescent *Pseudomonas* populations in the rhizosphere according to the type and number of plant-beneficial properties they harbor.

## EXPERIMENTAL PROCEDURES

### Soils

Four soils were collected in 2012: MS8 from Morens, county Fribourg, Switzerland (46° 52' 04.77'' N 6° 54' 15.83'' E), Bmo1 from Béligneux, Ain, France (45° 52' 22.28" N 5° 7' 53.21" E), Ysa5 and Ysa8 from Seyssel, Savoie region, France (respectively 45° 57' 2.42" N 5°51' 11.44" E and 45° 58' 30.42" N 5° 51' 2.43"E). MS8 and Bmo1 have a morainic geomorphological origin whereas Ysa5 and Ysa8 developed on sandstone material. Bmo1 and Ysa5 were cultivated with maize for at least 3 years, MS8 was an artificial grassland for at least 2 years, and Ysa8 is a natural grassland. Soils were taken from 10–30 cm depth (Table S1) at three locations 5–10 m apart, and were sieved at 0.5 mm.

### Plant experiment

Seeds of hybrid maize varieties DK315 (Monsanto SAS/Dekalb, USA) and PR37Y15 (Pioneer Semences SAS, France) were surface-sterilized by soaking one hour in sodium hypochlorite and one wash in 70% ethanol, and were rinsed three times with sterile distilled water. Three seeds of each maize variety were sown each in 2-$dm^3$ pots (2000 fresh g soil/pot; 3pots/cultivar) and soil water content was maintained at 20% w/w. Unplanted pots (n = 1) served as controls. Twenty-one days after sowing, rhizosphere soils and bulk soils were sampled (i.e. 4 root-adhering soils × 2 cultivars and 4 bulk soils). The root systems were shaken vigorously and rhizosphere extracts were prepared by putting each root system with adhering soil in 20 mL of 0.9% NaCl solution and shaking one hour at 150 rpm. Four bulk soil extracts were obtained using 10 g soil (in 20 mL).

### Metagenomic analysis and bioinformatic processing

The eight root systems and the four bulk soils were recovered and disposed in a 50mL Falcon tube and soaked in liquid nitrogen. Root systems or bulk soil were lyophilized and scratched. DNA extractions from 500mg of rhizosphere soils or bulk soil were performed using a FastDNA™ SPIN Kit for Soil kit (MP Biomedicals, USA). PCR primers 515/806 with barcode on the forward primer were chosen for the V4 variable region of the 16S rRNA gene *rrs* in a 30-cycle PCR (5 cycles implemented on PCR products), using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, with a final elongation step at 72°C for 5 min. PCR products were checked in 2% agarose gel to determine amplification success and relative band intensity. The 12 samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads and used to prepare a DNA library following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com; Shallowater, USA) on a MiSeq following the manufacturer's guidelines. Sequence data were

processed using the analysis pipeline of MR DNA. Briefly, sequences were depleted of barcodes, sequences <150 bp or with ambiguous base calls removed, the remaining sequences denoised, chimeras removed and operational taxonomic units (OTUs; defined at 3% divergence threshold) generated. Final OTUs were taxonomically classified using BLASTn against a curated database derived from Greengenes (DeSantis *et al.*, 2006), RDPII (http://rdp.cme.msu.edu) and NCBI (www.ncbi.nlm.nih.gov). Singleton assigned to OTUs were discarded from the analysis if not found in at least two different conditions, and rarefaction curves were done to estimate sample coverage. Sub-sampling was carried out to obtain the same number of sequences per condition (n = 38352).

**Isolation and characterization of *Pseudomonas* isolates**

For isolation of fluorescent *Pseudomonas*, the eight rhizosphere and four bulk soil extracts were serially diluted and 20 µL was mixed with 180 µL of King's $B^{+++}$ (Simon and Ridge, 1974) in 96-well microtiter plate following a most probable number (MPN) design with eight wells per dilution. Aliquots from each last positive well were plated on King'$B^{+++}$ agar. About 50 isolates were randomly selected for each of the 12 conditions (i.e. 4 soils × [2 cultivars and bulk soil]) and all colonies were purified three times, giving a total of 698 isolates. Genomic DNA was extracted for all isolates using NucleoSpin® 96 Tissue kits (Macherey Nagel, Germany) and identification performed by sequencing the housekeeping gene *rpoD* (accession numbers LN885567 to LN886065, EMBL-EBI database) using primers rpoDf/rpoDr (Frapolli *et al.*, 2007). When *rpoD* amplification failed, *rrs* was amplified with pA/pH (Edwards *et al.*, 1989) and sequenced (accession numbers: LN885368 to LN885566, EMBL-EBI database). *rpoD* sequences were aligned with MUSCLE (Edgar, 2004). Sequences were manually filtered to discard gaps and aligned regions of low quality. The phylogenetic trees were inferred with PHYML (Guindon *et al.*, 2010) with the GTR model and 500 bootstraps. Isolate redundancy was estimated at 30%, according to *rpoD* sequence similarity and functional profiles obtained.

**Screening of *Pseudomonas* isolates for plant-beneficial properties**

A total of 18 plant-beneficial properties were targeted. Molecular screening for plant-beneficial properties was performed by PCR targeting genes involved in production of 2,4 diacetylphloroglucinol (*phlD*), pyrrolnitrin (*prnD*), pyoluteorin (*pltC*), phenazines (overlapping region of *phzC*/*phzD*) and NO (*nirS*), as well as ACC deamination (*acdS*) and nitrogen fixation (*nifH*). All amplifications were performed with a thermocycler Mastercycler (Eppendorf, Germany). The reaction volumes contained 10× PCR buffer, 50 mM $MgCl_2$, 2 mM dNTP, 5% DMSO, 10 µM of each primer (Table S2), 1 unit of Taq polymerase (Invitrogen, Cergy-Pontoise, France) and 0.5 µL of DNA. Several strains harboring these genes were verified by sequencing and activity assay.

Screening for plant-beneficial activities targeted phosphate solubilizing activity, by measuring the degradation halo on a National Botanical Research Institute's Phosphate (NBRIP) agar after 6 days at 28°C, according to (Meyer *et al.*, 2011). Hydrogen cyanide production (indicated by color orange to red) was assessed after growth (3 days at 28°C) on King's $B^{+++}$ agar, using a Whatman filter paper n°1 previously soaked in 2% sodium carbonate

in 0.5% picric acid solution and placed in the lid of the Petri dish (subsequently sealed with parafilm). Production of extracellular protease and chitinase was assessed using respectively milk agar and minimum medium supplemented with colloidal chitin (Kim *et al.*, 2003).

Screening for production of the two auxinic phytohormones indole-3-acetic acid (IAA) and indol-3-butyric acid (IBA) and the five cytokinin phytohormones trans-zeatin, trans-zeatin riboside (ZR), kinetin, 6-benzylaminopurine (BAP) and isopentenyl adenosine (IPA) was done by UHPLC. Briefly, all isolates were grown in 2 mL of King's B medium supplemented with 250 mM of auxin precursor tryptophan and 0.1 mM of cytokinin precursor adenine (2 days at 28°C, 300 rpm). The cultures were centrifuged at 4500 rpm during 8 minutes and filtered at 0.2 µm. Supernatants were subjected to UHPLC separation on an Agilent 1290 Series instrument using a 100 × 3 mm reverse phase column (Agilent Poroshell 120 EC-C18, 2.7 µm particle size) with a diode array detector. Samples (10 µl) were loaded onto the column equilibrated with water and acetonitrile (98:2). Compounds were eluted by a two-step gradient increasing the acetonitrile concentration to 40% over a 6 min period, then to 100% over 4 min, followed by an isocratic step of 2 min, at a flow rate of 0.5 $ml.min^{-1}$. Hormones were detected with an Agilent 6530 Q-TOF mass spectrometer in positive electrospray ionization, based on comparison with commercial standards on both mass and UV (280 nm) chromatograms, along with accurate mass and UV spectra.

**Statistical analysis**

Heatmaps were analyzed using R "pheatmap" package (Kolde, 2012). Clustering analysis was performed using Euclidean distance method or Spearman correlation. For each condition, data were log-transformed for normal distribution and variances homogeneity and a two-way ANOVA and Tukey's HSD test were performed to detect soil or variety impact on fluorescent *Pseudomonas* population size. *Pseudomonas* proportions were compared with a $\chi^2$ test or Fisher's exact test when the expected values in any of the cells of a contingency table are below 5. *Pseudomonas* numbers were compared performing ANOVA and Fisher's LSD test. All analyses were performed at $P < 0.05$, using R software (R development Core Team, 2011). Results in text and figures are presented as means ± standard error.

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## SUPPLEMENTARY DATA

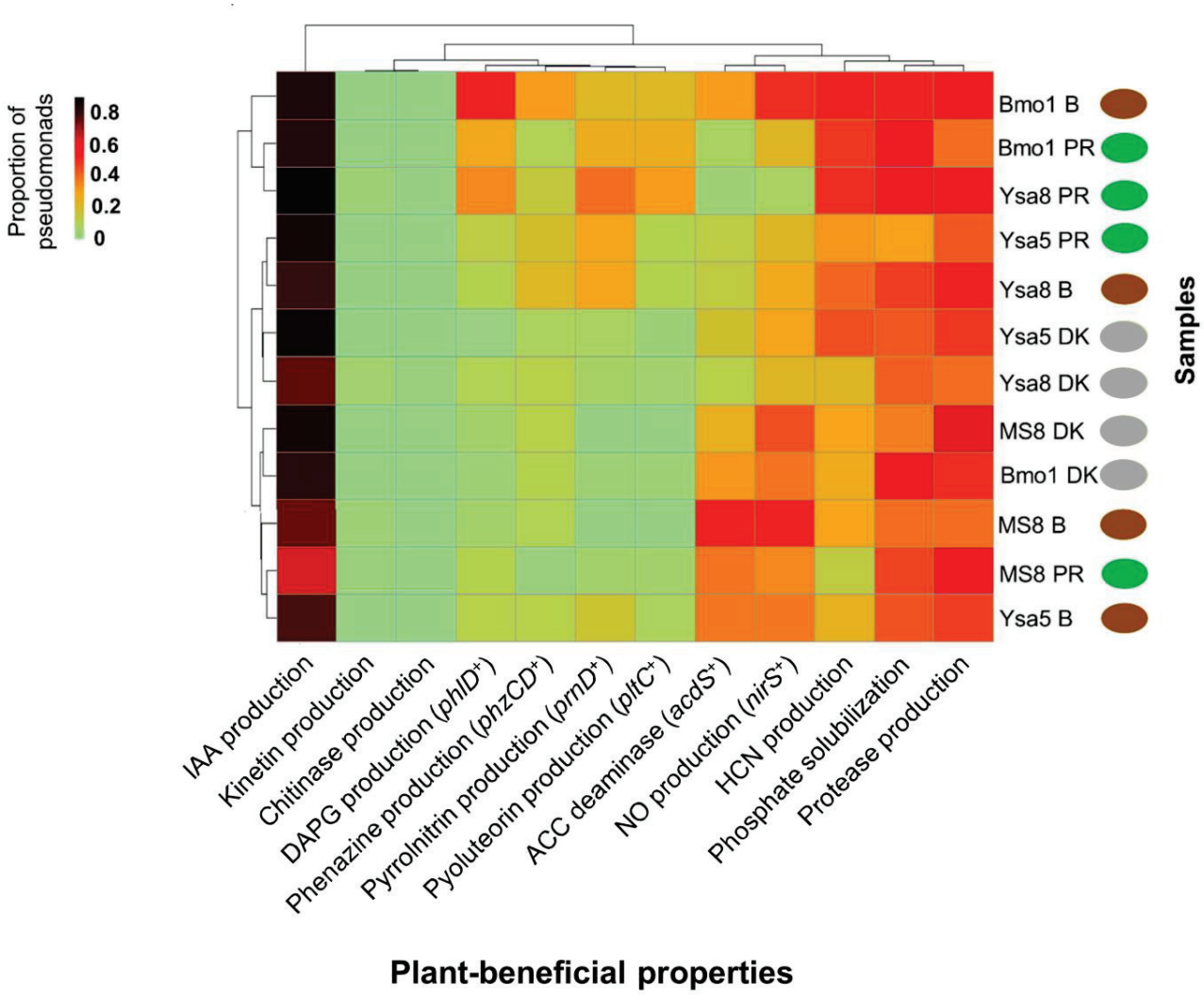



**Fig. S1: Prevalence of plant-beneficial properties in pseudomonads in bulk soil and rhizosphere of maize cultivars DK315 and PR37Y15 at 21 days in soils Bmo1, Ysa5, MS8 and Ysa8.** The heatmap represents the proportion of fluorescent pseudomonads harboring 12 plant-beneficial properties. Row clustering was performed using the Euclidean method, and column clustering using the correlation method. B, Bulk soil (brown circles) ; PR, cultivar PR37Y15 (green circles) ; DK, cultivar DK315 (grey circles). Compared with DK315 rhizosphere, the occurrence of *Pseudomonas* plant-beneficial properties in PR37Y15 rhizosphere was higher for the pyoluteorin gene *plt* and HCN production in soils Bmo1 and Ysa8 ($P < 0.010$), pyrrolnitrin gene *prnD* and DAPG gene *phlD* in all soils except MS8 ($P < 0.010$), and auxin production in soil Ysa8 ($P = 0.010$), and it was lower for ACC deaminase gene *acdS* in soil Bmo1 ($P < 0.010$) and auxin production in soil MS8 ($P < 0.001$) ($\chi^2$ tests, $P < 0.05$).


**Fig. S2: Co-occurrence of plant-beneficial properties harbored by pseudomonads.** For each comparison, Pearson correlation coefficient r and *P* value (with a red star when $P < 0.05$) are indicated.

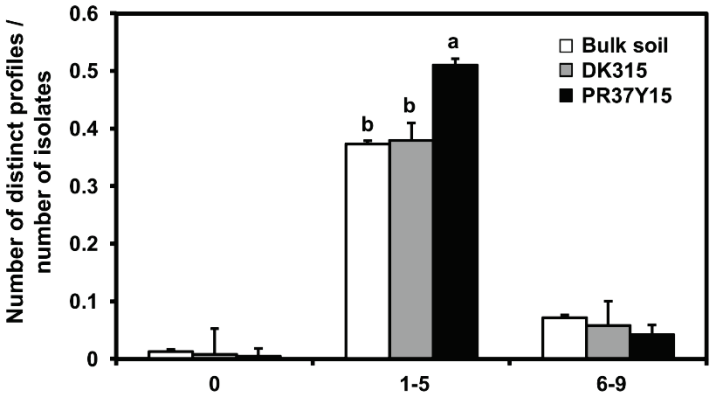



**Fig. S3: Functional profiles of *Pseudomonas* isolates according to the number of co-occurring plant-beneficial properties harbored, in bulk soil and in the rhizosphere of maize cultivars DK315 and PR37Y15.** Data from soils Bmo1, Ysa5, MS8 and Ysa8 were combined and error bars (standard errors) reflect the variability among the four soils. Error bars correspond to standard error. Statistical differences are indicated with letter a-b (ANOVA, Fisher's LSD test, $P < 0.05$).

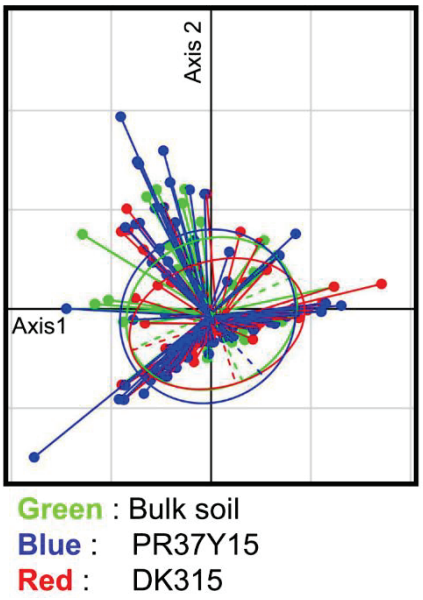



**Fig. S4: Factorial correspondence analysis (FCA) showing the repartition of the 698 functional profiles of *Pseudomonas* isolates according to conditions (bulk soil, rhizospheres PR37Y15, and DK315).** Axis 1 and axis 2 correspond to respectively 20.6 % and 19.1% of the total variance. Each point of the FCA corresponds to the profile of one isolate. Circles correspond to confidence ellipses.

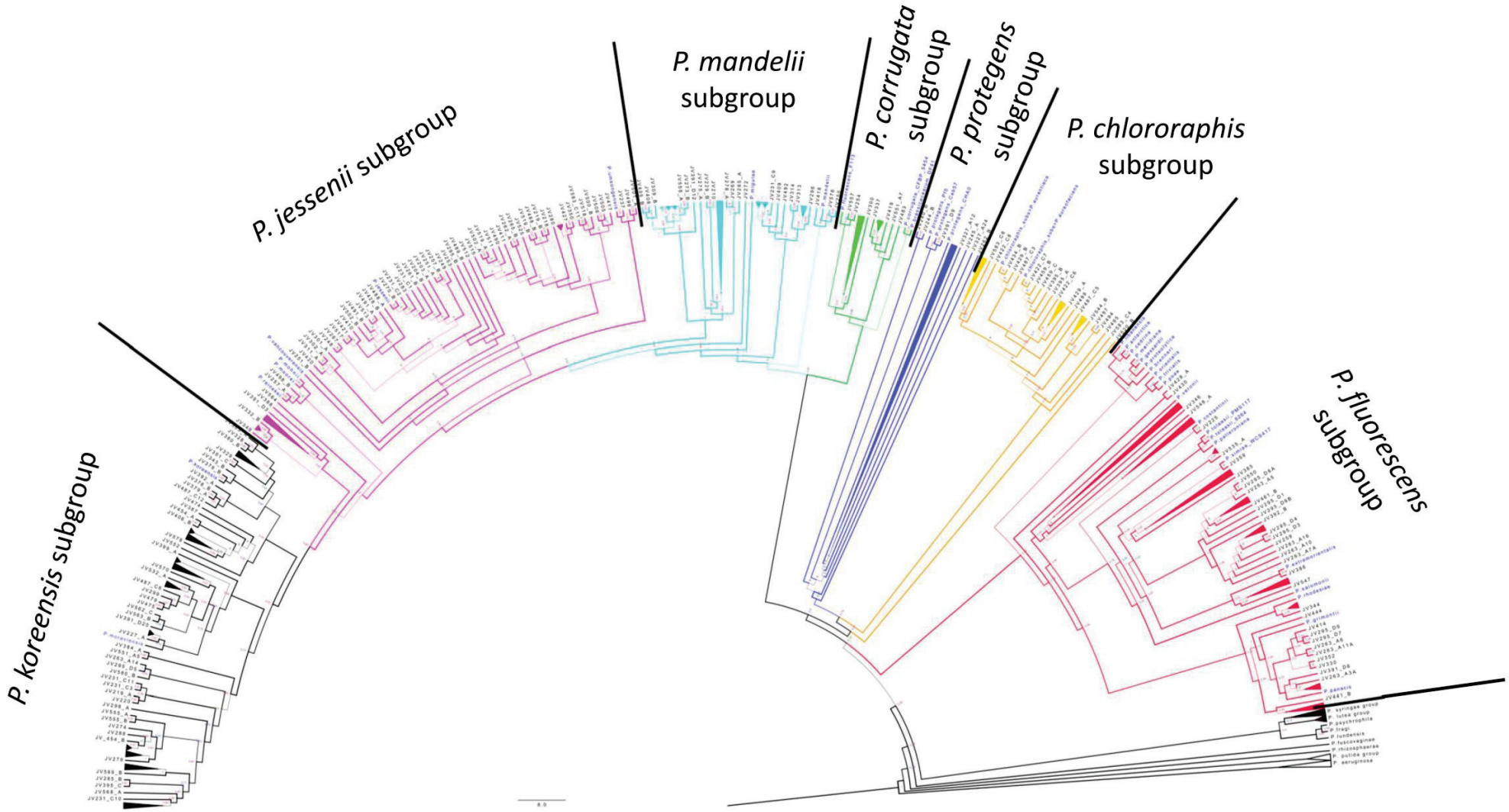



**Fig. S5: Phylogenetic tree of 498 isolates taxonomically characterized based on their *rpoD* sequence.** The phylogenetic tree analysis was based on the *rpoD* housekeeping gene, from 30 type strains (Mulet *et al.*, 2010), 9 non-type strains and a clade of 7 *P. aeruginosa* type strains (used for tree rooting). Other *Pseudomonas* groups such as the *P. syringae*, *P. putida* and *P. lutea* groups, as well as non-clustered *Pseudomonas* are represented. The maximum likelihood tree was inferred using PhyML and the GTR model, and nodal robustness was assessed using 500 bootstrap replicates. Red: *P. fluorescens* subgroup; Orange: *P. chlororaphis* subgroup; Blue: *P. protegens* subgroup; Green: *P. corrugata* subgroup; Light blue: *P. mandelii* subgroup; Pink: *P. jesseni* subgroup; Black: *P. koreenensis* subgroup.

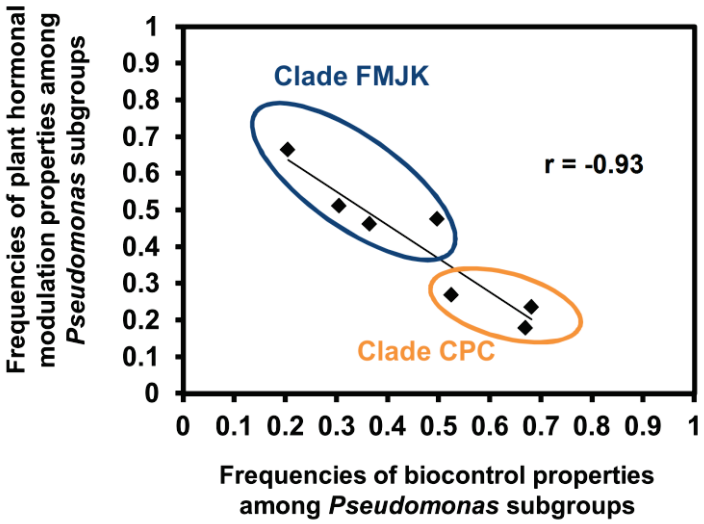



**Fig. S6: Relation between the proportion of pseudomonads harboring biocontrol properties and plant hormonal modulation properties according to the taxonomic subgroup they belong to.** A negative correlation was established according to the Pearson correlation test ($P$ = 0.001). Blue circle regrouped the FMJK clade and the orange one, the CPC clade.

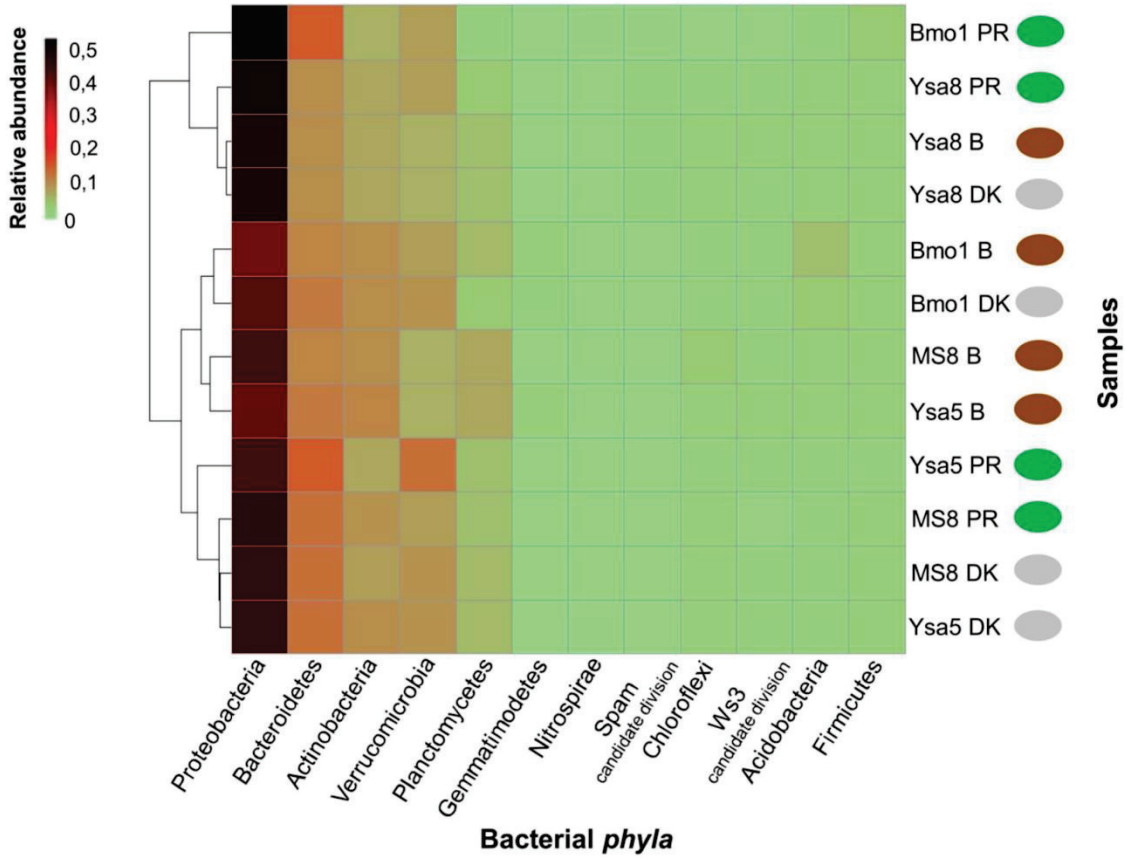



**Fig. S7: Relative abundance of bacterial *phyla* in bulk soil and rhizosphere of maize cultivars DK315 and PR37Y15 at 21 days in soils Bmo1, Ysa5, MS8 and Ysa8.** The heatmap represents the proportion of OTUs at the phylum level. Row clustering was performed using the Euclidean method. B, Bulk soil (brown circles); PR, cultivar PR37Y15 (green circles); DK, cultivar DK315 (grey circles).

**Table S1: Physical and chemical characteristics of soils used in this study**

| | MS8 | Ysa5 | Ysa8 | Bmo1 |
|---|---|---|---|---|
| Location | Morens (Switzerland) | Seyssel (France) | Seyssel (France) | Béligneux (France) |
| Vegetative cover | Grassland | Maize | Natural meadow | Maize |
| Land use | Maize for at least 2 years | Maize for at least 2 years | Never cultivated | Maize for at least 2 years |
| Geologic origin | Morainic | Sandstone | Sandstone | Morainic |
| Textural class | Sandy loam | Sandy loam | Loamy sand | Loamy sand |
| Clay (%) | 10.7 | 17.8 | 18.5 | 11.5 |
| Loam (%) | 29.6 | 25.9 | 25.5 | 26.2 |
| Sand (%) | 59.7 | 56.3 | 55.9 | 62.4 |
| pH (water) | 7.8 | 7.9 | 7.88 | 6.6 |
| Organic matter (%) | 1.3 | 2.2 | 3.8 | 2.4 |
| $CaCO_3$ total (%) | 6 | 2.8 | 5.8 | 1.4 |
| N total (%) | 0.13 | 0.16 | 0.17 | 0.17 |
| P total ($mg.kg^{-1}$) | 750 | 382 | 54 | 322 |
| K total ($mg.kg^{-1}$) | 150 | 223 | 136 | 158 |
| Ca total ($g.kg^{-1}$) | 8.47 | 6.86 | 8.05 | 2.14 |
| Mg total ($mg.kg^{-1}$) | 103 | 184 | 231 | 72 |
| Fe [$C_2H_4O_2$ 1:10] ($mg.kg^{-1}$) | 230 | 32.2 | 141 | ND |

**Table S2: List of primers used in this study**

| Traits | Targeted genes | Primers | Sequences [a] | References |
|---|---|---|---|---|
| DAPG production | *phlD* | B2BF | ACC CAC CGC AGC ATC GTT TAT GAG C | McSpadden Gardener *et al.* 2001 |
| | | BPR4 | CCG CCG GTA TGG AAG ATG AAA AAG TC | |
| ACC deaminase activity | *acdS* | accF5 | GGC AAC AAG MYS CGC AAG CT | Bouffaud (2011) |
| | | accR8 | CTG CAC SAG SAC GCA CTT CA | |
| Nitrogen fixation | *nifH* | PolF | TGC GAY CCS AAR GCB GAC TC | Poly *et al.* 2001 |
| | | PolR | ATS GCC ATC ATY TCR CCG GA | |
| NO production | *nirS* | cd3a | GTS AAC GTS AAG GAR ACS GG | Throbäck *et al.* 2004 |
| | | R3cd1322 | GAS TTC GGR TGS GTC TTG A | |
| Pyrrolnitrin production | *prnD* | prnD1 | GGG GCG GGC CGT GGT GAT GGA | de Souza *et al.* 2003 |
| | | prnD2 | YCC CGC SGC CTG YCT GGT CTG | |
| Pyoluteorin production | *pltC* | pltC1 | AAC AGA TCG CCC CGG TAC AGA ACG | de Souza *et al.* 2003 |
| | | pltC2 | AGG CCC GGA CAC TCA AGA AAC TCG | |
| Phenazin production | *phzC-phzD* | PHZ1 | AAC AGA TCG CCC CGG TAC AGA ACG | de Souza *et al.* 2003 |
| | | PHZ2 | AGG CCC GGA CAC TCA AGA AAC TCG | |
| Sigma 70 | *rpoD* | rpodf | ACT TCC CTG GCA CGG TTG ACC A | Frapolli *et al.* 2007 |
| | | rpodr | TCG ACA TGC GAC GGT TGA TGT C | |
| 16S ribosomal RNA | *rrs* | pA | AGA GTT TGA TCC TGG CTC AG | Edwards *et al.* 1989 |
| | | pH | AAG GAG GTG ATC CAG CCG CA | |

[a] IUPAC convention used for the description of degenerate DNA sequences : Y = C/T; S = G/C; R = A/G

# CHAPITRE 3

## DISTRIBUTION DES PROPRIETES PHYTOBENEFIQUES PARMI LA LIGNEE DES *PSEUDOMONAS FLUORESCENS*

## ***Préambule chapitre 3***

L'étude de la distribution de propriétés phytobénéfiques, présentée dans le chapitre précédent, a montré que deux clades appartenant au groupe des *P. fluorescens* pouvaient être distingués de part (i) le nombre de propriétés phytobénéfiques qu'ils possèdent, (ii) leur positionnement taxonomique et (iii) leur abondance au sein de la rhizosphère du maïs (**Chapitre 2**). Ainsi parmi les 18 fonctions phytobénéfiques recherchées, les isolats appartenant au clade CPC (composé des sous-groupes *P. chlororaphis, P. protegens et P. corrugata*) comportaient un nombre de propriétés phytobénéfiques plus important que ceux appartenant au clade FMJK (composé des sous-groupes des *P. fluorescens, P. mandelii, P. jesseni et P. koreensis*). Les fonctions retrouvées dans le clade CPC étaient principalement des propriétés impliquées dans le biocontrôle. Cette observation suggère que la distribution des fonctions serait en lien avec la taxonomie des *Pseudomonas* isolés de la rhizosphère du maïs. Etant donné la différence d'abondance de ces deux clades dans la rhizosphère, ceci suggère que des populations assurant certaines fonctions biologiques et de sous-groupes taxonomiques particuliers, pourraient être préférentiellement sélectionnées par la plante.

Afin de vérifier ces hypothèses, les objectifs de ce chapitre ont été (i) de dresser la phylogénie précise des *Pseudomonas* fluorescents afin (ii) de déterminer la distribution des propriétés phytobénéfiques au sein des groupes de *P. fluorescens* et *P. putida*, (iii) de mettre en évidence un lien entre la taxonomie des *Pseudomonas* et les propriétés phytobénéfiques possédées et (iv) d'évaluer si les fonctions phytobénéfiques associées préférentiellement à certains clades de *Pseudomonas* confèrent à ces derniers une meilleure efficacité écologique (phytostimulation/antagonisme envers des champignons phytopathogènes).

Afin de répondre à ces objectifs, une banque de génomes de *Pseudomonas* a été établie (à la date du 4 Septembre 2015), contenant 114 génomes appartenant aux groupes des *P. fluorescens* et des *P. putida*. L'ensemble des génomes a été replacé phylogénétiquement et 110 séquences de protéines ciblant des propriétés phytobénéfiques impliquées directement ou indirectement dans la mise en place d'un effet phytobénéfique ont été recherchées. 9 souches issues de la banque constituée dans le chapitre précédent et appartenant aux clades CPC, FMJK et au groupe des *P. putida* ont été séquencées et testées pour leur capacité à stimuler la croissance de la plante et à inhiber la croissance de microorganismes phytopathogènes *in vitro*.

Dans un premier temps, des incongruences ont été mises en évidence entre les noms d'espèces de certaines souches et leur position taxonomique au sein de la reconstruction phylogénique établie dans ce travail. De plus, nous proposons une révision de certains sous-groupes précédemment définis par Mulet *et al.* 2010 tel le sous-groupe des *P. chlororaphis* que nous proposons de séparer en celui des *P. protegens* et celui des *P. chlororaphis sensu-stricto*. Ce travail met en avant que (i) le nombre et (ii) le type de propriétés phytobénéfiques sont intimement liés à la taxonomie des *Pseudomonas*. De plus, certaines propriétés phytobénéfiques, notamment celles associées à la production de métabolites secondaires, semblent être plus souvent retrouvées dans le clade CPC, témoignant de potentielles acquisitions de ces propriétés par des ancêtres communs aux sous-groupes constituant ce clade. Toutefois, l'association préférentielle des propriétés phytobénéfiques à certains sous-groupes taxonomiques, n'est pas toujours rattachée à une meilleure efficacité de phytostimulation ou de biocontrôle, dans les conditions testées.

# Distribution des propriétés phytobénéfiques parmi les *Pseudomonas* fluorescents

## INTRODUCTION

Il y a plus d'un siècle, le genre *Pseudomonas* (Migula, 1894) avait une délimitation très large qui regroupait une majorité de bactéries qui répondaient aux critères suivants : bactéries aérobies strictes, sous forme de bacille et à coloration de Gram négative. La classification des individus appartenant à ce genre bactérien a depuis considérablement évolué avec l'apparition des techniques de classification bactérienne. La phylogénie des *Pseudomonas* a été réalisée en utilisant différents marqueurs moléculaires tels les gènes impliqués dans des fonctions cellulaires essentielles (gènes de « ménage »). Ainsi, l'une des premières phylogénies de *Pseudomonas* a été obtenue en utilisant le gène *rrs* codant l'ARNr 16S et a permis le reclassement de nombreuses espèces auparavant affiliées au genre *Pseudomonas* dans d'autres genres bactériens (Anzai *et al.*, 2000). D'autres marqueurs moléculaires ont été utilisés afin de retracer l'histoire évolutive des *Pseudomonas*, comme le gène *rpoB* (Ait-Tayeb *et al.*, 2005). L'utilisation de plusieurs marqueurs moléculaires par le biais de leur concaténation a alors permis l'accès à une phylogénie plus résolutive des *Pseudomonas* (Yamamoto *et al.*, 2000; Hilario *et al.*, 2004; Frapolli *et al.*, 2007; Mulet *et al.*, 2010; Gomila *et al.*, 2015).

Au sein des *Pseudomonas*, des groupes bactériens ont été établis comme celui des *P. fluorescens* ou encore celui des *P. putida* (Mulet *et al.*, 2010). Parmi ces deux groupes, certains *Pseudomonas* interagissent avec la plante de façon délétère (parasitisme, *P. corrugata* et *P. mediterranea ;* Catara *et al.*, 2002; Trantas *et al.*, 2015) ou bénéfique (coopération ; Ramette *et al.*, 2011). Dans les interaction bénéfiques, les bactéries accomplissent différents rôles, notamment protéger la plante vis-à-vis d'une large gamme de microorganismes phytopathogènes incluant des bactéries (Cronin *et al.*, 1997a), des champignons (Mazurier *et al.*, 2009), des nématodes (Cronin *et al.*, 1997b) ou des arthropodes (Kupferschmied *et al.*, 2013) *via* la production de nombreux métabolites secondaires comme le 2,4-diacétylphloroglucinol (DAPG), l'acide cyanhydrique (HCN), le 2-hexil, 5-propyl résorcinol (HPR), des phénazines (Phz), la pyrrolnitrine (Prn) ou encore la pyolutéorine (Plt) (Gross and Loper, 2009). D'autres *Pseudomonas* possèdent des propriétés phytobénéfiques impliquées dans l'amélioration de la nutrition du végétal comme la solubilisation du phosphate (Meyer *et al.*, 2011) ou encore la fixation libre de l'azote atmosphérique (Mirza *et al.*, 2006). Enfin, certains *Pseudomonas* fluorescents sont capables de moduler la balance hormonale de la plante en produisant des hormones de type auxine (Dimkpa *et al.*, 2012), oxyde nitrique (Molina-Favero *et al.*, 2007) ou encore d'interférer avec le voie de l'éthylène en consommant l'acide 1-aminocyclopropane-1-carboxylique (ACC, précurseur de l'éthylène végétal) grâce à la production de l'ACC désaminase (Redondo-Nieto *et al.*, 2012). L'analyse de la distribution de ces propriétés phytobénéfiques chez les *Pseudomonas* a permis d'identifier que certaines fonctions étaient préférentiellement associées chez certains individus comme par exemple la production de DAPG et d'HCN (Ramette *et al.*, 2003), ou encore la production de DAPG et d'auxine (Picard and Bosco, 2005). Avec une disponibilité toujours croissante du nombre de

génomes de *Pseudomonas* fluorescents, l'exploration du contenu génique de ces derniers est devenue plus rapide et permet dorénavant de dresser des profils potentiels de propriétés phytobénéfiques par clade taxinomique. Ainsi, associer des approches de génomique comparative et de phylogénie, permet l'étude de la distribution de ces propriétés phytobénéfiques de manière plus précise tout en tenant en compte de la proximité taxonomique des individus (Loper *et al.*, 2012) et de leur écologie (Bruto *et al.*, 2014).

Lassalle et collègues (2015) suggèrent que la spéciation des bactéries émergerait suite à des adaptations (acquisition de nouvelles fonctions) à de nouvelle niche écologique. Ces adaptations seraient maintenues dans le génome pour que la nouvelle espèce puisse se maintenir dans son nouvel habitat. Ces acquisitions lorsqu'elles sont très anciennes, sont retrouvées dans les génomes de clade sœur. Or, l'étude de la distribution de propriétés phytobénéfiques précédemment réalisée (**chapitre 2**) a montré que deux clades appartenant au groupe des *P. fluorescens* pouvaient être distingués de part (i) leur positionnement taxonomique, (ii) leur nombre de propriétés phytobénéfiques et de (iii) leur abondance au sein de la rhizosphère du maïs. Ainsi, le clade CPC (composé d'isolats appartenant aux sous-groupes *P. chlororaphis, P. protegens et P. corrugata*) comportait un nombre de propriétés phytobénéfiques plus important que le clade FMJK (regroupant des isolats appartenant aux sous-groupes des *P. fluorescens, P. mandelii, P. jesseni et P. koreensis*). De plus, les fonctions retrouvées dans le clade CPC étaient principalement des propriétés ayant un rôle écologique similaire, impliquées dans le biocontrôle. Notre hypothèse est que la spécialisation des *Pseudomonas* fluorescents vers des interactions de biocontrôle ou de phytostimulation résulterait d'un mécanisme évolutif de spéciation.

Aussi, le but de cette étude a été de déterminer si des spécificités écologiques pouvaient être liées à la taxonomie des *Pseudomonas* en regard des propriétés phytobénéfiques qu'ils possèdent. Pour cela, une banque de génomes de *Pseudomonas* a été constituée et les gènes impliqués dans la production de propriétés phytobénéfiques ont été recherchés *in silico*. L'histoire évolutive de certains gènes codant des propriétés phytobénéfiques a été retracée. En parallèle, 9 *Pseudomonas* isolés dans l'étude précédente (chapitre 2 ; 3 souches appartenant au clade CPC, 3 au clade FMJK, et 3 au groupe des *P. putida*) ont été séquencés et fonctionnellement caractérisés dans le but d'évaluer si les propriétés phytobénéfiques possédées par ces souches leurs conféraient une meilleure action en terme (i) de biocontrôle et/ou de (ii) phytostimulation.

## MATERIELS ET METHODES

### Microorganismes utilisés et conditions de culture

Les souches JV222, JV241A, JV395B, JV414, JV 449, JV497, JV551A1, JV551A3 et JV551A7 proviennent de la banque d'isolats réalisée dans le cadre des travaux présentés dans le chapitre précédent et ont différentes origines (Tableau S0). L'ensemble de ces souches a été cultivé dans du milieu King's B (Simon and Ridge, 1974) à 28°C sous agitation (150rpm).

Trois champignons (*Leptosphaeria maculans, Magnaporthe grisae, Thielaviopsis basicola*) et un oomycète (*Phytophthora alni*) phytopathogènes ont été cultivés sur milieu PDA (Potato Dextrose Agar, Conda) à 21°C.

**Séquençage de génomes et annotation**

L'extraction d'ADN génomique a été réalisée à partir de 4 mL de cultures des souches à l'aide du kit Nucleospin Tissue (Macherey Nagel, Hoerdt, France). Les ADN génomiques (~500ng/µL) ont été envoyés à la société Mr DNA (Mr DNA, Shallowater ; USA) afin de procéder au séquençage de génome par la méthode MiSeq Illumina 2x300pb. L'assemblage a été réalisé en se référant au génome complet et annoté de la bactérie modèle *Pseudomonas fluorescens* F113 (Redondo-Nieto *et al.*, 2012). Les génomes ont été automatiquement annotés *via* la plateforme MaGe. La recherche de gènes pouvant être potentiellement impliqués dans la biosynthèse de métabolites secondaires antimicrobiens a été entreprise sur les 9 souches séquencées en utilisant AntiSMASH 3.0.3 (« *Antibiotics & Secondary Metabolite Analysis Shell* » - Weber *et al.*, 2015). BAGEL 3 (van Heel *et al.*, 2013) a également été utilisé afin de prédire la présence d'éventuels gènes impliqués dans la biosynthèse de bactériocines. La prédiction d'îlots génomiques ainsi que la localisation à l'intérieur de ces îlots de gènes codant des fonctions phytobénéfiques ont également été recherchées *via* l'interface *Island Viewer* 3 (Dhillon *et al.*, 2015).

**Comparaison de génomes et constitution de la banque de génomes**

Un ensemble de 114 génomes de *Pseudomonas* (incluant les 9 génomes séquencés de cette étude et lorsque possible d'au moins 2 génomes représentant chacune des différentes espèces des clades FMJK et CPC, **Tableau 1**) a été sélectionné (sélection arrêtée au 4 Septembre 2015) et analysé. Un total de 110 protéines impliquées dans la production de propriétés phytobénéfiques a été recherché *via* TBLASTN, en prenant une identité protéique entre 40% et 50%, et une couverture de la séquence protéique minimale de 90% (**Tableau S1**). Les valeurs d'ANIb (*Average Nucleotide Identity*) ont été obtenues en utilisant BLAST comme décrit par Goris *et al.* (2007), celles d'ANIm en utilisant MUMmer comme indiqué par (Richter and Rosselló-Móra, 2009) et les fréquences tétranucléotidiques (TETRA) ont été calculées comme décrit par Teeling *et al.*, (2004). L'ensemble des calculs a été réalisé *via* le logiciel JSpecies. Le génome cœur et les gènes spécifiques de chacun des 9 génomes ont été obtenus *via* l'utilisation de la plateforme MaGe (Vallenet *et al.*, 2006).

**Analyses phylogénétiques**

Une analyse phylogénétique des 114 génomes a été réalisée en concaténant 4 gènes de ménage (*rrs*, *rpoD*, *rpoB* et *gyrB*). Les analyses des séquences concaténées ont été effectuées en utilisant l'interface utilisateur graphique multiplateforme SeaView (disponible à http://pbil.univ-lyon1.fr/) (Gouy *et al.*, 2010). Les séquences ont été alignées en utilisant MUSCLE (paramètres par défaut) (Edgar, 2004) et des arbres phylogénétiques ont été construits par la méthode du maximum de vraisemblance PhyML (version 3.0) (Guindon *et al.*, 2010) mais également par la méthode de parcimonie avec pour chacun des arbres 500 itérations.

**Tableau 1 : Souches utilisées dans cette étude**

| Strains | Accession number | Strains | Accession number | Strains | Accession number | Strains | Accession number |
|---|---|---|---|---|---|---|---|
| *E.coli* K12-ER3413 | GI:749198604 | *P.fluorescens* F113 | GI:378947941 | *P.monteilii* SB3101 | GI:567355861 | *Pseudomonas* sp. FGI182 | GI:568237447 |
| *P.abietaniphila* KF701 | GI:757729071 | *P.fluorescens* NZ007 | GI:396976438 | *P.moraviensis* R28-S | GI:597503881 | Pseudomonas sp. GM16 | GI:399016904 |
| *P.abietaniphila* KF717 | GI:752900911 | *P.fluorescens* NZ011 | GI:484123039 | *P.mosselii* SJ10 | GI:684194542 | Pseudomonas sp. GM21 | GI:398998719 |
| *P.agarici* NCPPB2289 | GI:396976195 | *P.fluorescens* PA3G8 | GI:742826561 | *P.parafulva* CRS01-1 | GI:730587126 | *Pseudomonas* sp. GM24 | GI:398992477 |
| *Pseudomonas* sp. JV241A | | *P.fluorescens* PA4C2 | GI:835986192 | *P.parafulva* YAB-1 | GI:902279470 | *Pseudomonas* sp. GM25 | GI:398981962 |
| P.*alkylphenolia* KL28 | GI:754072641 | *P.fluorescens* pf0-1 | GI:255961261 | *Pseudomonas* sp. PCL1751 | GI:763458725 | *Pseudomonas* sp. GM30 | GI:584600151 |
| *Pseudomonas* sp. JV551A7 | | *P.fluorescens* Pf29Arp | GI:662782635 | *P.plecoglossicida* DSM15088 | GI:607832837 | *Pseudomonas* sp. GM33 | GI:398161849 |
| *P.brassicacearum* DF41 | GI:591390487 | *P.fluorescens* Q2-87 | GI:423092759 | *P.plecoglossicida* NyZ12 | GI:752308899 | *Pseudomonas* sp. GM78 | GI:398250356 |
| *P.brassicacearum* NFM421 | GI:327374765 | *P.fluorescens* Q8R1-96 | GI:423694470 | *P.poae* RE*1-1-14 | GI:447915004 | *Pseudomonas* sp. MRSN12121 | GI:764708985 |
| *P.brassicacearum* PA1G7 | GI:742885813 | *P.fluorescens* R124 | GI:424920626 | *P.protegens* Cab57 | GI:751653884 | *Pseudomonas* sp. PAMC25886 | GI:372002201 |
| *P.brassicacearum* PP1-210F | GI:757730208 | *P.fluorescens* SBW25 | GI:229587578 | *P.protegens* CHA0 | GI:500239649 | *Pseudomonas* sp. PTA1 | GI:752854087 |
| *Pseudomonas* sp. JV395B | | *P.fluorescens* SS101 | GI:423689090 | *P.protegens* Os17 | GI:771839907 | *Pseudomonas* sp. R62 | GI:483843579 |
| *Pseudomonas* sp. JV497 | | *P.fragi* A22 | GI:378528797 | *P.protegens* pf-5 | GI:68342549 | *Pseudomonas* sp. TKP | GI:568136993 |
| *P.chlororaphis* 30-84 | GI:425896522 | *P.fragi* B25 | GI:378528907 | *P.protegens* st29 | GI:771846103 | *Pseudomonas* sp URMO17wk12:I12 | GI:654456350 |
| *P.chlororaphis* EA105 | GI:740584220 | *P.fuscovaginae* CB98818 | GI:396582125 | *P.psychrophila* DSM17535 | GI:856785763 | *Pseudomonas* sp UW4 | GI:426265132 |
| *P.chlororaphis* GP72 | GI:483167855 | *P.fuscovaginae* SE-1 | GI:478736782 | *P.psychrophila* HA-4 | GI:398363462 | *Pseudomonas* sp WCS374 | GI:646237114 |
| *P.chlororaphis* JD37 | GI:692336178 | *P.gingeri* NCPPB3146 | GI:396976647 | *Pseudomonas* sp. JV551A3 | | *P.stutzeri* DSM10701 | GI:395806679 |
| *P.chlororaphis* O6 | GI:389686655 | *P.kilonensis* DSM13647 | GI:918043351 | *P.putida* GB1 | GI:166857509 | *P.synxantha* BG33R | GI:391217940 |
| *P.chlororaphis* PA23 | GI:647802220 | *P.kilonensis* P12 | GI:918043348 | *P.putida* KT2440 | GI:26986745 | *P.thivervalensis* DSM13194 | GI:918043517 |
| *P.chlororaphis* PCL1606 | GI:787852299 | *P.lini* DSM16768 | GI:856777579 | *P.putida* PA14H7 | GI:742870580 | *P.thivervalensis* PITR2 | GI:918043659 |
| *P.corrugata* CFBP5454 | GI:575768925 | *P.lini* ZBG1 | GI:910873053 | *P.putida* UK4 | GI:749296184 | *P.tolaasii* 6264 | GI:426278775 |
| *P.corrugata* DSM7228 | GI:918043575 | *P.lundensis* DSM6252 | GI:856778358 | *P.putida* YL-1 | GI:655274719 | *P.tolaasii* PMS117 | GI:394306466 |
| *P.cremoricolorata* DSM17059 | GI:523392906 | *P.luteola* XLDN4-9 | GI:398329031 | *P.rhizosphaerae* DSM16299 | GI:692342259 | *P.trivialis* IHBB745 | GI:902687210 |
| *P.cremoricolorata* ND07 | GI:691224436 | *Pseudomonas* sp. JV414 | | *P.rhodesiae* FF9 | GI:698156157 | *P.umsongensis* 20MFCvi1.1 | GI:481753764 |
| *P.extremaustralis* 14-3substr14-3b | GI:371721769 | *P.mandelii* JR-1 | GI:635284334 | *Pseudomonas* sp. JV449 | | *P.umsongensis* UNC430CL58Col | GI:607821624 |
| *Pseudomonas* sp. JV222 | | *P.mandelii* PD30 | GI:696527166 | *P.simiae* MEB105 | GI:755328371 | *P.veronii* 1YdBTEX2 | GI:474502925 |
| *P.fluorescens* BBC6R8 | GI:562785069 | *P.mediterranea* CFBP5447 | GI:713120176 | *P.simiae* WCS417 | GI:740669164 | *P.veronii* R4 | GI:811166449 |
| *P.fluorescens* A506 | GI:387891255 | *P.mediterranea* TEIC1105 | IMG :2563366524 | *Pseudomonas* sp. JV551A1 | | | |
| *P.fluorescens* BRIP34879 | GI:440382951 | *P.monteilii* SB3078 | GI:567348216 | Pseudomonas sp. Ag1 | GI:395342341 | | |

En rouge : Génomes séquencés dans cette étude

Les séquences concaténées représentent approximativement 9600 nucléotides qui ont pu être comparées entre les différentes souches.

**Test d'antagonisme *in vitro***

Pour chacune des 9 souches, une suspension bactérienne (1µL à $10^8$ cellules en phase stationnaire de croissance) a été striée sur une longueur de 3 cm, à l'aide d'une oëse, à 2cm du centre d'une boîte de Petri contenant du milieu gélosé PDA. En parallèle, un disque mycélien de 6mm de diamètre provenant d'une culture fongique âgée d'une semaine a été déposé au centre de ces boîtes de Petri. Les cultures ont été incubées à 22°C jusqu'à ce que le champignon de la boite témoin (contenant le champignon sans bactérie) ait totalement envahi la boite. Des photographies de chaque boite ont été prises avec l'appareil Scan® 1200 (Interscience, Saint-Nom, France) afin de quantifier la croissance fongique. Les images obtenues sont traitées à l'aide du logiciel *ImageJ*. Les distances existant entre le centre et la périphérie de la zone de croissance mycélienne que ce soit du côté où le dépôt bactérien a été réalisé (BS = *Bacterial Side*) que du côté diamétralement opposé (FS = *Free Side*) ont été mesurées.

**Test de phytostimulation *in vitro***

*Préparation des plantules :* Des graines d'*Arabidopsis thaliana* (L.) Heynh génotype Col-0 ont été désinfectées par incubation dans une solution composée de 10% (v/v) d'eau de Javel à 9,6% de chlore actif et 0,5% (v/v) de Tween 20, sous agitation pendant 15 min à 1800rpm. Le surnageant a été retiré à la fin de l'agitation et une succession de cinq lavages dans de l'eau stérile a été effectuée. Les graines ont été mises 5 minutes sous agitation à 1800rpm. Suite au dernier lavage, 35 graines ont été disposées sur le milieu de culture gélosé (Mantelin *et al.*, 2006) contenant 1,2% (m/v) d'agar (Sigma ref : A1296, St Louis, MO, USA). Les boîtes ont été incubées à 4°C, à l'obscurité, pendant 2 jours (synchronisation de la levée de dormance) puis transférées dans un phytotron, préalablement réglé à 22°C avec un cycle jour/nuit de 16h/8h, pendant 7 jours.

*Inoculation bactérienne des plantules* : Les plantules âgées de 7 jours ont été transférées sur milieu de culture (Mantelin *et al.*, 2006) gélosé contenant 1,2% (m/v) d'agar (Sigma ref : A6686, St Louis, MO, USA). Des suspensions bactériennes de chacune des 9 souches ont été centrifugées puis rincées avec de l'eau stérile. La densité bactérienne a été ajustée de façon à obtenir une densité optique de 0,1 (soit $10^7$ UFC.mL$^{-1}$). La suspension bactérienne (5µL) est déposée à 1 mm de l'extrémité de la racine. L'ensemble des boîtes a été incubé 7 jours dans un phytotron à 22°C avec un cycle jour/nuit de 16h/8h. Après 7 jours d'incubation, les boîtes ont été scannées et le système racinaire des plantules d'*A. thaliana* analysé en utilisant le logiciel WinRhizo 2002c (Régent Instrument Inc., Québec City, Canada). Les biomasses fraiches des parties aériennes et racinaires des plantules ont été mesurées après 18 jours d'incubation.

***Analyses statistiques des données***

Les caractéristiques génomiques des 114 génomes (taille des génomes, %GC etc.) ainsi que les effets des 9 souches sur (i) les paramètres végétaux mesurés chez *A. thaliana* et (ii) sur la croissance mycélienne de différents pathogènes fongiques ont été comparés en réalisant une

analyse de variance (ANOVA) suivie d'un test de comparaison multiple HSD de Tukey. Le lien entre la taxonomie et les profils fonctionnels de chacune des souches analysées a été visualisé en réalisant un regroupement hiérarchique basé sur le calcul de distances euclidiennes entre les profils fonctionnels des souches de *Pseudomonas* selon leur appartenance aux différents sous-groupes de *P. fluorescens*. L'ensemble de ces analyses statistiques a été réalisé en utilisant le package ADE4 du logiciel R (Thioulouse *et al.*, 1997) et en se basant sur un seuil de significativité $P<0.05$.

# RESULTATS & DISCUSSION

## Génomique comparative et phylogénie des *Pseudomonas*

Les caractéristiques des 114 génomes de *Pseudomonas* (taille, %GC, nombres de séquences codantes, d'ARNt et d'ARNr) ont été obtenues et regroupées selon leur appartenance aux différents groupes ou sous-groupes de *P. fluorescens* et *P. putida* (**Tableau 2**). Le sous-groupe des *P. fragi* possède une taille de génomes significativement plus petite que les autres sous-groupes (5,13Mb ± 0,15Mb versus une taille moyenne 6,45Mb ± 0,49Mb pour les autres sous-groupes). Le %GC est significativement plus élevé chez les *P. protegens* et les *P. chlororaphis* que chez les autres sous-groupes (62.8% versus 60.6%). Le nombre de séquences codantes ainsi que les nombres d'ARNt et d'ARNr sont également plus élevés pour les sous-groupes *P. chlororaphis* et *P. protegens*.

**Tableau 2: Caractéristiques génomiques des groupes et sous-groupes de *Pseudomonas* fluorescents**

| | Genome size (Mb) | %GC | No of CDS | No of tRNA | No of rRNA | Number of strains |
|---|---|---|---|---|---|---|
| *P. fluorescens* group | 6,45 ± 0,49 | 60,51 ± 1,44 | 5692 ± 487 | 61 ± 7 | 10 ± 5 | 87 |
| *P. fluorescens* subgroup | 6,41 ± 0,47 a | 60,46 ± 0,42 b | 5661 ± 494 abc | 61 ± 7 b | 12 ± 5 | 26 |
| *P.fragi* subgroup | 5,13 ± 0,15 c | 58,06 ± 1,12 d | 4518 ± 178 d | 59 ± 6 b | 5 ± 2 | 5 |
| *P. mandelli* subgroup | 6,52 ± 0,29 a | 58,94 ± 0,27 d | 5837 ± 217 abc | 61 ± 4 b | 9 ± 4 | 10 |
| *P. jessenii* subgroup | 6,73 ± 0,55 a | 60,13 ± 0,05 bc | 5926 ± 440 abc | 61 ± 10 b | 12 ± 14 | 3 |
| *P. koreensis* subgroup | 6,40 ± 0,23 a | 59,70 ± 0,73 c | 5529 ± 262 bc | 60 ± 5 b | 7 ± 5 | 12 |
| *P. corrugata* subgroup | 6,50 ± 0,26 a | 60,85 ± 0,23 b | 5674 ± 257 abc | 59 ± 6 b | 10 ± 5 | 16 |
| *P. chlororaphis* subgroup | 6,82 ± 0,17 a | 63,11 ± 0,42 a | 6090 ± 292 ab | 65 ± 8 ab | 11 ± 6 | 10 |
| *P. protegens* subgroup | 6,90 ± 0,10 a | 63,33 ± 0,05 a | 6245 ± 200 a | 72 ± 2 a | 16 ± 2 | 5 |
| *P. putida* group | 5,76 ± 0,56 b | 62,54 ± 0,80 a | 5175 ± 638 cd | 69 ± 5 a | 15 ± 6 | 17 |

En bleu : Valeur minimale ; En rouge : Valeur maximale

En regroupant l'ensemble des génomes de cette étude selon leur appartenance aux deux clades définis dans le **Chapitre 2**, à savoir *P. corrugata*, *P. protegens* et *P. chlororaphis* (clade CPC) d'une part et *P. fluorescens*, *P. mandelii*, *P. jessenii* et *P. koreensis* (clade FMJK) d'autre part, il est observé que la taille moyenne des génomes appartenant au clade CPC (6.67 ± 0.27Mb) est significativement plus grande que celle des génomes appartenant au clade FMJK (6.44 ± 0.39Mb) ($P = 0.003$), que le nombre de séquences codantes l'est également (5900 ± 351 pour le clade CPC vs. 5681 ± 409 pour le clade FMJK – $P = 0.012$) ainsi que le %GC (61.88 ± 1.12 pour le clade CPC vs. 59.97 ± 0.76 pour le clade FMJK – $P < 0.001$).

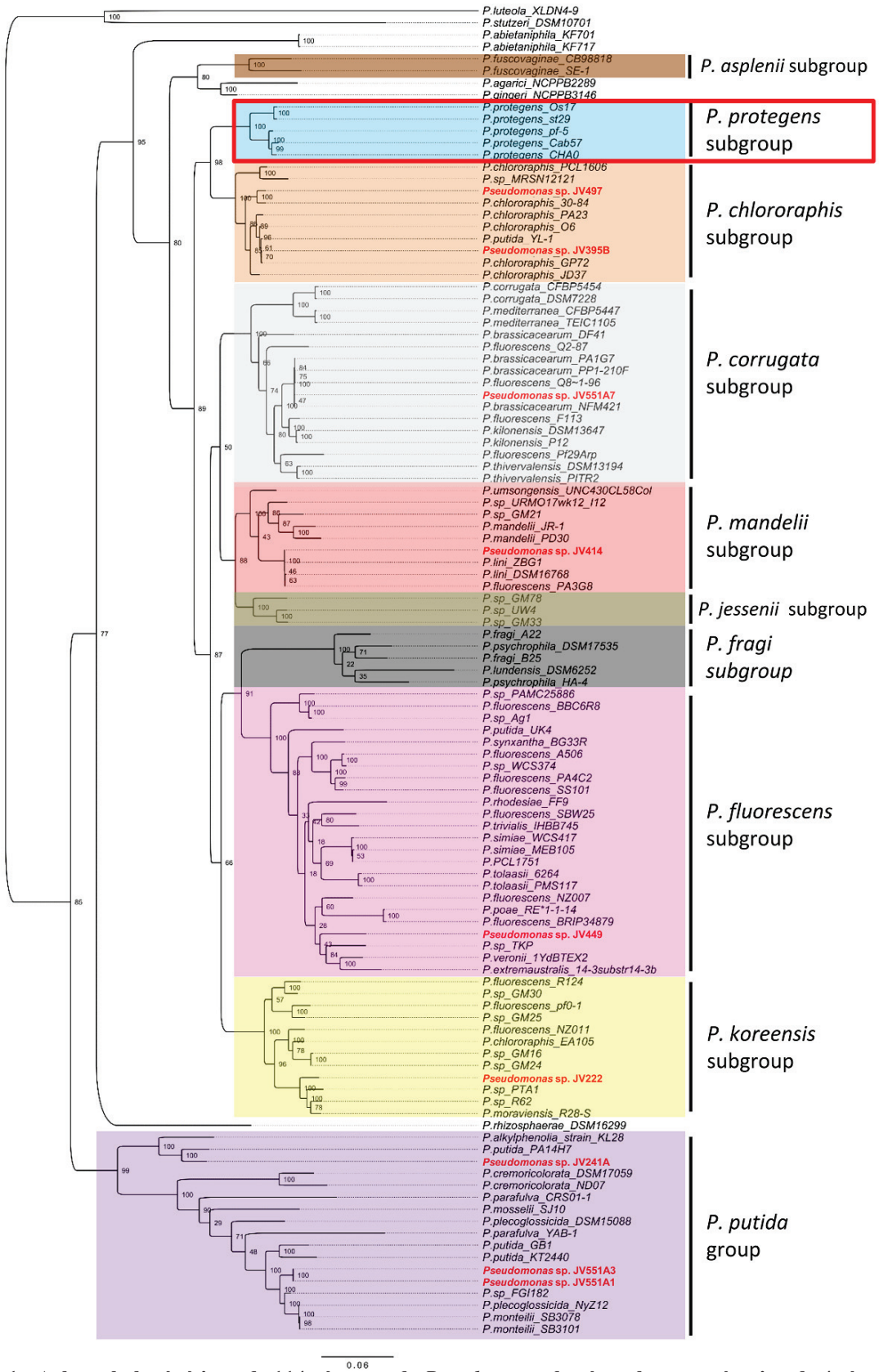



**Figure 1 : Arbre phylogénétique de 114 génomes de *Pseudomonas* basé sur la concaténation de 4 gènes de ménage (*rrs, gyrB, rpoB et rpoD*).** L'arbre a été généré par la méthode du maximum de vraisemblance en utilisant PhyML et le modèle GTR et 500 itérations. Les noms de souche inscrits en rouge correspondent aux 9 souches dont les génomes ont été séquencés au cours de cette étude. Les différentes couleurs correspondent aux différents sous-groupes et groupes de *Pseudomonas*. L'encadré rouge correspond au nouveau sous-groupe des *P. protegens* défini dans cette étude

La phylogénie des *Pseudomonas* a été réalisée *via* la concaténation de 4 gènes de ménage (*rrs*, *rpoD*, *rpoB* et *gyrB*), donnant lieu à une analyse sur une longueur de 9600 nucléotides. Aucune différence n'a été observée au niveau de la topologie des deux arbres générés (PhyML **Figure 1** et parcimonie **Figure S1**). La classification en groupes et sous-groupes établie par Mulet *et al.*, (2010) a été reportée sur les **Figures 1 et S1**. La topologie des arbres générés présente des divergences avec celles établies dans les études précédentes, en particulier au niveau du sous-groupe des *P. protegens* (**Figures 1 et S1)**. Cette différence s'explique par le fait que dans la présente étude le nombre de génomes de *Pseudomonas* fluorescents (groupe des *P. fluorescens* et *P. putida*) est 2 fois plus important que dans les études précédentes (114 vs. 59 - Mulet et al. 2010 ; ou vs. 58 - Gomila et al. 2015). De plus, la longueur des séquences concaténées dans cette étude et environ 2,5 fois plus longue que celles des deux études précédentes (3726nt pour Mulet et al. 2010 ; 3826nt pour Gomila et al. 2015). Ainsi, le nombre plus élevé de génomes appartenant à ces deux groupes analysés et la longueur du concaténa plus importante permettent une meilleure résolution de la phylogénie des *Pseudomonas* fluorescents. Toutefois, une analyse phylogénétique plus résolutive, basée sur l'alignement de l'ensemble des séquences protéiques orthologues des 114 génomes présentés dans cette étude, sera prochainement réalisée. L'aboutissement d'un arbre plus représentatif de la classification des *Pseudomonas* passe en premier lieu par une bonne assignation des souches nouvellement séquencées (Konstantinidis and Tiedje, 2005; Mulet *et al.*, 2010). Au cours de cette étude, nous avons observé qu'une souche possède un nom d'espèce qui n'est pas en adéquation avec sa position taxonomique : il s'agit de la souche *P. chlororaphis* EA105 (Spence *et al.*, 2014), qui n'est proche d'aucune souche de l'espèce *P. chlororaphis* et n'appartient pas au sous-groupe des *P. chlororaphis* mais à celui des *P. koreensis* (**Figure 1**).

Le regroupement des souches au sein d'un même taxon peut se calculer à partir des données de génome. Ainsi, le pourcentage moyen de nucléotides identiques entre deux souches peut être calculé *via* l'algorithme BLAST (ANIb) ou MUMmer (ANIm). Les ANI sont utilisés pour délimiter le seuil d'appartenance à une espèce entre deux ou plusieurs souches (> 95-96% Rosselló-Móra, 2006). Ces valeurs ont été calculées pour l'ensemble des 114 génomes (**Figure 2A-2B**). L'utilisation de ces valeurs ne permet pas une discrimination suffisante entre le groupe des *P. fluorescens* et celui des *P. putida* (**Figure 2A-2B**). En revanche, en se référant à un seuil de 84% pour l'analyse ANIb (**Figure S2-C**) et de 87,55% pour l'analyse ANIm (**Figure S3-C**), il est possible de redéfinir l'appartenance d'un génome à un sous-groupe de *P. fluorescens* particulier. Toutefois, ces seuils ne discriminent pas dans l'absolu l'appartenance à un sous-groupe. Par exemple, les souches appartenant au sous-groupe des *P. jessenii* présentent entre-elles des valeurs ANI supérieures au seuil de 85% (ANIb) et 88% (ANIm). De ce fait, les seuils établis dans cette étude ne sont pas assez élevés pour distinguer les *P. jessenii* des *P. mandelii* (**Figure S2-C et S3-C**). L'éventuelle fusion de ces deux sous-groupes pourrait être proposée, mais le nombre de génomes représentant le sous-groupe des *P. jessenii* reste trop faible pour pouvoir conclure. L'augmentation des valeurs seuils engendre une perte de regroupement des souches dans le sous-groupe de *P. fluorescens* (**Figure S2 - D et S3 - D**).

L'analyse des fréquences tétranucléotidiques (TETRA) correspond à la recherche de la fréquence de motifs de 4 nucléotides parmi les génomes (Noble *et al.* 1998). Des génomes proches devraient avoir des valeurs TETRA similaires. Le calcul de ces fréquences parmi les 114 génomes de cette étude ne permet pas une délimitation nette des deux groupes de *P. fluorescens* et *P. putida* (**Figure 2C**).

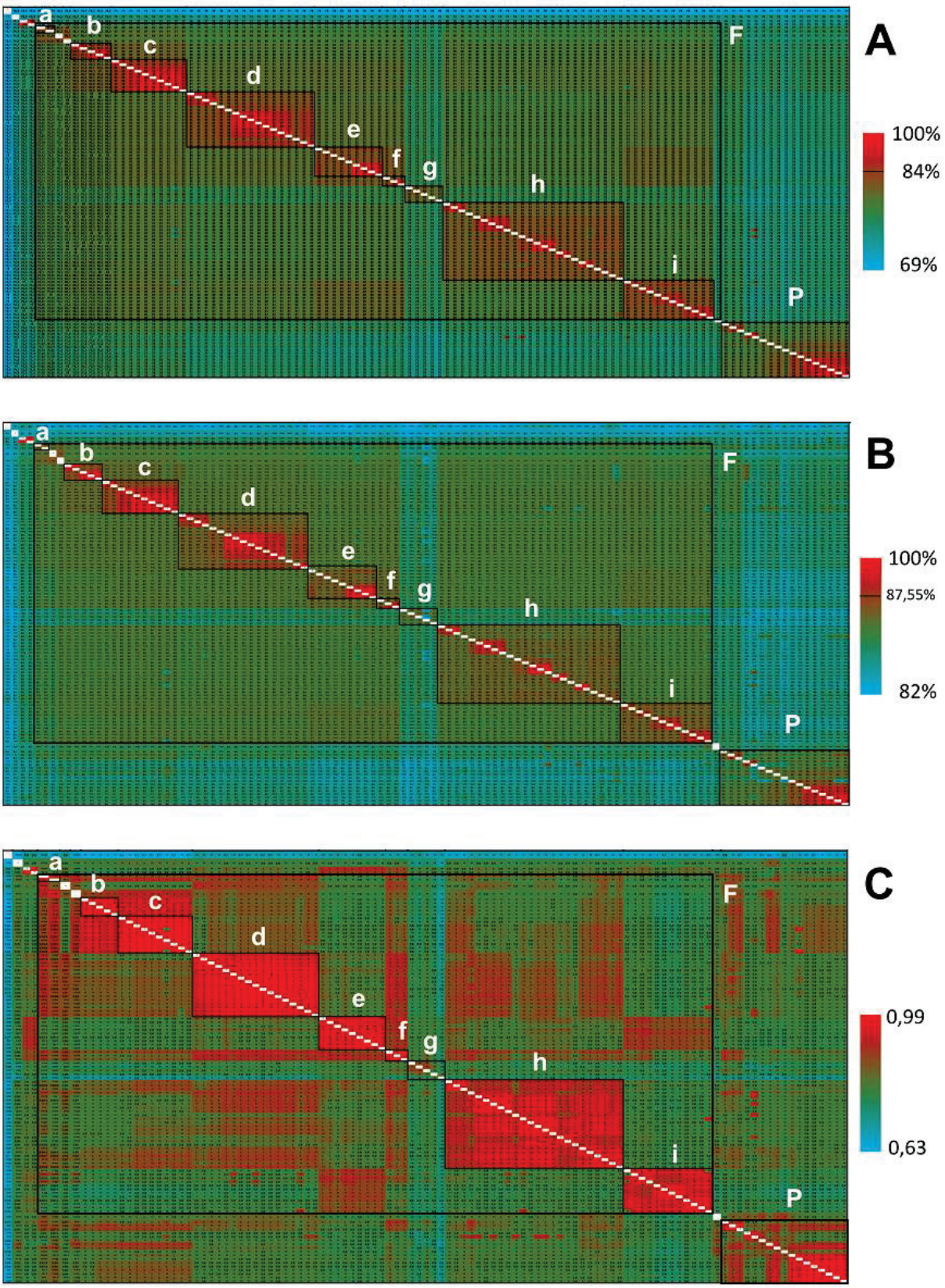



**Figure 2 : Représentation sous forme de carte thermique des indices ANIb (A), ANIm (B) et TETRA (C) des génomes de *Pseudomonas* étudiés.** Chacune des valeurs des indices calculées *via* JSpecies est reportée. Les encadrés notés « F » et « P » correspondent à la délimitation respective des groupe de *P. fluorescens* et *P. putida*. Les encadrés nommés de « a » à « i » correspondent aux délimitations entre les différents sous-groupes : a : *P. asplenii* ; b : *P. protegens* ; c : *P. chlororaphis* ; d : *P : corrugata* ; e : *P. mandelii* ; f : *P. jessenii* ; g : *P.fragi* ; h : *P. fluorescens* ; i : *P. koreensis*..

De même, la délimitation des sous-groupes est également imprécise avec une moins bonne discrimination des sous-groupes des *P. fragi*, *P. fluorescens* et *P. koreensis* (**Figure S4-B**). L'utilisation d'indices de type ANIb-ANIm à eux seuls ne permettent donc pas la stricte délimitation des sous-groupes de *Pseudomonas* comme celui des *P. jesseni*, *P. mandelii* et *P. fluorescens*. Quant à l'indice TETRA, une étude récente sur la phylogénie des *Pseudomonas* montre que l'utilisation de cet indice est adéquate pour juger de l'appartenance d'une souche au genre *Pseudomonas* (Gomila *et al.*, 2015) mais nos travaux montrent qu'elle ne le serait pas pour différentier des niveaux taxonomiques inférieurs.

***P. protegens* un sous-groupe à part en tiers parmi le groupe des *P. fluorescens.***

Dans cette étude, 9 sous-groupes ont été différenciés parmi le groupe des *P. fluorescens* (le groupe des *P. gessardii* n'a pas été inclus dans cette étude). Parmi ces sous-groupes, nous proposons la scission du groupe des *P. chlororaphis* en un premier sous-groupe regroupant les *P. protegens* (souches CHA0, Pf-5, Cab57, St29 et Os17) et un second correspondant aux *P. chlororaphis sensu stricto* (comportant les souches PCL1606, MRSN12121, JV497, 30-84, PA23, O6, YL-1, JV395B, GP72 et JD37) (**Figure 1**). Cette division témoigne (i) de la différentiation nette en deux branches de l'arbre au niveau de l'ancien groupe des *P. chlororaphis* (bootstrap = 98%), (ii) de la séparation de ces groupes selon leur ANIb et ANIm qui sont supérieurs aux seuils établis ci-dessus (ANIb-ANIm minimum de 88.89%-90.53% parmi les *P. protegens*, 89.42%-90.9% parmi les *P. chlororaphis*) (**Figure S2 et S3**) et (iii) de la présence d'un profil fonctionnel phytobénéfique commun chez le sous-groupe des *P. protegens* ($DAPG^+$, $HCN^+$, $Phz^-$ et $HPR^-$), différent du groupe des *P. chlororaphis sensu-stricto* ($DAPG^-$, $Prn^+$, $HCN^+$ et $HPR^+$) (**Tableau 3**).

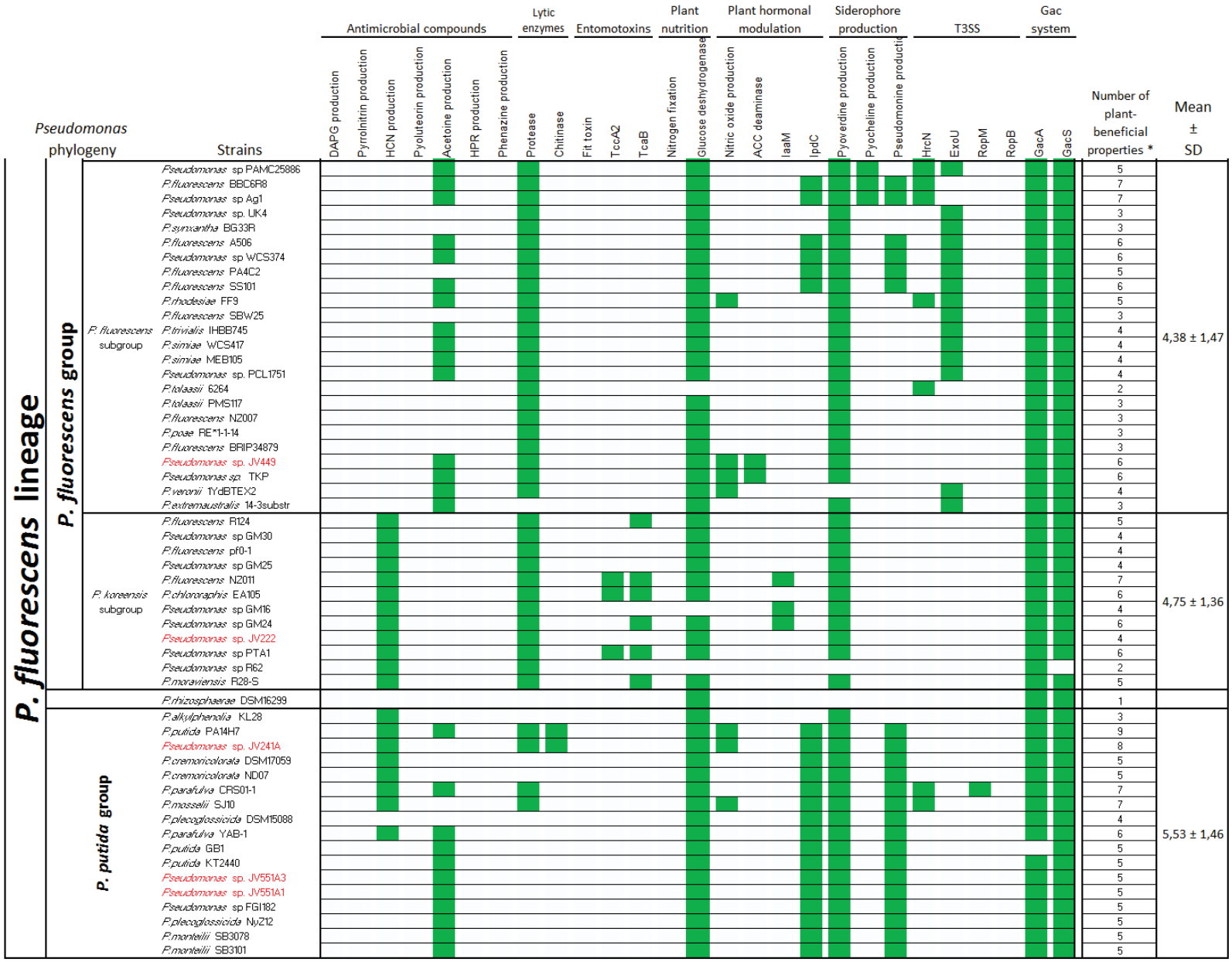
Le sous-groupe des *P. protegens* est composé de *Pseudomonas* appartenant à l'espèce « *protegens* » ($DAPG^+$, $Plt^+$) décrite par Ramette *et al.* (2011). C'est notamment le cas des souches CHA0, Pf-5 et Cab57 qui possèdent par ailleurs des pourcentages d'identités supérieurs à 95%, seuil de distinction des espèces établi par Richter and Rosselló-Móra (2009). En revanche, les souches Os17 et St29 n'appartiennent pas à l'espèce « *protegens* », du fait que (i) les pourcentages d'identité entre les 3 souches précédentes et celles-ci ne dépassent pas 90,7% d'identité et que (ii) ces deux souches sont $DAPG^+$ et $Plt^-$. Ainsi, il conviendrait de définir une nouvelle espèce pour les souches Os17 et St29.

***Distribution des propriétés phytobénéfiques parmi les Pseudomonas sp.***

110 gènes codant des protéines regroupant (i) des propriétés phytobénéfiques impliquées dans la phytoprotection, la modulation du système hormonal de la plante ou dans l'amélioration de sa nutrition (fixation d'azote, solubilisation du phosphate) (Vacheron *et al.*, 2013), (ii) les systèmes de sécrétion de type III qui ont été montrés comme jouant un rôle potentiel vis-à-vis de l'efficacité des propriétés phytobénéfiques (Almario *et al.*, 2014), ainsi que (iii) le système de régulation à deux composants GacA/GacS impliqué dans la régulation de la production de nombreux métabolites secondaires et d'exoenzymes (Laville *et al.*, 1992; Venturi, 2006) ont été recherchés (**Tableau 3**). Hormis la protéine NifH impliquée dans la fixation de l'azote atmosphérique, l'ensemble des protéines recherchées a été retrouvé au moins une fois dans l'ensemble des génomes de *Pseudomonas* étudiés. Certaines fonctions phyto-

**Tableau 3 : Distribution des propriétés phytobénéfiques selon la taxonomie des *Pseudomonas*.**

**Tableau 3 : Distribution des propriétés phytobénéfiques selon la taxonomie des Pseudomonas. (Suite)**




Ecrit en rouge : Les génomes séquencés dans cette étude

Les cases vertes représentent la présence de la propriété phytobénéfiques

* : Moyenne calculée en ne prenant pas en compte les T3SS et le système Gac

bénéfiques sont retrouvées spécifiquement dans un sous-groupe particulier comme la production de pyolutéorine et de pyochéline (Enantio-pyochéline) chez les *P. protegens*, la production de l'HPR chez les *P. chlororaphis* et la production de pseudomonine chez certains individus appartenant au sous-groupe des *P. fluorescens*. Par ailleurs, la production de pyrrolnitrine et de l'entomotoxine Fit semble produite exclusivement chez les *Pseudomonas* appartenant aux sous-groupes des *P. chlororaphis* et *P. protegens* à de rares exceptions près (*P. protegens* Os17 et *P. protegens* St29 sont dépourvus des enzymes impliquées dans la production de la pyrrolnitrine et *P. chlororaphis* JD37 ne possède pas les enzymes permettant la production de l'entomotoxine Fit) (**Tableau 3**).

Certaines fonctions semblent préférentiellement associées dans un même génome, comme la production de DAPG et d'HCN quel que soit le sous-groupe considéré, comme préalablement décrit chez de nombreux isolats de *Pseudomonas* fluorescents (Ramette *et al.*, 2003). D'autres, en revanche, se retrouvent associées chez tous les individus d'un même sous-groupe, comme par exemple la capacité de produire de la pyrrolnitrine et de l'HPR chez les *P. chlororaphis* (**Tableau 3**). Le système de régulation GacA/S est présent dans tous les génomes sauf pour celui de *Pseudomonas* sp. R62 (sous-groupe des *P. koreensis*) où GacS n'a pas été détecté. GacA est également absent au seuil de 40% d'identité protéique chez *P. putida* GB01 (**Tableau 3**). La capacité de solubiliser le phosphate peut être réalisée par la production d'acide gluconique *via* une glucose déshydrogénase (Gcd - quinoprotéine) munie d'un cofacteur de type pyrroloquinoline quinone (PQQ) (de Werra *et al.*, 2009; Meyer *et al.*, 2011). Cette enzyme est occurrente chez la quasi-totalité des *Pseudomonas* étudiés (**Tableau 3**). Cette forte occurrence peut être expliquée par le rôle pléiotrope de cette enzyme qui n'est pas affiliée strictement à la solubilisation du phosphate, puisqu'elle remplit également d'autres fonctions au sein du métabolisme primaire de la cellule (Werra *et al.*, 2009; Bruto *et al.*, 2014). La capacité de production d'auxines par la voie de la tryptophane monooxygénase (IaaM) est présente uniquement chez certains *Pseudomonas* appartenant aux sous-groupes des *P. chlororaphis* et *P. koreensis* alors que la production d'auxine par la voie de l'indole-pyruvate décarboxylase (IpdC) est présente chez certains *Pseudomonas* appartenant au sous-groupe des *P. fluorescens* et à de nombreux *Pseudomonas* appartenant au groupe des *P. putida* (**Tableau 3**). L'ensemble de ces observations est en accord avec une étude de génomique comparative menée sur 10 génomes de *Pseudomonas* appartenant au groupe des *P. fluorescens* et connus pour être associés à la plante (Loper *et al.*, 2012).

**Histoire évolutive des propriétés phytobénéfiques des *Pseudomonas* sp. – Cas des métabolites secondaires antimicrobiens.**

A partir de ces résultats, un scénario d'acquisitions et pertes des propriétés phytobénéfiques impliquées spécifiquement dans la production de métabolites secondaires antimicrobiens (DAPG, HCN, pyrrolnitrine, pyolutéorine, HPR et phénazine) (**Figure 3**) a été établi mais doit être validé par une étude de reconstruction de caractères ancestraux (prochainement menée avec l'aide de la plateforme Ibio de l'UMR5557).

Certaines de ces fonctions semblent spécifiques à certains sous-groupes des *P. fluorescens*, comme par exemple les gènes impliqués dans la production d'HPR (gènes *dar*) qui ne sont retrouvés que chez le sous-groupe des *P. chlororaphis sensu stricto* ou encore les gènes

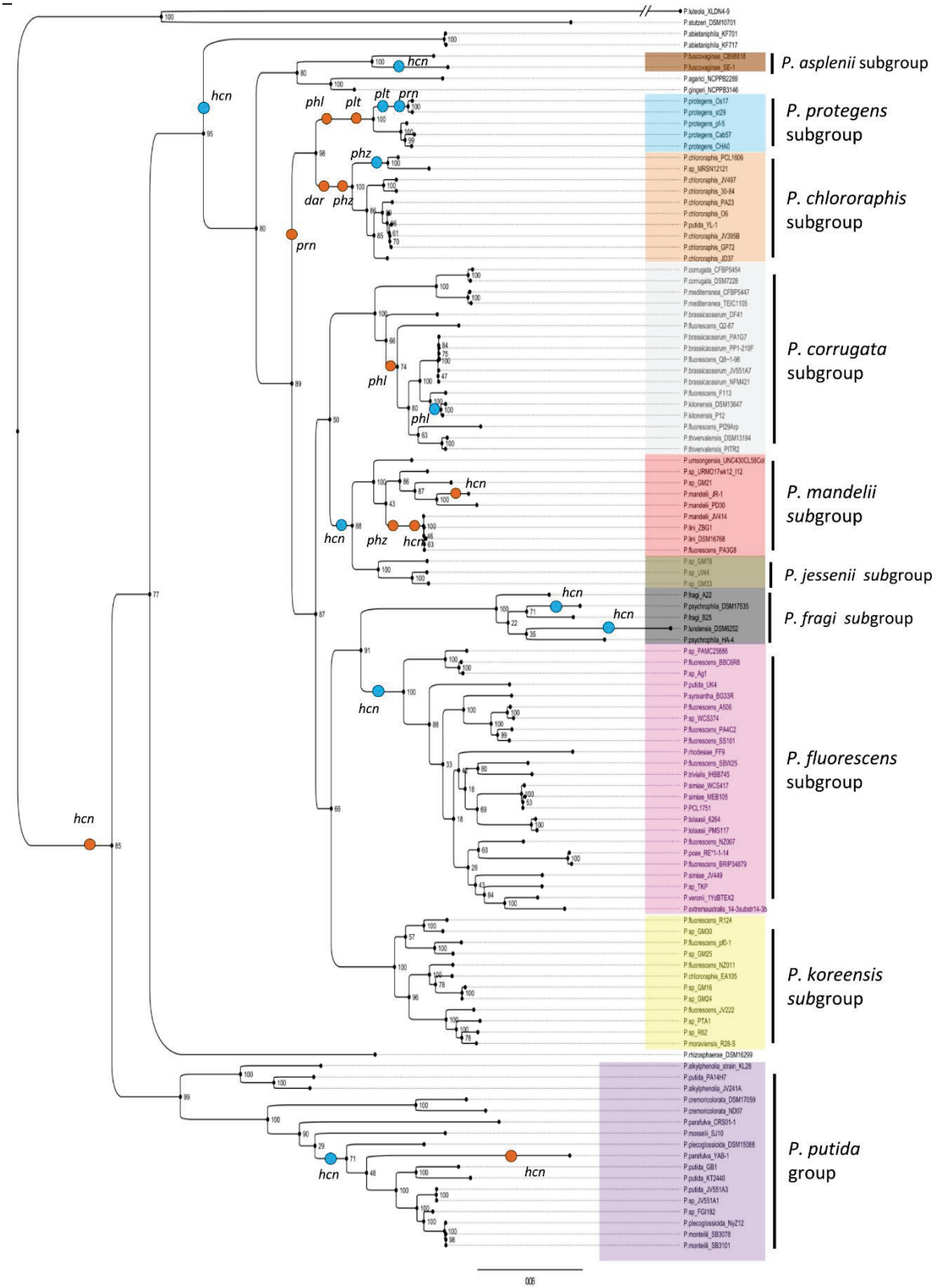



**Figure 3 : Scénario envisagé de pertes et acquisitions de propriétés impliquées dans la production de métabolites secondaires antimicrobiens parmi les *Pseudomonas*.** L'arbre phylogénétique correspond à celui de la Figure 1. Les points orange correspondent aux potentielles acquisitions, les points bleus aux potentielles pertes des gènes impliqués dans la production de métabolites secondaires antimicrobiens. *hcn* : opéron *hcn* regroupant les gènes *hcnABC* impliqués dans la production d'acide cyanhydrique (HCN) ; *phl* : opéron *phl* regroupant les gènes *phlACBD* impliqué dans la production du 2,4-diacétylphloroglucinol (DAPG) ; *prn* : opéron *prn* regroupant les gènes *prnABCD* impliqués dans la production de pyrrolnitrine ; *dar* : opéron *dar* regroupant les gènes *darABC* impliqués dans la production de 2-hexyl-5-propyl résorcinol (HPR) ; *plt* : opéron *plt* regroupant les gènes *pltABCDEF* impliqués dans la production de pyolutéorine ; *phz* : opéron *phz* regroupant les gènes *phzABCDEFG* impliqués dans la production de phénazine.

impliqués dans la production de pyolutéorine qui auraient été acquis par l'ancêtre commun du sous-groupe des *P. protegens*, puis perdu par l'ancêtre commun de *P. protegens* Os17 et *P. protegens* St29. Par ailleurs, les gènes impliqués dans la production de pyrrolnitrine (*prn*) pourraient avoir été acquis par l'ancêtre commun des sous-groupes des *P. protegens* et des *P. chlororaphis* (**Figure 3**). La production de pyrrolnitrine a d'ailleurs été décrite comme souvent transférée parmi les rhizobactéries, notamment entre des bactéries du genre *Burkholderia* et *Pseudomonas* (de Souza and Raaijmakers, 2003; Costa *et al.*, 2009). Les déterminants génétiques impliqués dans la production de DAPG (opéron *phl*) sembleraient avoir été acquis plusieurs fois dans l'histoire évolutive des *Pseudomonas*, comme proposé par Almario *et al.* (Thèse) où une première acquisition aurait eu lieu chez l'ancêtre commun de tous les *Pseudomonas* appartenant au sous-groupe des *P. protegens*, puis une seconde acquisition se serait produite chez l'ancêtre commun des *P. brassicacearum* (avec une perte possible de l'opéron *phl* chez la souche *P. kilonensis* P12) (**Figure 3**). En revanche, un scénario inverse a été précédemment décrit, où l'acquisition de l'opéron *phl* est un trait ancestral chez les *Pseudomonas* qui aurait été perdu dans de nombreux embranchements au cours de l'évolution (Moynihan *et al.*, 2009). De la même façon, l'opéron *phz* impliqué dans la production de phénazine aurait également été acquis à plusieurs reprises, chez l'ancêtre commun des *Pseudomonas* du sous-groupe des *P. chlororaphis* ainsi que chez l'ancêtre commun d'un des sous-embranchements au sein du sous-groupe des *P. mandelii*. Les gènes impliqués dans la production de phénazines sont considérés comme très conservés chez les *Pseudomonas* (Mavrodi *et al.*, 2010), suggérant une acquisition ancestrale au genre *Pseudomonas*. Cette hypothèse est supportée par le fait que l'opéron contenant l'ensemble des gènes nécessaires à la synthèse des phénazines est également retrouvé dans les génomes de *Pseudomonas aeruginosa* (Mavrodi *et al.*, 2001), appartenant à une lignée de *Pseudomonas* différente de celle des *P. fluorescens* (Mulet *et al.*, 2010) En revanche, si cette opéron est conservé chez les *Pseudomonas*, sa dissémination semble être plus dynamique dans le genre *Burkholderia* où il est suggéré que des évènements de transferts horizontaux ont eu lieu (Mavrodi *et al.*, 2010). Enfin, l'opéron *hcn* permettant la production du composé volatil antimicrobien HCN pourrait avoir été acquis très tôt dans l'histoire évolutive des *Pseudomonas*, chez l'ancêtre commun aux groupes des *P. putida* et *P. fluorescens*. Cet opéron, bien que rencontré chez un grand nombre de *Pseudomonas*, pourrait avoir été perdu à plusieurs reprises, notamment par les ancêtres des sous-groupes *P. fluorescens* et *P. mandelii* (**Figure 3**). Si ce scénario fait état de multiple pertes et acquisitions des propriétés phytobénéfiques parmi les *Pseudomonas* fluorescents, le nombre d'acquisition reste supérieur aux pertes lorsque l'on regarde la distribution de ces propriétés chez les protéobactéries (Bruto *et al.*, 2014).

**Nombre de propriétés phytobénéfiques et rôle écologique**

Une analyse de regroupement hiérarchique basée sur le calcul de distances euclidiennes a été réalisée afin d'évaluer s'il y avait une congruence entre la phylogénie et les fonctions phytobénéfiques présentes dans les génomes (**Figure 4**). Deux grands clades se distinguent, correspondant pour le premier, au clade CPC (contenant les sous-groupes *P. chlororaphis*, *P. protegens*, et *P. corrugata*), décrit lors de notre précédente étude (**Chapitre 2**). Le second clade correspond aux autres génomes (dont les génomes appartenant au clade FMJK contenant les

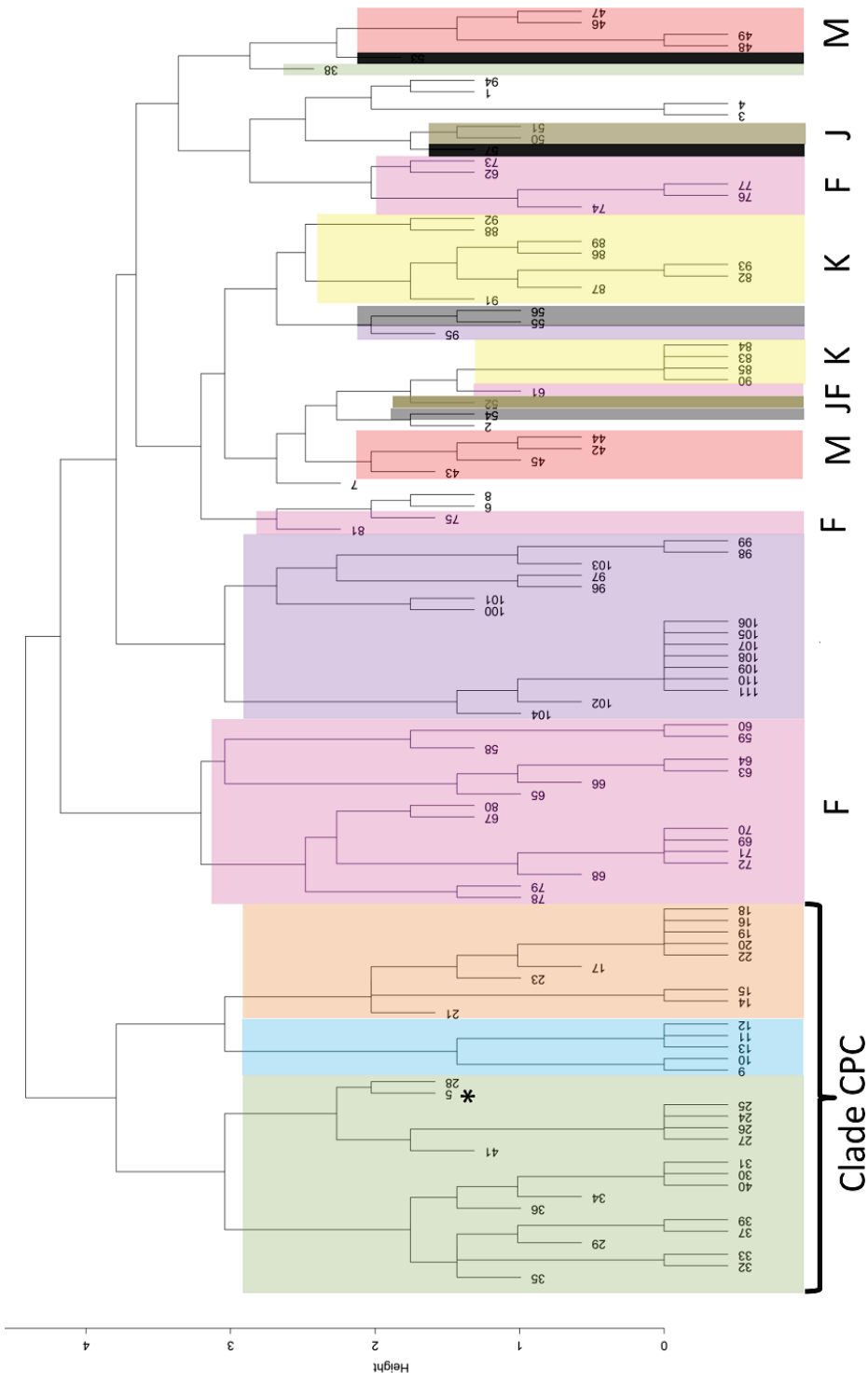



**Figure 4 : Visualisation du lien existant entre les sous-groupes de *Pseudomonas fluorescens* et les profils fonctionnels des 18 propriétés phytobénéfiques présentes au sein des génomes.** Analyse de regroupement hiérarchique basé sur un calcul de distances euclidiennes. Les différentes couleurs correspondent aux différents sous-groupes et groupes de *Pseudomonas*. Les lettres F, M, J et K correspondent respectivement aux sous-groupes des *P. fluorescens*, *P. mandelii*, *P. jessenii* et P. *korensis*, constituant le clade FMJK. Les numéros correspondent aux génomes étudiés, pour la correspondance entre n° et nom des souches se référer au **tableau S4**.

sous-groupes *P. fluorescens, P. mandelii, P. jesseni et P. koreensis*). Au sein de ce clade, aucun regroupement selon la phylogénie des *Pseudomonas* n'est observé, sauf pour le groupe des *P. putida* (**Figure 4**). Cela signifie que le lien entre profil fonctionnel et phylogénie serait plus important pour les souches du clade CPC que pour celles du clade FMJK ainsi que pour les autres sous-groupes restants. Il est possible que les gènes codant ces propriétés phytobénéfiques dans le clade des CPC fassent partie du génome cœur permettant une meilleure adaptation à la niche écologique du clade.

De plus, le nombre de propriétés phytobénéfiques est en moyenne plus important dans le clade CPC avec 8.97 ± 2.22 propriétés phytobénéfiques par génome que dans le clade FMJK qui n'en comporte que 4.52 ± 1.53, confirmant les résultats précédemment obtenus (**Chapitre 2**).

Neuf souches de *Pseudomonas* provenant de la banque d'isolats décrite dans le chapitre 2 et appartenant aux deux clades CPC, FMJK ainsi qu'au groupe des *P. putida* ont été séquencées. Il s'agit de 9 souches isolées de la rhizosphère du maïs ou du sol matriciel (**Tableau S2**). Leur caractérisation taxonomique a été réalisée *via* le replacement de ces souches dans l'arbre phylogénétique des *Pseudomonas* obtenus dans cette étude (**Figure 1**). Les caractéristiques de leurs génomes sont détaillées dans le **Tableau S3**. Les gènes codant les voies de biosynthèse de bactériocines et de métabolites secondaires impliqués dans le biocontrôle ont été recherchés (**Tableau 4)**.

**Tableau 4 : Propriétés phytobénéfiques possédés par les 9 souches de *Pseudomonas* séquencées**

| | Strain | DAPG production | Pyrrolnitrin production | HCN production | Pyoluteorin production | Acetoine production | HPR production | Phenazine production | Fit toxin | TccA2 | TcaB | TOTAL | Number of metabolite detected by AntiSMASH | Number of bacteriocin detected by BAGEL3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Clade FMJK | JV222 | | | ■ | | | | | | | | 1 | 7 | 4 |
| | JV414 | | | ■ | | | | ■ | | | | 2 | 11 | 3 |
| | JV449 | | | | | ■ | | | | | | 1 | 9 | 1 |
| Clade CPC | JV395B | | ■ | ■ | | | ■ | ■ | ■ | | | 5 | 16 | 0 |
| | JV497 | | ■ | ■ | | | ■ | ■ | ■ | | | 5 | 14 | 1 |
| | JV551A7 | ■ | | ■ | | ■ | | | | | ■ | 4 | 11 | 0 |
| *P. putida* group | JV551A1 | | | | | ■ | | | | | | 1 | 7 | 0 |
| | JV551A3 | | | | | ■ | | | | | | 1 | 9 | 0 |
| | JV241A | | | ■ | | | | | | | | 1 | 6 | 2 |

D'une façon générale, la diversité des métabolites antimicrobiens potentiellement produits est en moyenne plus importante dans les génomes de JV395B, JV497 et JV551A7 appartenant au clade CPC que dans les génomes des autres souches, exceptées pour les bactériocines dont les gènes de biosynthèse ont plus fréquemment été détectés dans les génomes de JV222, JV414 et JV449 appartenant tous les trois au clade FMJK (**Tableau 4**). La prédiction d'îlots génomiques ainsi que la localisation à l'intérieur de ces îlots de gènes codant des fonctions phytobénéfiques ont également été recherchées (**Figure 5**) afin de voir si ces gènes pouvaient être présents au sein d'éventuels éléments génétiques mobiles. Peu d'îlots

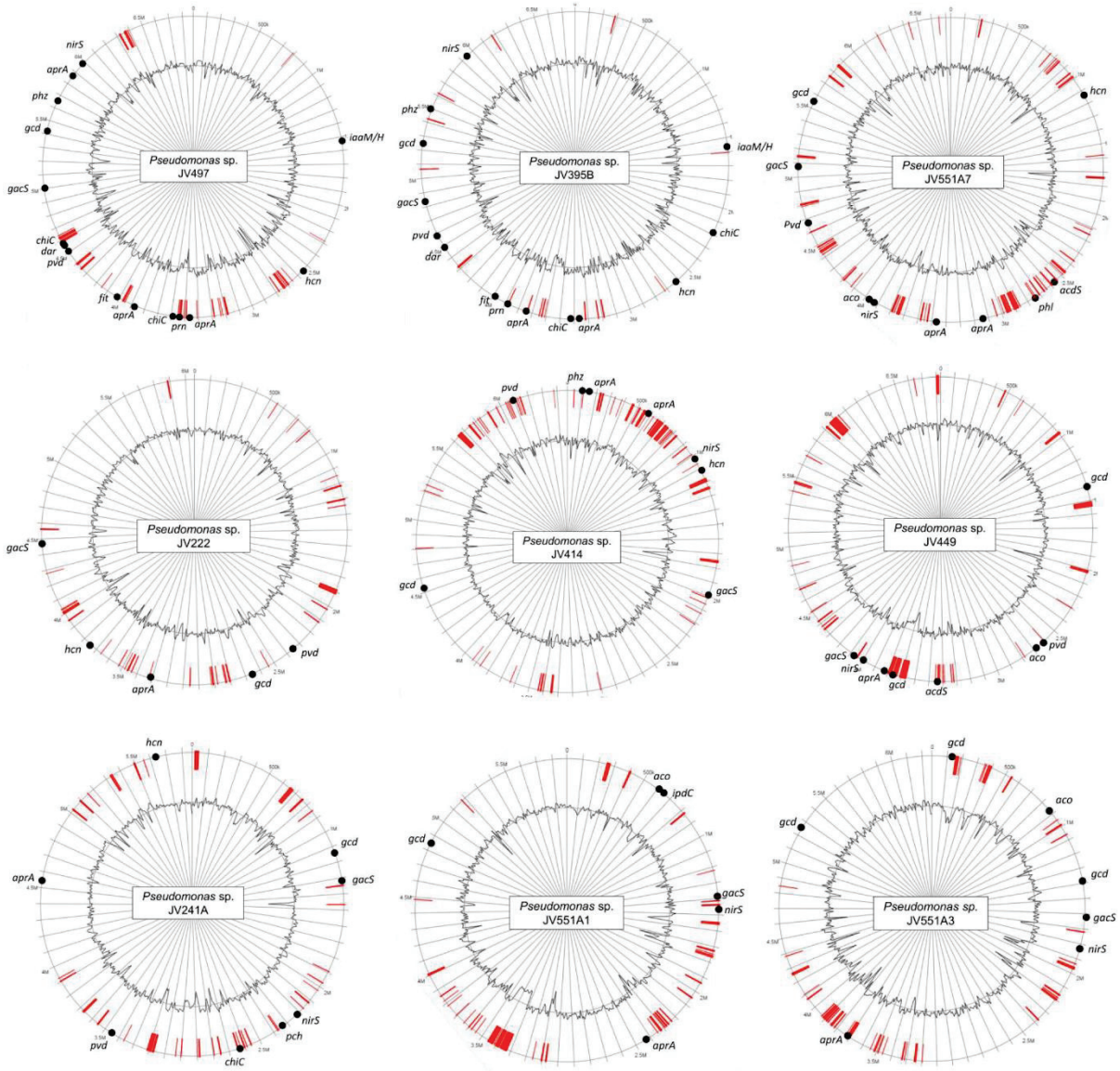



**Figure 5 : Distribution des propriétés phytobénéfiques au sein des 9 souches de *Pseudomonas* dont le génome a été séquencé et prédiction de potentiels îlots génomiques.** Les points noirs correspondent à la localisation des gènes codant de potentielles propriétés phytobénéfiques. Les traits rouges correspondent aux différents îlots génomiques putatifs prédits *via Island Viewer* 3, par trois différentes méthodes : SIGI-HMM, IslandPath-DIMOB et Islandpick. La correspondance des gènes est la suivante : *aco* : acétoine ; *acdS* : ACC désaminase ; *aprA* : protéase ; *chiC* : chitinase ; *dar* : 2-héxil, 5-propylrésorcinol (HPR) ;*fit* : entomotoxine Fit ; *gcd* : glucose déshydrogénase ; *gacS* : senseur du système Gac ; *hcn* : acide cyanhydrique (HCN) ; *iaaM/H* : tryptophane monooxygénase et indole-acétamide hydrolase (production d'auxine) ; *ipdC* : indole pyruvate décarboxylase (production d'auxine) ; *nirS* : nitrite réductase (production d'oxide nitrique) ; *pch* : pyochéline ; *phl* : 2,4-diacétylphloroglucinol (DAPG) ; *phz* : phénazine ; *prn* : pyrrolnitrine ; *pvd* : pyoverdine.

génomiques ont été prédits chez JV395B (*n*=18) comparativement à la souche JV414 pour laquelle 76 ilots génomiques ont été prédits. Dans l'ensemble des ilots détectés, différents types de gènes potentiellement impliqués dans la mobilisation de ces îlots, comme des intégrases, transposases, ou encore des systèmes de sécrétion de type IV, sont retrouvés. Ces gènes sont décrits pour être des entités fréquemment retrouvées dans les îlots génomiques (Juhas *et al.*, 2009; Juhas, 2015). Des gènes impliqués dans des propriétés phytobénéfiques ont également été retrouvés. Ainsi, l'opéron *prn* impliqué dans la production de pyrrolnitrine a été retrouvé sur des îlots génomiques de taille respective 6952pb, 6939pb et 5323pb chez les souches JV497, JV395B et PA-23 (sous-groupe des *P. chlororaphis*), mais cet opéron ne semble pas appartenir à des îlots génomiques chez *P. protegens* CHA0, Pf-5 et Cab57. L'ensemble de ces observations suggère une possible acquisition de cette fonction par transfert horizontal de gènes chez le sous-groupe des *P. chlororaphis*. L'opéron *phl* impliqué dans la production de DAPG est également retrouvé sur un îlot génomique (5780pb) dans le génome de JV551A7 appartenant au sous-groupe des *P. corrugata*. Dans le génome de JV449 (sous-groupe *P. fluorescens*), le gène *acdS* codant pour l'ACC désaminase est retrouvé à proximité (< 1000pb) d'un potentiel îlot génomique d'environ 9500pb.

Les 9 souches ont été testées pour leur capacité à moduler l'architecture racinaire et à influencer la croissance de la plante. Dans ce but, l'impact de l'inoculation de ces souches sur des plantules d'*A. thaliana* Col0 a été évalué en condition simplifiée. Des paramètres racinaires comme la longueur, la surface et le diamètre racinaires ont été mesurés ainsi que le nombre de ramifications et les biomasses fraiches et sèches des parties racinaires et aériennes (**Figure 6 A-D**). Seule l'inoculation de la souche JV497 appartenant au sous-groupe des *P. chlororaphis* module significativement l'architecture racinaire en augmentant l'ensemble des paramètres mesurés (**Figure 6**). De plus, après 16 jours de croissance, comparativement aux autres souches testées ainsi qu'aux plantes témoins non-inoculées, l'ensemble des plantes inoculées avec la souche JV497 se trouve de façon précoce en phase de floraison (**Figure 7**). Ces observations suggèrent que la souche pourrait produire des hormones de type gibbérellines connues pour stimuler la croissance végétative des plantes en modifiant leur perception du temps (Wilson *et al.*, 1992).

La capacité d'exercer une action antagoniste de ces 9 souches envers les microorganismes phytopathogènes *L. maculans*, *T. basicola*, *M. grisae* et *P. alni* a été testée *in vitro* en milieu gélosé (**Figure 8 A-D**). Toutes les souches testées présentent une action antagoniste plus ou moins forte envers les microorganismes phytopathogènes testés. La souche JV395B exerce toutefois une activité antagoniste significativement plus forte que les autres souches bactériennes, particulièrement envers *L. maculans*, *T. basicola* et *P. alni* (**Figure 8B et 8D**) où la croissance mycélienne est inhibée de part et d'autre du dépôt bactérien.

Que ce soit pour leur capacité à moduler l'architecture racinaire ou à inhiber la croissance de microorganismes phytopathogènes *in vitro*, aucun de ces deux clades CPC et FMJK (représentés par les 9 souches testées) ne montre une meilleure aptitude à effectuer l'une ou l'autre de ces actions.

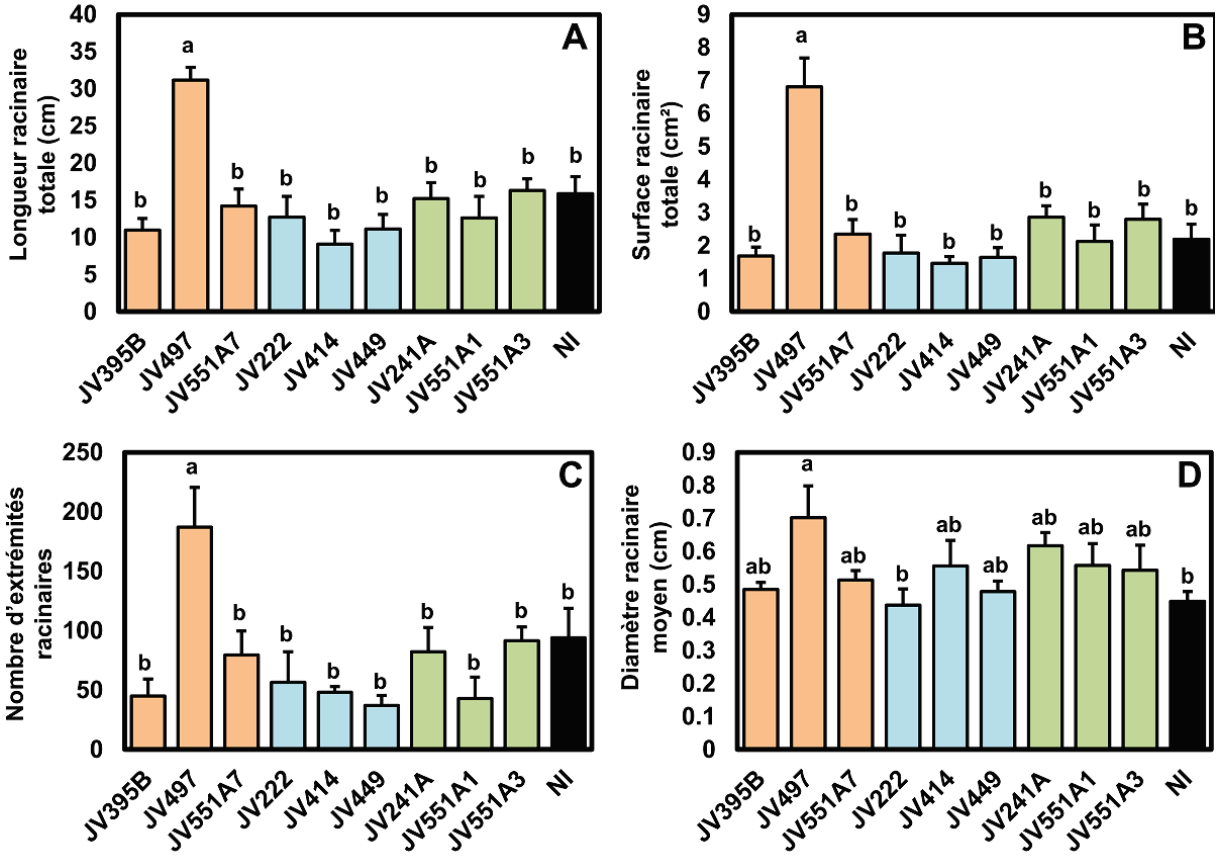



**Figure 6 : Effet des 9 souches de *Pseudomonas* séquencées sur l'architecture racinaire *d'Arabidopsis thaliana*.** A : Longueur racinaire totale ; B : Surface racinaire totale ; C : Nombre d'extrémités racinaires ; D : Diamètre racinaire moyen. L'appartenance des souches testées aux différents clades de *Pseudomonas fluorescens* définis dans le chapitre 2, est indiquée par des barres de couleurs différentes (en orange: clade CPC ; bleu: clade FMJK ; vert: groupe *P. putida* ; noir: condition non-inoculée). Les différences statistiques sont indiquées par les lettre a-b (ANOVA et test de comparaison multiple HSD de Tukey, $P<0.05$).

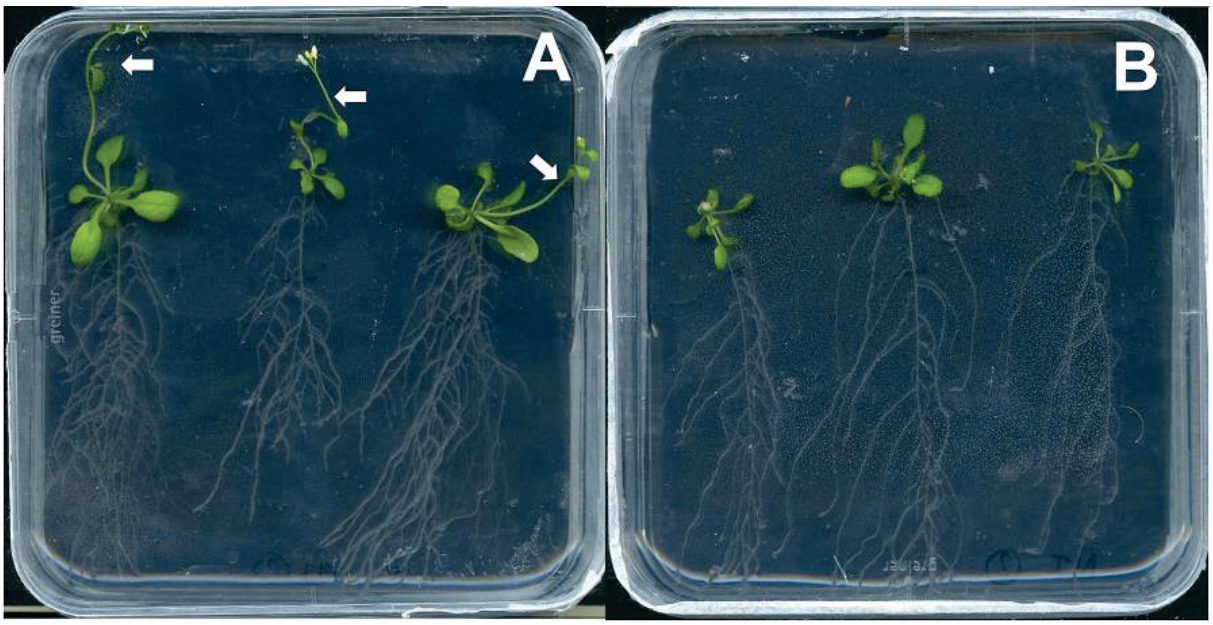



**Figure 7 : Effet de la souche JV497 sur la floraison d'*A. thaliana*.** A : *A. thaliana* inoculée par JV497 ; B : condition témoin non-inoculée. Les flèches blanches ciblent la hampe florale.

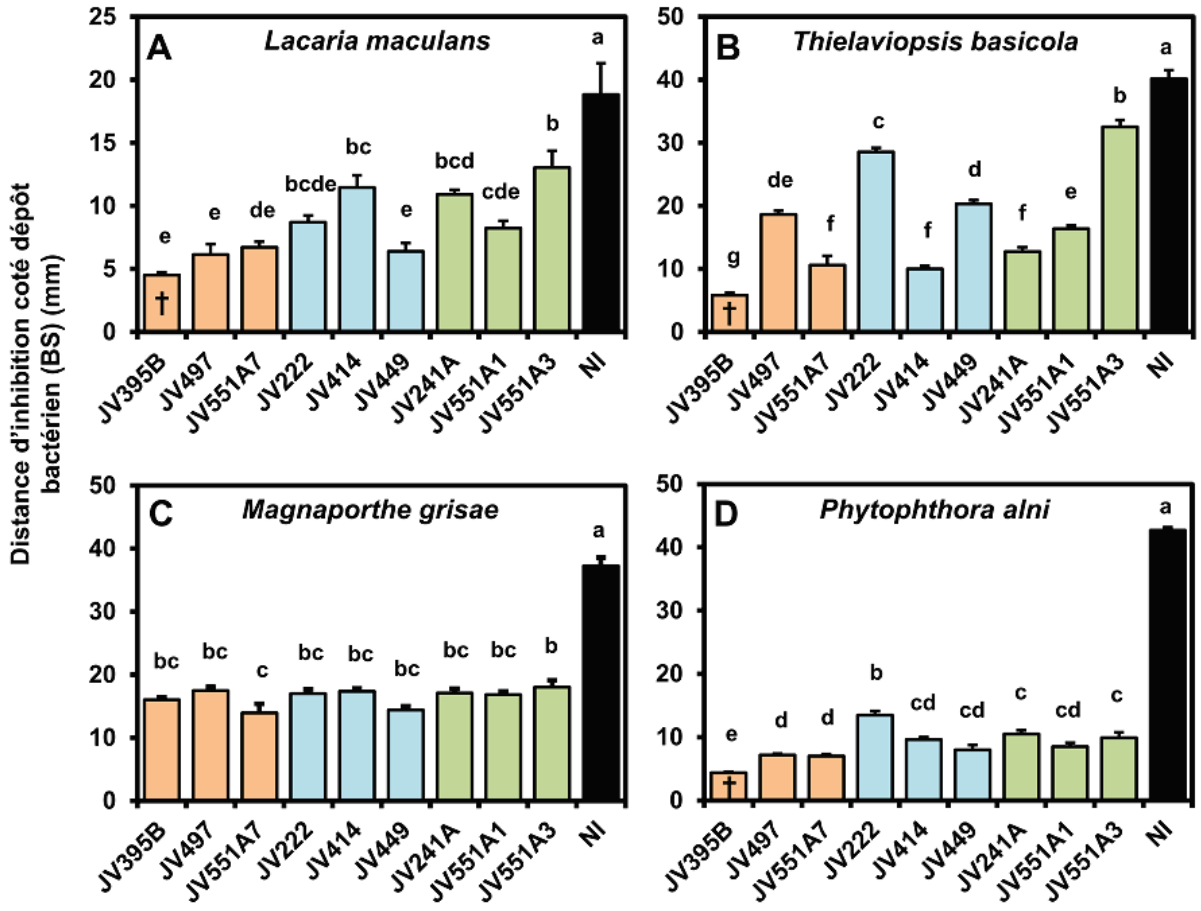



**Figure 8 : Inhibition de la croissance de champignons et d'oomycètes phytopathogènes par les 9 souches de *Pseudomonas* séquencées.**

A : test d'antagonisme contre *Lacaria maculans* ; B : test d'antagonisme contre *Thielaviopsis basicola* ; C : test d'antagonisme contre *Magnaporthe grisae* ; D : test d'antagonisme contre *Phytophthora alni*. L'appartenance des souches testées aux différents clades de *Pseudomonas fluorescens* est indiquée par des barres de couleurs différentes (en orange: clade CPC ; bleu: clade FMJK ; vert: groupe *P. putida* ; noir: condition non-inoculée). Les distances BS (côté du dépôt bactérien) sont reportées. † : Indique que l'effet inhibiteur de la souche sur la croissance mycélienne est également observé du côté diamétralement opposé à celui du dépôt bactérien. Les différences statistiques sont indiquées par les lettre a-b (ANOVA et test de comparaison multiple HSD de Tukey, $P<0.05$).

# CONCLUSION

Les acquisitions et pertes de propriétés phytobénéfiques peuvent moduler la colonisation de nouvelles niches écologiques et la fitness des *Pseudomonas* dans leurs environnements (Thomashow and Weller, 1988; Keel *et al.*, 1990; Wang *et al.*, 2000; Bruto *et al.*, 2014). Par conséquent, les évènements de pertes et d'acquisitions de gènes peuvent contribuer à la spéciation des bactéries, leur permettant d'évoluer dans une nouvelle niche écologique (Lassalle *et al.*, 2011, 2015). Dans le cas du groupe des *P. fluorescens*, plusieurs espèces peuvent faire partie d'un même sous-groupe taxonomique (Mulet *et al.*, 2010; Gomila *et al.*, 2015). Nos travaux suggèrent d'ailleurs que la distribution de propriétés phytobénéfiques semblerait d'avantage liée à un sous-groupe taxonomique qu'à des espèces particulières (**Figure 4**). Notre étude précédente a montré que les individus du clade CPC possédaient un nombre élevé de propriétés phytobénéfiques d'avantage associées à un mode d'action de type biocontrôle tandis que ceux du clade FMJK regroupaient des individus possédant moins de fonctions que le clade précédent, avec toutefois plus de propriétés phytobénéfiques affiliées à un mode d'action de type modulation hormonale chez la plante (**Chapitre 2**). La même tendance est visible parmi l'ensemble des génomes analysés dans cette étude et provenant d'individus ayant des écologies majoritairement en lien avec la plante.

Cependant, si les clades CPC et FMJK possèdent des profils fonctionnels majoritairement associés à un rôle écologique particulier, aucune différence d'efficacité entre individus de ces deux clades n'a été observée dans les tests d'antagonisme ou de phytostimulation réalisés dans cette étude. Ainsi, si certaines souches possèdent une grande diversité de traits phytobénéfiques impliqués dans la réalisation d'une fonction écologique particulière, cela ne garantit pas qu'elles auront une meilleure efficacité vis-à-vis de cette fonction.

Dans un contexte regroupant l'ensemble du microbiote racinaire, ces analyses réalisées au niveau des *Pseudomonas* suggèrent que différents types d'individus ayant des stratégies d'interactions différentes coexistent dans la rhizosphère : certaines vont proposer une plus grande variété de services (les 'poly-valents') et d'autres vont être spécialisés dans un petit nombre de services (les 'spécialistes') (Werner *et al.*, 2014). L'étude du **chapitre 2** appuie également cette théorie à l'échelle des *Pseudomonas* en mettant en évidence que la plante sélectionnerait préférentiellement des individus possédant entre 1 et 5 propriété phytobénéfiques, plutôt que ceux qui en possèdent, plus de 5. De plus, l'histoire évolutive des propriétés phytobénéfiques et particulièrement de celles impliquées dans la production de métabolites secondaires antimicrobiens montre une succession de pertes et d'acquisitions qui a modelé l'évolution adaptative des *Pseudomonas,* révélant le lien existant entre taxonomie et propriétés phytobénéfiques arborées par certains sous-groupes de *P. fluorescens* (en particulier au niveau du clade CPC).

# FIGURES ET TABLEAUX SUPPLEMENTAIRES

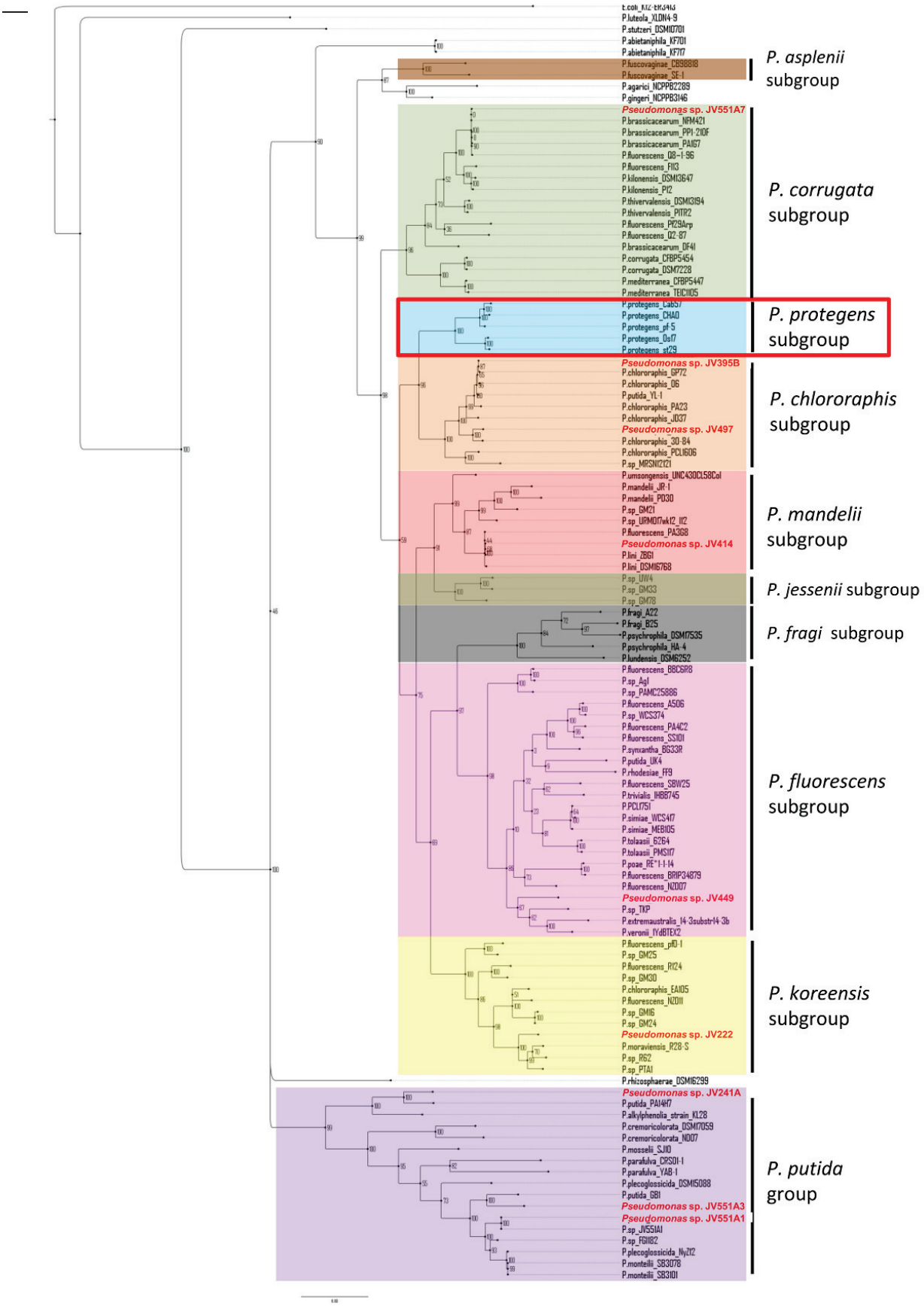



**Figure S1 : Arbre phylogénétique de 114 génomes de *Pseudomonas* basé sur la concaténation de 4 gènes de ménage (*rrs, gyrB, rpoB et rpoD*)**. L'arbre a été généré en utilisant la méthode de parcimonie, 500 itérations ont été réalisées. Les noms de souche inscrits en rouge correspondent aux 9 souches dont les génomes ont été séquencés au cours de cette étude. Les différentes couleurs correspondent aux différents sous-groupes et groupes de *Pseudomonas*. L'encadré rouge correspond au nouveau sous-groupe des *P. protegen*s définis dans cette étude.

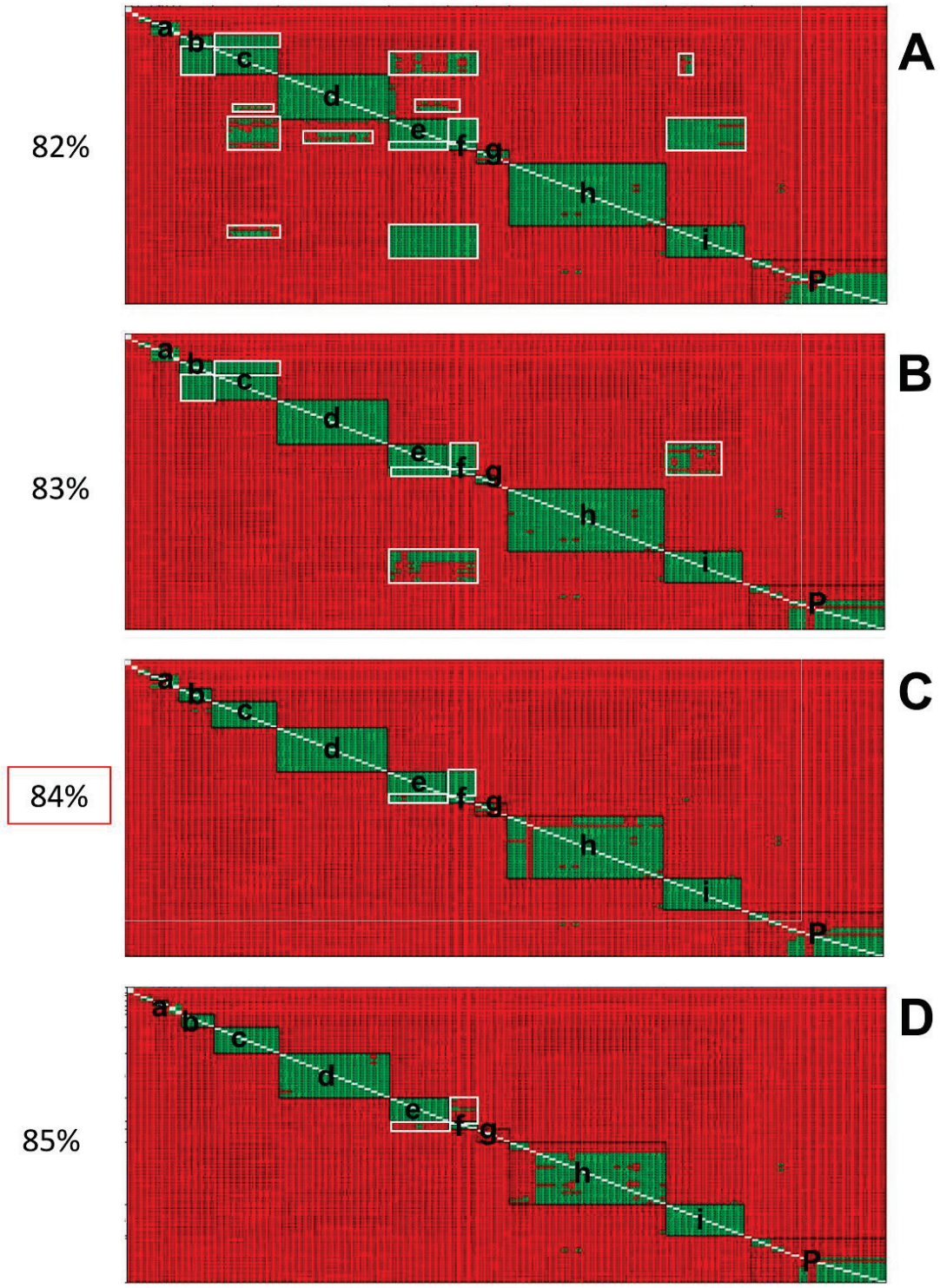



**Figure S2 : Définition du seuil ANIb permettant une délimitation des sous-groupes définis par Mulet *et al.* 2010.** Les différents sous-groupes sont délimités par des carrés de couleurs noirs. La couleur verte correspond à une valeur d'ANIb supérieure au seuil indiqué sur le côté de la figure. La couleur rouge correspond à une valeur d'ANIb inférieure au seuil indiqué sur le côté de la figure. Les encadrés blancs montrent des valeurs d'ANIb supérieures au seuil fixé mais n'étant pas en accord avec la définition des sous-groupes de *Pseudomonas*. Les encadrés nommés de « a » à « i » correspondent aux délimitations entre les différents sous-groupes : a : *P. asplenii* ; b : *P. protegens* ; c : *P. chlororaphis* ; d : *P : corrugata* ; e : *P. mandelii* ; f : *P. jessenii* ; g : *P. fragi* ; h : *P. fluorescens* ; i : *P. koreensis*.

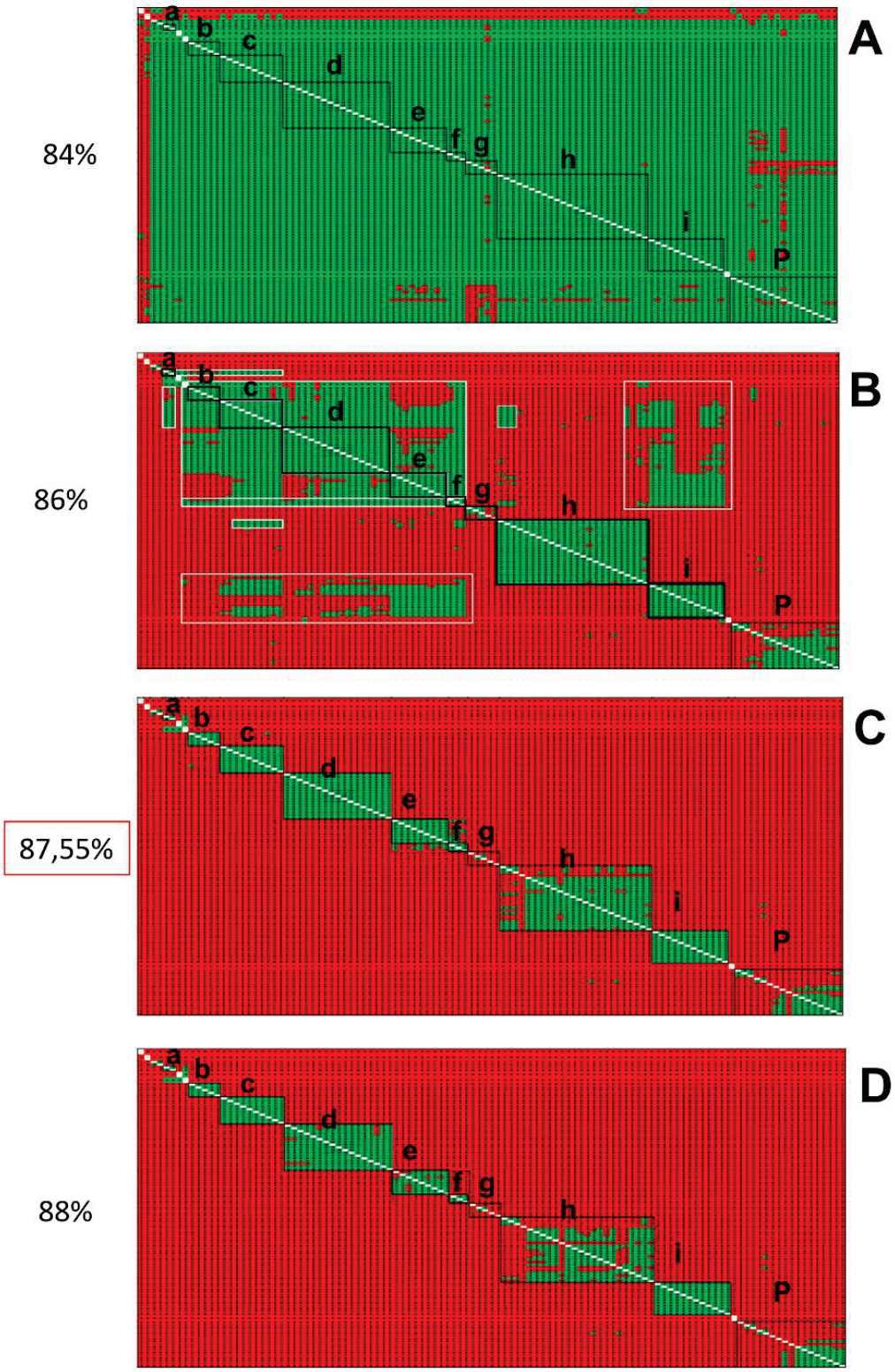



**Figure S3 : Définition du seuil ANIm permettant une délimitation des sous-groupes définis par Mulet *et al.* 2010.** Les différents sous-groupes sont délimités par des carrés de couleurs noirs. La couleur verte correspond à une valeur d'ANIm supérieure au seuil indiqué sur le côté de la figure. La couleur rouge correspond à une valeur d'ANIm inférieure au seuil indiqué sur le côté de la figure. Les encadrés blancs montrent des valeurs d'ANIm supérieures au seuil fixé mais n'étant pas en accord avec la définition des sous-groupes de *Pseudomonas*. Les encadrés nommés de « a » à « i » correspondent aux délimitations entre les différents sous-groupes : a : *P. asplenii* ; b : *P. protegens* ; c : *P. chlororaphis* ; d : *P : corrugata* ; e : *P. mandelii* ; f : *P. jessenii* ; g : *P. fragi* ; h : *P. fluorescens* ; i : *P. koreensis*.

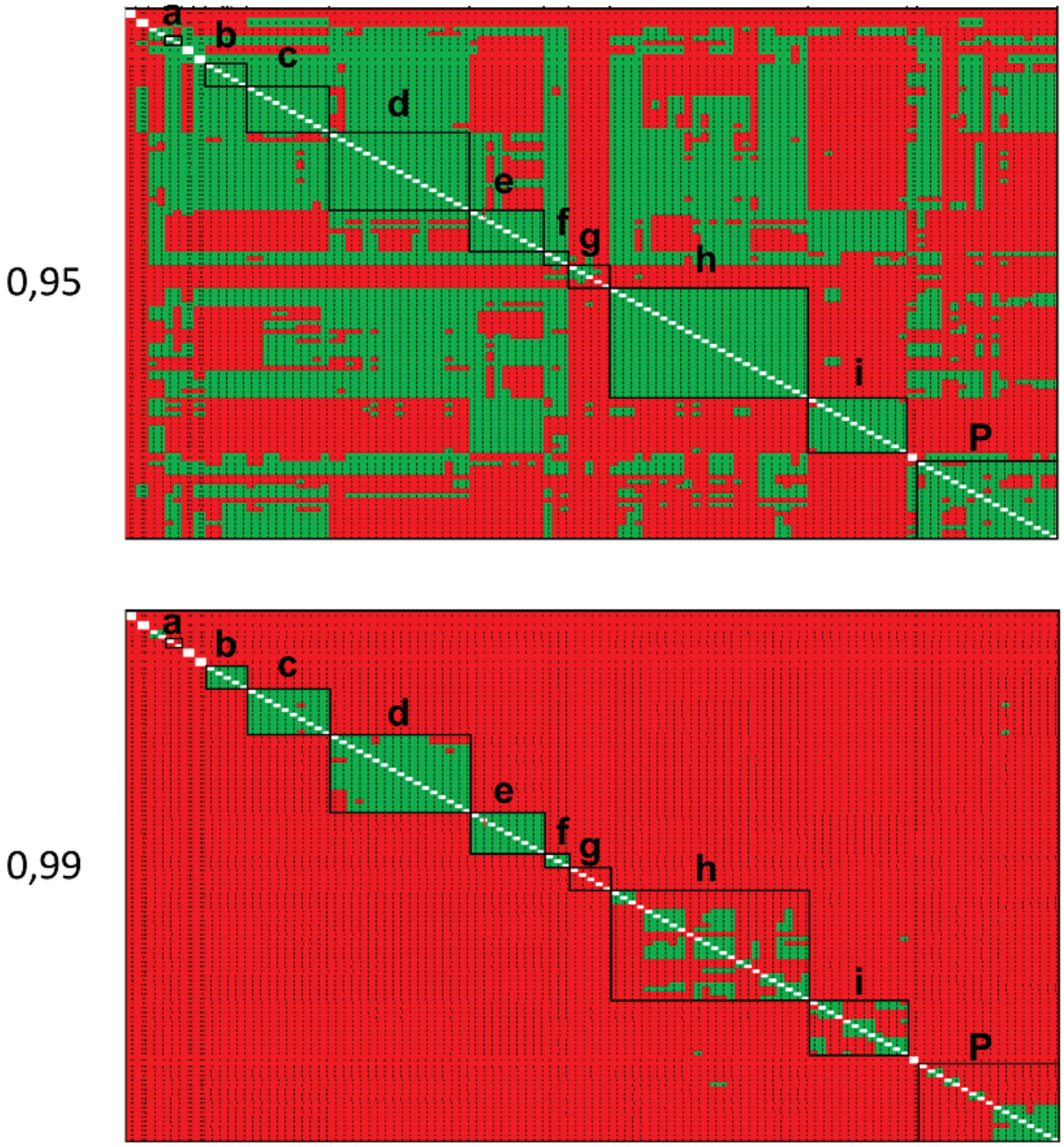



**Figure S4 : Représentation des valeurs de l'indice TETRA.** Les différents sous-groupes sont délimités par des carrés de couleurs noirs, basés sur les sous-groupes définis par Mulet *et al.* 2010. La couleur verte correspond à une valeur TETRA supérieure au seuil indiqué sur le côté de la figure. La couleur rouge correspond à une valeur TETRA inférieure au seuil indiqué sur le côté de la figure. Les encadrés nommés de « a » à « i » correspondent aux délimitations entre les différents sous-groupes : a : *P. asplenii* ; b : *P. protegens* ; c : *P. chlororaphis* ; d : *P : corrugata* ; e : *P. mandelii* ; f : *P. jessenii* ; g : *P. fragi* ; h : *P. fluorescens* ; i : *P. koreensis*.

**Tableau S1 : Liste des protéines recherchées parmi les génomes.**

| Protein name | Num accession | Function associated | Protein name | Num accession | Function associated | Protein name | Num accession | Function associated | Protein name | Num accession | Function associated |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PhlA | gi: 359760390 | DAPG production | AcoR | gi: 359761634 | Acetoine production | FitA | gi: 68344646 | Entomotoxin | PmsG | gi: 504531446 | Pseudo-monine production |
| PhlB | gi: 359760388 | | AcoA | gi: 387160042 | | FitB | gi: 68344647 | | PmsF | gi: 504531447 | |
| PhlC | gi: 359760389 | | AcoB | gi: 359761637 | | FitC | gi: 68344648 | | PmsD | gi: 387893961 | |
| PhlD | gi: 359760387 | | AcoC | gi: 387162310 | | FitD | gi: 68344649 | | PmsC | gi: 4150886 | |
| PhlE | gi: 359760386 | | AcoX | gi: 68343837 | | FitE | gi: 341580115 | | PmsE | gi: 4165301 | |
| PhlF | gi: 359760391 | | DarA | gi: 388461983 | HPR production | FitF | gi: 68344651 | | PmsA | gi: 1771426 | |
| PrnA | gi: 158514364 | Pyrrolnitrin production | DarB | gi: 388461984 | | FitG | gi: 68344652 | | PmsB | gi: 1771427 | |
| PrnB | gi: 517924158 | | DarC | gi: 388461985 | | FitH | gi: 68344653 | | PvdY | gi: 68345841 | Pyoverdin production |
| PrnC | gi: 158514366 | | DarS | gi: 388461986 | | PchA | gi: 500243140 | Pyochelin production | PvdZ | gi: 562780074 | |
| PrnD | gi: 158514367 | | DarR | gi: 388461987 | | PchB | gi: 341580168 | | PvdI | gi: 77382075 | |
| HcnA | gi: 359760299 | HCN production | PhzA | gi: 388550253 | Phenazine production | PchC | gi: 68345151 | | PvdJ | gi: 68345749 | |
| HcnB | gi: 359760300 | | PhzB | gi: 388550242 | | PchD | gi: 158523344 | | PvdK | gi: 77382077 | |
| HcnC | gi: 359760301 | | PhzC | gi: 388550198 | | PchR | gi: 68345158 | | PvdD | gi: 359759778 | |
| PltA | gi: 68344453 | Pyoluteorin production | PhzD | gi: 388550108 | | PchE | gi: 68345154 | | PvdE | gi: 359759784 | |
| PltB | gi: 68344454 | | PhzE | gi: 388550411 | | PchF | gi: 68345153 | | PvdP | gi: 359759788 | |
| PltC | gi: 68344455 | | PhzF | gi: 388550410 | | PchG | gi: 9950439 | | PvdA | gi: 359759801 | |
| PltD | gi: 4582975 | | PhzG | gi: 388550161 | | PchH | gi: 68345156 | | TccA2 | gi: 359760397 | Entomotoxin |
| PltE | gi: 68344457 | | AprA | gi: 359760874 | Protease | PchI | gi: 68345155 | | TcAB2 | gi: 749424465 | |
| PltF | gi: 68344458 | | ChiC | gi: 68343760 | Chitinase | NifH | gi: 588310886 | Nitrogen fixation | HrcN | gi: 359763499 | TSS3 |
| PltG | gi: 68344459 | | AcdS | gi: 359761421 | ACC deaminase | | | | ExoU | gi: 387164287 | |
| pltL | gi: 4582982 | | AcdR | gi: 51339974 | | IaaM | gi: 740796056 | Auxin production | RopM | gi: 397882711 | |
| pltR | gi: 4582980 | | NirS | gi: 359761676 | Nitrite reductase | IaaH | gi: 397891127 | | RopB | gi: 397882247 | |
| pltZ | gi: 55820064 | | NirK | gi: 500245035 | | IaaL | gi: 123973 | | | | |
| Gcd | gi: 359762612 | Phosphate solubilization | GacA | gi: 29826222 | Two-component | IpdC | gi: 356876418 | | | | |
| pqqC | gi: 359759574 | | GacS | gi: 48256736 | | IpdC | gi: 562781692 | | | | |

**Tableau S2 : Origines et appartenances taxonomiques des 9 souches séquencées.**

| Clade | Group or subgroup | Strains | Isolated from | Maize cultivar | Soil origins |
|---|---|---|---|---|---|
| CPC | *P. koreensis* subgroup | *Pseudomonas. sp.* JV222 | Bulk soil | | Morens - Swiss |
| | *P. mandelii* subgroup | *Pseudomonas. sp.* JV414 | Bulk soil | | Seyssel - France |
| | *P. fluorescens* subgroup | *Pseudomonas. sp.* JV449 | Rhizosphere | PR37Y15 | Seyssel - France |
| CPC | *P. chlororaphis* subgroup | *Pseudomonas. sp.* JV497 | Bulk soil | | Seyssel - France |
| | *P. chlororaphis* subgroup | *Pseudomonas. sp.* JV395B | Bulk soil | | Seyssel - France |
| | *P. corrugata* subgroup | *Pseudomonas. sp.* JV551A7 | Rhizosphere | PR37Y15 | Seyssel - France |
| | *P. putida* group | *Pseudomonas. sp.* JV241A | Rhizosphere | PR37Y15 | Morens - Swiss |
| | | *Pseudomonas. sp.* JV551A1 | Rhizosphere | PR37Y15 | Seyssel - France |
| | | *Pseudomonas. sp.* JV551A3 | Rhizosphere | PR37Y15 | Seyssel - France |

**Tableau S3 : Caractéristiques détaillés des génomes séquencés**

| Strains | Genome size (bp) | N50 | N75 | No CONTIGS | Largest contig | %GC | No of CDS | No of tRNA | No of rRNA |
|---|---|---|---|---|---|---|---|---|---|
| *Pseudomonas* sp. JV222 | 6 049 644 | 141 798 | 68 101 | 74 | 472 358 | 60,20 | 5963 | 60 | 7 |
| *Pseudomonas* sp. JV241A | 5 857 757 | 225 152 | 148 092 | 37 | 545 614 | 62.51 | 5653 | 63 | 10 |
| *Pseudomonas* sp. JV274 | 5 154 944 | 803 304 | 547 802 | 19 | 1 022 959 | 36,39 | 4813 | 85 | 11 |
| *Pseudomonas* sp. JV395B | 6 771 008 | 60 109 | 36 478 | 184 | 187 206 | 63,08 | 6515 | 60 | 8 |
| *Pseudomonas* sp. JV414 | 6 468 998 | 510 279 | 309 229 | 22 | 1 221 259 | 58,85 | 6182 | 59 | 8 |
| *Pseudomonas* sp. JV449 | 6 829 292 | 272 680 | 188 370 | 35 | 880 010 | 60,92 | 6558 | 55 | 9 |
| *Pseudomonas* sp. JV497 | 6 875 720 | 411 334 | 178 057 | 32 | 872 032 | 62,94 | 6515 | 59 | 8 |
| *Pseudomonas* sp. JV551A1 | 5 884 253 | 170 867 | 96 327 | 55 | 504 476 | 62,75 | 5782 | 64 | 7 |
| *Pseudomonas* sp. JV551A3 | 6 240 036 | 134 841 | 66 810 | 248 | 396 851 | 62,85 | 6116 | 66 | 12 |
| *Pseudomonas* sp. JV551A7 | 6 732 478 | 309 909 | 171 882 | 32 | 787 671 | 60,90 | 6470 | 55 | 8 |

**Tableau S4 : Correspondance des numéros de génomes en lien avec la figure 4**

| Strains | # | Strains | # | Strains | # |
|---|---|---|---|---|---|
| *P.luteola* XLDN4-9 | 1 | *P.fluorescens* Pf29Arp | 38 | *P.fluorescens* NZ007 | 75 |
| *P.stutzeri* DSM10701 | 2 | *P.thivervalensis* DSM13194 | 39 | *P.poae* RE*1-1-14 | 76 |
| *P.abietaniphila* KF701 | 3 | *P.thivervalensis* PITR2 | 40 | *P.fluorescens* BRIP34879 | 77 |
| *P.abietaniphila* KF717 | 4 | *P.umsongensis* UNC. | 41 | *Pseudomonas* sp. JV449 | 78 |
| *P.fuscovaginae* CB98818 | 5 | *Pseudomonas* sp URMO17wk12:I12 | 42 | *Pseudomonas sp.* TKP | 79 |
| *P.fuscovaginae* SE-1 | 6 | *Pseudomonas* sp GM21 | 43 | *P.veronii* 1YdBTEX2 | 80 |
| *P.agarici* NCPPB2289 | 7 | *P.mandelii* JR-1 | 44 | *P.extremaustralis* 14-3substr | 81 |
| *P.gingeri* NCPPB3146 | 8 | *P.mandelii* PD30 | 45 | *P.fluorescens* R124 | 82 |
| *P.protegens* Os17 | 9 | *Pseudomonas* sp. JV414 | 46 | *Pseudomonas* sp GM30 | 83 |
| *P.protegens* st29 | 10 | *P.lini* ZBG1 | 47 | *P.fluorescens* pf0-1 | 84 |
| *P.protegens* Pf-5 | 11 | *P.lini* DSM16768 | 48 | *Pseudomonas* sp GM25 | 85 |
| *P.protegens* Cab57 | 12 | *P.fluorescens* PA3G8 | 49 | *P.fluorescens* NZ011 | 86 |
| *P.protegens* CHA0 | 13 | *Pseudomonas* sp GM78 | 50 | *P.chlororaphis* EA105 | 87 |
| *P.chlororaphis* PCL1606 | 14 | *Pseudomonas* sp UW4 | 51 | *Pseudomonas* sp GM16 | 88 |
| *Pseudomonas* sp MRSN12121 | 15 | *Pseudomonas* sp GM33 | 52 | *Pseudomonas* sp GM24 | 89 |
| *Pseudomonas* sp. JV497 | 16 | *P.fragi* A22 | 53 | *Pseudomonas* sp. JV222 | 90 |
| *P.chlororaphis* 30-84 | 17 | *P.psychrophila* DSM17535 | 54 | *Pseudomonas* sp PTA1 | 91 |
| *P.chlororaphis* PA23 | 18 | *P.fragi* B25 | 55 | *Pseudomonas* sp R62 | 92 |
| *P.chororaphis* O6 | 19 | *P.lundensis* DSM6252 | 56 | *P.moraviensis* R28-S | 93 |
| *Pseudomonas* sp. YL-1 | 20 | *P.psychrophila* HA-4 | 57 | *P.rhizosphaerae* DSM16299 | 94 |
| *Pseudomonas sp.* JV395B | 21 | *Pseudomonas* sp PAMC25886 | 58 | *P.alkylphenolia* KL28 | 95 |
| *P.chlororaphis* GP72 | 22 | *P.fluorescens* BBC6R8 | 59 | *P.putida* PA14H7 | 96 |
| *P.chlororaphis* JD37 | 23 | *Pseudomonas* sp Ag1 | 60 | *Pseudomonas* sp. JV241A | 97 |
| *P.corrugata* CFBP5454 | 24 | *Pseudomonas* sp. UK4 | 61 | *P.cremoricolorata* DSM17059 | 98 |
| *P.corrugata* DSM7228 | 25 | *P.synxantha* BG33R | 62 | *P.cremoricolorata* ND07 | 99 |
| *P.mediterranea* CFBP5447 | 26 | *P.fluorescens* A506 | 63 | *P.parafulva* CRS01-1 | 100 |
| *P.mediterranea* TEIC1105 | 27 | *Pseudomonas* sp WCS374 | 64 | *P.mosselii* SJ10 | 101 |
| *P.brassicacearum* DF41 | 28 | *P.fluorescens* PA4C2 | 65 | *P.plecoglossicida* DSM15088 | 102 |
| *P.fluorescens* Q2-87 | 29 | *P.fluorescens* SS101 | 66 | *P.parafulva* YAB-1 | 103 |
| *P.brassicacearum* PA1G7 | 30 | *P.rhodesiae* FF9 | 67 | *P.putida* GB1 | 104 |
| *P.brassicacearum* PP1-210F | 31 | *P.fluorescens* SBW25 | 68 | *P.putida* KT2440 | 105 |
| *P.fluorescens* Q8~1-96 | 32 | *P.trivialis* IHBB745 | 69 | *Pseudomonas* sp. JV551A3 | 106 |
| *Pseudomonas* sp. JV551A7 | 33 | *P.simiae* WCS417 | 70 | *Pseudomonas* sp. JV551A1 | 107 |
| *P.brassicacearum* NFM421 | 34 | *P.simiae* MEB105 | 71 | *Pseudomonas* sp FGI182 | 108 |
| *P.fluorescens* F113 | 35 | *Pseudomonas* sp. PCL1751 | 72 | *P.plecoglossicida* NyZ12 | 109 |
| *P.kilonensis* DSM13647 | 36 | *P.tolaasii* 6264 | 73 | *P.monteilii* SB3078 | 110 |
| *P.kilonensis* P12 | 37 | *P.tolaasii* PMS117 | 74 | *P.monteilii* SB3101 | 111 |

En rouge : Génomes séquencés dans cette étude

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# CHAPITRE 4

## INTERACTIONS FONCTIONNELLES ET CONTRIBUTION RELATIVE DES FONCTIONS PHYTOBENEFIQUES A L'EFFET PHYTOSTIMULATEUR :

## CAS DE LA PGPR MULTI-FONCTION MODELE *PSEUDOMONAS FLUORESCENS* F113

## ***Préambule chapitre 4***

Les résultats obtenus dans le chapitre 2 ont montré que le maïs sélectionnait préférentiellement des *Pseudomonas* fluorescents selon le nombre de propriétés phytobénéfiques qu'ils possédaient. Les *Pseudomonas* majoritairement sélectionnés dans la rhizosphère du maïs possèdent jusqu'à 5 propriétés phytobénéfiques. L'analyse génomique a confirmé que certains sous-groupes taxonomiques de *Pseudomonas* possédaient un nombre plus élevé de propriétés phytobénéfiques co-occurrentes, notamment les souches appartenant au clade CPC (comportant les sous-groupes *P. chlororaphis*, *P. protegens* et *P. corrugata*). De plus, il a été proposé dans la littérature que l'ensemble des fonctions co-occurrentes dans un génome de PGPR participe, de façon concomitante ou non, aux effets bénéfiques observés sur la plante (Bashan *et al.* 2004). De ce fait, l'amélioration de la croissance et/ou de la santé du végétal résulteraient de la somme des contributions relatives des fonctions phytobénéfiques d'une PGPR (Bashan et de Bashan 2010).

Une approche ciblée sur une PGPR modèle a ainsi été envisagée afin de comprendre comment fonctionnent des propriétés phytobénéfiques lorsqu'elles co-occurrent au sein d'une même cellule. La PGPR multifonction modèle, *Pseudomonas fluorescens* F113, appartenant au clade CPC (plus particulièrement au sous-groupe des *P. corrugata*) et possédant 6 des fonctions phytobénéfiques recherchées dans le chapitre 2 ($DAPG^+$, $AcdS^+$, $Gcd^+$, $NirS^+$, $HCN^+$, $protéase^+$) a été utilisée dans ce travail. Les objectifs de ce chapitre ont été (i) de détecter d'éventuelles interactions fonctionnelles entre plusieurs propriétés phytobénéfiques co-occurrentes et (ii) de déterminer leur contribution à la capacité de colonisation racinaire ainsi que à l'effet phytobénéfique observé sur la plante.

Pour répondre à ces objectifs, ce chapitre est structuré en 2 parties. Dans la partie A, 4 propriétés phytobénéfiques impliquées dans l'amélioration de la nutrition ($Gcd^+$), la modulation phytohormonale ($AcdS^+$, $NirS^+$) et le biocontrôle ($DAPG^+$) ont été inactivées. Un mutant chez lequel les 4 fonctions ont été délétées a également été construit. Chez chacun de ces 5 mutants de délétion, l'ensemble des propriétés phytobénéfiques a été mesuré (sauf l'activité de la nitrite réductase). Cette approche *in vitro* a permis de visualiser les interactions fonctionnelles existant entre les propriétés phytobénéfiques co-occurrentes de *P. fluorescens*. Ces mutants ont ensuite été testés *in vitro* pour visualiser la contribution des propriétés mutées à l'effet observé sur la plante modèle *Arabidopsis thaliana*. Les résultats obtenus suggèrent que des interactions fonctionnelles ont lieu entre les 4 propriétés analysées, résultant en une modulation des activités phytobénéfiques de F113 entre-elles. De plus, chacune des propriétés phytobénéfiques étudiées ne contribuent pas de façon égale aux effets de modulation de l'architecture racinaire d'*A. thaliana*.

La partie B a consisté à déterminer la contribution relative des propriétés phytobénéfiques de F113 dans (i) la colonisation racinaire et (ii) la stimulation de la croissance du maïs, lors d'expériences réalisées en serre, dans du sol non-stérile. Les résultats obtenus montrent que l'ensemble des propriétés phytobénéfiques affecte la capacité de colonisation racinaire durant les 8 premiers jours après inoculation et que cet effet s'atténue au bout de 21 jours pour certaines propriétés. De plus, l'inoculation de ces mutants montre des patrons de stimulation du maïs (plus particulièrement de la modification de l'architecture racinaire) différents selon la propriété phytobénéfique mutée.

# PARTIE A

## *INTERACTIONS FONCTIONNELLES ET CONTRIBUTION RELATIVE DES PROPRIÉTÉS PHYTOBÉNÉFIQUES DE P. FLUORESCENS F113*

# Crosstalk between plant beneficial properties in the multi trait PGPR *Pseudomonas* fluorescens F113 affects root architecture of *Arabidopsis thaliana*

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## ABSTRACT

Fluorescent pseudomonads are playing key role in plant-bacteria interaction due to the multiple plant-beneficial properties they harbored. The relative contributions of plant-beneficial properties were investigated in the multi-plant-beneficial property strain, *Pseudomonas* fluorescens F113. To this end, several deletion mutants were constructed: Δ*phlD* (2,4-diacetylphloroglucinol [DAPG] deficient), Δ*acdS* (ACC deaminase activity deficient), Δ*gcd* (glucose dehydrogenase deficient, impaired in phosphate solubilization), Δ*nirS* (nitrite reductase deficient) and the quadruple mutant (deficient for all plant-beneficial properties targeted). Plant-beneficial activities were assayed for all mutants and revealed that phosphate solubilization and denitrification interfered with the production of 2,4-diacetylphloroglucinol (DAPG). Contrariwise, the DAPG production impacted positively phosphate solubilization. Inoculation of the F113 wild type strain on *Arabidopsis thaliana* Col0 inhibited primary root elongation, increased root hair length and plant-ethylene emission. Mutant strain inoculations revealed that the relative contribution of each of the plant-beneficial properties differed according to the plant trait measured. Plant effects did not correspond to the sum of the relative contribution of each plant-beneficial property. We showed that the contribution of these plant-beneficial properties to the root hair elongation ranged between 22% and 64% of the F113 wild type effects according to the plant-beneficial property considered. The current work underlined that cross-talk between plant-beneficial properties modulates their potential activities and generates specific root system architecture effects.

# INTRODUCTION

Plant growth and development are influenced by a myriad of abiotic and biotic factors. Among the latter, microorganisms found in the rhizosphere – also called the rhizomicrobiota – may affect plant growth and development (Mendes *et al.*, 2013; Vacheron *et al.*, 2013). Plant interactions with these microorganisms may be deleterious concerning plant pathogen microorganisms (Raaijmakers *et al.*, 2009), or beneficial when interacting with growth-promoting rhizobacteria (PGPR), a group of bacteria that stimulates plant growth, health and/or development (Nelson, 2004; Berg, 2009).

Among PGPR, fluorescent *Pseudomonas* constitutes a bacterial group well described in plant-bacteria interactions (Almario *et al.*, 2014; Weller, 2015). They were mostly studied for their biocontrol properties (Couillerot *et al.*, 2009), but some are capable of stimulating plant growth, both through direct and indirect effects on the host plant (Combes-Meynet *et al.*, 2011; Walker *et al.*, 2012). These PGPR effects are governed by plant-beneficial properties carried by these rhizobacteria, such as production of antimicrobial compounds like pyoluteorin (Maurhofer *et al.*, 2004), pyrrolnitrin (Ligon *et al.*, 2000), phenazines (Thomashow and Weller, 1988), cyanhydric acid (HCN, Voisard *et al.*, 1989), cyclic lipopeptids (CLps) (Raaijmakers *et al.*, 2006). Another molecule produced by some *Pseudomonas* is the 2,4-diacetylphloroglucinol (DAPG) a well described antimicrobial compound involved in many antagonism interactions against plant pathogens such as nematodes, protozoans, oomycetes, fungi or bacteria (Jousset *et al.*, 2006; Couillerot *et al.*, 2009; Lanteigne *et al.*, 2012) and implicated in the soil suppressiveness (Stutz *et al.*, 1986; Haas and Défago, 2005; Almario *et al.*, 2014). DAPG may also be considered as a signal which can activate the induce systemic resistance (ISR) in plant (Iavicoli *et al.*, 2003) and can change the root system architecture of plant using plant auxin-dependent hormonal pathways (Brazelton *et al.*, 2008). The fluorescent *pseudomonas* may also enhance plant nutrition increasing the availability of some nutrient such as phosphate that is essential for plant growth (Katiyar and Goel, 2003; Richardson *et al.*, 2009). Finally, fluorescent *Pseudomonas* may modulate directly the plant hormonal balance producing plant hormones as auxins (for example IAA; Indole-3-acetic acid), cytokinins (such as trans-zeatin ribose, isopentenyl adenosin, dihydrozeatin riboside) (García de Salamone *et al.*, 2001), or indirectly degrading the precursor of ethylene (1-aminocyclopropane-1-carboxylic acid - ACC) *via* the production of ACC deaminase (Shaharoona *et al.*, 2006). *Pseudomonas fluorescens* F113 was isolated from sugar beet and had been described as a biocontrol strain against a wide range of plant pathogen included oomycetes (*Pythium ultimum*, *Phytophtora cactorum*), fungi (*Fusarium oxysporum, Phoma betae, Rhizopus stolonifer*), bacteria (*Erwinia carotovora*) and even nematodes (*Globodera rostochiensis*) (Fenton *et al.*, 1992; Shanahan *et al.*, 1992, Cronin *et al.*, 1997a; Cronin *et al.*, 1997b; Delany *et al.*, 2001; Barahona *et al.*, 2011). The analysis of the F113 genome showed that this rhizobacteria harbors several key genetic determinants encoding plant-beneficial properties as described above in particular *phlD* (encoding a type III polyketide synthase involved in the production of DAPG), *acdS* (encoding the ACC deaminase), *gcd* (encoding a glucose dehydrogenase in particular involved in the phosphate solubilization) or *nirS* (encoding a nitrite reductase involved in nitric oxide (NO) production) (Redondo-Nieto *et al.*, 2013). *P.* fluorescens F113 was also tested for its capacity to stimulate maize growth alone or in combination with other microorganisms (Walker *et al.*, 2012). In this

way, this fluorescent *Pseudomonas* strain can be qualified as a multi-traits PGPR. In several model PGPR, the ecological role of each of their plant-beneficial properties was most of the time characterized independently. How all these co-occurring plant-beneficial properties interact and contribute to the beneficial effect on the host plant remains poorly understood.

This work aims to study a PGPR model strain harboring co-occurring plant-beneficial properties, in order to address the question of how plant growth-promotion properties interact with each other and what the resulting effects on plant are. To explore this issue, plant-beneficial properties from *P.* fluorescens F113 were disrupted one after another in order to (i) analyze the functional relationships between its plant-beneficial activities and to (ii) determine if all its co-occurring properties contribute equally or not to the beneficial effects of the strain on plant.

## MATERIAL AND METHODS

### Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in (**Table 1)**. *P.* fluorescens F113 wild type (WT) and its mutants were incubated at 28°C in King's B broth media (Simon and Ridge, 1974) supplemented when required with gentamycin (15 µg.mL$^{-1}$) or kanamycin (50 µg.mL$^{-1}$) . *Escherichia coli* was incubated at 37°C in Lysogeny Broth (Bertani, 1951) supplemented with gentamycin (15 µg.mL$^{-1}$) or ampicillin (75 µg.mL$^{-1}$) and kanamycin (50 µg.mL$^{-1}$) when necessary. In order to assess the effects of DAPG on the growth of F113 WT and its mutants, DAPG (Santa Cruz Biotechnology, USA) was added in King's B broth medium at 3 different concentrations (0.1 mg.mL$^{-1}$, 0.5 mg.mL$^{-1}$, and 1 mg.mL$^{-1}$).

### Growth kinetic assays

Bacterial growth was monitored during 24 h at 28°C by measuring optical density (OD) in a Bioscreen C analysator (Labsystems, Finland) with one OD measured every 15min. Growth kinetic assays were performed three times independently with 5 technical repetitions.

### Construction of deletion mutants

To construct F113 mutants impaired in plant-beneficial properties, about 700-pb fragments were amplified from regions upstream and downstream the plant-beneficial genes of interest using specific primers (**Table S1**) and subcloned into pGEM®-T easy (Promega, Madison, USA). The upstream and downstream DNA fragments were digested with respectively AatII/MunI and SacII/SacI for Δ*phlD* and Δ*gcd*, AatII/PvuII and SacII/SacI for Δ*acdS*, PvuII/MunI and SacII/SacI for Δ*nirS* and then cloned one after another into pCM184, on both sides of the kanamycine resistance encoding gene (Marx and Lidstrom, 2002). All restriction enzymes were supplied by ThermoFisher Scientific (USA). All plasmid amplifications were done using *E. coli* JM109 chemically competent cells (Promega, Madison, USA). For validation of the correct insertion of upstream and downstream DNA fragments into pCM184, sequencing using respectively primers S1F/S1R and S2F/S2R (**Table S1**) was performed. pCM184 plasmids harboring upstream and downstream regions of the plant-beneficial genes of interest were transformed into *P. fluorescens* F113 by electroporation

**Table 1: Bacterial strains and plasmids used in this study**

| Bacterial strain or plasmid | Relevant Characteristics[a] | Source or reference |
|---|---|---|
| ***Bacterial Strains*** | | |
| *Pseudomonas fluorescens* | | |
| F113 | Wild-type, Phl$^+$, *acdS*$^+$, *gcd*$^+$, *nirS*$^+$ HCN$^+$ | Shanahan *et al* 1992 |
| Δ*phlD* | F113 strain *phlD* gene deleted | This study |
| Δ*acdS* | F113 strain *acdS* gene deleted | This study |
| Δ*gcd* | F113 strain *gcd* gene deleted | This study |
| Δ*nirS* | F113 strain *nirS* gene deleted | This study |
| Δ*phlD* Δ*acdS* Δ*gcd* Δ*nirS* | F113 quadruple mutant | This study |
| Δ*phlD*Comp | Δ*phlD* with restored DAPG production capacity | This study |
| *Escherichia coli* | | |
| JM109 | Host strain for the plasmids transformation | Promega |
| ***Plasmids*** | | |
| pGEM-T easy vector | Cloning vector; Amp$^r$; | Promega |
| pBBR1-MCS5 | Cloning vector used for complementation, Gm$^r$ | Kovach *et al.* (1995) |
| pCM184 | Cloning vector used for homologous recombination, Amp$^r$, Km$^r$, Tc$^r$ | Marx and Lidstrom (2002) |
| pCM157 | Containing Cre recombinase activity, Amp$^r$, Tc$^r$ | Marx and Lidstrom (2002) |
| pGEM-T easy:*phlD* | *phlD* cloned into pGEM-T easy, Amp$^r$ | This study |
| pGEM-T easy:UP*phlD* | Upstream sequence of *phlD* cloned into pGEM-T easy, Amp$^r$ | This study |
| pGEM-T easy:DW*phlD* | Downstream sequence of *phlD* cloned into pGEM-T easy, Amp$^r$ | This study |
| pGEM-T easy:UP*acdS* | Upstream sequence of *acdS* cloned into pGEM-T easy, Amp$^r$ | This study |
| pGEM-T easy:DW*acdS* | Downstream sequence of *acdS* cloned into pGEM-T easy, Amp$^r$ | This study |
| pGEM-T easy:UP*gcd* | Upstream sequence of *gcd* cloned into pGEM-T easy, Amp$^r$ | This study |
| pGEM-T easy:DW*gcd* | Downstream sequence of *gcd* cloned into pGEM-T easy, Amp$^r$ | This study |
| pGEM-T easy:UP*nirS* | Upstream sequence of *nirS* cloned into pGEM-T easy, Amp$^r$ | This study |
| pGEM-T easy:DW*nirS* | Downstream sequence of *nirS* cloned into pGEM-T easy, Amp$^r$ | This study |
| pCM184:*phlD* | Upstream and downstream sequence of *phlD* into pCM184, Amp$^r$, Km$^r$, Tc$^r$ | This study |
| pCM184:*acdS* | Upstream and downstream sequence of *acdS* into pCM184, Amp$^r$, Km$^r$, Tc$^r$ | This study |
| pCM184:*gcd* | Upstream and downstream sequence of *gcd* into pCM184, Amp$^r$, Km$^r$, Tc$^r$ | This study |
| pCM184:*nirS* | Upstream and downstream sequence of *nirS* into pCM184, Amp$^r$, Km$^r$, Tc$^r$ | This study |
| pBBR1-MCS5:*phlD* | *phlD* cloned into pBBR1-MCS5, Gm$^r$ | This study |

[a]: Amp$^r$ Ampicillin resistant; Km$^r$ Kanamycin resistant; Tc$^r$ Tetracycline resistant, Gm$^r$, Gentamycin resistant

(

(Højberg *et al.*, 1999). Inactivation mutants resulting from a double recombination event of upstream and downstream regions were selected on King's B medium containing kanamycin (50 µg.mL$^{-1}$) and screened for plant-beneficial gene deletion by PCR with specific primers (**Table S1**). Then, the plasmid pCM157 containing the *cre-lox* recombinase gene was introduced into the *P. fluorescens* F113 kanamycin resistant mutants harboring in order to remove the antibiotic marker. To check the loss of the kanamycin cassette, mutant strains were screened for their inability to grow on King's B agar supplemented with kanamycin (50µg.mL$^{-1}$) and by PCR using external primers (**Table S1**).

**Complementation of *phlD* deletion mutant**

For complementation of the *phlD* deletion mutant, the coding region of the *phlD* gene of *P.* fluorescens F113 WT was amplified by PCR using primers phlDCompF1 and phlDCompR1 (**Table S1**) and subcloned into pGEM-T easy. The *phlD* gene was extracted from pGEM-T easy using ApaI/SacI digestion and inserted into the multiple cloning site of vector pBBR1MCS-5, under control of the lac promoter, resulting in plasmid pBBR1-MCS5:*phlD*. This plasmid was introduced into Δ*phlD* by electroporation (Højberg *et al.*, 1999).

**DAPG quantification by HPLC**

DAPG production was measured by HPLC according to the method developed by Bonsall *et al.* (1997). Briefly, all strains were grown in 4 mL of yeast malt broth medium (3g of Difco yeast extract, 3g of malt extract, 5g of peptone, 10g of glucose, and 1 liter of deionized distilled water) during 4 days at 28°C with agitation at 300 rpm. Cultures were centrifuged at 4000*g* during 8 min. The supernatant was filtered at 0.2 µm and was submitted to a liquid/liquid partition with ethyl acetate. The organic phase was evaporated to dryness and the remaining pellet was suspended in 1.0 ml of methanol. Samples were analyzed with an Agilent 1200 series HPLC (Agilent Technologies, Santa Carla, USA) equipped with a degasser (G132A), a quaternary pump module (G1311A), an automatic sampler (G1329A) and a diode array detector (DAD G1315B). Separation of compounds was performed at room temperature with a C18 reverse-phase column. For each sample, 20µL were injected and eluted at 1ml/min using a step-by-step gradient increasing acetonitrile (ACN) proportion in water: the gradient started at 40% of ACN over 4 min then rose from 40 to 64% in 7.5 min, then came to 75% at 16.5 min and ended at 100% at 18.5min (100% maintained for 3 min before decreasing to 40% for 5 min). Chromatograms were recorded at 270nm (maximum of absorbance of DAPG). The chemstation Agilent software was used for integration of chromatograms, and quantitation of DAPG was done according to a standard curve with a chemical standard (Sigma-Aldrich). This experiment was done three times independently.

**ACC deaminase activity assay**

The ACC deaminase activity of strains was performed as described by Blaha *et al.*, (2006). The method is based on the quantification of α-ketobutyrate produced by the deamination of ACC into α-ketobutyrate by the enzyme ACC deaminase. Briefly, 4 mL of King's B medium was inoculated with the tested strains. Cultures were incubated overnight at

28°C with shaking. Bacterial cells were harvested by centrifugation (8000*g*) for 10min at 4°C. The pellet were washed twice in 5 mL of DF salt minimal medium (Penrose and Glick, 2003) and suspended in 10mL of DF salt minimal medium supplemented with 3mM ACC as sole nitrogen source to induce *acdS* transcription. These cultures were incubated for 24h with shaking at 28°C and then centrifuged, as described above. Cells were washed twice in 1mL 0.1 M Tris HCl, pH 7.6 then in 800 µL of 0.1 M Tris HCl pH 8.5 and lysed with the addition of 15 µL of toluene. This mixture was shaking for 1 min in a MM200 Retsch mixer mill (Bioblock, Vaulx Milieu, France) to ensure complete cell lysis. The amount of α-ketobutyrate produced by the samples was then determined with a colorimetric method (absorbance at 540 nm), by comparison with a standard curve, according to Penrose and Glick, (2003). Total protein content in the assay was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL). ACC deaminase activity was assessed three times for each strain. The data are expressed in nmol of ketobutyrate.$h^{-1}$.mg of protein$^{-1}$. This experiment was done three times independently.

**Phosphate-solubilizing assay**

The phosphate-solubilizing ability of strains was evaluated by measuring tricalcium phosphate solubilization halos on a National Botanical Research Institute's Phosphate (NBRIP) agar medium. Strains were cultured for 1 day at 28°C in King's B broth and then deposited 4 times (5µL each) on NBRIP agar medium. Solubilization halos were measured after 6 days of incubation at 28°C using ImageJ software. This experiment was done three times independently.

**Inoculation effect on *Arabidopsis* root system architecture**

The seeds of *Arabidopsis thaliana* Ecotype Columbia0 (Col0) were disinfected for 20min in a solution containing 900µL of sterilized water, 100µL of bleach concentrated to 9.6% (active chlorine) and 3 drops of Tween 20 (wetting agent). Seeds were washed five times in sterile sterilized water and disposed in square Petri dishes (120 x 120 mm) containing solid and sterile plant culture media (0,5mM $CaSO_4 2(H_2O)$, 2mM $KNO_3$, 0,5mM $MgCl_2 6(H_2O)$, 1mM $KH_2PO_4$, 0,05mM $Na_2FeEDTA$, 2,5mM MES, 0,03µM $6(NH_4)Mo_7O_{24} 5(H_2O)$,1µM $CuSO_4 2(H_2O)$, 1µM $ZnSO_4 7(H_2O)$,15µM $MnCl_2$ $4(H_2O)$, 50µM $H_3Bo_3$, 1,2 % of agar, pH5,7) and placed in the dark at 4°C for 48 h (synchronization of dormancy). Then, seeds were transferred to a phytotron set to the following parameters: 21°C/days; 17°C/night, 13h light period, 9500lux. After 7 days, plantlets were transferred to a new plant agar medium containing F113 and mutant strains. Briefly, F113 and mutant strains were grown overnight in King's B medium and centrifuged at 3000 rpm. The pellet was washed with sterilized water. Bacteria ($10^7$ cells.$mL^{-1}$) were incorporated in plant agar medium and 50 mL were introduced in square Petri dishes. Inoculated plantlets were transferred to a phytotron set to the following parameters: 21°C/days; 17°C/night, 13h light period, 9500lux. Four *Arabidopsis* plantlets were disposed per Petri dish and 6 plates were prepared (i.e. 24 plants per conditions). The 7 conditions tested were *A. thaliana* inoculated with F113 WT, with the 4 simple mutants, with the quadruple mutant and the non-bacterized condition. This experiment was made thrice independently with 5 plants per square Petri dishes with 3 technical repetitions (15 plants per conditions). After 7

days, inoculated and non-inoculated plants were scanned in order to analyze the primary root elongation and root hair length using ImageJ software.
The relative contribution of each tested plant-beneficial property in the modulation of the *Arabidopsis* root architecture was calculated as follow:

$$(1)\ F113\ WT\ effect - control\ plant\ effect = x$$

$$(2)\ \left(\frac{F113\ WT\ effect - deleted\ "gene"\ Mutant\ effect}{x}\right) \times 100 = deleted\ gene\ contribution(\%)$$

**_Arabidopsis_ ethylene emission**

Measurements of *in vivo* ethylene emission by *Arabidopsis* plantlets were performed using infrared laser spectroscopy coupled to photo-acoustic detection (Cristescu *et al.*, 2012) (ETD-300, SensorSense, Nijmegen, Holland). Practically, we carefully poured 600µL of sterile plant culture medium (as described above) inoculated or not with the various F113 strains at a final density of $2x10^7$ cells.$mL^{-1}$ in a 2ml sterile Eppendorf tube. Once solidified, we carefully introduced two wild type *Arabidopsis* plantlets aged of seven days and closed the lid. Lids were punched to allow a regular breathing of the plants. This system was then transferred to a phytotron (MLR-352-PE, Panasonic, Gunma, Japan) set to the following parameters: 21°C (day); 17°C (night), 13h light period, 9500lux. No measure was made during 48h to allow plant acclimation in order to recover stress associated to the transfer. Prior ethylene emission measurement, 200µl of plant liquid medium was added in each tube in order to keep the head space humid, and therefore protecting the plant from the dry air coming from the compressed air bottle. Then, the lids of the tubes were removed and replaced by a rubber plug. Needles were pushed through the plug to allow the headspace to be flushed to the ethylene detector by compressed air. Every 1h30, the ethylene content in the headspace was measured while the tubes remained in the phytotron. That measurement was performed during two to three consecutive days to collect enough data on ethylene content during the day. At the end of the experiment, plant fresh weight was measured to allow data normalization. Raw results were exported into a spreadsheet in which they were normalized and processed to obtain at least 10 different measures of ethylene content in presence of light in each condition.

**Statistical analysis**

Regarding assays on each plant-beneficial function, data were log-transformed in order to respect normal distribution and variances homogeneity and a one-way ANalysis Of VAriance and a Tukey's HSD test post hoc were performed to detect significant differences between strains. Root hair length data were compared using Kruskal-Wallis tests followed by post-hoc non-parametric multiple comparisons. Primary root length data were square-root transformed in order to respect variance homogeneity and normality and a one-way ANOVA coupled with Tukey's HSD test was performed. Ethylene emission data were expressed related to non-inoculated condition and compared using ANOVA coupled with Tukey's HSD test. All analyses were performed at $P < 0.05$, using R studio software (RStudio Team, 2015). Results in figures are presented as means ± standard error.

# RESULTS

## Mutant growth characteristics

Colonies of F113 WT and its related mutants are orange-brown on King'B and LB agar, after 48h of incubation at 28°C, except for the Δ*phlD* and quadruple mutants, which were not able to produce DAPG. The latter displayed an intensive green fluorescent pigmentation under UV light (**Figure S1**). Colonies of the Δ*gcd* and quadruple mutants, which were not able to solubilize phosphate were significantly larger than the other strains on NBRIP agar media (respectively 6.6± 0.4 mm and 7.0± 0.3mm against 5.1 ± 0.3mm for all the other strains). Moreover, colony aspect of Δ*gcd* and quadruple mutant on NBRIP media appeared yellow and mucoid.

Kinetic growth in King's B broth was followed for each strain (**Figure 1**). Slopes of the exponential growth were similar between the mutants and the F113 WT (**Table S2**) except for the quadruple mutant, which displayed a higher slope value in the exponential growth phase. Δ*acdS*, Δ*gcd*, Δ*nirS* and F113 WT displayed similar parameters in the stationary growth phase whereas the Δ*phlD* and quadruple mutants showed a higher optical density in stationary growth phase than the other strains (**Figure 1**). Potential DAPG toxicity was evaluated measuring bacterial growth with or without DAPG (**Figure S2**). On one hand, the complemented *ΔphlD* mutant had a similar growth curve to F113 WT (**Figure S2A**). On the other hand, the addition of DAPG reduced significantly the optical density in the stationary growth stage of F113 WT (starting from 0.1 µg.mL$^{-1}$ of DAPG) and no concentration effect was observed (**Figure S2B**). The growth of Δ*phlD* was negatively affected by the addition of DAPG, starting from 0.5 mg.mL$^{-1}$ of DAPG (**Figure S2C**). The optical density in the stationary growth stage was significantly lower when Δ*phlD* was grown in presence rather than in absence of DAPG (**Figure S2C**). The Δ*phlD* growth was similar in presence of 0.5 µg.mL$^{-1}$ and 1µg.mL$^{-1}$ of DAPG (**Figure S2C**). Contrasted effects were observed for the complemented Δ*phlD* mutant. The addition of DAPG (from 0.5 µg.mL$^{-1}$) slowed down the exponential growth stage of the complemented Δ*phlD* mutant, but the optical density reached in the stationary growth stage was similar to that of F113 WT (**Figure S2D**).

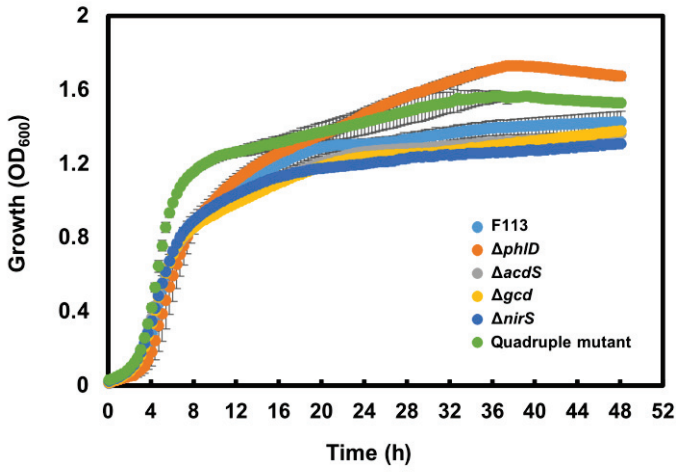


**Figure 1: Growth curve of the *P. fluorescens* wild-type F113 and its plant-beneficial mutants in King's B media at 28°C.** The data represent the means (standard errors) of five replicate cultures. The experiment was done three time independently.

**Determination of plant-beneficial activities of *P. fluorescens* F113 and its derivative plant-beneficial mutants**

In order to evaluate potential crosstalk each plant-beneficial properties may have on another, activities were assayed for each plant-beneficial property, for each mutant strains. First of all, DAPG production was assessed using HPLC for F113 WT and its related plant-beneficial mutants. The Δ*phlD* and the quadruple mutant were functionally validated for their inability to produce DAPG (**Table 2**). The lack of ACC deaminase activity (*acdS*) had no significant effect on DAPG production (**Table 1**) contrariwise to the lack of glucose dehydrogenase activity (*gcd*) which showed a significant increase of DAPG production; 82 times more DAPG was detected in the Δ*gcd* supernatant than in F113 WT. The deletion of *nirS* also increased the DAPG production (1.7 fold more than F113 WT), but lesser than the deletion of *gcd*.

Concerning the ACC deaminase activity, the Δ*acdS* and quadruple mutants were functionally validated for their inability to degrade ACC to α-ketobutyrate. The ACC deaminase activity increased significantly 1.7 more for Δ*gcd* and 1.3 more for Δ*nirS* than in F113 WT (**Table 2**). ACC deaminase activity from Δ*phlD* was not significantly different to F113 WT (**Table 2**).

For the phosphate solubilization capacity, the Δ*gcd* and quadruple mutants were not able to solubilize phosphate (**Table 2**). The Δ*phlD* showed a decrease of this ability to solubilize phosphate (1.7 times less than the F113 WT) (**Table 2**). Other simple mutants have the same phosphate solubilization capacity than F113 WT (**Table 2**).

**Table 2: Effect of deleted F113 plant-beneficial functions on plant-beneficial activity.**

| Strains | DAPG production † | ACC deaminase activity ‡ | Phosphate solubilization ∞ |
|---|---|---|---|
| F113 WT | 0.81 ± 0.16 c | 3.58 ± 0.52 a | 10.37 ± 3.26 ab |
| Δ*phlD* | ND[θ] d | 3.20 ± 0.86 a | 5.99 ± 0.93 c |
| Δ*acdS* | 1.36 ± 0.46 bc | ND b | 11.64 ± 2.02 a |
| Δ*gcd* | 66.53 ± 3.67 a | 5.99 ± 1.34 a | ND d |
| Δ*nirS* | 1.39 ± 0.35 b | 4.66 ± 1.32 a | 9.51 ± 2.00 b |
| Quadruple mutant | ND d | ND b | ND d |

† : DAPG production unit of measure: $\mu g.mL^{-1}.OD^{-1}$

‡ : ACC deaminase acitivity unit of measure: µmol of α-ketobutyrate. $h^{-1}$. mg of $protein^{-1}$

∞ : Phosphate solubilization unit of measure: mm

θ : Non detected

Data were presented as Mean ± SD. Letters indicate statistical relations between strain for each tested plant-beneficial activities (ANOVA and Tukey's HSD test, $P < 0,05$)

**Effect of *P. fluorescens* F113 and mutants on *A. thaliana* Col0 ethylene emission**

Plant ethylene emission was assessed by photoacoustic spectrophotometry. The ethylene amount was expressed by comparing condition where F113 WT or mutants were inoculated to *A. thaliana* versus the non-inoculated condition. Plant ethylene emissions were

significantly higher than non-inoculated plant for F113 WT (1.5 fold), and Δ*acdS* (2 fold). In contrast, when Δ*phlD* was inoculated, plant ethylene emission was significantly lower than F113WT, (1.7fold), Δ*acdS* (2.6 fold), and Δ*gcd* (1.4 fold) (**Figure 2**). The plant ethylene emission when the quadruple mutant was inoculated did not significantly differ from that of the non-inoculated condition and mutants except for Δ*acdS* and the F113WT.

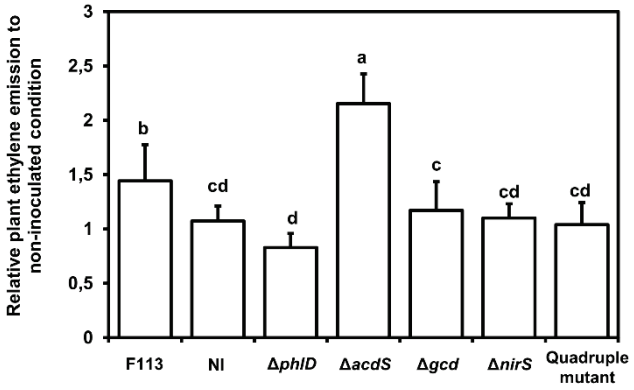


**Figure 2: Plant ethylene emission in response to inoculation with F113 WT and its plant-beneficial mutants.**

NI: Non-inoculated condition. Conditions harboring the same letter were not significantly different (ANOVA, Tukey's HSD test, $P < 0.05$).

## Effect of *P. fluorescens* F113 and mutants on primary root and root hair elongation of *A. thaliana* Col0

The inoculation of *A. thaliana* was done in plant media agar containing $10^7$ bacteria.mL$^{-1}$. F113 WT, simple and quadruple mutants were tested and primary root elongation was measured (**Figure 3-A**). First, F113 WT inoculation inhibited significantly primary root length of *A. thaliana* (**Figure 3-A**). The primary roots of the non-inoculated plants were 4 times longer than the F113 WT inoculated plants. When *A. thaliana* Col0 was inoculated with Δ*phlD,* the same plant phenotype was observed than for F113 WT inoculated plants, implying that DAPG production did not contribute to the primary root length inhibition (**Figure 3-A**). In contrast, the reduction of primary root length was lost when Δ*acdS*, Δ*gcd*, Δ*nirS* and the quadruple mutant were inoculated compared to the F113 condition. (**Figure 3-A**). Therefore, *acdS*, *gcd* and *nirS* contributed equally to primary root inhibition (**Figure 3-A**).

Differential effects between inoculated and non-inoculated plants were found for root hair elongation (**Figure 3-B**). First of all, *A. thaliana* inoculation with F113 WT led to a significant enhancement of root hair elongation (2.4 times more than the non-inoculated condition). Inoculation with the quadruple mutant generated the same plant phenotype than the non-inoculated condition, suggesting that the deleted plant-beneficial properties were responsible for the root hair stimulation obtained in F113 WT inoculated plants (**Figure 3-B)**. The relative contribution of each plant-beneficial property was not the same (**Figure 3-B)**. For Δ*phlD* inoculated plants, root hair length was significantly lower than for F113 WT inoculated plants (-20%), but maintained a higher root hair length than non-inoculated plants (+116%) (**Figure 3-B)**. Compared to the WT, *phlD* contributes to the root hair elongation for 32%. In Δ*acdS* and Δ*gcd* inoculated plants, root hair length also decreased by respectively -13.6% and -20.9% compared to the F113 WT inoculated plants but still remained higher than non-

inoculated ones (+133% and +112%) (**Figure 3-B)**. The relative contribution to the root hair elongation of these two plant-beneficial properties amounted to 22% for *acdS* and 34% for *gcd*. Δ*nirS* inoculation led to a significant reduction of 40.6% of the root hair length compared to F113 WT inoculated plants and this inoculated mutant effect still remained higher than the non-inoculated plants (+60.6%) (**Figure 3-B)**.

To summarize, F113 plant-beneficial properties did not contribute in the same way to the plant phenotype observed.

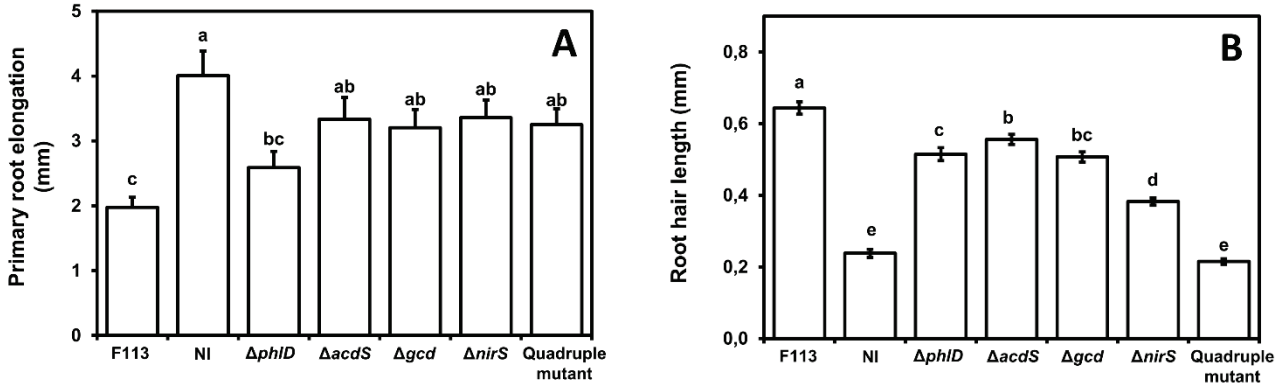


**Figure 3: Effect of the *Arabidopsis thaliana* inoculation of F113 and its mutants on the primary root elongation (A) and root hair length (B).**
NI: Non-inoculated condition. Conditions harboring the same letter were not significantly different (ANOVA, Tukey's HSD test, $P < 0.05$)

## DISCUSSION

F113 plant-beneficial mutants were constructed in order to determine (i) the interaction between co-occurring plant-beneficial properties and (ii) the relative contribution of the latter on *A. thaliana* root parameters and ethylene emission.

Many of our observations led us to think that DAPG may be toxic to F113 WT. The addition of DAPG to F113WT culture media induced a lag phase at the beginning of growth curve and a reduction of OD600 obtained in stationary growth phase (**Figure S1-B**). Moreover, the addition of DAPG involved same lag phase in Δ*phlD* and also reduced the stationary growth phase. However, Δ*phlD* stationary growth stage was still upper than F113 WT one, suggesting that this difference may be not only due to DAPG toxicity but could also be attributed to the consequent metabolic cost of DAPG production (Zha *et al.*, 2006).

Specific patterns of co-occurring plant-beneficial properties were observed frequently among fluorescent pseudomonads such as DAPG and HCN productions (Ramette *et al.*, 2003), DAPG and auxin productions (Picard and Bosco, 2005), DAPG and pyoluteorin productions (Loper *et al.*, 2012). Co-occurrences of plant-beneficial properties were also study among *Proteobacteria* (Bruto *et al.*, 2014). This preferential plant-beneficial property association may reflect functional interactions between them. In the current study, the model strain F113 which harbors multiple plant-beneficial properties (Redondo-Nieto *et al.*, 2013) was used in order to evaluate functional interactions between plant-beneficial properties. First, the disruption of *gcd* encoding a quinoprotein glucose dehydrogenase (Duine *et al.*, 1979), involved in phosphate

solubilization (de Werra *et al.*, 2009; Miller *et al.*, 2010; Meyer *et al.*, 2011), increased dramatically the production of DAPG. Quite similar findings were evidenced for a *gcd* mutant in *P. protegens* CHA0 which also increased the production of an antimicrobial compound, the pyoluteorin (de Werra *et al.*, 2009). The glucose dehydrogenase had pleiotropic roles in bacterial cells. One of them is its involvement in carbon source utilization and energy supply. In F113 WT cells containing a functional Gcd, one part of glucose-6P is transformed into gluconic acid, which is exported out of the cells for phosphate solubilization and the other part is imported inside the cytoplasm and incorporated into the central metabolism (**Figure 4-A**). Thus, when *gcd* is deleted we can hypothesize that more glucose-6P is provided to the glycolysis, producing on one hand more ATP for the cell and on the other hand a higher amount of pyruvate that is then incorporated to the tri-carboxylic acid cycle to produce higher amount of acetyl CoA, the precursor of DAPG (Bangera and Thomashow, 1999; Clifford *et al.*, 2015) (**Figure 4-B**). In order to support this model, the expressions of genes and enzyme activities involved in the glucose catabolism pathway have to be measured in F113 WT and in its Δ*gcd* mutant.

In this study, we also find that the nitrite reductase (*nirS*) seems to be involved in inhibition of DAPG production, but to a lesser extent than does the glucose dehydrogenase. This functional interaction was not yet characterized but may involve modification of the reducing power state of the bacterial cell.

We also find that DAPG production impacted positively phosphate solubilization. Considering the model established in **Figure 4**, it could be hypothesized that DAPG may affect medium acidification by modulating *gcd* gene expression. It had been described that the expression of *acdS* was regulated by both ACC and leucine concentration in *Pseudomonas* sp. UW4 (formerly *Enterobacter clocae* UW4) (Li and Glick, 2001) but also by the oxygen content in *Azospirillum lipoferum* 4B (Prigent-Combaret *et al.*, 2008). However, in the current study, no F113 plant-beneficial property was found to interact with the ACC deaminase activity (*acdS*). In F113, crosstalk between plant-beneficial properties might mainly involve metabolic interactions than direct expression regulation of plant-beneficial property pathways as documented for the fine tuning regulation between pyoluteorin and DAPG production in *P. protegens* Pf-5 and *P. protegens* CHA0 (Schnider-Keel *et al.*, 2000; Kidarsa *et al.*, 2011; Clifford *et al.*, 2015).

Despite the fact that the relative contribution of co-occurring plant beneficial properties in biocontrol performance (such as the production of antimicrobial compounds) have been well studied (Voisard *et al.*, 1989; Iavicoli *et al.*, 2003; Romanowski *et al.*, 2011; Calderón *et al.*, 2013), the contribution of co-occurring plant-beneficial properties in plant beneficial growth promotion effect was poorly documented. Recently, Bashan and de-Bashan (2010) hypothesized that each co-occurring plant-beneficial property was involved, simultaneously or successively, in improvement of the plant-growth and this improvement may result from the sum of each co-occurring plant-beneficial properties additive hypothesis". In this line, the contribution of each co-occurring plant-beneficial properties harbored by F113 WT was assessed considering two *Arabidopsis* root parameters, (i) the primary root length and (ii) the root hair length. Mutant inoculations revealed that each plant-beneficial property did not contribute equally to root architecture modulation (**Figure 3**). The primary root elongation in *A. thaliana* is controlled by multiple plant hormones such as auxin (controlling cell division

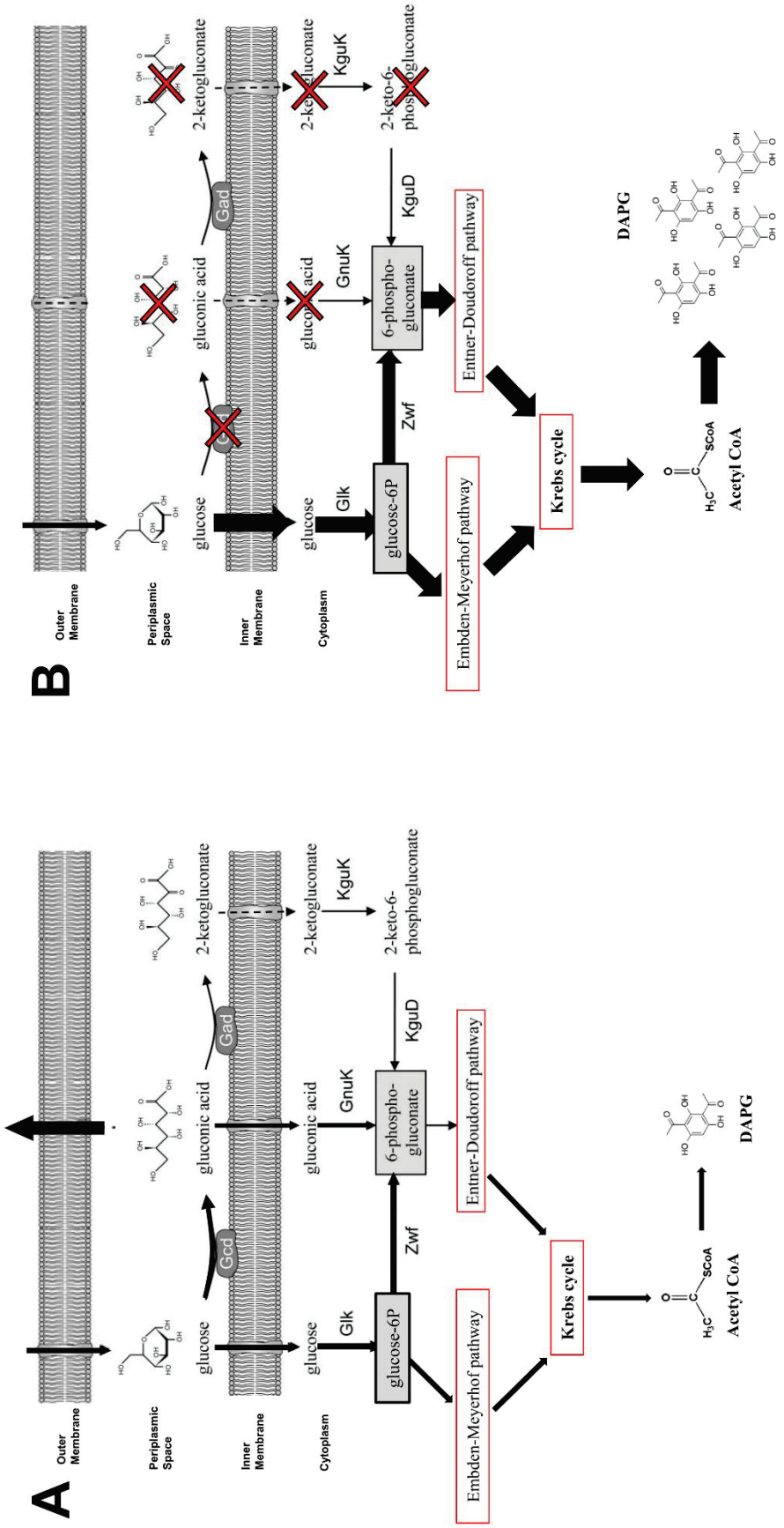


**Figure 4: Model of metabolic regulation involved in DAPG production in *P. fluorescens* F113.**
A: In F113 WT ; B: In F113 *gcd* mutant. Glucose catabolism in pseudomonads based on studies with *P. protegens* CHA0 (Werra *et al.*, 2009; Miller *et al.*, 2010). Enzymes involved in glucose metabolism are abbrev*i*ated using the same nomenclature as de Werra *et al.* (2009). The corresponding enzyme functions and F113 gene names are indicated in parenthesis: Gcd (glucose dehydrogenase - *gcd*); Glk (glucokinase - *PSF113_1279*), Gad (Gluconate dehydrogenase - *PSF113_4842*); Zwf (glucose-6-phosphate-1-dehydrogenase - *PSF113_1290* and *PSF113_2674*); GnuK (gluconokinase - *PSF113_1311*); KguD (2-ketogluconate 6-phosphate reductase - *kguK*); KguK (2-ketogluconate kinase – *kguK*).

and elongation) or ethylene (controlling root cell elongation) (Swarup *et al.*, 2007; Jung and McCouch, 2013; Street *et al.*, 2015). DAPG was found to inhibit primary root elongation using the plant-auxinic pathway (Brazelton *et al.*, 2008). Primary root was still found inhibited when each mutant and especially the DAPG mutant were inoculated, implying that other F113 compounds inhibit primary root elongation. Moreover, the inoculation of all mutants had induced similar plant-ethylene emission than non-inoculated condition, except Δ*acdS* (**Figure 2**). This mutant was unable to degrade the ethylene precursor ACC, which resulted in an over-emission of plant-ethylene. Strangely, plant ethylene emission remained higher when F113 WT was inoculated than non-inoculated condition. It is possible that the production of ethylene caused by bacterial recognition remains higher than the capacity of F113 WT to degrade plant ACC and may explain in part the primary root inhibition (**Figure 3-A**). If *Arabidopsis* primary root elongation was inhibited in inoculated conditions, root hair elongation was enhanced (**Figure 3-B**). Root hair elongation was activated by auxin compound in *A. thaliana* (Spaepen and Vanderleyden, 2011) but F113 did not produce indol-3-acetic acid or other auxinic compounds by a tryptophan dependent pathway (data not shown). When the fourth plant-beneficial properties studied were deleted, root hair length was similar to the non-inoculated condition, showing that at least one of these plant-beneficial properties is involved in this plant phenotype (**Figure 3-B**). F113 plant-beneficial properties did not contribute equally to stimulate root hair length. The most significant effect on root hair length was obtained with Δ*nirS*. The nitrite reductase (NirS) in F113 belongs to denitrification pathway, which allows the reduction of nitrate ($NO_3^-$) to dinitrogen ($N_2$) with nitric oxide as intermediate. A part of nitric oxide produced may leave the bacterial cells (Choi *et al.*, 2006) and may reach the plant cells where it would affect the development of lateral roots and root hair elongation (Lombardo *et al.*, 2006). Thereby, the potential nitric oxide produced by F113 had an important contribution to root hair elongation (**Figure 3-B**). Plant-beneficial properties contributed to the root hair elongation at different levels of contribution, but contrariwise to the "additive hypothesis", the plant observed effects do not resulted from the sum of the relative contribution of each plant-beneficial property, probably due to the crosstalk existing between co-occurring plant-beneficial properties.

To summarize, this work underlines that distinct patterns of plant-beneficial properties harbored by PGPR strains may lead to specific crosstalk between these properties, resulting in precise plant effects.

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# SUPPLEMENTARY DATA

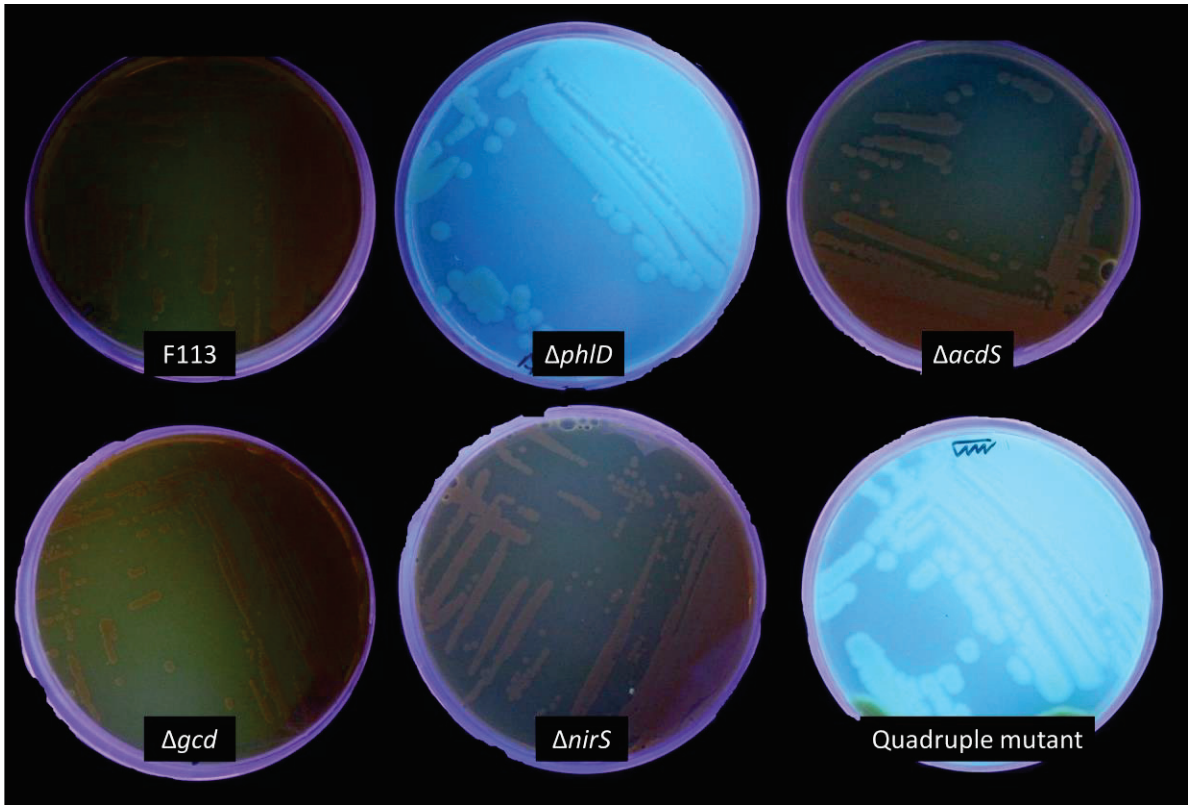


**Figure S1: Effect of *phlD* deletion on *Pseudomonas fluorescens* F113 UV fluorescence.**

Strains were grown on King's B media for one week and plates were incubated on UV light. A: F113 WT; B: Δ*phlD*; C: Δ*acdS*; D: Δ*gcd*; E: Δ*nirS*; F: quadruple mutant.

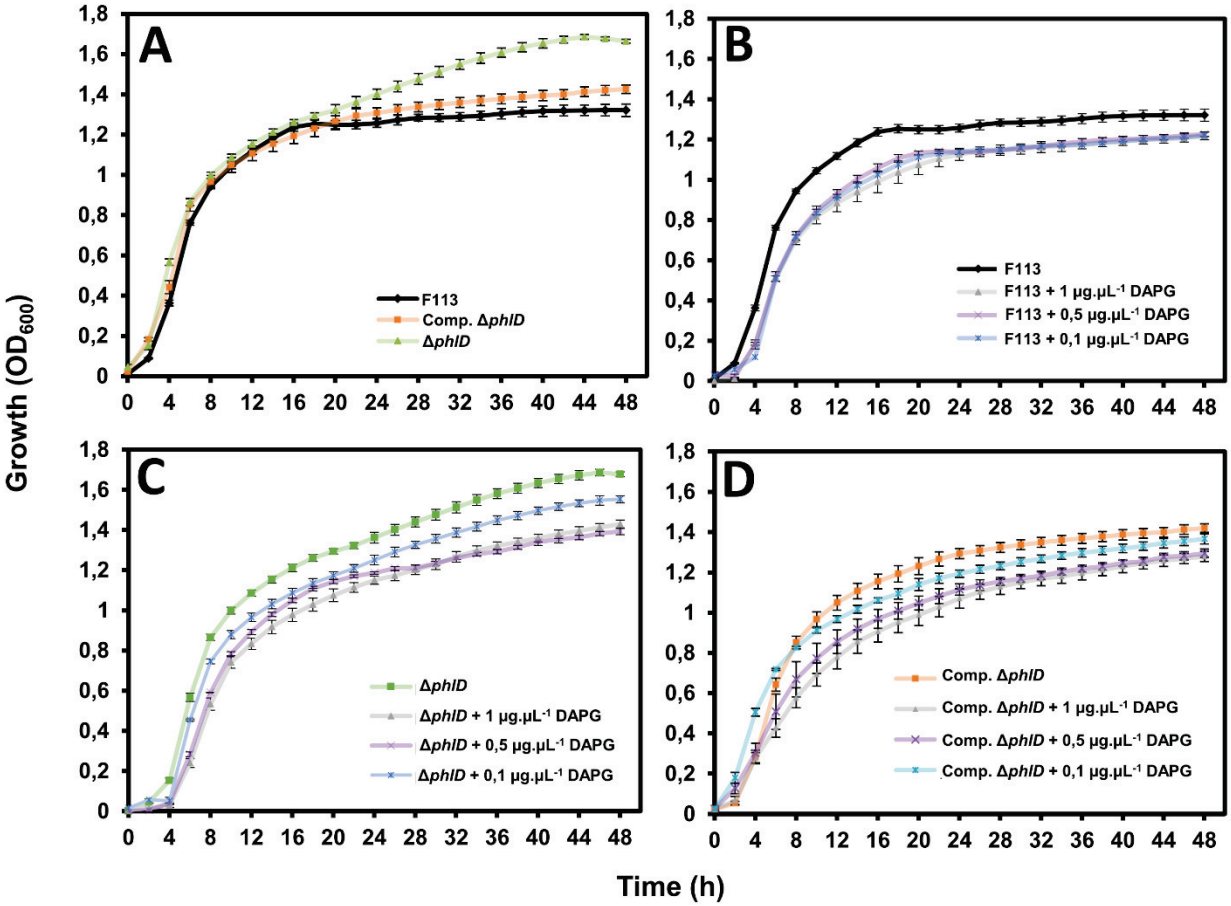


**Figure S2: Effect of the addition of DAPG on growth curves of *P. fluorescens* wild-type F113, Δ*phlD* mutant, Δ*phlD* mutant with restored DAPG production.**

Growth curves of F113 WT, Δ*phlD* and complemented Δ*phlD* (**A**). Effect increasing DAPG concentrations on F113 WT (**B**), Δ*phlD* (**C**) and complemented Δ*phlD* (**D**). Comp.: Complemented

# PARTIE B

## *IMPACT DES FONCTIONS PHYTOBENEFIQUES DE P. FLUORESCENS F113 SUR LA COLONISATION RACINAIRE ET LA CROISSANCE DU MAÏS*

# The main plant-beneficial functions of *Pseudomonas fluorescens* F113 differently affected its ability to colonize maize roots and to stimulate maize growth

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## ABSTRACT

Fluorescent pseudomonads are well known for their involvement in biocontrol or plant growth improvement. The importance of *Pseudomonasfluorescens* F113 plant-beneficial functions for maize root colonization as well as the implication of these plant-beneficial functions in the effect on maize growth were investigated using *P. fluorescens* F113 mutant strains Δ*phlD* (deficient in 2,4 diacetylphloroglucinol (DAPG) production), Δ*acdS* (deficient in 1-aminocylclopropane-1-carboxylic acid (ACC) deaminase activity), Δ*gcd* (deficient in glucose dehydrogenase involved in phosphate solubilization), Δ*nirS* (deficient in nitrite reductase) and the quadruple mutant Δ*phlD*Δ*acdS*Δ*gcd*Δ*nirS*. The ability of all plant-beneficial mutants to colonize maize root was affected at 8 days post inoculation (dpi), and even more drastically for Δ*acdS*, Δ*nirS* and the quadruple mutant at 20 dpi. The deletion of these two functions did not modified aerial plant parts of maize but had a major impact on the root system architecture, decreasing the number of root tips (-62% and -45%, respectively), root fresh biomass (-31% and -38%), total root length (-64% and -59%) and total root surface (-50% and -50%), and increasing root diameter (+39% and +26%) compared to F113 wild type. This study evidences for the first time the differential contribution of 4 plant-beneficial functions harbored by *P. fluorescens* F113 on (i) colonization of maize roots and (ii) maize growth in pot experiments with non-sterile soil.

# INTRODUCTION

Plant-growth promoting rhizobacteria (PGPR) are root-colonizing bacteria able to procure a better growth and health for plants according to various mechanisms (Richardson *et al.*, 2009; Vacheron *et al.*, 2013). Among these mechanisms, PGPR may modulate plant hormonal signalization by producing plant hormones such as auxins, cytokinins, gibberellins, nitric oxid (NO) (Bashan and de-Bashan, 2010), or by secreting the 1-aminocyclopropane carboxylic acid (ACC) deaminase which degrades the plant ethylene precursor (ACC), resulting in the decrease of ethylene content in plant (Glick *et al.*, 1998). PGPR may also help plant nutrition by solubilizing inorganic compounds such as phosphate, or other mineral elements (Vessey, 2003; Adesemoye and Kloepper, 2009). Some PGPR, in particular fluorescent pseudomonads producing antimicrobial compounds such as 2,4-diacetylphloroglucinol (DAPG), are able to protect plants against plant pathogen microorganisms (Couillerot *et al.*, 2009; Lanteigne *et al.*, 2012) and also to activate plant defenses (Iavicoli *et al.*, 2003). Production of DAPG may also modify root system architecture acting as a plant hormone signal (Leij *et al.*, 2002; Brazelton *et al.*, 2008). Among fluorescent pseudomonads, some strains may harbor several of these plant beneficial properties.

*Pseudomonas fluorescens* F113, isolated from sugar beet rhizosphere (Shanahan *et al.*, 1992), is well known for its biocontrol activity against a broad range of plant pathogen microorganisms (Cronin *et al.*, 1997; Don Cronin *et al.*, 1997; Dunne *et al.*, 1998). The inoculation of this PGPR on field-grown sugar-beets had no effect on enzyme activities involved in P and N nutrient cycling and on soil chemistry in the rhizosphere (Naseby *et al.*, 1998) as well as on the diversity of rhizobia populations nodulating red clover in a crop rotation system (Walsh *et al.*, 2003). Indigenous fluorescent pseudomonads populations were overall weakly affected by the inoculation of F113 (Moënne-Loccoz *et al.*, 2001). The impact of F113 inoculation on plant growth was also investigated on sugar beet (Moënne-Loccoz *et al.*, 1998) and maize (Walker *et al.*, 2012) in field experiments. If no impact of *P. fluorescens* F113 inoculation was observed for sugar beet, *P. fluorescens* F113 appeared to enhanced total root length and root numbers in maize cultivar PR37Y15.

The potential success of plant growth stimulation by PGPR primarily relies on their root colonization ability. *P. fluorescens* F113 can colonize maize root and persist over 5 weeks (Von Felten *et al.*, 2010). It has been showed that the presence of flagella in *P. fluorescens* F113 was required for successful root colonization of alfalfa (Capdevila *et al.*, 2004). Root colonization by *P. fluorescens* F113 is repressed by three independent systems of regulation including the Gac system, the Wsp system and the *sadB* gene (Navazo *et al.*, 2009). Mutants of these pathways were described as hyper-colonizing strains but were unable to produce antimicrobial compounds resulting in a loss of their biocontrol ability (Barahona *et al.*, 2010). Furthermore, natural phase variation occurs in *P. fluorescens* F113 during rhizosphere colonization involving site-specific recombinases Sss and XerD that switch off the Gac system (Martínez-Granero *et al.*, 2006). The production of DAPG has also been described as not involved in the ability of this rhizobacteria to colonize sugar beet roots (Carroll *et al.*, 1995). In addition to the ability to produce DAPG (*phlD* being a key determinant of its biosynthesis), *P. fluorescens* F113 harbors other genetic determinants involved in plant-beneficial properties such as *gcd* gene encoding a glucose dehydrogenase involved in phosphate solubilization (Miller *et al.*, 2010), *acdS* gene

encoding the ACC deaminase enzyme (Bruto *et al.*, 2014) and *nirS* encoding the nitrite reductase involved in NO production (Choi *et al.*, 2006). The contribution of these plant-beneficial properties in root colonization and plant growth promotion are unknown.

The aim of this study was to evaluate the importance of 4 main plant-beneficial functions harbored by the model strain *P. fluorescens* F113 in its ability to colonize roots and to provide beneficial effects on root and shoot parameters of the maize cultivar PR37Y15. To this end, the ability of F113 simple mutants Δ*phlD*, Δ*acdS*, Δ*nirS* and Δ*gcd* and of a quadruple mutant Δ*phlD*Δ*acdS*Δ*gcd*Δ*nirS* to colonize maize roots were compared to that of the parental strain F113, at 8 and 20 days post inoculation (dpi). Plant-beneficial mutants were also inoculated in order to evaluate their capacity to modulate maize root architecture and aerial plant parts when compared to F113 and non-inoculated conditions. We provide evidence that all plant-beneficial functions did not contribute equally to the colonization of maize roots and enhancement of maize growth.

## MATERIAL AND METHODS

### Bacterial inoculum preparation

The *P. fluorescens* F113 wild type and its deletion mutants Δ*phlD,* Δ*acdS*, Δ*gcd*, Δ*nirS* and the quadruple mutant (Δ*phlD*Δ*acdS*Δ*gcd*Δ*nirS*, constructed previously Vacheron *et al. in preparation*) were incubated overnight at 28°C in King's B broth medium. The bacterial solution was washed twice using sterilized water and adjusted to $10^7$ bacteria per milliliter.

### Plant experiments

The semi-late maize hybrid PR37Y15 (Pioneer Semences, Aussonne, France) was used in this study. Maize seeds were surfaced disinfected using a solution of sodium hypochlorite (3.4%). Briefly, seeds were soaked in the sodium hypochlorite solution during one hour with agitation. Five washes were performed with sterile water. Four maize seeds were sown in non-sterile soil taken from La Côte Saint-André (LCSA) near Lyon (France; El Zemrany *et al.*, 2006) and placed in $2dm^3$ pots filled with 2.0kg of sieved soil (5mm) with a water content of 20% (v/v). The moisture was maintained at 20% during the experiment. Seeds (4 per pot) were inoculated with 100µL of bacterial inoculum solution, corresponding to $10^6$ bacteria, and recovered by the soil. Ten pots per conditions were performed.

### Root colonization

The measurement of root colonization was assessed at 0, 8 and 20 dpi taking 10 seeds for each condition at each sampling time. Inoculated seeds or plants were harvested and disposed in a 50mL Falcon tube and then soaked in liquid nitrogen. Seeds and root systems were lyophilized and then scratched in order to extract DNA from the seed surface and root adhering soil. DNA extractions from 500mg of rhizosphere soils or seeds teguments were performed using a FastDNA™ SPIN Kit for Soil kit (MP Biomedicals, USA). DNA was also extracted from 1mL of inoculum solution in order to ascertain the cell concentration of inocula.

### Real-time PCR condition

The seed and root bacterial colonization was assessed by real-time PCR as described by Von Felten *et al.*, (2010). Real-time PCR were performed using the LightCycler® FastStart DNA Master SYBR® Green I kit and a LC-480 LightCycler (Roche Applied Science, Indianapolis, IN). 5ng of extracted DNA were used to assess the abundance of seed and root colonization by the F113 parental and mutant strains using F113_1_for and F113_1_rev primers with a detection limit of $10^4$ cells/seed and $10^4$ cells/g of rhizosphere (Von Felten *et al.*, 2010). Real-time PCR quantification data were converted to gene copy number per gram of lyophilized root as described by (Couillerot *et al.*, 2012).

### Plant growth promotion improvement

Inoculum impact on plant growth was assessed at 20dai by characterizing maize root system architecture using WinRhizo (Reagent Instruments, Quebec City, Quebec, Canada). Leaf surface was measured with the WinFolia image analysis software (Regent Instruments, Quebec City, Quebec, Canada). Chlorophyll content was measured with the chlorophyll content meter (Hansatech instruments model CL-01, Amesbury, USA). Root and shoot fresh and dry biomasses were weighed. 10 plants were analyzed per conditions.

### Statistical and data analysis

The comparison of root colonization between strains at the same sampling time was analyzed by ANOVA and Tukey's HSD post hoc. The comparison of root colonization across the time for each strain was analyzed by ANOVA and Fisher's LSD test. The effects of strain inoculation on plant growth parameters were compared using ANOVA and Tukey's HSD post hoc and represented using star plot representation. All data were presented as mean ± standard error.

## RESULTS

### The *acdS* and *nirS* encoding functions affect the fitness of *P. fluorescens* F113 in maize rhizosphere

The inoculum solution concentration was controlled and appeared to be identical for each strain tested (from 7.57±0.16 to 7.79±0.13 log cells/mL) (**Figure S1**). Thereby, an inoculation of $5.10^6$ bacteria in 100µL was performed on maize seeds. At the day of inoculation (0dpi), no significant differences were found between all tested strains (**Figure 1**). At 8dpi, all mutant strains were less abundant in the rhizosphere than the F113 WT (**Figure 1**). The Δ*phlD* and quadruple mutants were about 7.9 and 4.8 times less abundant in the rhizosphere than the F113 WT strain whereas the Δ*acdS,* Δ*gcd,* and Δ*nirS* were respectively 2.4, 2.5, and 3.4 times less abundant. Therefore, Δ*acdS* and Δ*gcd* mutants were better root-colonizer than Δ*phlD*. At 20dpi, there was no significant difference in the rhizosphere colonization between F113 and the Δ*phlD* and Δ*gcd* mutants. In contrast, Δ*acdS*, Δ*nirS* and the quadruple mutants were less abundant in the rhizosphere compared to F113. The F113 WT strain and the Δ*acdS* rhizosphere abundance decreased significantly from day 20 (**Figure 1**). The rhizosphere abundance of

Δ*phlD* decreased significantly from day 8 and stayed constant up to 20dpi. For Δ*gcd*, Δ*nirS* and the quadruple mutant, their rhizosphere abundance started to significantly decrease at day 8 and still declined at day 20 (**Figure 1**).

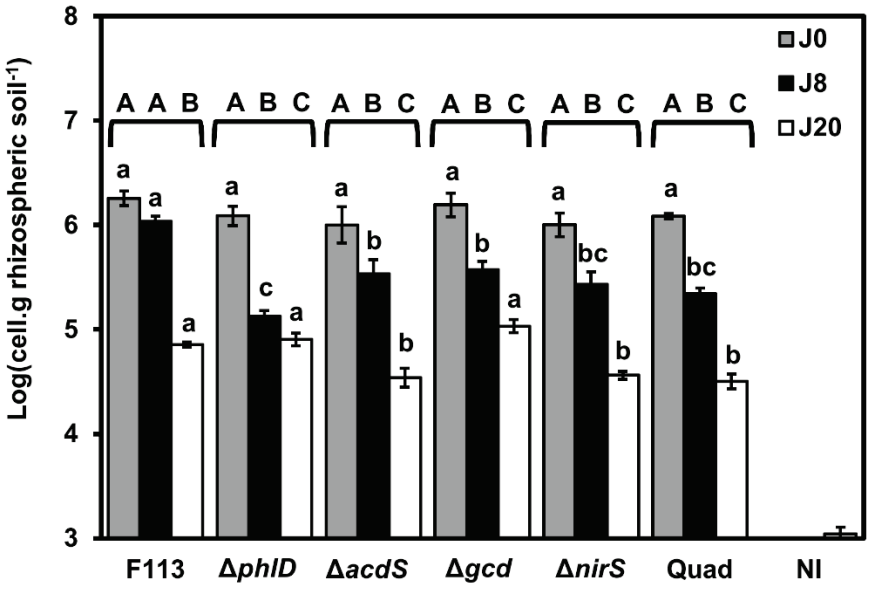


**Figure 1: Comparison of the maize root colonization levels between *P. fluorescens* F113 and its plant-beneficial mutants.**

Error bars correspond to standard error. NI: Non-inoculated condition ; Quad: Quadruple mutant. Statistical differences for the root colonization across the time are represented with letters a-c. Statistical differences between strains are represented with letters α-γ.

## The *acdS*, *nirS* and *gcd* encoding functions affect maize growth

The F113 WT strain and related plant-beneficial mutants were inoculated on maize seeds as described above, and 15 plant parameters (7 shoots parameters, 7 root parameters and R/S ratio) were measured in order to determine which plant-beneficial functions contribute to the modulation of maize growth and development (**Figure 2** and **table S1**). For each F113 mutant, parameter profiles were compared to that obtained with F113 inoculation (**Figure 2**). Profiles observed with the Δ*phlD* mutant inoculation were similar to that of F113 inoculation. For all plant parameters assessed, no significant differences were observed between F113 and this mutant (**Table S1**). Differences between these 3 mutants and F113 WT inoculation occurred with significant modifications in root system architecture compared to F113 WT, that is, for root-surface (Δ*acdS* and Δ*nirS*: -50%; Δ*gcd*: -20%), root length (Δ*acdS*: -64%*;* Δ*nirS*: -59%; Δ*gcd*: -26%). Moreover, the inoculation of Δ*acdS* and Δ*nirS* mutants presented a significant decrease compared to F113 WT for root tips number (Δ*acdS*: -62%*;* Δ*nirS*: -45%) and fresh root weight (Δ*acdS*: -31%*;* Δ*nirS*: -38%) (**Figure 2**, **Table S1**). No significant differences occurred in shoot parameters when Δ*acdS* and Δ*nirS* were inoculated (**Figure 2**, **Table S1)**. The profile of the quadruple mutant tended to be similar to those of Δ*acdS* and Δ*nirS* mutants (**Figure 2**, **Table S1**).

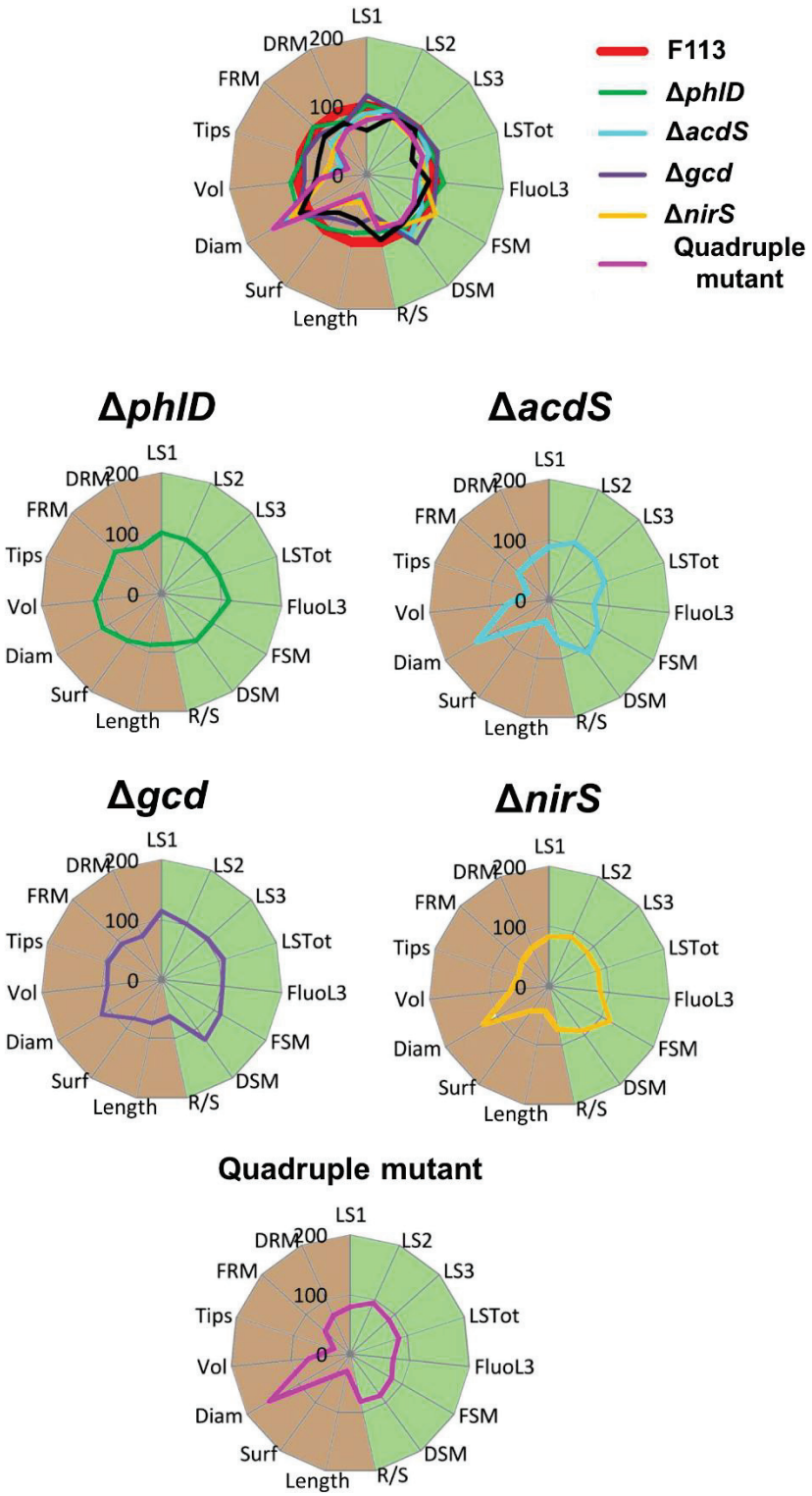


**Figure 2: Comparison of the effects of *P. fluorescens* F113 plant-beneficial mutants on maize growth.**
Effects on plant parameters of mutants were reported against data from the *P. fluorescens* F113 wild type strain and expressed in %.
Shoot measured parameters are colored green on the star plot. LS: Leaf surface; LSTot: Total leaf surface; FluoL3: Chlorophyll content in leaf number 3; FSM: Fresh shoot mass; DSM: Dry shoot mass. Root measured parameters are colored brown on the star plot. Length: total root length, Surf: Total root surface; Diam: Root diameter; Vol: root volume; Tips: Root tips; FRM: Fresh root mass; DRM: Dry root mass. The R/S ratio represents the ratio between the dry root biomass and the dry shoot biomass in the star plot.

The plant-growth promotion of F113 and its mutants was also analyzed related to the non-inoculated condition (**Figure S2**). F113, and the Δ*phlD* and Δ*gcd* mutants presented quite similar plant growth promotion profiles with an enhancement of total leaf surface (respectively, +44%, +43% and +56%) due to a specific increase of the first leaf surface (respectively, +55%, +58% and +78%) (**Table S1 – Figure S2**). F113 and the Δ*phlD* mutant had also a significant effect on root architecture (with an increase of total root length of +50% and +31%, respectively, and of total root surface of +46% and +41%, respectively). The inoculation of Δ*acdS* and Δ*nirS* mutants caused a significant reduction of the number of root tips and total root length. The quadruple mutant had a similar plant-growth promotion profile to that of the Δ*acdS* and Δ*nirS* mutants.

## DISCUSSION

In the present study, we use deletion mutants of well described plant-beneficial properties, DAPG production (Δ*phlD*), ACC deamination (Δ*acdS*), phosphate solubilization (Δ*gcd*) and NO production (Δ*nirS*) and a quadruple mutant of these four plant-beneficial properties to determine their relative contribution to maize root colonization and F113 fitness in maize rhizosphere, and their impact on plant growth compared to the wild type strain and non-inoculated condition. Our results show that Δ*acdS* and Δ*nirS* were affected in their ability to remain in the maize rhizosphere after 20 days (**Figure 1**). Both mutants had also a different impact on the maize root system architecture compared to the wild type strain (**Figure 2**).

Root colonization was assessed by qPCR targeting *P. fluorescens* F113 (Von Felten *et al.*, 2010) or its plant-beneficial related mutants. Root colonization of the wild-type *P. fluorescens* F113 strain decreased from 8dpi and then was maintained up to 20dpi (**Figure 1**). The density level of *P. fluorescens* F113 found at 20dpi was similar to levels obtained with another maize cultivar in field experiment (Von Felten *et al.*, 2010). *P. fluorescens* F113 inoculation provided an enhancement of root length and surface which is congruent with previous study made on the same maize cultivar in field experiment (Walker *et al.*, 2012). *P. fluorescens* F113 was also able to increase the first leaf surface in tested conditions. This is the first time that *P. fluorescens* F113 was shown to stimulate the growth of maize aerial plant part. The stimulation of leaf surface and root elongation was lost when maize seeds were inoculated with the Δ*acdS,* the Δ*nirS* or the quadruple mutant (**Figure S2**), suggesting that the ACC deaminase and nitrite reductase activities contribute to the positive effects of F113 on maize root and aerial parts.

The Δ*acdS* strain was validated in a previous work for its inability to degrade ACC (Vacheron *et al. in preparation*). As proposed by Glick *et al.*, (1998), the $AcdS^+$ strain *P. fluorescens* F113 may catabolize ACC exuded by plant roots, acquiring carbon (α-ketobutyric acid) and nitrogen ($NH_4^+$) sources, unlike its $AcdS^-$ mutants. In this way, *acdS* deletion may impact the fitness of *P. fluorescens* F113, resulting in a decrease of maize root colonization (**Figure 1**). The lost of root architecture effects in presence of the Δ*acdS* mutant was consistent with results from Glick *et al.*, (1994) who reported that the inoculation of a *Pseudomonasputida* GR 12-2 *acdS* mutant resulted in the decrease of canola root elongation compared to the parental strain. Similar observations were made in presence of *acdS* mutants of *Burkholderia phytofirmans* PsJN and *Enterobacter cloacae* UW4 (*novo Pseudomonassp.* UW4; Li *et al.*,

2000; Sun *et al.*, 2009). The gain of ability to produce ACC deaminase activity and to degrade ACC conferred to *Pseudomonasprotegens* CHA0 (former *P. fluorescens* CHA0) the ability to increase canola root length such as a better biocontrol efficiency (Wang *et al.*, 2000). ACC is the precursor of ethylene, a hormone inhibiting root elongation; lowering the plant ACC content through its bacterial deamination may result in the decrease of plant ethylene effects on root architecture (Glick *et al.*, 1998). Thus, the observed root effects of the F113 Δ*acdS* mutant were consistent with an enhanced emission of the plant ethylene hormone as evidenced previously with measurement of ethylene emission in the model plant *Arabidopsis thaliana* (Vacheron *et al., in preparation*). Moreover, the Δ*acdS* mutant inoculation showed a lower level of chlorophyll content in plants that might be explained by an increase of plant ethylene amount that could promote chlorophyll degradation (Knee, 1991) by inducing the biosynthesis of chlorophyllase able to degrade chlorophyll (Trebitsh *et al.*, 1993)

Similar root colonization patterns and plant growth effects were found when the Δ*nirS* mutant instead of the Δ*acdS* mutant was inoculated (**Figure 1**). These results are consistent with those obtained in other *P. fluorescens* strains (Ghiglione *et al.*, 2000). Microaerophilic or anaerobic conditions may be encountered in the rhizosphere (Højberg *et al.*, 1999). In those conditions, respiration of nitrogen-oxidized forms, notably $NO_3^-$ and $NO_2^-$, allows the bacteria to get the energy needed to ensure their survival. In that way, the lower root colonization observed with the Δ*nirS* mutant may be explained by a difference in the energetic yields obtained in the rhizosphere between the F113 and the Δ*nirS* mutant. *P. fluorescens* F113 may obtain energy from all steps of the denitrification process whereas in Δ*nirS*, only the first steps of this process would provide energy (Ghiglione *et al.*, 2002). The low number of lateral roots (i.e. number of tips) observed when Δ*nirS* was inoculated compared to F113 may result from its lower root colonization ability and from the loss of its ability to produce NO, a volatile hormone which plays a key role in the modulation of the root system architecture, and especially in the control of lateral root emergence (Molina-Favero *et al.*, 2008).

The Δ*phlD* mutant shared the same root colonization patterns at 20dpi and effects on tested plant parameters than F113, in the tested conditions. On the contrary, the Δ*gcd* mutant induced significant lower root length and surface compared to F113 (**Figure 2**, **Table S2**). The Δ*gcd* mutant was able to produce a high amount of DAPG in *in vitro* conditions, due to cross interactions between DAPG production and the glucose dehydrogenase-driven metabolism of glucose in *P. fluorescens* F113 (Vacheron *et al. in preparation*). DAPG is well known for its antimicrobial activity against fungal and bacterial phytopathogens (Weller *et al.*, 2007) but it is also relevant in the modulation of the root system architecture by inducing root branching (Leij *et al.*, 2002; Brazelton *et al.*, 2008). Despite potential higher production of DAPG in the Δ*gcd* mutant, the number of lateral roots was similar in F113, and in both Δ*phlD* and Δ*gcd* in tested conditions. This discrepancy can result from the repression of the transcription of the F113 *phl* operon in presence of maize root exuded metabolites (de Werra *et al.*, 2008, 2011). The role of DAPG production in root colonization was assessed by measuring the number of Δ*phlD* cells in maize rhizosphere. At 8dpi, the level of Δ*phlD* cells was the same as the quadruple mutant. However, root colonization of both these mutants remained lower than other mutants and the parental strain (**Figure 1**). In this study, DAPG production appears to play a role in colonization of maize roots. On the contrary, same root colonization levels were observed between *P.*

*fluorescens* F113 and its DAPG mutant in wheat or sugar beet rhizospheres (Carroll *et al.*, 1995; Combes-Meynet *et al.*, 2011).

Root colonization by the quadruple mutant was similar to Δ*acdS* and Δ*nirS* at 8dpi and 20dpi (**Figure 1**). The deletion of both of these genes in the quadruple mutant does not have an even more deleterious effect on root colonization than those obtained with the corresponding single mutants. Considering plant-growth parameters, the plant growth promotion pattern obtained with the quadruple mutant (**Figure 2**) related to the *P. fluorescens* wild type strain was similar with those obtained with Δ*acdS* and Δ*nirS*. However, for some plant traits, differences were observed even if not significant, implying that cumulative effects of the loss of plant-beneficial properties might exist (**Figure 2** and **Figure S2**).

This report underlines that all plant beneficial properties harbored by PGPR have to be taken into account when analyzing the plant response effects of the inoculation of a PGPR. To our knowledge, this is the first study that reports the impact of four key plant-beneficial properties co-occurring in a PGPR on maize growth in non-sterile soil.

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# SUPPLEMENTARY DATA

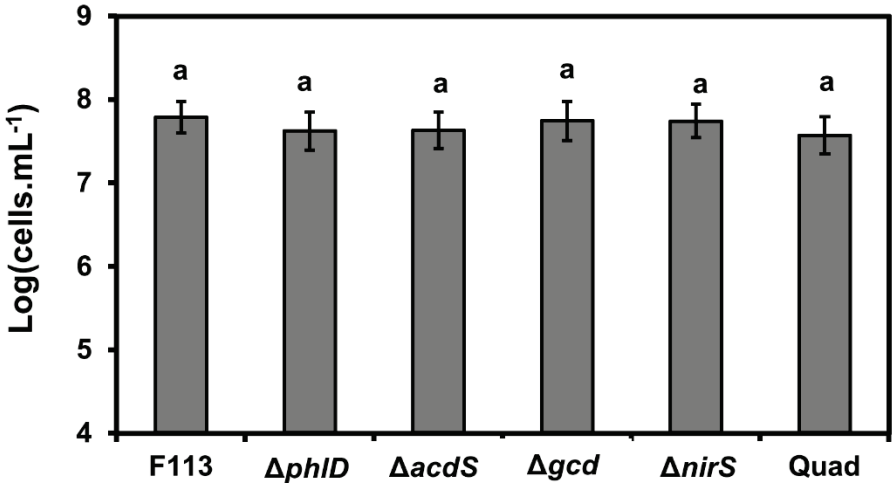


**Figure S1: Inoculum concentrations of *P. fluorescens* F113 and its plant-beneficial mutants.**
No significant difference was found between the initial concentrations of inoculum of *P. fluorescens* F113 and its plant-beneficial mutants (ANOVA; $P = 0.86$).

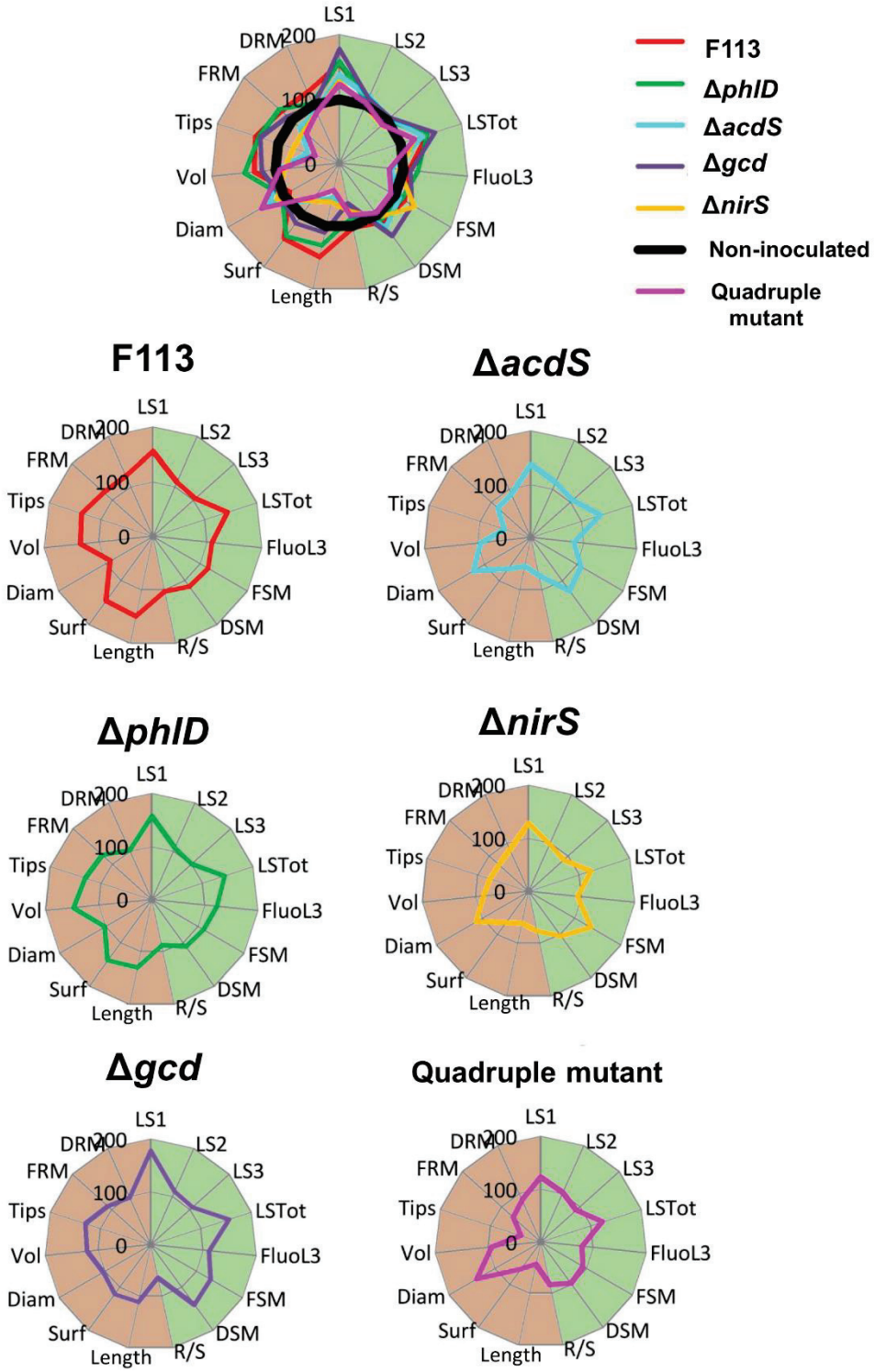


**Figure S2: Effect of *P. fluorescens* F113 and its plant-beneficial mutants on maize growth.**

Effects on plant parameters of F113 and its mutants were reported against data from the (non-inoculated condition and expressed in %. Shoot measured parameters are colored green on the star plot. LS: Leaf surface ; LSTot : Total leaf surface ; FluoL3 : Chlorophyll content in leaf number 3 ; FSM : Fresh shoot mass ; DSM : Dry shoot mass. Root measured parameters are colored brown on the star plot. Length: total root length, Surf: Total root surface; Diam: Root diameter; Vol: root volume; Tips: Root tips; FRM: Fresh root mass; DRM: Dry root mass. The R/S ratio represents the ratio between the dry root biomass and the dry shoot biomass in the star plot.

**Table S1: Summary of statistical differences between F113 and its plant-beneficial mutants according to the plant parameter measured.**

| | Leaf 1 surface | Leaf 2 surface | Leaf 3 surface | Total leaf surface | Chloro-phyll content | Fresh shoot mass | Dry shoot mass | R/S ratio [1] | Total root length | Total root surface | Root diameter | Root volume | Root tips | Fresh root mass | Dry root mass |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F113 | a | a | a | a | ab | ab | ab | ab | a | a | b | ab | a | a | a |
| Δ*phlD* | a | a | a | a | a | ab | ab | ab | a | a | ab | a | a | a | ab |
| Δ*acdS* | ab | a | a | ab | b | ab | ab | ab | bc | bc | ab | ab | b | b | ab |
| Δ*gcd* | a | a | a | a | ab | ab | a | b | b | bc | ab | ab | a | ab | ab |
| Δ*nirS* | ab | a | a | ab | ab | a | ab | ab | bc | bc | ab | b | b | b | b |
| Quad[2] | ab | a | a | ab | b | ab | ab | ab | c | c | a | ab | b | b | b |
| NI[3] | b | a | a | b | ab | b | b | a | bc | bc | ab | ab | a | ab | ab |

[1]: R/S ratio: dry root biomass / dry shoot biomass

[2]: Quadruple mutant

[3]: Non-inoculated condition

Same letter indicates that no significant difference was found between strains according to the plant parameter considered.

# CHAPITRE 5

# DISCUSSION GÉNÉRALE ET PERSPECTIVES

Ces travaux de thèse se sont intéressés aux propriétés phytobénéfiques portées par les *Pseudomonas* fluorescents et plus particulièrement à l'importance de la multiplicité des fonctions phytobénéfiques présentes chez certains individus appartenant à ce groupe bactérien dans leur écologie et leurs interactions avec la plante hôte. Trois approches différentes et complémentaires ont été réalisées dans le but d'étudier la distribution de ces propriétés phytobénéfiques dans ce groupe. La première a reposé sur une analyse « globale » de la distribution de propriétés phytobénéfiques au niveau de la rhizosphère du maïs parmi les populations de *Pseudomonas* fluorescents sélectionnées par la plante. La seconde approche a consisté en une analyse bio-informatique de l'étude de la distribution de ces propriétés dans les génomes de *Pseudomonas* fluorescents disponibles dans les banques de données en regard de la phylogénie des *Pseudomonas*. Enfin, une approche « modèle-centrée » autour de la PGPR multifonction *Pseudomonas fluorescens* F113 a été réalisée dans le but de comprendre (i) les interactions fonctionnelles existant entre ses propriétés phytobénéfiques co-occurrentes et (ii) la contribution de chacune de ses propriétés dans l'effet bénéfique observé sur la plante.

## ***Détermination du statut « multifonctions » des Pseudomonas fluorescents***

A travers les différents chapitres de ce manuscrit, la notion de bactérie phytobénéfique multifonction a été employée pour des individus possédant un large éventail de propriétés phytobénéfiques (> 5 propriétés). Toutefois, ce terme est à considérer de façon relative. En effet, les isolats multifonctions de *Pseudomonas* fluorescent obtenus dans le **chapitre 2** sont qualifiés de multifonctions de par le nombre de fonctions qu'ils possèdent parmi celles que nous avons recherchées. Le choix des fonctions phytobénéfiques recherchées s'est basé sur 2 critères majeurs. Le premier a été établi suite à une étude bibliographique inventoriant les propriétés phytobénéfiques les plus décrites chez les *Pseudomonas* fluorescents en interaction avec la plante et chez d'autres bactéries ayant un statut écologique de PGPR clairement défini. Pour le second, il fallait que la détection de ces propriétés puisse être réalisée de manière haut-débit soit par (i) détection moléculaire ou par (ii) un système de détection biochimique. Le respect de ces deux critères nous a mené à rechercher parmi une banque de 700 isolats, 18 propriétés phytobénéfiques (**chapitre 2**). Toutefois, nous sommes conscients que d'autres propriétés phytobénéfiques prévalent et n'ont pu être recherchées. La classification que nous avons réalisée des isolats présente donc un biais lié au choix de fonctions ciblées. Certains isolats n'ayant que 0 ou 1 propriété phytobénéfique parmi celles que nous avons recherchées peuvent posséder d'autres propriétés que nous n'avons pas recherchées. De la même façon, il est également envisageable de passer à côté de fonctions non-décrites à ce jour.

L'étude des *Pseudomonas* fluorescents multifonctions présents dans la rhizosphère est également sujette aux limites technologiques actuelles. Pour caractériser les fonctions d'un individu et connaitre le nombre de fonctions phytobénéfiques possédées, il est nécessaire à l'heure actuelle d'utiliser des méthodes culture-dépendantes. Ces méthodes présentent un biais certain quant à la représentation de la diversité et de l'abondance de ces individus dans la rhizosphère. Toutefois, même avec l'avènement du séquençage haut-débit et des approches de métagénomique, il est actuellement difficile voire impossible de déterminer dans un environnement précis, le patrimoine génique de chacune des populations d'un système analysé.

De plus, nous sommes conscients que la présence de ces déterminants génétiques décrits comme étant impliqués dans des propriétés phytobénéfiques n'est pas liée de façon absolue au statut écologique des individus qui les possèdent. Par exemple, la modulation de la quantité d'éthylène dans la plante, une phytohormone volatile aux rôles multiples pour le végétal,

comme l'activation des défenses ou le contrôle de croissance racinaire est une propriété commune aux bactéries phytopathogènes (*Ralstonia solanacearum*, *Dickeya dadantii*) et aux PGPR (*Pseudomonas fluorescens* F113, *Azosprillum lipoferum* 4B). Ainsi, les PGPR désaminant l'ACC (le précurseur de l'éthylène) sont capables de diminuer la concentration de l'éthylène végétal intracellulaire, levant ainsi l'inhibition de la croissance racinaire et permettant ainsi de stimuler sa croissance (Shaharoona *et al.* 2006). Les bactéries ayant un statut écologique de phytopathogène pourraient également utiliser cette même enzyme pour diminuer la concentration intracellulaire d'éthylène dans la plante, afin de diminuer les défenses de cette dernière lors de l'infection. Au sein du genre *Pseudomonas*, le gène *acdS* codant l'ACC désaminase est en effet présent chez de nombreux *Pseudomonas* phytopathogènes du groupe des *P. syringae* (**Chapitre 3** ; Joardar *et al.* 2005 ; Bruto *et al.* 2014).

## ***Co-occurrence et distribution des propriétés phytobénéfiques chez les Pseudomonas fluorescents***

Des fonctions phytobénéfiques peuvent être présentes au sein d'un même individu (voir **Chapitre 1**). On parle alors de fonctions phytobénéfiques co-occurrentes. Ces travaux de thèse nous ont permis d'étudier la distribution de ces fonctions au sein du groupe bactérien des *Pseudomonas* fluorescents. Nous avons constaté des combinaisons préférentielles de fonctions phytobénéfiques, récurrentes ches les *Pseudomonas* fluorescents (**Chapitre 2)**. Par exemple, la présence du gène *phlD* impliqué dans la production de DAPG et la capacité de produire de l'HCN est une cooccurrence retrouvée fréquemment dans les génomes de *Pseudomonas* fluorescents (Ramette *et al.* 2003 et **chapitre 2 et 3**). Si des associations préférentielles entre fonctions existent, d'autres associations peuvent être rares comme la co-occurrence entre la production de DAPG et de phénazine. Ce phénotype a été détecté pour un petit nombre d'individus parmi la banque d'isolats créée dans le **Chapitre 2** (3%), mais cette association n'est décrite dans aucun des génomes de *Pseudomonas* séquencés (**Chapitre 3**). Hernández-León et collègues (2015) ont recensé un isolat, *P. fluorescens* UM16 présentant les gènes *phlD* et *phzCD*. Comme précisé dans cette étude, et pour nos isolats ayant ce même phénotype, une validation fonctionnelle est nécessaire afin de valider la production effective de ces deux métabolites antimicrobiens (en mettant en œuvre des méthodes de chromatographie liquide haute performance). Ces deux métabolites secondaires ont été décrits comme souvent impliqués dans des effets d'inhibition de phytopathogènes du fait de leurs actions antifongiques et antibiotiques (Chin-A-Woeng *et al.* 1998 ; Thomashow et Weller 1988 ; de Souza *et al.* 2003 ; Isnansetyo *et al.* 2003). Si la production de ces deux métabolites est confirmée, il conviendra dans un premier temps de tester les isolats pour leur potentiel de biocontrôle contre une batterie de microorganismes phytopathogènes que ce soit *in vitro* ou par des tests d'infection sur plantes. Dans un second temps, la faible occurrence des individus portant ces deux fonctions est intéressante et soulève la question de savoir pourquoi cette co-occurrence est si peu fréquente. Une première hypothèse serait que cette faible occurrence est due au biais lié au milieu de culture classiquement utilisé pour l'isolement de telles souches. Une seconde hypothèse pourrait être la présence d'effets antagonistes entre ces deux types de molécules. Pourtant, l'équipe de recherche de Linda Thomashow et de David Weller ont inséré l'ensemble des gènes nécessaire à la synthèse de phénazine dans la souche productrice de DAPG *P. fluorescens* Q8r1-96 et ont montré que la production de ces deux métabolites se fait de façon séquentielle (pic de production à 24h pour le DAPG, 40h pour les phénazines) et que le gain de cette fonction chez la souche Q8r1-96 entraine une meilleure efficacité de protection de la plante contre

*Rhizoctonia solani* AG-8 (Huang *et al.* 2004). De ce fait, il serait intéressant chez un isolat présentant ces deux fonctions de comprendre l'expression et la régulation de ces deux voies métaboliques *via* des analyses de transcriptomiques et également d'analyser la contribution relative de ces deux fonctions dans leur capacité à inhiber la croissance de microorganismes phytopathogènes.

La présence de propriétés préférentiellement associées (ou non) au sein des génomes nous interroge sur les facteurs influençant leur distribution chez les *Pseudomonas* fluorescents. L'étude génomique des *Pseudomonas* fluorescents réalisée dans le **Chapitre 3** met en lumière des associations de fonctions phytobénéfiques qui semblent liées à la taxonomie des *Pseudomonas*. Ainsi, le principal facteur influençant la distribution des propriétés phytobénéfiques serait le signal phylogénétique. De nombreuses études ont montré que la distribution des gènes pouvait être influencée par la proximité phylogénétique entre organismes (Comas *et al.* 2007) dont notamment des gènes impliqués dans des fonctions phytobénéfiques (Bruto *et al.* 2014). Pour valider cela à l'échelle du groupe des *Pseudomonas* fluorescents, des indices statistiques décrivant le poids de la phylogénie sur la distribution des gènes phytobénéfiques doivent être calculés comme par exemple l'indice *D* de Fritz et Purvis (2010) basé sur un jeu de donnée binaire (présence/absence de fonctions phytobénéfiques). L'utilisation de cet indice a déjà été réalisée afin de visualiser l'importance du signal phylogénétique dans la distribution de fonctions phytobénéfiques à l'échelle des protéobactéries et a montré que certaines de ces fonctions sont influencées plus ou moins fortement par la phylogénie des protéobactéries (Bruto *et al.* 2014). Ainsi, le calcul de cet indice pourrait être intéressant pour juger de l'importance du signal phylogénétique sur la distribution des propriétés phytobénéfiques à l'échelle du groupe des *Pseudomonas* fluorescents. L'élaboration d'un scénario de perte et d'acquisition de propriétés phytobénéfiques a été réalisée dans le chapitre 3 sur la base de la distribution des propriétés phytobénéfiques selon la taxonomie. Il convient bien entendu de tester ce scénario par des méthodes de reconstruction de caractères ancestraux, nécessitant préalablement la génération de chacun des arbres relatifs aux gènes phytobénéfiques étudiés (Bruto *et al.* 2014).

## ***Contribution des propriétés phytobénéfiques co-occurrentes à l'effet sur la plante***

La co-occurrence de fonctions phytobénéfiques a soulevé de nombreuses questions quant au fonctionnement de ces fonctions co-occurrentes et de leur contribution dans l'effet phytobénéfique observé. L'hypothèse « d'additivité » des fonctions énoncées par Yoav Bashan en 2004 considérait que l'ensemble des fonctions co-occurrentes dans un génome de PGPR participe, de façon simultanée ou successive, à l'amélioration de la croissance et de la santé de la plante. Nous avons entrepris d'apporter des réponses concernant cette hypothèse *via* une approche de perte de fonctions chez *P. fluorescens* F113 (**Chapitre 4**). Ce modèle bactérien comporte selon l'analyse génomique réalisée dans le **Chapitre 3**, 9 propriétés phytobénéfiques. Parmi ces propriétés, 4 ont été choisis pour leur capacité de stimulation de la croissance du végétale : la production de DAPG (*phlD*), d'oxide nitrique (*nirS*), de l'ACC désaminase (*acdS*) et la solubilisation du phosphate (*gcd*).

Nous avons montré que les 4 propriétés phytobénéfiques ciblées chez F113 ne contribuent pas toutes de façon égale à l'effet phytobénéfique observé chez *A. thaliana*. De plus, l'effet observé sur la plante ne résulte pas de l'addition des contributions relatives de chacune des fonctions phytobénéfiques comme suggéré en 2010 par Bashan et deBashan. La

présence d'interactions fonctionnelles entre les fonctions co-occurrentes peut affecter leur effet sur la plante. Par exemple, le mutant *gcd* surproduit du DAPG en condition *in vitro*. Cette surproduction peut masquer en partie la contribution du gène *gcd* à l'effet phytostimulateur, lorsque ce dernier est inoculé sur la plante. Il serait nécessaire de confirmer *in planta* la surproduction du DAPG lorsque le mutant Δ*gcd* est inoculé sur la plante, comme décrit par Bonsall *et al.* (2007). L'étude de l'expression des gènes phytobénéfiques de F113 par RT-PCRq pourrait être également envisagée afin de déterminer si les interactions fonctionnelles observées dans ce travail impliquent des régulations à un niveau transcriptionnel des gènes codant ces propriétés.

Nous avons également observé que la contribution de deux profils fonctionnels identiques (sur la base des fonctions phytobénéfiques que nous avons étudiés) peut conférer des rôles écologiques différents aux souches qui les possèdent. En effet, lors de la caractérisation fonctionnelle des 9 souches séquencées (**Chapitre 3**), les souches JV395B et JV497 appartenant toutes les deux au sous-groupe des *P. chlororaphis* (*sensu-stricto*) possèdent les mêmes propriétés phytobénéfiques et n'ont pourtant pas le même rôle écologique : la souche JV497 stimule la rhizogenèse et avance le stade de floraison chez *A. thaliana*, alors que la souche JV395B n'a montré aucun effet. Inversement, la souche JV395B est la souche la plus efficace concernant l'inhibition des microorganismes phytopathogènes testés et présente des effets d'inhibition plus important que JV497. Ainsi, il est possible que des profils identiques puissent conduire à des rôles écologiques différents. Il serait intéressant chez ces 2 isolats de comparer l'expression de leurs fonctions phytobénéfiques et d'étudier des mécanismes de régulation pouvant être impliquées dans le contrôle de leur expression.

Toutefois l'hypothèse énoncée par Yoav Bashan n'est pas fausse mais doit être considérée non-pas à l'échelle d'un individu, mais à celle de l'ensemble des populations présentes dans le rhizomicrobiote, comme discuté précédemment (**Chapitre 1 partie A)** avec des interconnexions existant probablement à l'échelle des groupes fonctionnels.

## ***Quels rôles écologiques pour les bactéries rhizosphériques multifonctions ?***

L'un des constats que nous avons réalisé au cours de ces travaux de thèse est que même si les individus multifonctions sont présents en faible abondance dans la rhizosphère, ils existent et doivent avoir un impact sur la plante ou sur le rhizomicrobiote. Ce constat soulève alors la question suivante: *Quels rôles ont ces bactéries rhizosphériques multifonctions dans le fonctionnement microbien de la rhizosphère ?*

Au sein du rhizomicrobiote, des souches appartenant à différents genres, espèces et ayant une action bénéfique sur le végétal coexistent (Upadhyay et al, 2009) et peuvent exercer leurs fonctions phytobénéfiques. Des populations de PGPR contribuant à une même fonction phytobénéfique (comme par exemple, la production de DAPG, la solubilisation du phosphate, la production d'auxines etc.) appartiennent de ce fait à un même groupe fonctionnel. Comme décrit plus haut, certains groupes fonctionnels peuvent également comprendre des PGPR et des non-PGPR (comme par exemple le groupe fonctionnel *acdS*). De ce fait, l'effet sur la plante résulte de l'activité de ces groupes fonctionnels, générée par la contribution de chacun des individus constituant ces groupes. Les groupes fonctionnels phytobénéfiques ont souvent été étudiés de façon indépendante avec par exemple des études sur le groupe fonctionnel des fixateurs d'azotes (Wartiainen *et al.* 2008) ou encore des producteurs de DAPG (Frapolli *et al.*, 2012). Pourtant, des interactions entre microorganismes ont lieu à l'intérieur mais également entre différents groupes fonctionnels. Premièrement, certaine fonctions phytobénéfiques sont

décrites pour présenter une régulation auto-induite de leur production comme le DAPG ou la pyolutéorine, connus pour auto-induire les gènes permettant leur biosynthèse (Schnider keel *et al.* 2000, Kidarsa *et al.* 2011). Ainsi, il est possible que la production d'un de ces deux composés par une souche régule l'expression chez d'autres souches productrices au sein de ce groupe fonctionnel (exemple du DAPG – Maurhofer *et al.* 2004). Deuxièmement, certaines propriétés phytobénéfiques comme la production d'auxine chez *Azospirillum* est induite en présence de DAPG (Combes-Menet 2011), rendant ainsi possible pour une souche productrice de DAPG de moduler un groupe fonctionnel comme celui des producteurs d'auxines en agissant sur l'expression des gènes de biosynthèse possédés par certains individus de ce groupe. Par ailleurs, la communication cellulaire *via* le *Quorum Sensing* reste également un moyen clé de modulation des groupes fonctionnels que ce soit *via* la régulation de l'expression de gènes à un niveau intra ou intergroupes fonctionnels (Boyer *et al.* 2009 ; Pierson *et al.*, 1994, Selin *et al.* 2012 ; Veselova *et al.* 2006). Ces connexions intra ou inter groupes fonctionnels peuvent également se faire non pas directement au niveau de la modulation de l'activité de ces groupes, mais indirectement *via* les interactions de compétitions/antagonisme entre individus constituant le rhizomicrobiote.

Le résultat majeur de ces travaux est que la plante au niveau de sa rhizosphère, ne sélectionne pas préférentiellement des *Pseudomonas* fluorescents possédant un grand nombre de fonctions phytobénéfiques. Cette observation nous a conduits à proposer un modèle de fonctionnement des groupes fonctionnels phytobénéfiques dans la rhizosphère. Une bactérie qui possède de nombreuses fonctions phytobénéfiques (polyvalente), appartient à chacun des groupes fonctionnels relatifs aux propriétés phytobénéfiques qu'elle possède. Ainsi, cette bactérie participerait à la modulation de l'ensemble des groupes fonctionnels auxquels elle appartient et pourrait être ainsi considérée comme une bactérie indispensable dans le contrôle du fonctionnement de la rhizosphère (**Figure 1**).

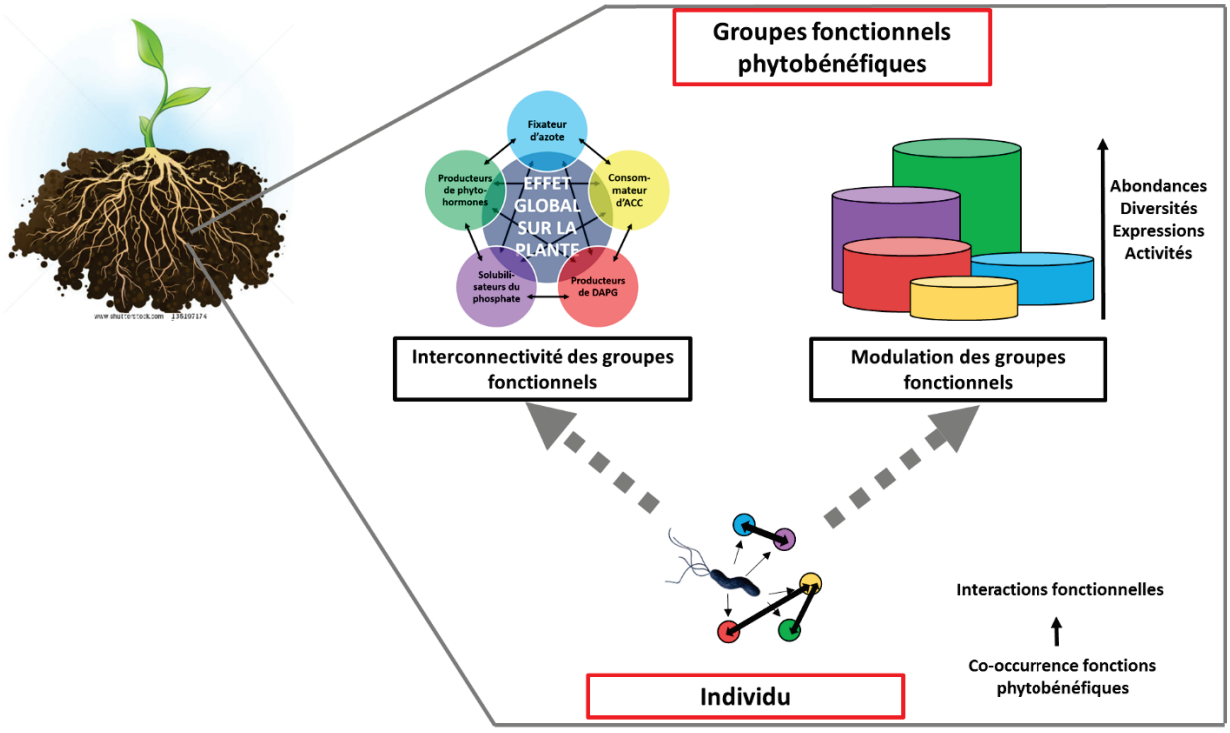


**Figure 1 : Modèle hypothétique du rôle des bactéries multifonctions dans le fonctionnement de la rhizosphère.** Les flèches grises en pointillées représentent l'effet des bactéries multifonctions sur l'interconnectivité des groupes fonctionnels et leur modulation

## ***Perspectives de travail***

Ces travaux de thèse ont montré l'importance des propriétés phytobénéfiques de *P. fluorescens* F113 sur le phénotype de la plante, en particulier en modulant son architecture racinaire. Les perspectives de travail à **court terme** sont de mieux caractériser l'effet des fonctions phytobénéfiques de F113 sur le phénotype végétal en regardant l'impact de ces fonctions sur (i) le transcriptome et (ii) le métabolome de la plante. Concernant l'étude du transcriptome végétale, elle pourrait être réalisée par des méthodes de séquençage des ARNm du maïs suite à l'inoculation des souches mutées de F113. Cette approche pourrait être complétée par des études centrées autour du modèle *Arabidopsis*, permettant de visualiser l'impact de chacun des mutants de F113 sur les voies de signalisations impliquées dans le contrôle du développement d'*A. thaliana via* l'utilisation de plantes modifiées comme (*A. thaliana* DR5::*gfp* rapportrice de l'accumulation d'auxine (Ottenschläger *et al.* 2003), ou encore les mutants affectés dans la réponses auxiniques aux1-100 et axr1-3 (Desbrosses *et al.* 2009). Ces travaux seront en partie amorcés en Janvier 2016 dans le cadre d'un stage de Master 2 Recherche. Cette approche nous permettra à terme de mieux comprendre l'impact des fonctions phytobénéfiques sur le maïs à différentes échelles (transcriptomique, métabolique et phénotypique).

A **moyen terme**, il est possible de mieux caractériser l'interaction fonctionnelle entre les propriétés phytobénéfiques co-occurrentes chez ce modèle bactérien par l'utilisation des doubles et triples mutants de ces propriétés. Ces mutants ont été obtenus lors de la thèse et l'analyse de leur activité est déjà amorcée. Par ailleurs, il serait intéressant de réaliser une approche d'inoculations croisées de ces mutants afin de voir si une complémentation fonctionnelle (coopération) entre deux individus dont il manque à chacun une des propriétés phytobénéfiques, restaure l'effet observé sur la plante inoculée par la souche sauvage que ce soit *in vitro* sur l'arabette (**Chapitre 3 Partie A**) ou sur du maïs dans du sol non-stérile (**Chapitre 3 Partie B**) (**Figure 2**). Un consortium comportant le quadruple mutant ainsi que les 4 mutants simples pourrait être également testé. Ces expériences apporteraient une compréhension supplémentaire du fonctionnement des propriétés phytobénéfiques co-occurrente à l'échelle d'une population clonale.

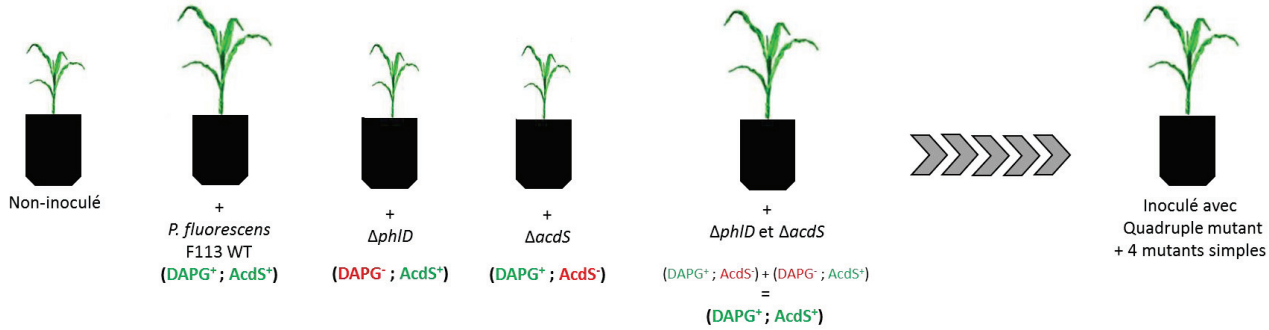


**Figure 2 : Ebauche de plan expérimental permettant l'étude du fonctionnement des propriétés phytobénéfiques à l'échelle d'une population clonale.** En couleur sont représentés les phénotypes des souches testées. En gras est indiqué le phénotype « potentiel » résultant des coinoculations des différentes souches.

A **long terme**, la caractérisation du rôle écologique des individus multifonctions est une piste de recherche intéressante à la fois (i) d'un point de vue fondamental car ellepermettrait d'améliorer la compréhension du fonctionnement microbien de la communauté bactérienne

rhizosphérique et du rôle joué par certains individus (les polyvalents *versus* les spécialistes) dans la rhizosphère et l'interaction avec la plante, (ii) qu'appliqué.

Une approche visant à valider le rôle clé des individus multifonctions serait de réaliser une approche simplifiée en inoculant dans la rhizosphère d'une plante, une bactérie multifonction au sein d'un rhizomicrobiote artificiel, à l'image des travaux réalisés sur le comportement écologique des espèces microbiennes invasives dans une communauté artificielle (van Elsas *et al.* 2012). La communauté artificielle rhizosphérique serait composée d'un nombre réduit d'individus (environ 100 individus) ne possédant pas un nombre élevé de propriétés phytobénéfiques. Le choix de ces individus serait réalisé en fonction de (i) leurs profils fonctionnels possédés (synonyme d'appartenance à ces groupes fonctionnels), (ii) leur diversité taxonomique, et (iii) afin qu'une redondance fonctionnelle soit établie. Ainsi, cette ébauche de plan expérimental permettrait de valider si la présence de ces individus multifonctions (i) module l'expression des gènes phytobénéfiques (relatif aux groupes fonctionnels correspondants), (ii) modifie l'abondance et la diversité des individus composant ces groupes fonctionnels et (iii) affecte la physiologie de la plante hôte (en terme de réponse phénotypique, métabolique et transcriptomique) (**Figure 3**). Des plans expérimentaux plus complexes faisant varier le type de sol, le génotype de plante et bien entendu la communauté utilisés (en termes de diversité taxonomique, fonctionnelle, mais également en termes d'abondance) pourront être envisagés.

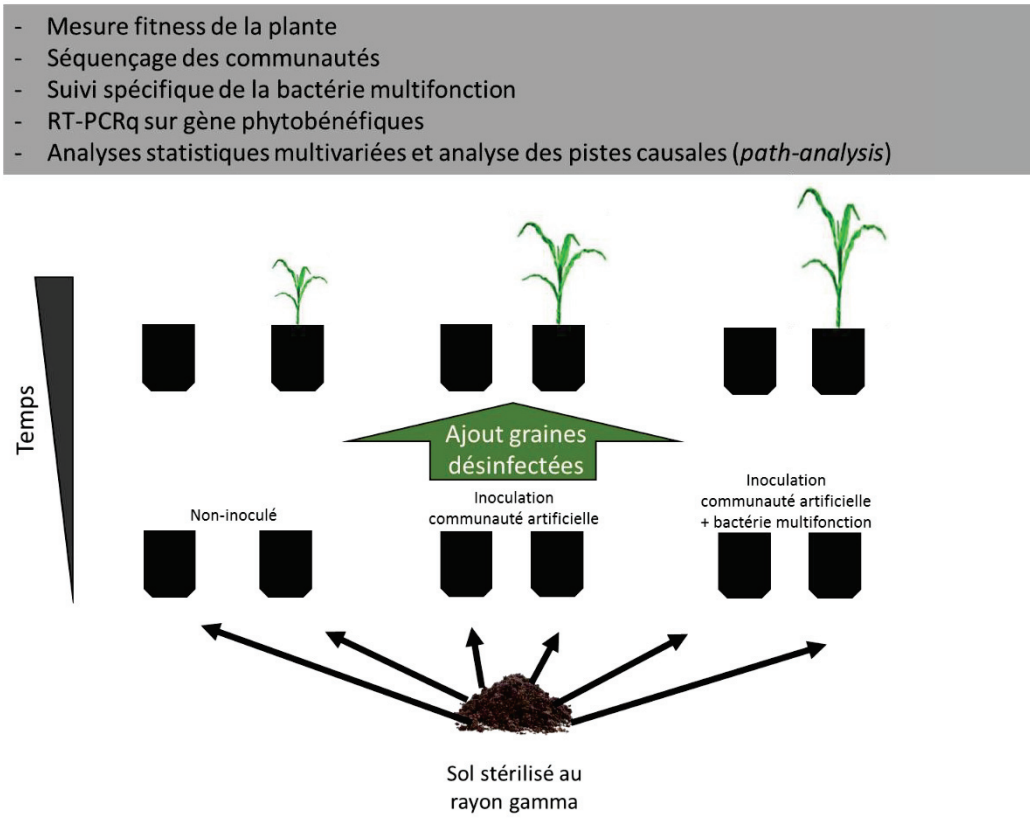


**Figure 3 : Approche impliquant une communauté rhizosphérique artificielle permettant l'étude de l'importance des bactéries multifonctions dans l'interaction avec la plante et avec les groupes fonctionnels d'une communauté bactérienne rhizosphérique artificielle.**

La compréhension des systèmes biologiques représente un enjeu agricole majeur en vue de l'utilisation de microorganismes pour une agriculture durable. En effet, la caractérisation du rôle de ces individus permettrait (i) d'optimiser la dose de ces microorganismes à apporter ou (ii) d'orienter la recherche de nouvelles souches microbiennes vers des individus ne possédant pas nécessairement un nombre élevé de fonctions phytobénéfiques mais capables de s'établir de façon efficace au sein du rhizomicrobiote. Il serait en effet important de privilégier des individus capables d'interagir positivement avec l'ensemble des groupes fonctionnels présents dans la rhizosphère permettant ainsi de favoriser l'expression de leurs propriétés phytobénéfiques et leur effets de phytostimulation et phytoprotection. D'un point de vue fondamental, une meilleure compréhension de l'écologie de ces microorganismes permettrait de décrypter le fonctionnement des interactions présentes dans le système biologique qu'est la rhizosphère, ce qui permettrait de confirmer que les théories de l'Ecologie générale et fonctionnelle sont également transposables à l'Ecologie Microbienne.

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# ANNEXES

## PRODUCTIONS SCIENTIFIQUES

∴ Annexe 1 – Valorisation agronomique de deux agents microbiens vis-à-vis de la protection de la mâche (*Valerianella locusta*) contre *Thievialopsis basicola*

∴ Annexe 2 – Plant growth-promoting properties of *Pseudomonas* biocontrol agent producing 2,4-diacetylphloroglucinol

∴ Annexe 3 – Alleviation of abiotic and biotic stresses in plant by *Azospirillum*

# ANNEXE 1

## VALORISATION AGRONOMIQUE DE DEUX AGENTS MICROBIENS VIS-A-VIS DE LA PROTECTION DE LA MACHE (*VALERIANELLA LOCUSTA*) CONTRE *THIEVIALOPSIS BASICOLA*

**Participation :** Isolement, obtention des profils phytobénéfiques, confrontation envers des microorganismes phytopathogènes, traitement statistique.

# Valorisation agronomique de deux agents microbiens vis-à-vis de la protection de la mâche (*Valerianella locusta*) contre *Thievialopsis basicola*

## INTRODUCTION

*Thielaviopsis basicola* (Berk. et Br.) Ferraris est un champignon phytopathogène tellurique appartenant à la division des ascomycètes et est retrouvé aussi bien dans des sols cultivés que non-cultivés (Yarwood, 1981). Ce champignon est responsable de la maladie engendrant la pourriture noire des racines (137 genres; Huang and Kang, 2010) chez de nombreuses plantes, de familles différentes, et d'intérêts économiques certains comme la lentille (Bowden *et al.*, 1985), le pois, le haricot (Burke and Kraft, 1974), le coton (Allen, 1990), le tabac (Delon, 1987) mais également les salades (Coumans *et al.*, 2011) dont la mâche.

Sur le territoire français en 2014, la mâche a été cultivée sur une superficie de 7851 ha avec une production de 43 041 tonnes (Données Agreste-Juillet 2015). 90% de cette production est réalisée dans le Val de Loire (Données Agreste 2012). Depuis ces 15 dernières années, *T. basicola* cause d'importants dégâts rendant difficile la commercialisation du produit. Actuellement, la solarisation est une solution employée pour lutter contre ce phytopathogène. Toutefois, son efficacité n'est pas toujours satisfaisante et son impact sur le fonctionnement des processus microbiens opérant dans le sol est dévastateur (Roux-Michollet *et al.*, 2008). De ce fait, il est urgent de trouver des solutions alternatives efficaces de luttes contre ce champignon, dans le pathosystème *T. basicola*-*Valerianella locusta*.

Dans d'autres pathosystèmes, comme par exemple le pathosystème *T. basicola* – *Nicotiana tabacum* (tabac), des moyens de lutte biologique impliquant des bactéries du genre *Pseudomonas* ont été développés (Keel *et al.*, 1990; Wirthner *et al.*, 1992; Maurhofer *et al.*, 1995). Certains sols présentent une faible sévérité de la maladie (pourriture noire des racines) alors que le champignon est présent et que les conditions environnementales sont réunies pour son développement (Almario *et al.*, 2014). Ces sols sont ainsi qualifiés de « sols résistants ». Cette résistance naturelle a été octroyée à la présence de populations bactériennes particulières, celles des *Pseudomonas* fluorescents producteurs de 2,4-diacétylphloroglucinol (DAPG), une molécule ayant un rôle d'antimicrobien mais également capable d'éliciter les défenses de la plante (Stutz *et al.*, 1986; Keel *et al.*, 1990; Iavicoli *et al.*, 2003). Toutefois, d'autres métabolites produits par les *Pseudomonas* fluorescents (l'HCN, ou les phénazines …) peuvent jouer un rôle dans la résistance des sols à la maladie (Mazurier *et al.*, 2009).

Dans l'optique d'étoffer les solutions de lutte envers *T. basicola* sur la culture de la mâche, des microorganismes du groupe des *Pseudomonas* fluorescents producteurs de métabolites secondaires antimicrobiens pourraient s'avérer être de bons candidats de lutte biologique. Ainsi, deux souches parmi la banque d'isolats obtenue dans le chapitre 2 ont été sélectionnées pour être envoyées au CTIFL de Carquefou afin de procéder à des essais de protection de la mâche vis-à-vis de la pourriture noire des racines provoquée par *T. basicola,* en conditions semi-contrôlées.

Cette partie débute en dressant les profils fonctionnels des 2 isolats retenus dans cette étude ainsi qu'en présentant les résultats des essais *in vitro* d'antagonisme vis-à-vis de différents

phytopathogènes dont *T. basicola* réalisés au Laboratoire d'Écologie Microbienne, puis fait état dans un second temps des essais préliminaires réalisés en serre au CTIFL sous la direction de Céline Ade (Responsable des programmes d'expérimentation sur mâche et radis, CTIFL Centre de Carquefou).

# MATERIELS ET METHODES

## Matériels biologiques

Les souches *Pseudomonas* Ps1 et Ps2, ont été cultivées dans du milieu King's B et incubées à 28°C sous agitation à 150 rpm. Les microorganismes phytopathogènes bactériens, *R. solanacearum* GMI1000, *D. dadantii* A3937 et *P. syringae* DC3000 ont été cultivés à 28°C dans du milieu Lysogeny Broth (LB) sous agitation à 150 rpm.

Les microorganismes phytopathogènes testés *in vitro* (*P. alni*, *T. basicola* et *F. graminearum*) ont été cultivés sur du milieu PDA (*Potato Dextrose Agar*, Conda) à 22°C. Concernant les tests sur plantes, *T. basicola* a été mis en culture à partir d'une souche isolée de mâche issue de la mycothèque du CTIFL de Carquefou. Pour ce faire, des spores de *T. basicola* conservées à -80°C ont été disposées au centre d'une boite de Petri contenant du milieu PDA. Les boîtes ont été incubées à 22°C pendant 1 semaine.

Les variétés de mâches utilisées dans les essais réalisés au CTIFL étaient la variété Verte de Cambrai lors de l'essai Printemps 2014 et la variété Trophy lors de l'essai réalisé durant l'hiver 2014-2015.

## Test d'antagonisme *in vitro*

*Envers des champignons et oomycète phytopathogènes*

La suspension bactérienne (1 µL à $10^8$ cellules en phase stationnaire de croissance par mL) a été striée sur une distance de 3cm, à l'aide d'une oëse, à 2 cm du centre de la boîte de Petri contenant un milieu gélosé PDA. Un disque mycélien de 6 mm de diamètre a ensuite été déposé au centre de la boîte de Petri. Les boîtes ont été incubées à 22°C et l'incubation est arrêtée lorsque le champignon de la boite témoin (champignon sans bactérie) a totalement envahi la boîte (Jour 13). Des photos sont prises tous les 2 jours pour quantifier la croissance fongique. Les images obtenues sont traitées à l'aide du logiciel *ImageJ*. Les distances existant entre le centre et la périphérie du champignon que ce soit du côté où le dépôt bactérien a été réalisé (BS = *Bacterial Side*) que du côté diamétralement opposé (FS = *Free Side*) (**Figure 1**) ont été mesurées.

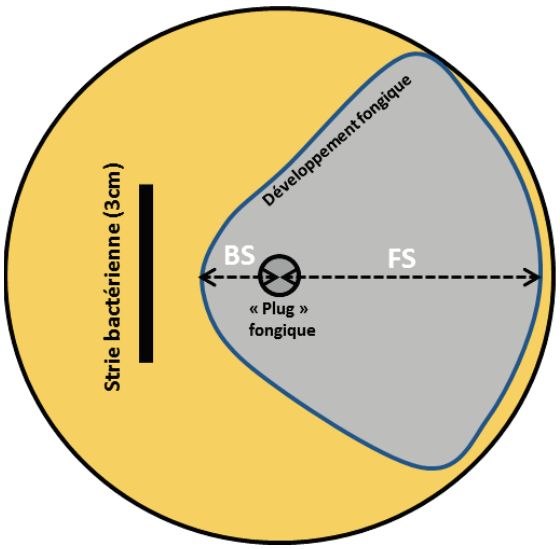


**Figure 5 :** ***Principe de la mesure de la croissance fongique lors des tests d'antagonisme in vitro.***
BS : « Bacterial side »
FS : « Free side »

*Envers des bactéries phytopathogènes*

Les pré-cultures bactériennes (phytopathogènes) sont ajustées à une $DO_{600nm}$=0.1 dans 20mL de milieu LB juste tiède. Le milieu inoculé est ensuite coulé dans une boite de Petri 90x90. Cinq gouttes de 5µL de suspension bactérienne de la souche Ps1 ou de Ps2 (environ $10^8$ UFC.mL$^{-1}$) sont déposées par boites (3 boites par souches). Les boites sont incubées pendant 48h à 28°C. Les halos d'inhibition ont directement été mesurés *via* l'utilisation d'une règle graduée.

**Essais de phytoprotection de *Valerianella locusta* vis-à-vis de *Thielaviopsis basicola* (CTIFL)**

*Plans expérimentaux des essais*

L'essai n°1 (Printemps 2014) a été mis en place dans la serre à plant du CTIFL et a été disposé selon un dispositif en blocs de Fischer à 4 répétitions, avec 12 modalités. Chaque répétition d'une modalité est constituée de 5 godets, remplis d'un mélange de terreau (1/3) et de sable (2/3) et semés avec 5 graines/godet. Un total de 240 godets (répartis dans 4 blocs de 48 terrines) a été préparé. Trois traitements ont été testés : (i) Cas d'un sol fortement contaminé : *T. basicola* 900 spore.mL$^{-1}$ avec inoculation 8j avant semis (ii) Cas d'un sol après une désinfection: *T. basicola* 300 spore.mL$^{-1}$avec inoculation 8j avant semis ou (iii) avec inoculation le jour du semis.

L'essai du printemps 2014 a été répété en 2015 avec quelques ajustements apportés aux protocoles. Le contrôle des conditions climatiques dans la serre lors de l'essai 1 a rendu l'interprétation des résultats complexes à cause de fortes fluctuations de température. De ce fait, l'essai 2 a été réalisé en chambre climatique (21°C/18°C, 4h/20h, 60% d'humidité). Il a été disposé selon un dispositif en blocs de Fischer à 4 répétitions, avec 6 modalités. Chaque répétition d'une modalité est constituée de 16 godets semés avec 2 graines/godet, soit un total de 384 godets. Un seul traitement a été testé, le cas d'un sol fortement contaminé (*T. basicola* 900 spore.mL$^{-1}$ avec une inoculation 8j avant semis).

*Inoculations bactérienne et fongique*

L'inoculum de *T. basicola* a été préparé par récupération des spores après 10 à 15 jours de culture sur milieu carotte. L'inoculum a été réalisé en utilisant de l'eau stérile (5 mL) pour la récolte des spores de l'agent infectieux. Sa concentration a été ajustée avec de l'eau stérile à une concentration de 1,2x10$^4$ et 3,6x10$^4$ spore.mL$^{-1}$ pour respectivement 300 spore.g$^{-1}$ de sol et 900 spore.g$^{-1}$ de sol. *T. basicola* a été inoculé dans chaque godet par une application de 5mL de la suspension de spores 8 jours avant le semis ou le jour du semis selon l'essai.

Les concentrations bactériennes ont été mesurées par l'intermédiaire d'une cellule de Malassez pour obtenir une concentration de 2,0x10$^7$ UFC.mL$^{-1}$. Un contrôle de la concentration bactérienne de l'inoculum a été réalisé par dénombrement sur boites. Les deux souches bactériennes ont été inoculées le jour du semis par application de 50 µL d'une solution concentrée à 2,0x10$^7$ UFC.mL$^{-1}$ par graine de mâche (soit $n$=10$^6$ UFC). Les inoculations fongiques et bactériennes ont été remplacées par de l'eau pour les témoins.

*Acquisition des données*

Après la levée des mâches, des notations régulières (environ tous les 15 jours) ont été réalisées sur le système racinaire selon une échelle de sévérité de symptômes (**Figure 2**).

A la fin de l'essai, 2 mâches par godet ont été prélevées pour mesurer les matières fraîches et sèches de la partie aérienne et racinaire. La température et l'humidité relative dans la serre ont été enregistrées quotidiennement.

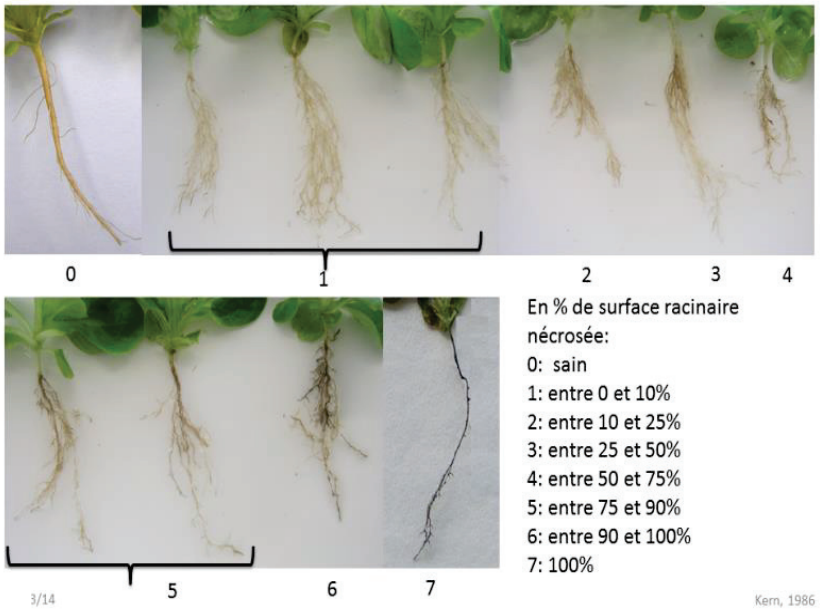


**Figure 6 : Echelle de notation des nécroses du système racinaire provoquées par *T. basicola* sur la mâche.** Adapté de Stutz *et al.* 1986

## Analyses des données

Concernant les tests d'antagonismes vis-à-vis des bactéries, champignons et oomycète phytopathogènes, les données ont été comparées en réalisant une analyse de variance (ANOVA) suivie d'un test de comparaison multiple HSD de Tukey. Pour l'état sanitaire des racines de mâche, un indice de pathogénicité (IP) ainsi que la sévérité conditionnelle (SC) de l'attaque ont été calculés à partir des observations visuelles :

$$IP = \frac{\sum_0^7 numéro\ de\ la\ classe\ i * nombre\ de\ mâches\ en\ classe\ i}{nombre\ de\ mâches\ observées\ au\ total}$$

$$SC = \frac{\sum_1^7 numéro\ de\ la\ classe\ i * nombre\ de\ mâches\ en\ classe\ i}{nombre\ de\ mâches\ atteintes\ observées}$$

Les variables matières fraîche, sèche, IP et SC sont soumises à une analyse de variance puis à une comparaison de moyennes (test de Newman et Keuls).

# RESULTATS & DISCUSSION

## Sélection des candidats bactériens

Deux souches du genre *Pseudomonas* parmi une banque de 700 isolats, nommées Ps1 et Ps2, ont été retenues sur la base de leurs profils fonctionnels et leurs propriétés antagonistes comportant la production de DAPG, pyolutéorine, phénazine et de d'HCN (composé volatile) vis-à-vis de microorganismes phytopathogènes. L'activité antagoniste de ces deux souches a été testée *in vitro* contre différents microorganismes phytopathogènes incluant trois bactéries (*D. dadantii* A3937, *P. syringae* DC3000 et *R. solanacearum* GMI1000), deux champignons (*T. basicola* et *F. graminearum*) et un oomycète (*Ph. alni*) (**Figures 3** et **4**).

La souche Ps1 exerce sur les bactéries phytopathogènes une action antagoniste plus forte ou égale à celle exercée par des PGPR de références productrices de DAPG comme *P. fluorescens* F113, *P. protegens* CHA0 et *P. protegens* Pf-5 (**Figure 3-A, 3-C et 3-E**). En revanche, la souche Ps2 possède une activité antagoniste seulement envers *D. dadantii* A3937 (**Figure 3-A**).

L'inhibition de la croissance fongique a été mesurée du côté du dépôt de la souche bactérienne ainsi que du côté diamétralement opposé. Les résultats présentés dans la **figure 3-B, 3-D et 3-F** correspondent aux observations effectuées au bout de 13 jours de croissance mycélienne. La souche Ps1 inhibe le développement mycélien de *T. basicola* du côté du dépôt bactérien (inhibition significative de la croissance fongique de 62% par rapport au témoin contenant le champignon sans bactérie), mais elle n'a pas d'impact sur la croissance fongique du côté opposé au dépôt bactérien (**Figure 3-B**), suggérant une action d'antagonisme par diffusion d'un métabolite antimicrobien à travers le milieu gélosé.

En revanche, la souche Ps2 inhibe la croissance de *T. basicola* du côté du dépôt bactérien (85% par rapport au témoin) et du côté opposé (78% par rapport au témoin) (**Figure 3-B et 4-A**). Pour la souche Ps2, trois modes d'action possibles peuvent être suggérés : le premier est l'action d'un ou plusieurs métabolites antimicrobiens diffusant à travers la gélose et ayant un effet dévastateur sur la croissance mycélienne au point de l'inhiber complètement ; le second mode d'action envisagé est celui d'un ou plusieurs composés volatils antimicrobiens ; le troisième mode d'action impliquerait la combinaison des deux modes d'actions décrits précédemment. Ce troisième mode d'action serait le plus probable, car il a déjà été démontré qu'une action couplée de deux métabolites antimicrobiens, un composé volatil (HCN) et l'autre non (DAPG), est impliquée dans la protection du tabac contre *T. basicola,* par *P. protegens* CHA0, dans du sol artificiel (Voisard *et al.*, 1989). Par ailleurs, diverses familles de composés volatils sont connues pour leurs différents rôles dans l'interaction tripartite plante-phytopathogène-bactérie biocontrôle (Audrain *et al.*, 2015; Hernández-León *et al.*, 2015).

Les 3 souches de référence possèdent la capacité de produire du DAPG et de l'HCN. Cependant, leur capacité d'inhibition de la croissance mycélienne de *T. basicola* n'est pas la même, avec une inhibition de la croissance fongique par rapport au témoin s'échelonnant de 30% pour F113 à 80% pour Pf-5. Ces observations suggèrent que d'autres modes d'action peuvent être responsables de ces différences. Il peut s'agir de la production de pyrrolnitrine et de pyolutéorine produites par les souches Pf-5 et CHA0 mais pas par F113, par exemple (Wirthner *et al.*, 1992; Loper *et al.*, 2012).

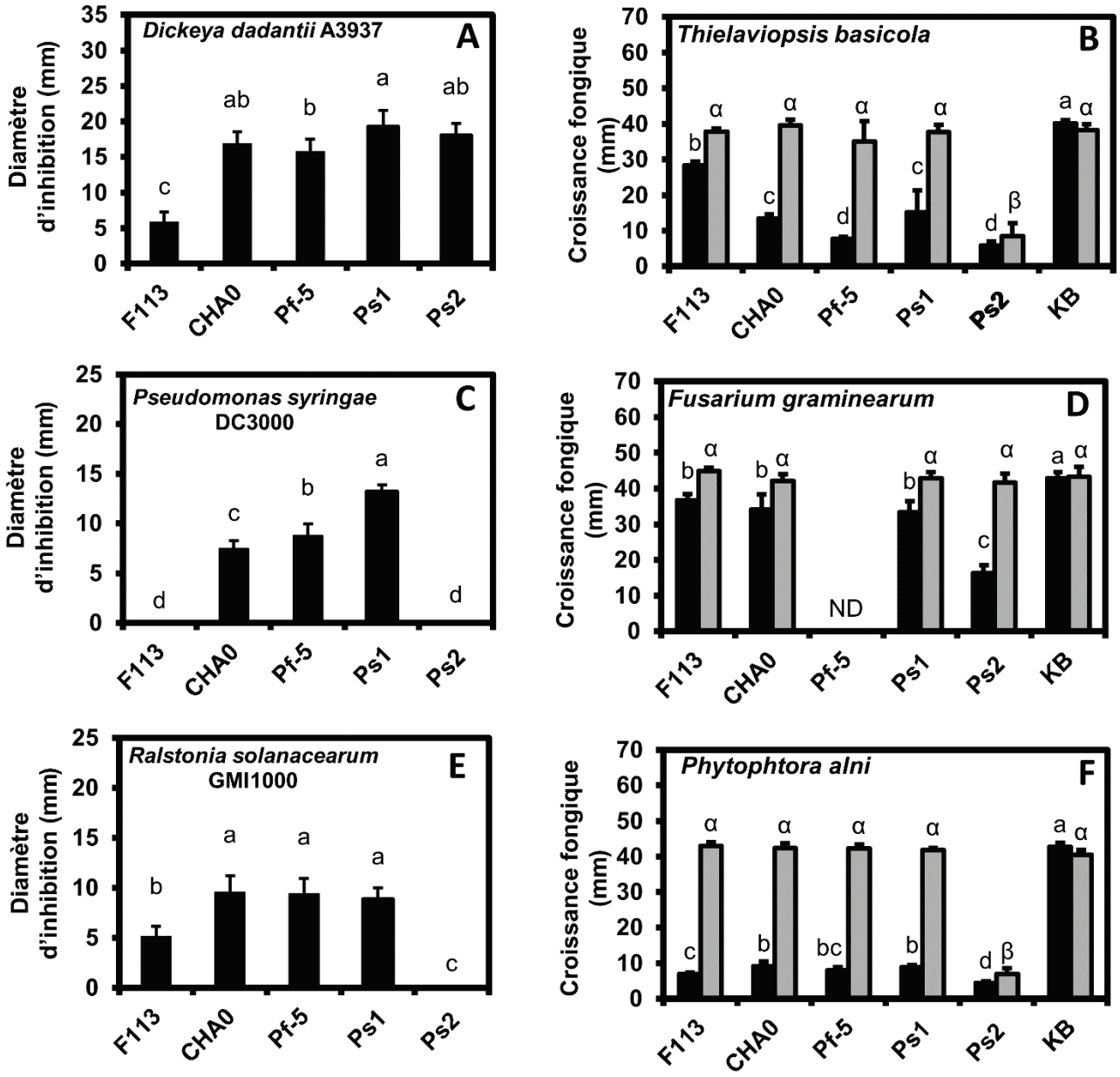


**Figure 7 : Effet des souches bactériennes candidates sur la croissance de bactéries, champignons et oomycètes phytopathogènes**

A, C et E : Tests d'inhibitions des bactéries phytopathogènes. B, D et F : Test d'inhibition des champignons et oomycète phytopathogènes. Les barres noires correspondent aux mesures de la croissance fongique réalisées du côté du dépôt bactérien (BS). Les barres grises correspondent aux mesures de la croissance fongique réalisées du côté diamétralement opposé au dépôt bactérien.
Les barres d'erreurs correspondent à l'erreur standard. Les traitements possédant les mêmes lettres ne sont pas statistiquement différents (ANOVA and Tukey's HSD test, $P < 0,05$).

Ps1 et Ps2 exercent également une action antagoniste significative envers *Ph. alni*, avec une inhibition plus sévère de la croissance de cet oomycète en présence de la souche PS2 (**Figure 3-F**). En revanche, concernant *F. graminearum*, seule la souche Ps2 inhibe significativement la croissance fongique (du côté du dépôt bactérien uniquement) (**Figure 4-B**). Les souches biocontrôle de référence ont également une faible incidence sur l'inhibition de la croissance de *F. graminearum*. Cette absence d'effet pourrait être liée à la production par ce

champignon d'un composé capable de contrôler la production de métabolites antimicrobiens chez les souches de référence testées. En effet, des études ont montré l'inefficacité des souches productrices de DAPG à inhiber la croissance fongique du phytopathogène *Fusarium oxysporum* et ont identifié que l'acide fusarique produit par ce champignon réprimait l'expression de l'opéron *phl* (Duffy and Défago, 1997; Schnider-Keel *et al.*, 2000; Notz *et al.*, 2002). Par ailleurs, l'acide fusarique produit par ces champignons semblent également réprimer l'expression de gènes impliqués dans la production d'autres métabolites secondaires antifongiques comme les phénazines chez *Pseudomonas chlororaphis* PCL1391 (van Rij *et al.*, 2004).

Les deux souches d'intérêt ayant montré un effet significatif sur la croissance de *T. basicola* ainsi que sur celle d'autres microorganismes phytopathogènes, elles ont donc été sélectionnées pour mener des essais préliminaires en serre de protection de plants de mâche vis-à-vis de *T. basicola* (CTIFL de Carquefou).

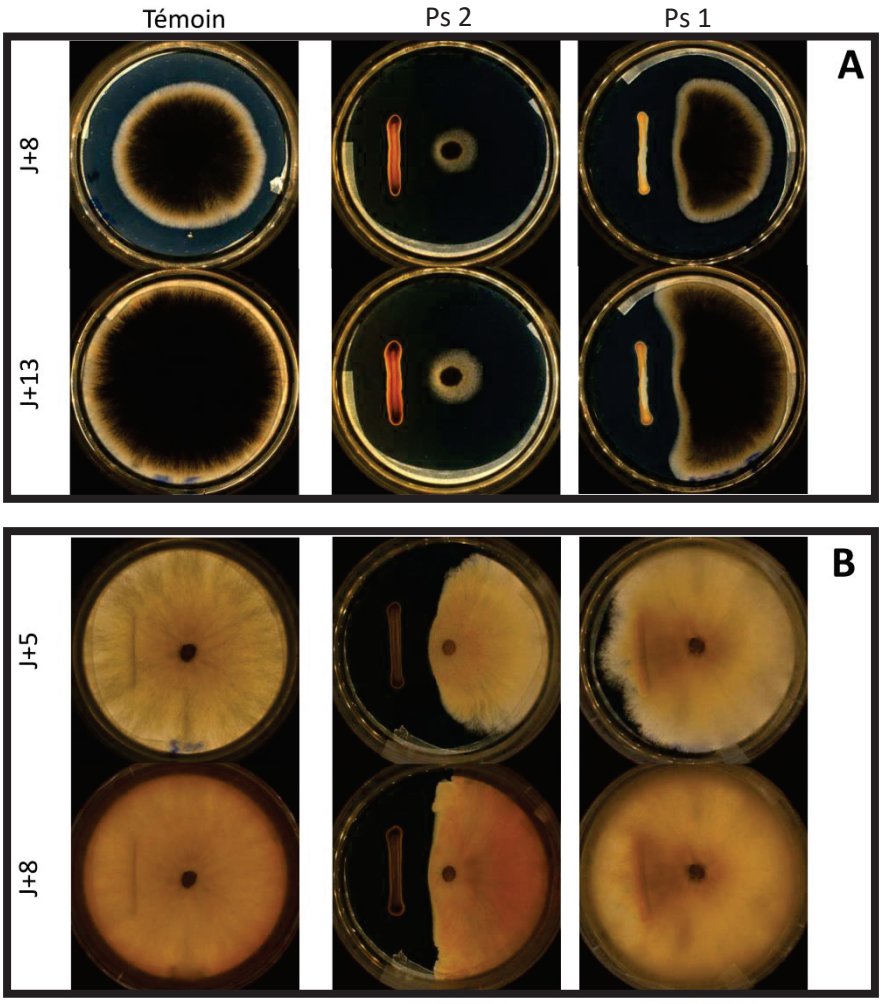


**Figure 8 : Photographies montrant l'inhibition de la croissance de *T. basicola* (A) et *F. graminearum* (B) par 2 souches bactériennes candidates.**
A : Croissance de *T. basicola* à J+8 et J+13 en présence des souches Ps1 et Ps2.
B : Croissance de *F. graminearum* à J+5 et J+8 en présence des souches Ps1 et Ps2.

**Résultats des essais de phytoprotection de *V. locusta* vis-à-vis de *T. basicola***

*Essai n°1 – Printemps 2014*

Le premier essai s'est déroulé en serre au printemps 2014. Les relevés de température et d'humidité montrent de fortes variations de ces 2 paramètres du 10 au 20 mai. Durant cette période les plants de mâche ont subi un fort stress hydrique, provoquant l'apparition de symptômes de flétrissement et nécroses sur les parties aériennes.

En comparant les valeurs d'Indice de Pathogénicité (IP) et de Sévérité Conditionnelle (SC) obtenus avec une infection de 300 spore.g$^{-1}$ de sol de *T. basicola*, aucune différence significative n'a été constatée avec les témoins non-inoculés par le phytopathogène ou par les bactéries (**Figure 5**).

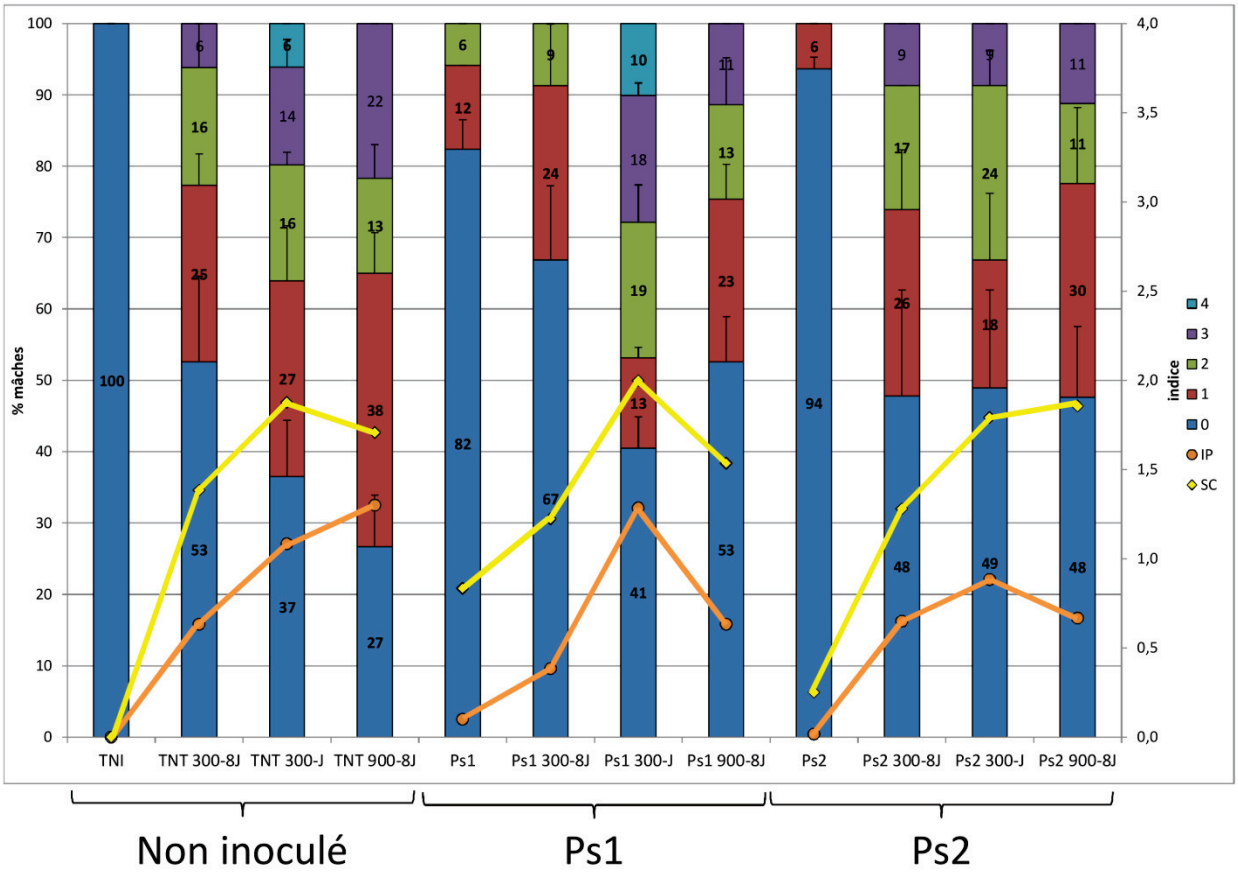


**Figure 9 : Etat sanitaire des racines à la récolte dans l'essai n°1**
Moyennes de 4 répétitions de 15 mâches classées de 0 à 7 selon l'échelle de notation des nécroses. IP : Indice de Pathogénicité, SC : Sévérité Conditionnelle. Les chiffres indiqués dans les barres d'histogrammes correspondent au pourcentage de mâches. Les barres d'erreurs correspondent à l'écart type. TNI : Traitement non-inoculé ; TNT: Traitement non traité avec les souches candidates ; Ps1 : Traitement inoculé avec la souche Ps1 le jour du semis ; Ps2 Traitement inoculé avec la souche Ps2 le jour du semis ; 300-8J : 300 spore.g$^{-1}$ de sol inoculées 8 jours avant semis ; 300-J : 300 spore.g$^{-1}$ de sol inoculées le jour du semis ; 900-8J : 900 spore.g$^{-1}$ de sol inoculées 8 jours avant semis.

De plus, aucune différence significative n'a été détectée entre les conditions inoculées avec 300 et 900 spore.g$^{-1}$ de sol et ce quel que soit la date d'inoculation du champignon ($P >$

0.05). Une réduction de l'IP est observée lorsque les souches Ps1 et Ps2 sont inoculées dans la condition où 900 spores.g$^{-1}$ de sol sont apportées 8 jours avant semis par rapport à la condition témoin correspondante sans inoculation bactérienne ($IP_{Ps1\text{-}900\text{-}8J}$ = 0.63 ; $IP_{Ps2\text{-}900\text{-}8J}$ = 0.62 ; $IP_{TNT900\text{-}8J}$ = 1.27). Pour autant, la SC n'est pas différente ($SC_{Ps1\text{-}900\text{-}8J}$ = 1.53 ; $SC_{Ps2\text{-}900\text{-}8J}$ = 1.85 ; $SC_{TNT900\text{-}8J}$ = 1.72) (**Figure 5**). Cela signifie que lorsque les souches Ps1 et Ps2 sont inoculées, il y a moins de plante présentant des symptômes (diminution de l'IP), mais que les plantes atteintes présentent la même sévérité de symptômes (SC stable). Les poids secs ont également été mesurés, mais aucun effet de l'inoculation n'a été observé (données non-montrées).

*Essai n°2 – Hiver 2014-2015*

Le second essai s'est déroulé en chambre climatique durant l'hiver 2014-2015. Seule la condition mimant le cas d'un sol fortement contaminé a été conservée (900 spore.g$^{-1}$ de sol, inoculées 8 jours avant le semis). Lors de cet essai, après dénombrement sur gélose, il a été constaté que la concentration initiale de l'inoculum était très inférieure à celle initialement prévue (2x10$^4$ UFC.mL$^{-1}$ au lieu des 2x10$^7$ UFC.mL$^{-1}$).

Les IP obtenus sont similaires que ce soit avec ou sans bactérie (TNT), ce qui signifie que le nombre de plantes infectées est le même ($IP_{Ps1\text{-}900\text{-}8J}$ = 0.85 ; $IP_{Ps2\text{-}900\text{-}8J}$ = 0.75 ; $IP_{TNT900\text{-}8J}$ = 0.85). De plus, la SC n'est pas significativement différente de celle du témoin traité avec 900 spore.g$^{-1}$ de sol ($SC_{Ps1\text{-}900\text{-}8J}$ = 1.60 ; $SC_{Ps2\text{-}900\text{-}8J}$ = 2.16 ; $SC_{TNT900\text{-}8J}$ = 1.72) (**Figure 6**). Les poids secs et frais ont également été mesurés, mais aucun effet de l'inoculation bactérienne n'a été observé (données non-montrées).

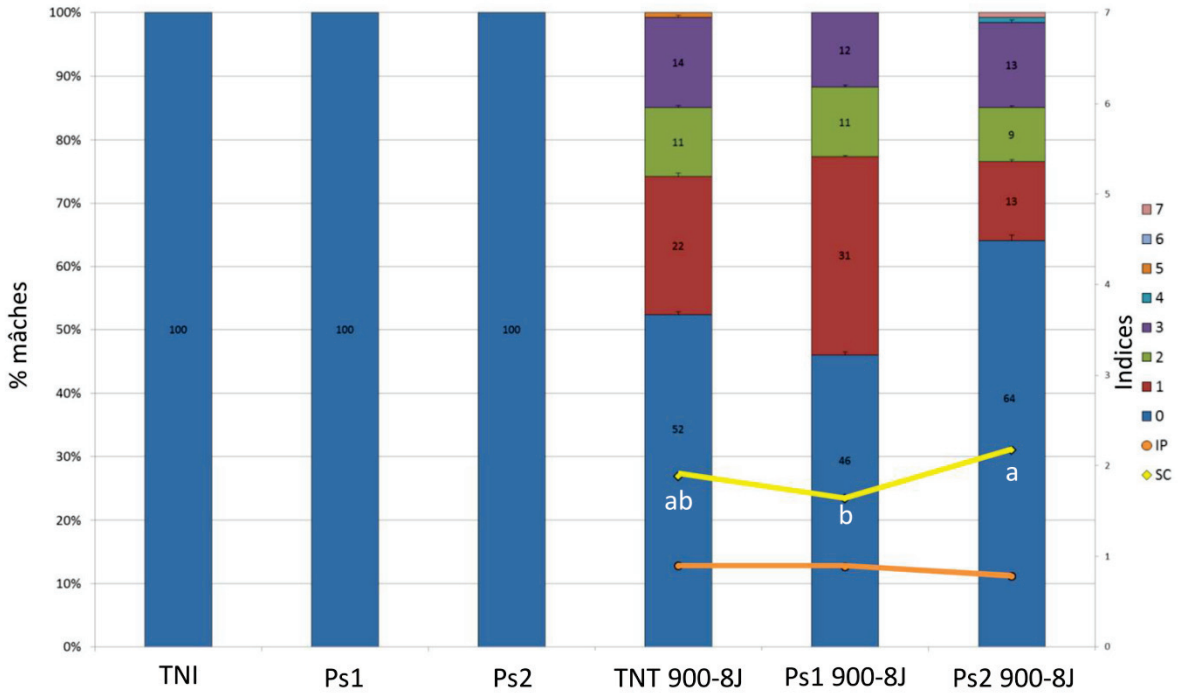


**Figure 10 : Etat sanitaire des racines à la récolte dans l'essai n°2**
Moyenne de 4 répétitions de 32 plantes classées de 0 à 7 selon l'échelle de notation des nécroses. IP : Indice de Pathogénicité, SC : Sévérité Conditionnelle. Les chiffres indiqués dans les barres d'histogrammes correspondent au pourcentage de mâches. Deux lettres différentes symbolisent une différence significative entre modalités au risque de 5% (test de comparaison de moyenne avec correction de Bonferroni). Les barres d'erreurs correspondent

à l'erreur standard. TNI : Traitement non-inoculé ; TNT: Traitement non traité avec les souches candidates ; Ps1 : Traitement inoculé avec la souche Ps1 le jour du semis ; Ps2 Traitement inoculé avec la souche Ps2 le jour du semis ; 300-8J : 300 spore.g$^{-1}$ de sol inoculées 8 jours avant semis ; 300-J : 300 spore.g$^{-1}$ de sol inoculées le jour du semis ; 900-8J : 900 spore.g$^{-1}$ de sol inoculées 8 jours avant semis.

## CONCLUSION

Deux souches bactériennes ont été sélectionnées d'après les fonctions biocontrôle qu'elles possèdent. Ces deux souches ont présenté une capacité d'antagonisme différente envers le champignon phytopathogène *T. basicola*. En effet, la souche PS2 montre une inhibition de la croissance mycélienne du champignon importante en condition *in vitro*, alors que la souche PS1 n'est capable d'inhiber la croissance du champignon qu'à faible distance (**Figure 3-B** et **Figure 4-A**). Ces deux souches ont été proposées pour évaluer leur capacité à réduire les symptômes de maladie causés par *T. basicola* sur la mâche, lors de tests préliminaires menés au CTIFL. Ces tests ont montré un effet identique et positif des souches testées lors d'une forte contamination du sol par ce champignon (900 spore.g$^{-1}$ de sol). Cependant, le 2$^{ème}$ essai mené dans des conditions de température et d'humidité mieux contrôlées n'a pas fonctionné du fait d'un problème de concentration insuffisante de l'inoculum bactérien.

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# ANNEXE 2

## ARTICLE DE CONGRES PUBLIE EN 2015 DANS LE CADRE DU CONGRES « *NATURAL PRODUCTS AND BIOCONTROL 2012* »

# Plant growth-promoting properties of *Pseudomonas* biocontrol agent producing 2,4-diacetylphloroglucinol

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## 1 - Introduction

Suppressive soils correspond to field locations where low root disease severity is observed (Weller *et al.*, 2002). This trait involves biocontrol microbial populations, which limit the survival or the virulence of plant pathogens or parasites. Well-known biocontrol microorganisms belonging to the fluorescent *Pseudomonas* group were isolated from suppressive soils, such as *Pseudomonas protegens* CHA0 (isolated from a soil suppressive against black root rot of tobacco caused by *Thielaviopsis basicola*) or *Pseudomonas fluorescens* Q2-87 (isolated from a soil suppressive to against take-all of wheat caused by *Gaeumannomyces graminis var. tritici*) (Frapolli *et al.*, 2012). Biocontrol of soilborne plant diseases involves the production of antibiotics by fluorescent pseudomonads (Couillerot *et al.*, 2009; Weller *et al.*, 2007). Indeed, fluorescent pseudomonads produce a wide range of antimicrobials, such as phenazines, pyoluteorin, pyocyanin, pyrrolnitrin, hydrogen cyanide, 2,4-diacetylphloroglucinol (DAPG), or surfactants with antifungal properties such as viscosinamides (Dwivedi and Johri 2003; Thrane *et al.*, 2000).

DAPG-producing pseudomonads inhabit most cropped soils (not only suppressive soils). DAPG is a broad spectrum antimicrobial metabolite, both acting on eukaryotic and prokaryotic phytoparasites (bacteria, fungi, nematodes, chromista) (Couillerot *et al.*, 2009; Jousset *et al.*, 2006; Meyer *et al.*, 2009). It inhibits soilborne pathogens but also triggers ISR systemic resistance pathways in plant, which renders the host less susceptible to pathogen infection (Weller *et al.*, 2012). DAPG is synthesized by condensation of 3 malonyl-CoA and their cyclisation to phloroglucinol, a step catalysed by the type-III polyketide synthase PhlD. The transacetylases PhlA, PhlB and PhlC are responsible for the two acetylation reactions leading to synthesis of DAPG from phloroglucinol (Yang and Cao, 2012). Genes encoding those enzymes belong to a *phl* cluster along with two specific regulator genes (*phlF* and *phlH*), *phlG* encoding a hydrolase and *phlE* encoding a transporter (Haas *et al.*, 2000).

Recently, high-throughput diversity analyses of the rhizosphere microbiome of suppressive soils evidenced that soil disease suppression may not be restricted to a single bacterial group, but could involve an entire community of microorganisms, including perhaps rhizobacteria that are not acting as biocontrol agents (Kyselková *et al.*, 2009; Mendes *et al.*, 2011; Sanguin *et al.*, 2008). Among indigenous populations from suppressive soils, species of *Azospirillum*, a key genus of phytostimulating rhizobacteria, were found colonizing roots jointly with DAPG-producing pseudomonads (Kyselková *et al.*, 2009; Sanguin *et al.*, 2009).

We hypothesize that besides their ability to regulate populations of phytopathogenic microorganisms in many soils, DAPG-producing pseudomonads have the capacity to enhance the growth and development of plant, in both an indirect and a direct fashion. To address this hypothesis, we developed two strategies. First, we studied the impact of DAPG and DAPG-producing *Pseudomonas* on the phytostimulating bacterium *Azospirillum brasilense* Sp245, which is able to stimulate plant growth through the production of indole-3-acetic acid (IAA) (Dobbelaere *et al.*, 1999). This auxin controls a wide variety of processes in plant development and plant growth; it stimulates the formation of lateral roots, decreases primary root length and increases root hair formation (Dobbelaere *et al.*, 1999; Patten and Glick, 2002; Spaepen *et al.*, 2008). Second, we explored the bacterial diversity of maize rhizosphere to isolate *Pseudomonas* strains possessing genes of the *phl* operon (designed as $Phl^+$) and harboring several distinct plant growth-promotion properties (production of phytohormones, phosphate solubilisation, etc.) and thus able to stimulate plant growth directly.

## 2 – Indirect plant growth-stimulating properties of DAPG-producing pseudomonads

### 2.1 –DAPG at signal concentration stimulates the expression of *Azospirillum*'s phytostimulation-relevant genes

The effects of high and low concentrations of DAPG on the physiology of a plant-beneficial bacterium, *A. brasilense* Sp245 were analyzed. At high concentrations (500 µM), DAPG inhibits the growth of *A. brasilense* Sp245 by inducing damages to the cytoplasmic membrane (Couillerot *et al.*, 2011). At lower concentrations, DAPG induces cytoplasmic accumulation of bacterioruberin-type carotenoids and poly-□-hydroxybutyrate(PHB)-like granules. The $Phl^+$ strain, *P. fluorescens* F113 producing DAPG, is able to inhibit the growth of Sp245, whereas its Phl-negative mutant F113G22 cannot, when performing *in vitro* growth inhibition plate assays and simultaneous inoculations of *Pseudomonas* and *Azospirillum* strains on wheat roots (Couillerot *et al.*, 2011). At low concentrations, DAPG acts as a signal, stimulating the expression of phytostimulation-relevant genes in strain Sp245 (Combes-Meynet *et al.*, 2011). More precisely, a promoter trapping approach was developed to select, among a random library of *A. brasilense* Sp245 promoter fusions upstream the *egfp* reporter gene, DAPG-induced clones by flow cytometry. Two DAPG concentrations were tested: 10 µM DAPG, a concentration evidenced in rhizosphere soil (Bonsall *et al.*, 1997) and 0.01 µM DAPG, a 1000-fold lower concentration, similar to the concentration of other rhizosphere signals. Flow cytometry cell sorting gave 1,920 clones at 0.01 µM DAPG and 960 at 10 µM DAPG. DAPG induction of the most fluorescent clones was confirmed in microtiter plates leading to the identification of 158 clones induced in presence of 0.01 µM DAPG and 60 clones in presence of 10 µM DAPG (Combes-Meynet *et al.*, 2011). Sequencing of the 96 most induced clones lead to the identification of 52 promoter regions. Several genes are involved in stress response, which may be important to allow *Azospirillum* cells located in the vicinity of DAPG-producing *Pseudomonas* to face up to inhibitory concentrations of DAPG (Couillerot *et al.*, 2011). Among those clones, was found a gene coding a putative enoyl-CoA hydratase/isomerase potentially involved in the biosynthesis of PHB, which favors *A. brasilense* survival under stress conditions

(Kadouri *et al.*, 2003; Kamnev *et al.*, 2008). Other genes identified are involved in cell motility, biofilm formation and root colonization like the polyphosphate kinase gene *ppk* and the flagellar hook protein gene *flgE*. DAPG also induced the expression of genes involved in stimulus sensing and signal transduction like putative hybrid sensor kinase/response regulator and adenylate/guanylate cyclase genes. Surprisingly, DAPG was shown to induce Sp245 genes involved in plant growth promotion, like *nifX* implicated in nitrogen fixation, *ppdC* encoding the phenylpyruvate decarboxylase required for IAA production, and *nirK* encoding a nitrite reductase required for nitric oxide production (Combes-Meynet *et al.*, 2011).

### 2.2 – The DAPG-producing P. fluorescens F113 stimulates the expression of Azospirillum phytostimulation-relevant genes on wheat roots

As *P. fluorescens* cells are able to inhibit the growth of *Azospirillum* cells when the two PGPR are in close contact with each other (Couillerot *et al.*, 2011), successive inoculations of wheat plantlets with *Azospirillum* and two days later with *Pseudomonas* was performed in order to limit antagonistic interactions between the two PGPR (Combes-Meynet *et al.*, 2011). Under these conditions, which favored the signaling effect of DAPG produced by F113 on *Azospirillum* cells, the enhanced expression of Sp245 phytostimulation-relevant genes (like *nirK, ppdC*) was confirmed (Figure 1). This demonstrates that DAPG can trigger gene expression of plant-beneficial functions in neighboring rhizobacteria.

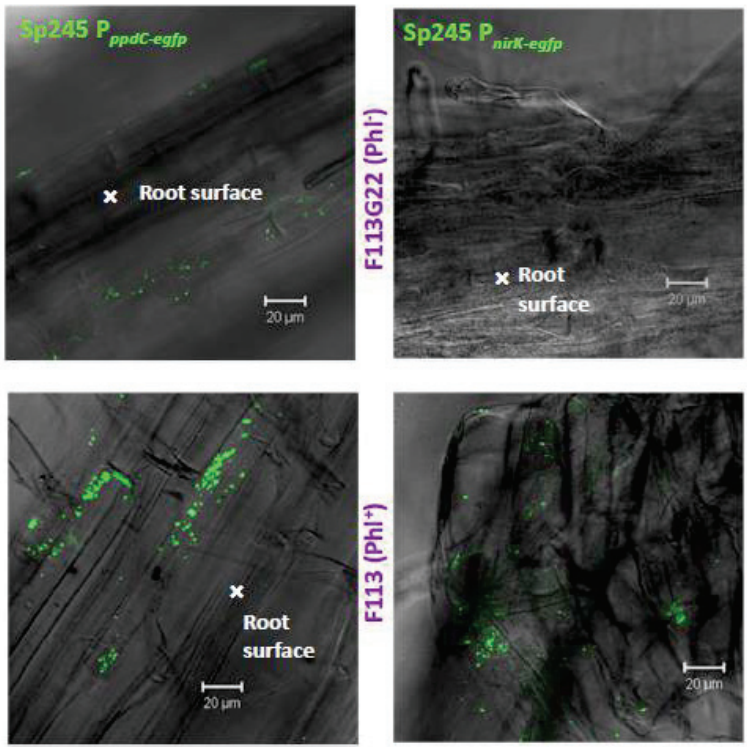


**Figure 1 : Confocal laser scanning microscope images of *A. brasilense* Sp245 clones expressing the *ppdC-egfp* and the *nirK-egfp* fusions on wheat roots, at 7 d post inoculation with the Phl-negative mutant F113G22 and the wild-type *P. fluorescens* F113.** Observations were made using a 510 Meta microscope (Carl Zeiss S.A.S., Oberkochen, Germany) equipped with an argon-krypton laser, detectors and filter sets for green fluorescence (i.e. 488 nm for excitation and 510-531 nm for detection). Cells expressing EGFP are green and grey backgrounds correspond to the root image formed by the transmitted light. The same detector amplification gains were used for semi-quantitative comparison of fluorescence levels in the treatments. Images are representative of the analysis of at least 10 images per condition. Enhanced fluorescence of the phytostimulation-relevant gene fusions is observed in presence of the DAPG-producing F113 strain.

### 2.3 – The DAPG-producing P. fluorescens F113 enhances plant growth promotion by A. brasilense Sp245

The successive inoculations of wheat plantlets with *A. brasilense* Sp245 and two days later with the Phl$^+$ *P. fluorescens* F113 resulted in enhanced stimulation of wheat growth compared to single *Azospirillum* inoculation or successive inoculation with the Phl-negative mutant F113G22 (Figure 2) (Combes-Meynet *et al.*, 2011). By contrast, simultaneous inoculation of *A. brasilense* Sp245 and *P. fluorescens* F113 on wheat plantlets did not lead to plant growth stimulation due to the growth inhibition of *Azospirillum* cells by *P. fluorescens* F113 in those conditions of inoculation (Couillerot *et al.*, 2011). Thus, DAPG-producing pseudomonads can have positive indirect effects on plant growth by controlling the positive interaction of phytostimulating PGPR with the host plant.

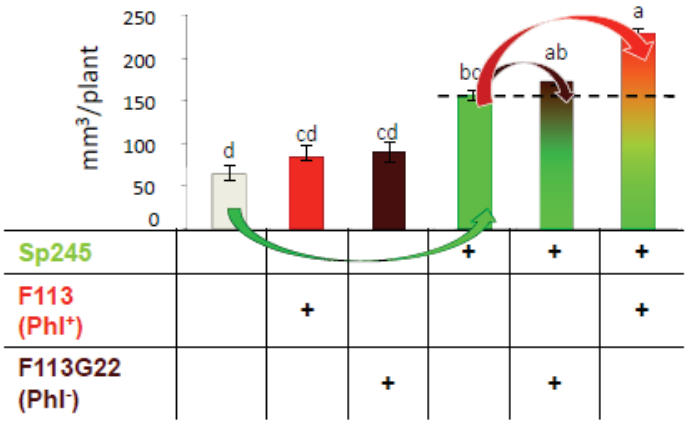


**Figure 2: Effects of single or double successive inoculations of wheat with *A. brasilense* Sp245, DAPG-producing *P. fluorescens* F113, or its Phl-negative mutant F113G22, on root volume, at 7 days (mean ± standard error, n = 5).** Statistical differences between treatments are indicated with lowercase letters (analysis of variance and Fisher's least significant difference tests; $P < 0.05$). A. brasilense Sp245 significantly increases wheat root volume and synergistic effect of the Phl+ P. fluorescens F113 and A. brasilense Sp245 on plant growth are observed.

## 3 – Direct plant growth properties of DAPG-producing pseudomonads

Besides the potential indirect plant growth-promoting effects of DAPG-producing *Pseudomonas*, comparative genomic approaches evidence that DAPG-producing *Pseudomonas* strains harbor a huge diversity of plant growth promotion properties (like production of phytohormones, phosphate solubilisation, nitrogen fixation) and thus might be able to stimulate plant growth directly (Loper *et al.*, 2012; Redondo-Nieto *et al.*, 2013). Moreover, DAPG has been recently identified as a signal affecting root system architecture of plant: at around 10 µM, DAPG increases the number of lateral roots in tomato (Brazelton *et al.*, 2008). It appears obvious that DAPG-producing *Pseudomonas* are able to directly stimulate the growth of Poaceae and Fabaceae (Boruah and Kumar, 2002; De Leij *et al.*, 2008; Walker *et al.*, 2012). In order to isolate new plant growth stimulating Phl$^+$ *Pseudomonas*, two maize varieties, DK315 and PR37Y15, were grown in 3 distinct soils from Switzerland and France, and the maize rhizosphere soils were screened for the presence DAPG-producing *Pseudomonas* that are potentially able to stimulate directly the growth of plants. Fluorescent pseudomonads (n=216), possessing *phl* genes (potential DAPG producers) and harboring other plant beneficial traits like ACC deaminase activity, indolic and NO production, phosphate solubilisation, nitrogen

fixation, and exopolysaccharide (EPS) production were identified using PCR amplification of target genes and biochemical assays (Figure 3).

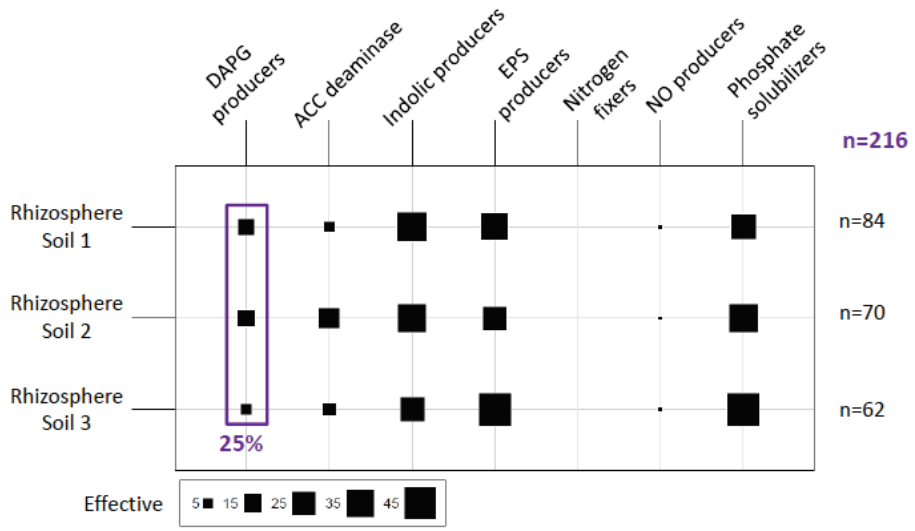


**Figure 3: Distribution of fluorescent pseudomonads harboring beneficial functions depending on soil. Square size corresponds to the proportion of *Pseudomonas* isolates that possess the associated beneficial functions.** Phl$^+$ *Pseudomonas* represent approximately 25% of all isolates (n=216). More Phl$^+$ *Pseudomonas* are present in Rhizosphere soil 1 and Rhizosphere soil 2 than in Rhizosphere soil 3 ($P < 0.05$).

Many soil isolates (n=36) displayed at least three distinct plant beneficial traits. This survey shows that similar distribution of plant beneficial traits was observed among isolates from one soil to another (Figure 3). These preliminary results indicate that co-occurrence of plant beneficial properties is widespread among fluorescent *Pseudomonas*. One can suggest that co-occurrence of plant beneficial functions in PGPR might have been selected by the plant during it co-evolution with the soil rhizobiome.

## 4 - Conclusion & Perspective

Besides their well-known ability to benefit plant health, DAPG-producing pseudomonads have the capacity to enhance the growth and development of plant, in both (i) a direct fashion, by producing diverse plant growth regulators and enhancing plant nutrition and (ii) an indirect fashion, by stimulating the expression of plant beneficial traits in phytostimulating rhizobacteria (Figure 4). This work reveals that the antimicrobial compound DAPG is a key cell–cell signalling molecule in the rhizosphere, which might regulate gene expression in rhizosphere microbial populations and possibly the interactions of these populations with the host plant. This highlights a novel concept that antimicrobials constitute a large collection of cell-signalling molecules (Fajardo and Martínez, 2008; Yim *et al.*, 2007). Social relations between different types of PGPR are thus important to take into account when studying the cooperation of PGPR with plants and developing efficient bacterial inoculant consortia.

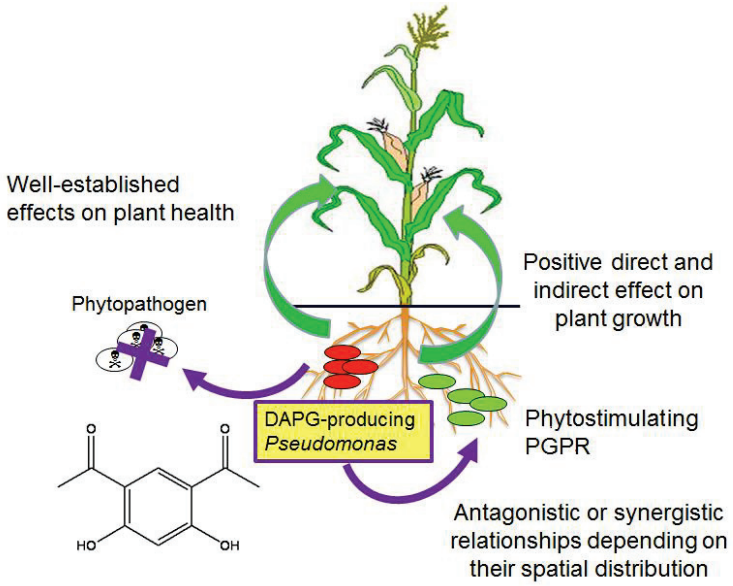


**Figure 4 : Besides their ability to regulate populations of phytopathogenic microorganisms in many soils, DAPG-producing *Pseudomonas* strains have the capacity to enhance the growth and development of plant, in both a direct and an indirect fashion by enhancing the expression of plant beneficial functions in *Azospirillum* phytostimulating PGPR. Depending on the local distribution of *Azospirillum* and *Pseudomonas* populations on roots, antagonistic or synergistic relationships may occur between different types of PGPR populations.**

*Key Words : Biocontrol, fluorescent Pseudomonas, 2,4-diacetylphloroglucinol, Plant Growth-Promoting Rhizobacteria, rhizosphere signal.*

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# ANNEXE 3

## CHAPITRE DE LIVRE SUR L'IMPACT D'*AZOSPIRILLUM* DANS L'AMELIORATION DE LA RESISTANCE AU STRESS BIOTIQUES ET ABIOTIQUES CHEZ LA PLANTE.

# Alleviation of abiotic and biotic stresses in plants by *Azospirillum*

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**Abstract**

In the face of global changes, plants must adapt to a wide range and often combined biotic and abiotic stresses that seriously impaired plant growth and development. Plants develop complex strategies to deal with water stress conditions, soil fertility losses, soil pollutions, pests and disease. Emerging evidence suggest the involvement of common hormonal players in plant defense signaling pathways triggered in response to biotic and abiotic stresses. Besides plant strategies, plant growth-promoting rhizobacteria (PGPR), which colonize the root system and establish cooperative interactions with plants can improve their growth and help them to adapt to and cope with multiple stresses including drought, salinity, heavy metal pollutions, and parasites. Accordingly, PGPR supply added values to the plant defense strategies by expressing many relevant functions for modulating the plant hormonal balance, increasing nutrients supply to the plant, improving the functional and physical properties of protective barriers against plant parasites. Among PGPR, *Azospirillum* strains were long viewed as biofertilizers and less as biocontrol agents. It is becoming evident that *Azospirillum* is able to protect plants against a myriad of detrimental conditions. This review provides an update of works regarding the ability of *Azospirillum* strains to alleviate plant stress and brings out the relevant involved plant-beneficial functions. Developing PGPR based bio-inoculants is a promising strategy to improve the growth and health of crops and develop sustainable agriculture.

**Keywords:** plant stress alleviation, plant growth-promoting rhizobacteria, *Azospirillum*, plant hormonal balance modulation, plant nutrition improvement, biocontrol, induced systemic resistance.

## 1. Introduction

As principal producers of organic matter, plants play a major role in ecosystem dynamics at both micro and macroscopic scales. Through their roots, plants strongly influence the physical structure and chemical composition of the soil compartment (Hiltner 1904). The region of soil directly under the influence of roots, called the rhizosphere, is colonized by a huge abundance and diversity of microorganisms in continuous interactions with plants (Hartmann *et al.* 2008). A root system releases a large quantity of amino acids, sugars and organic compounds by a phenomenon called root exudation (Haichar *et al.* 2014). Root exudates constitute a real "buffet" for rhizospheric microorganisms (López-Guerrero *et al.* 2013), and according to their chemical compositions they modulate the structure, abundance and diversity of microbial communities in the rhizosphere. These microorganisms associated to the roots, including fungi, protozoa, nematodes, and bacteria, constitute the rhizo-microbiote. These microorganisms can establish a wide range of ecological interactions with plants, ranging from parasitism to symbiosis, and passing by commensalism (Hartmann *et al.* 2008).

Plants and their rhizo-microbiote must cope with environmental condition changes and can suffer from different biotic (diseases, competition for resources) and abiotic (drought, cold, acidification) stresses. In a context of global changes caused by human activities (urbanization, pollution and deforestation), stresses to which plants must adapt are more diverse, important and frequent (IPCC 2001). Since the middle of the twentieth century, synthetic fertilizers and pesticides have considerably improved crop yields. This "green revolution" has allowed the world population to increase. However, chemical inputs and intensive use of soils caused environmental pollutions and soil fertility troubles (Gerhardson 2002, Ramirez *et al.* 2012, Eisenhauer *et al.* 2012). The world population still continues to grow rapidly. Increasing agricultural production is thus desirable, but the potential for expanding agriculture area is limited and chemical inputs are harmful to animals and humans and can accumulate in the environment. Environmentally friendly solutions, based on the use of microorganisms as biofertilizers and biocontrol agents, exist to improve crop yields (Berg and Smalla 2009). However, these solutions are rarely used by farmers due to their small commercial scale. Developing these solutions requires better understanding the interactions between plants and their rhizo-microbiote, especially under biotic and abiotic stress conditions.

Modifications of environmental conditions can dramatically affect plant development and deeply modify the structure and diversity of root-associated microbiotes (Compant *et al.* 2010). Plants set up various physiological responses to limit the effects of stress on their development. The rhizo-microbiote can be directly affected by biotic and abiotic stresses, but also indirectly through the physiological modifications induced by stresses in plants. Conversely, the rhizo-microbiote can also modify plant physiology and help it to tolerate stresses. Indeed, cooperative microorganisms have plant beneficial properties like inducing plant systemic defense, improving mineral nutrition and controlling plant pathogen development (Vacheron *et al.* 2013). Plant beneficial properties are recovered especially in Plant Growth-Promoting Rhizobacteria (PGPR), which do not belong to a unique phylogenetic clade (Bruto *et al.* 2014). PGPR establish associative symbiosis with plant and colonize their roots without inducing the formation of a symbiosis-dedicated organ.

Several studies demonstrate the ability of PGPR to alleviate biotic and abiotic stresses affecting plants (Dimkpa *et al.* 2009). The most studied PGPR belong to the genus *Bacillus*, *Pseudomonas*, and *Enterobacter* (Babalola and Akindolire 2011). Bacteria of the genus *Azospirillum* are less known as being able to protect plants against stresses, but they have several plant beneficial functions potentially efficient to alleviate biotic and abiotic plant stress (Wisniewski-Dyé *et al.* 2011). These include i) modification of the plant hormonal balance (auxin production, ethylene modulation) resulting in root system augmentation; ii) improvement of mineral nutrition by siderophores production and fixation of atmospheric nitrogen; iii) production of antimicrobial compounds and inducing plant systemic defenses.

This chapter focuses on the technical methods that bring to light the beneficial traits of PGPR and, in particular, of bacteria from *Azospirillum* genus for their role in alleviating biotic and abiotic plant stresses.

## 2. Plant responses to biotic and abiotic stresses

### a- Responses to biotic stresses

Mechanisms of plant defense against biotic stresses are complex and consist of several layers of defense. The recognition of Pathogen-Associated Molecular Patterns (PAMPs) can lead to the induction of protective responses in plants, such as callose deposition, oxidative bursts, production of antimicrobial compounds, and programmed cell death. This response corresponds to PAMP-Triggered Immunity (PTI). If pathogens are able to secrete effectors that suppress PTI, disease occurs resulting in Effector-Triggered Susceptibility (ETS). If pathogens secrete effectors that are recognized by the plant, plant disease resistance occurs resulting in Effector-Triggered Immunity (ETI). The latter involves the recognition of an avirulent (Avr) factor from the pathogen by a plant resistance protein (R). This leads to a hypersensitive response (HR), corresponding to a localized cell death that prevents root invasion of plant tissue by the pathogen.

Plants respond to pathogen attack by synthesizing pathogenesis related (PR) proteins. They encode enzymes like chitinases and glucanases that can hydrolyze the cell walls of fungal pathogens (Mauch *et al.* 1988). In addition, plants also use the proteasome system to degrade proteins impaired by cellular stress. Ubiquitin is used to trigger this response (Dreher and Callis 2007). It acts as a covalent molecular tag to target proteins that must be degraded. After delivery to the proteasome, the poly-ubiquitylated substrates can be de-ubiquitylated and cleaved in small peptides to release free amino-acids.

The induction of many defense related genes is often linked to the increase of plant endogenous content of salicylic acid (SA), a key signaling molecule involved in plant defense against pathogens, and the establishment of the Systemic Acquired Resistance (SAR), which renders the plant more resistant to subsequent attacks by pathogens.

Besides SA, reactive oxygen species (ROS) produced in part from mitochondria and nitric oxide (NO) contents strongly increase following pathogen recognition. All of these signaling molecules coordinate defense responses in plant (Alvarez 2000, Neill *et al.* 2002, Laloi *et al.* 2004, Wendehenne *et al.* 2004, Delledonne 2005, Torres and Dangl 2005, Amirsadeghi *et al.* 2007).

Another key hormone, ethylene (ET), plays a major role in plant responses to pathogens. ET is both a plant growth regulator and a stress hormone. ET is produced endogenously by plants and in soil and plays a key role in inducing multifarious physiological changes in plants at molecular level. Under stress, the endogenous production of ET is accelerated substantially. This hormone stimulates the transcription of numerous defense-related genes (Dreher and Callis, 2007). Transcription factors of the EIN3 (Ethylene-insensitive 3) family play a major role in the regulation of plant defense responses (van Loon *et al.*, 2006). Jasmonate (JA) promotes resistance to microbial pathogens and to insects. JA and ET mostly operate synergistically to activate the expression of defense related genes, and share a similar target gene network (Schenk *et al.* 2000). Contrariwise, SA and JA mostly operate in opposite ways. Certain key regulators play a pivotal role in the balance between SA and JA pathways (Li *et al.* 2006). For instance, it has been shown in *Arabidopsis* that the transcription factor WRKY70 acts as a positive regulator of SA-dependent defenses and a negative regulator of JA-dependent defenses whereas the Mitogen Activated Protein Kinase 4 (MPK4) acts inversely. The Nonexpressor of Pathogenesis-Related genes 1 (NPR1) also plays an important role in regulating the SA and JA pathways. It is important to note that the expression of PR1 is typically used as a marker of the induction of SA-dependent defense pathway (Martin *et al.* 2003), while the plant defensive PDF1.2 is one of the most useful markers for the induction of SA-independent defense pathway (Pieterse *et al.* 2002).

Numerous studies provide evidence that plant defense signaling networks are extremely complex. The involvement of auxin, abscisic acid (ABA) and gibberellic acid also act as important components of the signaling network involved in the regulation of defense responses against various pathogens (Bari and Jones 2009). Defense signaling networks activated by the plant depend on the life modes of pathogens. Briefly, SA is mainly involved in the activation of defense responses against biotrophic and hemi-biotrophic pathogens whereas JA and ET are usually involved with defense against necrotrophic pathogens. Since pathogens are able to produce phytohormones, numerous recent studies suggest that pathogens can manipulate the defense-related regulatory network of plants. Thus plant pathogens seem to manipulate components of hormone biosynthesis and signaling machinery leading to hormone imbalances and alterations in plant defense responses.

**b. Responses to abiotic stresses**

Plants have developed common strategies to cope with abiotic stresses like drought, flooding, salinity, chilling, or high temperatures. One common mechanism involves the accumulation of compatible solutes, like glycine betaine (amine), trehalose (sugar), proline (amino acids), etc (Ashraf and Foolad 2007, Seki *et al.* 2007).

In many plant species, the quaternary amine, glycine betaine, is synthesized from choline at high level in response to various types of abiotic stresses, and protects plants against water deficiency, frost and salinity (Chen and Murata 2002). It acts by stabilizing the quaternary structures of enzymes and proteins, and protecting cell membranes.

Other compatible solutes like mannitol, proline and sorbitol are produced for scavenging ROS, which are produced by plants experiencing drought, salt and temperature stresses. ROS can directly damage cellular components, and the accumulation of compatible solutes may protect cells against increased levels of ROS, thereby resulting in the protection of plants against stress-induced damages (Chen and Murata 2002). Accordingly, up-regulation of anti-oxidative enzymes, like superoxide dismutase, is a general response to different abiotic stress conditions.

Polyamines such as putrescine, spermidine and spermine also accumulate in response to abiotic stresses. Transcriptomic analyses in the model plant *Arabidopsis*, the utilization of polyamine-overproducing transgenic plants or, contrariwise, of mutants deficient in polyamine biosynthesis permit to evidence the involvement of polyamines in the tolerance of plants to different kind of stresses (Alcázar *et al.* 2006). It has been suggested that they may act as ROS scavenging molecules and as membrane protectors.

In response to water deficit or salinity stress, plants increase the synthesis of osmolytes (Farooq *et al.* 2009), thereby leading to increased osmotic potentials within cells and adaptation of the plant to drought. ABA plays a prominent role in plant responses to drought. It can directly affect ion transport in guard cells, thereby controlling stomatal aperture and plant transpiration (Roelfsema *et al.* 2004). In addition, it induces the expression of drought stress-related genes. The level of this hormone increases in response to drought and salt stresses (Seki *et al.* 2007).

The levels of several other hormones are induced in response to abiotic stresses. This is the case of the ET stress hormone, whose synthesis increases when plants are exposed to different types of abiotic stresses. As mentioned previously, the EIN3 transcription factor acts as a positive regulator that turns on a complex upstream network of signaling responses thereby leading to the activation of numerous defense reactions (cell wall modification, oxidative burst). Increased levels of NO synthesis and subsequently the up-regulation of NO-dependent defense genes are observed in response to drought, high or low temperature, salinity, heavy metals and oxidative stress (Arasimowicz and Floryszak-Wieczorek 2007).

Increasing evidence suggests that crosstalk exists between plant responses to biotic and abiotic stresses. Signaling pathways regulated by ABA, and ET, play key roles in this crosstalk (Fujita *et al.* 2006). The generation of ROS appears as a key convergent response mechanism between biotic and abiotic stresses (Mittler *et al.* 2004, Fujita *et al.* 2006). Transcriptomic analyses have thus revealed that a large set of genes that encode ROS-scavenging enzymes are commonly induced when plants are subjected to abiotic and biotic stress treatments (Fujita *et al.* 2006). Plants respond to biotic and abiotic stresses by changing their physiology and metabolism, in order to limit the negative effects of stresses on plant tissues. Root-associated microbial communities might also help them. Some rhizobacteria are indeed capable of alleviating biotic and/or abiotic stresses.

## 3. Plant stress alleviation by rhizobacteria

### a- Rhizobacteria able to reduce stress in plants

Rhizobacteria, including PGPR, dispose of a wide range of beneficial functions that may increase plant growth under stress condition (Dimkpa *et al.* 2009). A broad taxonomic and functional diversity occurs in the plant rhizosphere (Bouffaud *et al.* 2014) and may affect plant fitness under stress condition (drought, salinity, pollutions, parasite attacks, etc., Table 1). PGPR are found in all clades of Proteobacteria especially in Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria, and in Firmicutes such as in Actinobacteria. Hence, there are no specific media or a unique way to isolate PGPR able of alleviate stress in plant. Functional approaches can be developed to select potential PGPR.

One of the most common used methods is firstly to isolate bacteria and then to test whether they share biocontrol effects against plant pathogens and/or have plant growth stimulation properties under abiotic stresses like drought, salinity or heavy metal stress. Mayak *et al.* (2004) screened bacteria that produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which is known for lowering the plant content of the stress hormone ET and enhancing plant growth under salt stress. *Achromobacter piechaudii* strains were isolated from salty soils on solid DF medium containing ACC as the sole source of nitrogen, and then tested for their capacity to reduce salt stress effects on tomato seedlings (Mayak *et al.* 2004). The same method was employed to isolate fluorescent pseudomonads, which alleviate salinity stress on canola (Jalili *et al.* 2009) and drought stress on pea (Arshad *et al.* 2008).

Another way to select bacteria that may have the potential to alleviate abiotic stress is to isolate strains, which are able to grow in extreme environmental conditions. For instance, a *Bacillus sp*. strain, isolated from desert soils, alleviates drought stress in lettuce by stimulating the symbiotic interaction between lettuce and arbuscular mycorrhizal fungi (Vivas *et al.* 2003). *Bacillus* and *Arthrobacter* strains, isolated from wheat rhizosphere in salt-infested zone, allow salt stress alleviation in wheat (Upadhyay *et al.* 2009). Numerous PGPR were also isolated from heavy metal polluted soils and are able to enhance both plant growth and plant development under heavy metal stress conditions, such as in the presence of arsenic (Reichman 2014), cadmium (Guo et Chi 2014), or both zinc and cadmium (Pereira *et al.* 2014).

Certain plant beneficial microorganisms can protect plants against diseases caused by fungi, and bacteria, such as against virus (Ryu *et al.* 2004), insects (Kupferschmied *et al.* 2013) and nematodes (Kerry 2000). Mechanisms involved in disease suppression by PGPR can be indirect through the activation of plant defenses or direct by competitive or antagonistic interactions against plant pathogens. PGPR can produce various compounds with detrimental effects on plant pathogens. Fluorescent *Pseudomonas* is one of the most widely studied bacterial lineages for antibiotic-based biocontrol activities. They can produce 2,4-diacetylphloroglucinol (DAPG), a well-known antibiotic involved in soil disease suppressiveness (Almario *et al.* 2013, 2014). Other *Pseudomonas* anti-microbial compounds are pyoluteorin active against *Pythium*

(Maurhoffer *et al.* 1994), phenazines against *Gaeumannomyces graminis* (Thomashow and Weller 1988), pyrrolnitrin against *Rhizoctonia solani* (Ligon et al 2000), and cyclic lipopeptides (Raaijmakers *et al.* 2006). Biocontrol strains can be isolated from suppressive soils on semi- or selective media (Stutz *et al.* 1986, Frapolli *et al.* 2010). Another way for PGPR to alleviate plant pathogen-induced stress is to produce lytic enzymes like chitinase, cellulase, and pectinase. Media containing colloidal chitin, methyl cellulose, or pectin can be used to isolate potential PGPR strains able to produce these enzymes, respectively (Siddikee *et al.* 2010).

Besides their ability to inhibit the growth of soil-borne pathogens, some PGPR are able to stimulate plant natural defenses, by a mechanism called induced systemic resistance (ISR) (Pieterse *et al.* 2014). ISR corresponds to a plant immune response that allows plants to express a stronger defense reaction when further exposed to pathogen attack. The bacteria trigger a plant-mediated resistance response in aboveground plant parts while inoculated on roots. Induced accumulation of pathogenesis-related (PR) proteins like PR-1, PR-2, chitinases, and some peroxidases are often observed in plants (Maurhofer *et al.* 1994, Pieterse *et al.* 1996, Park and Kloepper 2000, Ramamoorthy *et al.* 2001). However, certain PGPR do not induce PR proteins (Hoffland *et al.* 1995, van Wees *et al.* 1997) but rather increase accumulation of peroxidase, phenylalanine ammonia lyase, phytoalexins, polyphenol oxidase, and/or chalcone synthase (Van Peer *et al.* 1991, Ongena *et al.* 2000, Chen *et al.* 2000). They can stimulate callose deposit whereas no callose accumulation is observed in plants treated only with a pathogen (Tortora *et al.* 2012). Callose accumulation contributes to the reinforcement of the cell wall at the sites where the pathogen attacks. PGPR-elicited ISR has been demonstrated in many dicotyledonous plant species, including *Arabidopsis*, bean, carnation, cucumber, radish, tobacco, and tomato (van Loon *et al.* 1998) but less in monocotyledonous plants. PGPR strains belonging to the genera *Pseudomonas*, *Bacillus*, and *Azospirillum* have been reported to elicit growth promotion and ISR (Gutiérrez Mañero *et al.* 2001; Kloepper *et al.* 1980; Kloepper *et al.* 2004; van Peer *et al.* 1991). However, little information is available regarding the plant metabolic pathways involved in the systemic responses elicited by PGPR strains.

Among biocontrol rhizobacteria, *Azospirillum* was shown to be a good candidate for the mitigation of biotic and abiotic stresses.

### b- *Azospirillum* strain ability to reduce stress in plant

- Alleviation of biotic stresses by *Azospirillum*

Some *Azospirillum* strains are able to suppress soil-borne pathogens. *Azospirillum* can reduce the incidence and severity of damping off caused by *Rhizoctonia solani* (Gupta *et al.* 1995), foliar diseases of tomato caused by *Pseudomonas syringae* pv. *tomato* (Bashan and de-Bashan 2002; Romero *et al.*, 2003), crown gall disease (Bakanchikova *et al.* 1993), *Cucumis sativus* disease (Hassouna *et al.* 1998), bacterial leaf blight of mulberry (Sudhakar *et al.* 2000), *Prunus cerasifera* disease (Russo *et al.* 2008), anthracnose symptoms on strawberry plants (Tortora *et al.* 2012); and can enhance disease resistance in rice (Yasuda *et al.* 2009).

Different methods are employed to test the biocontrol activity of *Azospirillum* strains. These methods differ according to both (i) inoculum preparation protocols and (ii) pathogen

inhibition assays. *Azospirillum* inoculum may be prepared using complex media like Nutrient Broth (Russo *et al.* 2008) or synthetic media like Nitrogen free base or AB malate medium (Sankari *et al.* 2011, Tortora *et al.* 2011, Tortora *et al.* 2012). For inoculum preparation, a washing step is often used to remove culture medium residues and bacterial metabolites. This washing step is mostly realized with phosphate buffer (pH 6,8 – 7) (Tortora *et al.*2011, Tortora *et al.* 2012, Sankari *et al.* 2011) or with water (Russo *et al.* 2008). Different protocols may be used to bring out the biocontrol activities of *Azospirillum*. First, the antagonistic action of *Azospirillum* against microbial plant pathogens can be evaluated *in vitro* by confronting *Azospirillum* and plant pathogen strains, on agar plate, and quantifying the growth inhibition of the plant pathogen (Russo *et al.* 2008, Totora *et al.* 2011). In case of fungal pathogens, a plug of fungal mycelium can be laid on the center of an agar plate, and the biocontrol strain deposited few centimeters from the plug. At different incubation times, the mycelial growth rate can be recorded, both on the side where the bacterial inoculum was deposited, and on the diametrically opposite side. The 2 growth rates can be compared in order to evaluate the ability of a strain to inhibit the growth of the fungus. In case of bacterial pathogens, they can be spread on an agar plate and the biocontrol strain spotted on the surface of the agar. At different incubation times, growth diameters can be measured. Second, the antagonistic action of *Azospirillum* can be evaluated in plant assays performed in gnotobiotic or more complex conditions (Tortora *et al.* 2011). The timing, localization and mode of application of *Azospirillum* inoculum, such as the density level of the bacterial inoculum, are key elements that influence the biocontrol activity of *Azospirillum* strains (Tortora *et al.* 2011, 2012). Disease symptom scales based on the number and size of necrotic area are established to evaluate plant health in biocontrol assays. The antifungal activity of *Azospirillum brasilense* strains REC2 and REC3 against *Colletotrichum acutatum* was evaluated on strawberry plants grown in hydroponic conditions. These two strains were mixed together and inoculated in the hydroponic system. Results showed that these two strains of *Azospirillum* confer a reduction of symptoms on strawberry plants, which had previously been inoculated with them before the addition of the fungal pathogen (Tortora *et al.* 2011). Biocontrol activity had also been tested in sterile artificial substrate, and *Azospirillum* was inoculated by watering plants with bacterial solution (Tortora *et al.* 2012). Natural organic substrates or autoclaved soils were used to test respectively the biocontrol activity of *A. brasilense* Sp245 and another strain of the same species against respectively *Rhizoctonia* spp. and *Pratylenchus brachyurus* (Russo *et al.* 2008, Dias-Arieria et al 2012). *Azospirillum* was inoculated directly on the seed (Dias-Arieria *et al.* 2012) or by watering the plantlet (Russo *et al.* 2008). Bashan and collaborators (2014) recently reviewed inoculation methods and formulation technologies for *Azospirillum*.

- Alleviation of abiotic stresses by *Azospirillum*

Environmental conditions and their fluctuation may be stressful for plant growth and development. Cereals require a large amount of water for their growth and development, and water can become the main factor limiting plant development. The inoculation of *Azospirillum* strains was shown to alleviate drought stress in maize (Bano *et al.* 2013), wheat (Creus *et al.* 2004) and rice (Ruíz-Sánchez *et al.* 2011). As noted above, water deficiency leads to the accumulation of free amino acids, proline, soluble proteins and soluble sugars in plant tissues (Mohammadkhani and Heidari, 2008). Bano *et al.* (2013) tested the capacity of an inoculated

*A. lipoferum* strain to alleviate drought stress on maize, and the impact of the mode of application of *Azospirillum* on plant metabolite accumulation. *Azospirillum* supply was realized either by soaking seeds in *Azospirillum* cell suspension or by applying the bacterial inoculum in the close vicinity of roots. The water stress simulation was conducted by maintaining soil moisture content at 15% for the drought stress condition against 19% in the well-watered condition. Better drought stress resistance was obtained when *Azospirillum* was applied whatever the bacterial inoculation mode (Bano *et al.* 2013). Another way to apply water stress conditions on plants could be to decrease water irrigation by 50% after 2 weeks of well-watered condition (Ruíz-Sánchez *et al.* 2011). Using this condition, the authors showed that beneficial effects of *A. brasilense* on most of the physiological and biochemical traits of rice plants were only clearly visible when the plants were mycorrhized (Ruíz-Sánchez *et al.* 2011).

Soil salinity may also strongly affect plant development. To simulate salt stress conditions, different protocols have been used. Bacilio *et al.* (2004) used autoclaved sand and watered wheat seedlings with either 80 mM or 160 mM NaCl. *A. lipoferum* JA4 was shown to enhance plant growth (higher height and dry weight of shoots and roots) under continuous irrigation with 160 mM NaCl compared to the un-inoculated control (Bacilio *et al.* 2004). In another study, the salinity stress was applied by watering barley plants grown in natural soil with either 250 mM or 350 mM NaCl (Omar *et al.* 2009). Omar and collaborators (2009) employed two different wheat cultivars, one sensitive and one resistant. *A. brasilense* NO40 addition was shown to enhance growth and salt tolerance of the sensitive barley cultivar by increasing pigment contents, reducing accumulation of the osmoregulator proline, and the activities of antioxidant enzymes (Omar *et al.* 2009). Salinity stress can also be obtained using irrigation with diluted seawater solutions (Alamri and Mostafa 2009). These authors compared the effect of *Azospirillum* inoculation on wheat watered with seawaters at a final concentration of 4650 and 9300 ppm saline, and with tap water (140 ppm saline) as a control. Results demonstrate the ability of *A. brasilense* Sp-248 to reduce the deleterious effects of saline stress on wheat growth (Alamri and Mostafa 2009). In addition, this *A. brasilense* strain shares a relatively high tolerance to saline irrigation.

Soil chemical composition in terms of nitrogen content and heavy metal concentrations, etc. can also be stressful for plants. Esquiviel-Cote *et al.* (2010) showed the potential of *A. lipoferum* to stimulate plant growth under different nitrogen levels (0 kg $N.ha^{-1}$, 170 kg $N.ha^{-1}$ and 340 kg $N.ha^{-1}$). A better effect was observed when *A. lipoferum* AZm5 was inoculated in presence of a moderate nitrogen amount of 170 kg $N.ha^{-1}$ (Esquiviel-Cote *et al.* 2010). *Azospirillum* allows a better plant resistance to heavy metal stress (Belimov *et al.* 2004). Indeed, in pot and field experiments, *Azospirillum* seed inoculation improved the growth of barley plants in Pb- and Cd-contaminated soils. The presence of *Azospirillum* also prevented the accumulation of Pb and Cd in barley plants, thereby mitigating their toxic effects (Belimov *et al.* 2004).

*Azospirillum* is a good candidate to ensure the alleviation of biotic and / or abiotic stress. Mechanisms involved in the establishment of stress resistance in plants by *Azospirillum* and the molecular responses of the host plant are increasingly studied.

## 4. Main mechanisms of stress alleviation

The principal mechanisms permitting alleviation of biotic stresses consist of antagonism, competition and induction of plant defense against pathogens (Figure 1). Mechanisms involved in alleviating abiotic stresses consist principally of modifications of the plant hormonal balance and increased nutrient availability for plants (Figure 2) (Compant *et al.* 2005).

### a- Mechanisms recovered in Rhizobacteria

- Against biotic stresses:

Antagonism consists of the inhibition of plant pathogen development. In Rhizobacteria, this phenomenon is mediated by a large range of compounds including DAPG, phenazines, pyrrolnitrin, pyolutheorin, cyclic lipopeptides, extracellular enzymes and volatile compounds (Figure 1).

One of the most studied and well-documented compounds is DAPG, which is produced by certain species of fluorescent pseudomonads (Bruto *et al.*, 2014). It is a polyketide antibiotic efficient against bacteria, fungi, nematodes, algae and protozoans (Haas and Défago 2005, Jousset *et al.* 2006, Meyer *et al.* 2009). DAPG acts through membrane permeabilization and destabilization and triggers oxidative burst in the target cell. This oxidative burst causes inactivation of V-ATPase and the disturbance of cell respiration that leads to a loss of cell homeostasis (Kwak et al 2011). Other PGPR belonging to *Pseudomonas*, *Bacillus* and *Burkholderia* genera are able to produce hydrogen cyanide (HCN), a volatile compound with antimicrobial activity (Reetha *et al.* 2014, Ryall *et al.* 2008, Voisard *et al.* 1989). HCN may pass through the plant pathogen membrane and inhibits the cytochrome C oxidase and several other metalloenzymes (Blumer and Hass 2000). Phenazines have a wide spectrum of action. Their mechanisms of action, at the cellular level, are still not well resolved. Given the molecular structure of these compounds, it can be suggested that, after their diffusion through the membrane of the target cell, they can act as electron acceptors and shunt the respiratory chain. This interference leads to the production of hydrogen peroxide ($H_2O_2$) and superoxide ion ($O_2^-$), and cell death (Delaney *et al.* 2001). *Pseudomonas* phenazine producers are protected by their high superoxide dismutase activity (Delaney al. 2001). Indeed, it has been demonstrated that the addition of sublethal doses of phenazines in the liquid cultures of *Mycosphaerella graminicola* induced a strong increase of the activity of catalase, superoxide dismutase and peroxidases, allowing the fungus to survive in the presence of these antibiotics (Levy *et al.* 1992). Pyrrolnitrin is an antimicrobial compound produced by numerous bacteria like *Pseudomonas* and *Burkholderia* (Costa *et al.*, 2009). Pyrrolnitrin is produced from tryptophan and targets the mitochondrial electron transport system with the inhibition, among others, of succinate and NADH oxidase activities (Tripathi and Gottlieb, 1969). Cyclic lipopeptides (Clp) are composed of fatty acid tails linked to a short oligopeptide, which is cyclized to form a lactone ring between two amino acids. Clp like iturin and viscosin are surface-active agents causing membrane permeabilization in fungi, bacteria, oomycetes, protozoans and nematodes (Raaijmakers *et al.* 2006; Souza *et al.* 2003; Ongena and Jacques 2008).

PGPR, especially *Bacillus* and *Pseudomonas* strains, can produce extracellular enzymes such as chitinase or glucanase, which degrade components of the membrane and cell wall of microbial pathogens (Siddiqui *et al.* 2005, Maksimov *et al.* 2011, Kim *et al.* 2014). Moreover, certain PGPR can quench quorum-sensing (QS) signals and affect QS-dependent pathogenicity in plant pathogenic bacteria by degrading or mimicking their acyl-homoserine lactones, thereby blocking the expression of virulence functions (Faure and Dessaux 2007, Boyer and Wisniewski-Dyé 2009, Helman and Chernin 2014).

The ability of rhizobacteria to produce insecticidal toxins is more and more sought after (Wu *et al.* 2008, Péchy-Tarr *et al.* 2008, Lacey and Georgis 2012). Those rhizobacteria are taxonomically diverse. The most studied and used toxins for biological control of insects are the *Bacillus thuringiensis* toxins (Bravo *et al.* 2011). These toxins induce pore formation in the lipidic membrane of midgut insect cells (Vachon *et al.* 2012). Other biocontrol bacteria are known to be entomotoxin producers, like bacteria belonging to fluorescent pseudomonads (Kupferschmied *et al.* 2013, 2014, Ruffner *et al.* 2013). One of these toxins, "fit", is increasingly studied. This insecticidal toxin is produced by some strains belonging to the *Pseudomonas protegens* and *P. chlororaphis* subgroups (Péchy-Tarr *et al.* 2008, 2013, Kupferschmied *et al.* 2013, 2014). The mode of action of this toxin is still not entirely resolved.

Root surfaces and surrounding soil are areas where resources are abundantly available for microbial growth (Figure 1). These various and suitable nutrients attract numerous microbes including PGPR and pathogens. Efficient competiveness of PGPR for resources and ecological niches along roots is a fundamental mechanism by which they can protect plants against pathogens. The strong rhizo-competence of PGPR permits their fast and durable colonization of roots. Indeed, numerous PGPR can move rapidly along roots, thanks to their flagellar mobility (de Weert *et al.* 2002). Moreover they produce exopolysaccharides (EPS), lipopolysaccharides (LPS), pili or other fimbriae permitting root adherence and recognition between the host plant and the PGPR (Albareda *et al.* 2006, Rodrígues-Navarro *et al.* 2007). Furthermore, root associated rhizobacteria share adequate enzymatic machinery to degrade toxic root exudate compounds (Bais *et al.* 2004). The ability of PGPR to efficiently colonize roots makes these microbes very well adapted to the rhizosphere life and competitive against pathogens. The rhizo-microbiote might act as a biological barrier against pathogens. Accordingly, parallels have recently been made with the intestinal microbiota and its barrier-protective function (Mendes *et al.* 2013, Ramirez-Puebla *et al.* 2013). Rhizobacteria can competitively use a great variety of molecules as carbon, iron or nitrogen sources by producing high affinity transporters for these sources, and thereby scavenging away nutrients from plant pathogens. Competition for iron resources and production of siderophores by PGPR has been studied, revealing the importance of this property in biotic stress alleviation by PGPR (Crowley 2006, Dimkpa *et al.* 2009)

Rhizobacteria can reduce the activity of pathogens not only by antagonism or competition but also by activating plant defenses (Figure 1). As previously mentioned, this phenomenon is called ISR. ISR has been widely described in literature (van Loon 2007). ISR induction is mediated by different kinds of elicitors such as LPS, flagellin (Meziane *et al.* 2005) or secreted molecules like DAPG or siderophores (Bakker *et al.* 2007). Elicitor recognition by

host plant receptors triggers a local and systemic response, leading to the activation of JA and ET pathways (Iavicoli 2003, Djavaheri *et al.* 2007). PGPR-triggered plant responses depend on the type of pathogens (Vleesschauwer *et al.* 2008).

- Against abiotic stresses

PGPR may interact with the plant-specific mechanisms related to abiotic stress resistance. PGPR effects involve multiple changes in plant metabolism and signaling networks (Lugtenberg and Kamilova 2009, Friesen *et al.* 2011). Modifications in phytohormone content and/or signaling have been reported (Dodd *et al.* 2010), such as decreased ET production *via* bacterial ACC deaminase activity (Glick *et al.* 1998, Belimov *et al.* 2009, Bresson et al 2013), changes in cytokinin–ABA balance (Figueiredo *et al.* 2008, Cohen *et al.* 2009) or changes in auxin signaling (Persello-Cartieaux *et al.* 2003, Contesto *et al.* 2010) (Figure 2). Phytohormones production by PGPR can compensate for the reduction in plant growth caused by temperature stress, drought stress, heavy metals, salt stress and some other unfavorable environmental conditions.

Multiple combinations of traits can participate in plant strategies for dealing with drought, including those that allow drought escape or drought resistance (Verslues and Juenger, 2011). Several PGPR represent an added value to these strategies. For instance, some rhizobacteria help plants to maintain a favorable water status under water deficit (Creus *et al.*, 2004), by enhancing the development of the root system (Marulanda *et al.*, 2010). PGPR that produce ACC deaminase conferred resistance to drought stress in plants (Mayak et al 2004, Glick et al 2007). Under stress conditions, including drought, the plant hormone ethylene endogenously regulates plant homeostasis and results in reduced root and shoot growth (Figuereirredo et al 2008). However, degradation of the ET precursor ACC by bacterial ACC deaminase releases plant stress and rescues normal plant growth (Figuereirredo et al 2008). Some PGPR improve plant enzyme activity, such as catalase or superoxide dismutase, which alleviates the oxidative damage induced by drought (Kohler *et al.* 2008, Wang *et al.* 2012). Finally, PGPR have been shown to increase drought-response transcript abundances (Timmusk and Wagner 1999, Wang *et al.* 2005, 2012). Rhizobacteria often induce modifications in phytohormone signaling (Yang *et al.* 2009), which may mediate effects on meristem activity and identity (Hayat *et al.* 2010). For example, the most noticeable phenological change observed on *Arabidospsis* plants inoculated with *Phyllobacterium brassicacearum* STM196 was a significant delay in flowering time corresponding with a prolonged vegetative phase of PGPR-inoculated plants under drought conditions. The intensity of changes was more pronounced under drought and led to better plant tolerance to drought (Bresson et al 2013). Under drought stress, inoculated Arabidopsis showed increased ABA levels. An accumulation of ABA or enhancement of sensitivity to this hormone in the leaf cells, is leading to the induction of plant genes inducing a reduction of transpiration through reduced leaf conductance following stomata closure (Harb *et al.* 2010). Moreover, a decrease in transpiration by stomatal closure can be followed on a longer timescale by a reduced plant growth rate (Westgate and Boyer 1985). Auxin plays a role in the regulation of leaf and floral initiation and of the position of lateral organs (Reinhardt *et al.* 2000). But, STM196 is not a high auxin producer (Contesto *et al.* 2010) and, thus, cannot supply plant roots with exogenous auxin. However, Bresson and

colleagues (2013) observed that inoculation with STM196 changed auxin distribution within Arabidopsis roots towards apices, which probably explains the positive effect of STM196 on lateral root development (Contesto *et al.* 2010). In addition, other hormonal pathways are modified by STM196, including ET, which participates in root hair elongation in vitro (Contesto *et al.* 2008, Galland *et al.* 2012).

Phosphorus (P) is a major essential macronutrient for biological growth and development. Soluble P is often the limiting mineral nutrient for biomass production in natural ecosystems only taken up in monobasic ($H_2PO^{-4}$) or dibasic ($HPO_2^{4-}$) soluble forms (Glass *et al.* 1989), and the elevated levels of heavy metals in soil interfere with P uptake and lead to plant growth retardation (Zaidi *et al.* 2006). PGPR can either convert these insoluble phosphates into available forms through acidification, chelation, exchange reactions, and release of organic acids (Chung *et al.* 2005, Richardson *et al.* 2009) or mineralize organic phosphates by secreting extracellular phosphatases (Gyaneshwar *et al.* 2002; van der Heijden *et al.* 2008, Richardson *et al.* 2009) (Figure 2). An increase in P availability to plants through the inoculation of phosphate-solubilizing bacteria has been reported in pot experiments and under field conditions (Pal 1998; Zaidi *et al.*, 2003). In addition, fixation of atmospheric nitrogen is a metabolic virtuosity of endophytes and rhizobacteria and their colonization offers benefit to the host (Dobbelaere *et al.*, 2003).

For survival in metal contaminated soil, bacteria are coding an arsenal of functions, by which they can immobilize or transform metals rendering them inactive, to tolerate the uptake of heavy metal ions. The mechanisms are generally exclusion of metal by membrane permeability barrier or by active export of metal from the cell, physical sequestration of metal by binding extracellular polymers or extra cellular sequestration, detoxification where metal is chemically modified to render it less active (Rouch *et al.* 1995, Lièvremont et al 2009). For instance, binding of metals to anionic functional groups from microbial origin (i.e., sulfhydryl, carboxyle, hydoxyle, salfonate, amine and amide groups) immobilizes the metal and prevents its entry into the plant roots. Similarly, metal-binding extracellular polymers, comprising polysaccharides, proteins, humic substances, may detoxify metals by chelating the heavy metals (Pulsawat *et al.* 2003). The bacterial siderophores and organic acids can also reduce the metal bioavailability and toxicity by chelating the metal ions (Tripathi *et al.* 2005, Dimkpa *et al.* 2008).

**b- Mechanisms recovered in *Azospirillum***

- Against biotic stresses

*Azospirillum* is a good candidate to alleviate plant pathogen diseases as previously shown. The mechanisms involved in these biotic stress suppressions can be direct, by releasing secondary metabolites, which have an antagonistic activity against plant pathogens. *Azospirillum* can produce bacteriocins, which are generally considered as proteinaceous-toxins (Tapia-Hernández *et al.* 1989, Bashan and de-Bashan 2002). These bacteriocins act on the bacterial membrane. They bind to membrane receptors and cause pore formations, thereby leading to cell lysis. The phenylacetic acid (PAA) produced by *A. brasilense* seems to exert

antimicrobial activity against *Rhizoctonia solani, Pythium ultimum, Phytophtora capsici* and *Pseudomonas syringae* (Somers *et al.* 2005). However, the molecular mechanisms involved are still not elucidated. Gonçalves *et al.* (1998) showed that certain *Azospirillum* strains might produce HCN as *Pseudomonas* biocontrol strains. Therefore, it is possible to consider the antibiosis action of this volatile compound against potential plant pathogen agents in *Azospirillum*. Antagonism mechanisms implemented by the secretion of secondary metabolites are not the only way used by *Azospirillum* to control plant pathogens.

Another way for *Azospirillum* to control soil borne diseases is to produce siderophores with high-affinity iron-binding activity. Some of these siderophores seem to have antimicrobial activity (Tortora *et al.* 2011). This property may be used by *Azospirillum* to compete with other microbes, including pathogens, for access to iron, and thereby to limit their growth (Shah *et al.* 1992, Tortora *et al.* 2011).

*Azospirillum* may also protect plant against pathogen agents by an indirect process involving the induction of plant defense, notably ISR. The exopolysaccharides (EPS) produced by *Azospirillum* strains A7, A18, A26 and A37 were described to induce ISR and protect rice plant against *Pyricularia oryzae* (Sankari *et al.* 2011). Recognition of *Azospirillum* EPS by Plant Pattern Receptor (PRR) in plant cells might generate a regulation cascade inducing the transcription of plant defense genes (Boller and He 2009, Pieterse *et al.* 2014). Indeed, the protection of strawberry plants against *Colletotrichum acutatum* by endophytic *Azospirillum* PGPR is associated to enhanced content of plant phenolic compounds, a transient accumulation of SA, callose accumulation, and the induction of defense-related genes (Tortora *et al.*, 2012). Regarding the protection effects of another endophytic strain, *Azospirillum* sp. B510, Yasuda and collaborators (2009) compared the responses of *Oryza sativa* cv *Nipponbare* challenged with *Xanthomonas oryzae* in presence and absence of the PGPR. B510 had no antagonist activity on *Xanthomonas in vitro*, but activated the innate-immune system of the host-plant. The authors analyzed, using real-time PCR, the effect of B510 on the expression of pathogenesis-related genes involved in the SA response (OsPR-1a, OsPR-1b and WRKY45) and in the JA response (OsPR-4). The results (i.e. B510 down-regulation of OsPR-1a and OsPR-4 and no effect on OsPR-1b and WRKY45) suggest that B510 rather down-regulated SA signaling pathway (Yasuda *et al.* 2009). Similar plant responses were observed with *Pseudomonas fluorescens* WCS417r, which triggers defense responses in *A. thaliana* through a pathway independent of SA and PR gene activation but dependent of JA and ET signaling (Pieterse *et al.* 1996). Ramos and collaborators (2008) also demonstrate that PGPR plant protection in pathogen-challenged plants is inversely related to SA production. Among the three PGPR studied, *Azospirillum brasilense* Sp7 caused the highest increase in SA, but showed the lowest level of defense of *A. thaliana* against *P. syringae* pv. *tomato* DC3000.

- Against abiotic stresses

As described above, some PGPR harbor the *acdS* gene encoding ACC deaminase. This enzyme is involved in the biotic and abiotic stress alleviation in plant by modulating plant ET amount. This plant hormone has pleiotropic effects on different processes as on both plant growth and plant development (e.g. seed germination, root elongation, fruit development, etc.), and response to environmental stresses (Glick 2014). A model was proposed to explain how

bacteria harboring the ACC deaminase activity could lower ET amount in plant cells (Glick *et al.* 1998). This model is valid for certain *Azospirillum* strains that have ACC deaminase activity (Blaha *et al.* 2006, Prigent-Combaret *et al.* 2008). The ACC deaminase degrades ACC, the ET precursor. Abiotic stresses induced the production of two peaks of ET in the plant (Glick *et al.* 2007). These authors suggest that ACC deaminase activity can lower the second ET concentration peak, also called the "deleterious" peak. In this way, ET concentration decreases allowing the plant to grow under abiotic stress (Jalili *et al.* 2008).

*Azospirillum* may also modify plant morphology, in particular through the production of hormones, to alleviate water-stress by increasing xylem vessel area and stem hydraulic conductivity in tomato (Romero *et al.* 2014). Wider xylem vessels were found when *A. brasilence* Sp245 was inoculated on wheat seedling under water and osmotic stresses (Pereyra *et al.* 2012). Cell morphological changes often implicate a modification of plant hormonal balances. Auxins (especially Indole-Acetic Acid – IAA) induce vascular differentiation in plant (Lovisolo *et al.* 2002). In this manner, the production of IAA by *Azospirillum* may lead to modifications of the vascular vessel morphology. Another plant hormone is involved in stress response, like ABA, which is involved in response to abiotic stresses like drought or cold stress (Cohen *et al.* 2008, 2009). During drought stress, stomatal closure is induced by ABA, thereby minimizing water loss by plant transpiration (Mishra *et al.* 2006). *Azospirillum lipoferum* and *A. brasilense* Sp245 strains produce ABA, and they might permit alleviation of water stress by inducing stomatal closure (Cohen *et al.* 2008, 2009, 2014).

Nutrients and / or mineral elements deficiency are responsible for plant developmental disorder and decreasing plant growth, as described above. The availability of some essential mineral elements such as iron, phosphorus or nitrogen can be insufficient to allow appropriate plant development. These elements may be in weak concentrations in soils; or they may be present in sufficient concentrations but not available to the plant roots. Nitrogen deficiency may be reduced by the process of biological nitrogen fixation (BNF), performed by nitrogenase activity harboring by diazotrophic bacteria like *Azospirillum* (Figure 2). In the case of the inoculation of *Azospirillum amazonense*, a significant improvement of nitrogen nutrition in rice was observed through the use of a nitrogen isotope ($^{15}N$) (Rodrigues *et al.* 2008). The authors were able to show that the improvement in growth is mainly due to atmospheric nitrogen fixation made by the inoculum. Some works evidence that bacterial nitrogen fixation might improve nitrogen nutrition in plant, however, the accurate mechanisms involved in the beneficial interaction between plant and the diazotrophic PGPR are not yet well known (Richardson *et al.* 2009). Similar to nitrogen, phosphorus is an essential element for plants. However, its bioavailability is very low in soils due to its presence as insoluble and complexed forms (Meyer *et al.* 2011). As mentioned above, one of the main mechanisms harbored by PGPR to solubilize phosphorus is based on soil acidification *via* the release of acid compounds as gluconic acid, citric acid, oxalic acid (Richardson *et al.* 2009). However, rhizosphere acidification could be obtained by indirect mechanisms. Inoculation with *A. brasilense* Cd increased rhizosphere acidification, and it was suggested that auxins produced by the *Azospirillum* PGPR may stimulate the plasma membrane H-ATPase thereby leading to the transport of protons across the cell wall (Carrillo *et al.* 2002). Lastly, *A. brasilense* may increase

root iron absorption by releasing bacterial siderophores that can be recognized by plant iron receptors and used to enhance iron content in plant (Barton *et al.* 1986).

Among the specific PGPR-mediated mechanisms identified is the enhancement of wheat growth by *Azospirillum* sp. strains under various drought intensities, which was associated with better maintenance of plant water status as a result of increased cell wall elasticity (Creus *et al.*, 2004). The bacterial production of exopolysaccharides that is a physiological response of *A. brasilense* to salt stress (Chowdhury et al 2007), may protect the plant against drought stress. EPS of bacteria are highly hydrated compounds and can enhance water retention in area surrounding roots (Naseem and Bano 2014). However exopolysaccharides overproduction in *A. brasilense* may not necessarily stimulate plant growth promotion in standard plant-growth conditions (Volfson *et al.* 2013).

## 5. Concluding remarks

Plants adapt to fluctuating environmental changes by modifying their physiology and development. PGPR may help plants to cope with the biotic and abiotic stresses through the expression of a myriad of plant-beneficial functions. Important highlighted issues are:

- Crosstalk exists between plant signaling pathways induced in responses to biotic and abiotic stresses.
- PGPR including *Azospirillum* are able to mediate enhanced resistance to biotic stressors, as well as to increase tolerance to abiotic stresses in host plants.
- PGPR may elicit different plant pathways simultaneously, conferring additive responses that are more effective than single-elicited pathways.
- PGPR harbor coocurrent plant beneficial properties. The identification of bacterial strains that have the potential to provide cross-protection against multiple stress factors in crops would be highly valuable for developing sustainable agriculture.

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## Figure

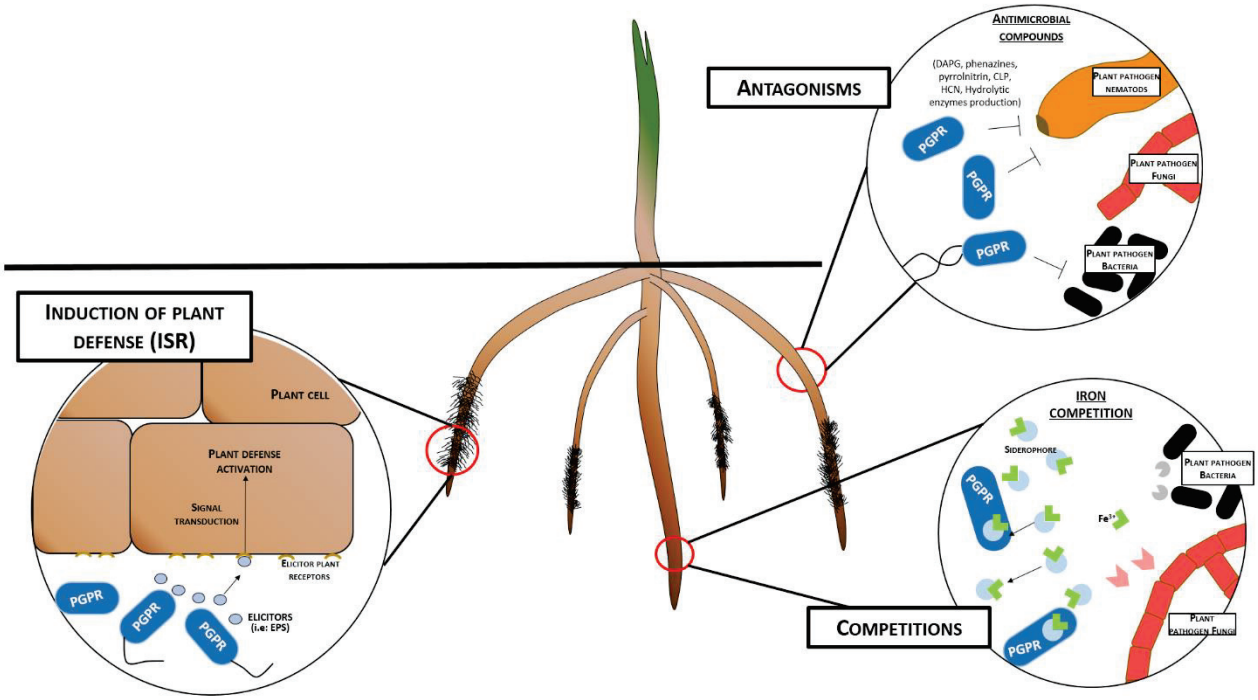


**Figure 1: Main bacterial mechanisms involved in alleviation of biotic stresses in plants by *Azospirillum* strains and other PGPR**

The mechanisms involved in biotic stress alleviation in crops by PGPR can be separated in three main processes. Antagonism interactions can take place between PGPR and diverse plant pathogens like fungi, bacteria, nematodes. This involves the production of antimicrobial components and hydrolytic enzymes by PGPR. Competition for essential elements like iron through siderophore production by PGPR may lead to growth limitation of the plant pathogen. PGPR can also induce plant defenses (i.e. ISR) by producing plant defense elicitors like exopolysaccharides.

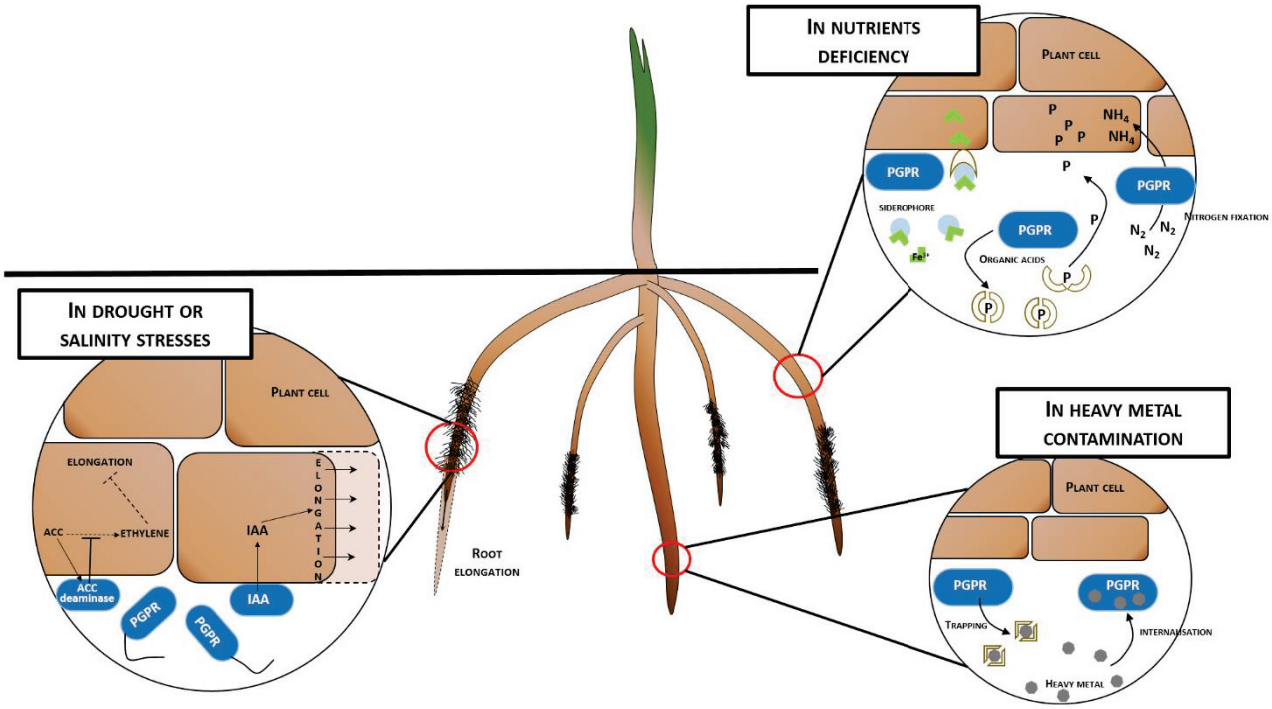


**Figure 2: Main bacterial mechanisms involved in alleviation of abiotic stresses in plants by *Azospirillum* strains and other PGPR**

PGPR can modify the plant hormonal balance (in particular the ethylene or auxin signaling pathway), thereby favoring the host plant resistance to drought or salinity. PGPR may improve plant nutrient acquisition especially when nitrogen, phosphate or iron are scarce. They can ensure phosphate solubilization by releasing organic acids, improve plant nitrogen inputs by fixing the atmospheric dinitrogen and/or facilitate iron acquisition through the production of siderophores. PGPR can play a role in alleviation of heavy metal stress in plants by trapping heavy metals outside the bacterial cell or by internalizing and detoxifying them.

Table 1: List of microbes identified for their ability to alleviate plant stress

| Stress type | Bacteria inoculated | Plant species | Reference |
|---|---|---|---|
| ***Abiotic stresses*** | | | |
| Arsenic | *Bradyrhizobium japonicum* CB1809 | Sunflower (*Helianthus annus*)<br>Wheat (*Triticum aestivum*) | Reichman 2014 |
| Cadmium | *Rhizobium sp.* | Italian ryegrass (*Lolium multiflorum*)<br>Soybean (*Glycine max*) | Guo and Chi 2014 |
| Cadmium and lead | *Arthrobacter mysorens* 7<br>*Agrobacterium radiobacter* 10<br>*Flavobacterium sp.* L30<br>*Azospirillum lipoferum* 137 | Barley (*Hordeum vulgare*) | Belimov *et al.* 2004 |
| Cadmium and Zinc | *Rhodococcus erythropolis* EC 34<br>*Achromobacter* sp. 1AP2<br>*Microbacterium* sp. 3ZP2 | Clover *(Trifolium repens)* | Pereira *et al.* 2014 |
| Drought | *Achromobacter piechaudii* | Tomato (*Lycopersicon esculentum*) | Mayak *et al.* 2004 |
| Drought | *Pseudomonas sp.* | Pea (*Pisum sativum*) | Arshad *et al.* 2008 |
| Drought | *Bacillus sp.* | Lettuce (*Lactuca sativa*) | Vivas *et al.* 2003 |
| Drought | *A. lipoferum* | Maize (*Zea mays*) | Bano *et al.* 2013 |
| Drought | *A. brasilense* Sp245 | Wheat (*T. aestivum*) | Creus *et al.* 2004 |
| Drought | *A. brasilense* AZ-39 | Rice (*Oryza sativa*) | Ruíz-Sánchez *et al.* 2011 |
| Drought | *A. brasilense* Sp245 | *Arabidopsis thaliana* | Cohen *et al.* 2014 |
| Drought | *P. mendocina* Palleroni | Lettuce (*Lactuca sativa*) | Kohler *et al.* 2008 |
| Drought | Consortium of *B. cereus* AR156, *B. subtilis* SM21, and *Serratia sp.* XY21 | Cucumber (*Cucumis sativus*) | Wang *et al.* 2012 |
| Drought | *Phyllobacterium brassicacearum* STM196 | *Arabidopsis thaliana* | Bresson *et al.* 2013 |
| Nitrogen deficiency | *A. brasilense* VS9<br>*A. lipoferum* AZm5 | Tomato (*L. esculentum*) | Esquivel-Cote *et al.* 2010 |
| Insoluble phosphate | *Bacillus sp.* | Maize (*Z. mays*)<br>Fingermillet (*Elosine coracana*)<br>Amaranth (*Amaranthus hypochondriacus*)<br>Buckwheat (*Fagopyrium esculentum*)<br>Frenchbean (*Phaseolus vulgaris*) | Pal *et al.* 1998 |
| Salt | *P. fluorescens*<br>*P. putida* | Canola (*Brassica napus*) | Jalili *et al.* 2008 |
| Salt | *Arthrobacter sp.*<br>*Bacillus sp.* | Wheat (*T. aestivum*) | Upadhyay *et al.* 2009 |
| Salt | *Brevibacterium epidermidis* RS15<br>*Micrococcus yunnanensis* RS222<br>*B. aryabhattai* RS341 | Canola (*B. napus*) | Siddikee *et al.* 2010 |
| Salt | *A. brasilense* NO40 | Barley (*H.vulgare*) | Omar *et al.* 2009 |
| Salt | *A. lipoferum* JA4 | Wheat (*T. aestivum*) | Bacilio *et al.* 2004 |
| Salt | *A. brasilense Sp-248* | Wheat (*T. aestivum*) | Alamri and Mostafa 2009 |
| ***Biotic stresses*** | | | |
| *Botrytis cinerea* | *B. thuringiensis*UM96 | *Medicago truncatula* | Martínez-Absalón *et al.* 2014 |
| *Colletotrichum acutatum* | *A. brasilense REC2 and REC3* | Strawberry (*Fragaria x ananassa*) | Tortora *et al.* 2011 |
| *Gaeumannomyces graminis* | *P. fluorescens 2-79* | Wheat (*T. aestivum*) | Thomashow and Weller 1988 |
| | *P. fluorescens 2-79*<br>*P. aureofaciens 30-84* | Wheat (*T. aestivum*) | Mazzola *et al.* 1992 |
| *Pratylenchus brachyurus* | *A. brasilense* | Maize (*Z. mays*)<br>Soybean (*G. max*) | Dias-Arieria *et al.* 2012 |
| *Pythium sp.* | *P. protegens CHA0* | Cucumber (*Cucumis sativus*) | Maurhoffer *et al.* 1994 |

| | | | |
|---|---|---|---|
| | | Cress (*Lepidium sativum*) | |
| *Pythium ultimum* | *P. fluorescens* P60 | Wheat (*T. aestivum*) | de Souza *et al.* 2003a |
| *Pythium sp.* | *P. fluorescens* SS101 | Wheat (*T. aestivum*) | de Souza *et al.* 2003b |
| *Albugo candida* | | | |
| *Phytophthora infestans* | | | |
| *Ralstonia solanacearum* | *Acinetobacter sp*. Xa6 | Tomato (*L. esculentum*) | Xue *et al.* 2009 |
| | *Enterobacter sp.* Xy3 | | |
| *Rhizoctonia solani* | *P. fluorescens HC1-07* | Wheat (*T. aestivum*) | Yang *et al.* 2014 |
| | *Azospirillum sp.* | Tomato (*L. esculentum*) | Gupta *et al.* 1995 |
| | *Azotobacter chroococcum* | | |
| *Rhizoctonia* sp. | *A. brasilense Sp245* | *Prunus cerasifera* | Russo *et al.* 2008 |
| *Erwinia carotovora* | *Pseudomonas sp.* B10 | Potato *(Solanum tuberosum)* | Kloepper *et al.* 1980 |
| *Fusarium oxysporum* | *Pseudomonas sp.*WCS417r | Carnation *(Dianthus caryophyllus)* | van Peer *et al.* 1990 |
| *Pseudomonas syringae* | | | |
| *Xanthomonas campestris* | *A. brasilense Sp7* | Tomato (*L. esculentum*) | Romero *et al.* 2003 |
| | *A. sp. BNM-65* | | |
| *Agrobacterium tumefasciens* | *A. brasilense Sp7* | Grapevines (*Vitis*) | Bakanchikova *et al.* 1993 |
| | *A. brasilense 94-3* | | |
| *Pyricularia oryzae* | *A. strains A7, A18, A26 and A37* | Rice (*O. sativa*) | Sankari *et al.* 2011 |
| *Fusarium oxysporum* | *A. brasilense SBR* | Cucumber (*C. sativus*) | Hassouna *et al.* 1998 |
| *Rhizoctonia solani* | *Azotobacter chroococcum* ZCR | | |
| *Pythium sp.* | *Klebsiella pneumoneae* KPR | | |
| *Magnaporthe oryzae* | *Azospirillum. sp.* B510 | Rice (*O. sativa*) | Yasuda *et al.* 2009 |
| *Xanthomonas oryzae* | | | |
| *Thielaviopsis basicola* | *P. protegens CHA0* | Tobacco (*Nicotiana tabacum*) | Voisard *et al.* 1990 |
| *Meloidogyne incognita* | *P. protegens CHA0* | Tomato (*L. esculentum*) | Siddiqui *et al.* 2005 |