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Mémoire présenté par

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**en vue de l'obtention du diplôme**
**d'Habilitation à Diriger les Recherches**

# Gestion du potentiel phytostimulateur du microbiote rhizosphérique : cas des communautés végétales plurispécifiques en milieu méditerranéen et sub-tropical

UMR "Laboratoire des Symbioses Tropicales et Méditerranéennes" - Montpellier

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# CURRICULUM VITAE


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Situation professionnelle actuelle :
chargé de recherche 1ère classe à l'Institut de Recherche pour le Développement (IRD)
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## A - Cursus universitaire

**1998/2001**
Doctorat de Sciences Agronomiques, écologie microbienne du sol (Ecole Nationale Supérieure d'Agronomie et des Industries Alimentaires-Institut National Polytechnique de Lorraine ENSAIA-INPL, Nancy), avec allocation ministérielle de recherche, mention très honorable
Titre : " Impact des rhizodépôts du maïs sur la structure des communautés bactériennes telluriques", directeur de thèse : Pr. E. Benizri

**1998**
Diplôme d'Etudes Approfondies de Sciences Agronomiques, option biotechnologies végétales (ENSAIA), mention bien
Titre : " Mise au point d'un protocole de cryoconservation de suspensions cellulaires de *Quillaja saponaria* ", encadrant du stage Pr. M. Henry (Faculté de Pharmacie, Nancy)

**1995/1998**
Elève ingénieur agronome de l'ENSAIA

**1992/1995**
Elève en Classes Préparatoires aux Grandes Ecoles, lycée Albert Châtelet, Douai

**1992**
Baccalauréat série D, mention très bien, lycée Henri Wallon, Valenciennes

# CURRICULUM VITAE

## B - Expériences professionnelles

**depuis janvier 2009**
Chargé de recherche 1ère classe à l'IRD et affectation à Montpellier au LSTM. Thèmes de recherche : écologie microbienne de l'effet mycorhizosphère, notamment associé aux végétaux exotiques et invasifs, diversité moléculaire des communautés bactérienne et mycorhizienne, diversité fonctionnelle des communautés bactériennes, sélection et inoculation de bactéries phytostimulatrices, phytostabilisation de déblais miniers.

**avril 2004 / décembre 2008**
Recruté chargé de recherche 2ème classe à l'IRD et affecté à Dakar-Sénégal du 16/05/04 au 31/08/08. Thème de recherche : effets des modalités de fertilisation organique sur les communautés bactériennes dénitrifiantes des sols agricoles en milieu sub-tropical.

**décembre 2001 / décembre 2003**
Contrat post-doctoral de deux ans sur le projet européen EcoSafe, Université de Lyon 1, UMR CNRS-5557 Ecologie Microbienne
Thème de recherche : impact de l'inoculation sur maïs et blé de bactéries phytostimulatrices du genre *Azospirillum* sur les communautés microbiennes du sol. Référents : Pr. Y. Moënne-Loccoz, Dr. S. Nazaret, Dr. R. Bally.

**septembre 1998 / décembre 2001**
Doctorat de Sciences Agronomiques, Laboratoire Agronomie Environnement de l'ENSAIA. Thème de recherche : impact des rhizodépôts du maïs et en particulier des exsudats sur la structure des communautés bactériennes de la rhizosphère. Directeur de thèse : Pr. E. Benizri.
Monitorat à l'IUT du Montet (Nancy), 2ème année agronomie ; 3×96 heures annuelles de Travaux Pratiques (clefs de détermination insectes-champignons, essais comparatifs *in vitro* de fongicides, essais de fertilisation en serre, sorties et observations sur parcelles agricoles…), référents : Dr. A. Blouet, Dr. F. Lasserre.

**janvier 1998 / juin 1998**
Stage de DEA de Sciences Agronomiques (validé aussi comme stage de 3ème année élève ingénieur agronome) au Laboratoire de Botanique de la Faculté de Pharmacie de Nancy. Thème du stage : mise au point d'un protocole de cryoconservation dans l'azote liquide de suspensions cellulaires de *Quillaja saponaria* productrices de métabolites secondaires d'intérêt pharmaceutique (saponines). Référents : Pr. M. Henry, Pr. F. Bourgaud (ENSAIA).

**juin 1997 / août 1997**
Stage élève ingénieur 2ème année à l'Institut pour la Nutrition des Plantes (Institut für Pflanzenernährung, Hohenheim Universität), Stuttgart-Allemagne. Thème du stage : caractérisation éco-physiologique de la fertilisation azotée du maïs. Référent : Pr. V. Römheld.

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# II - Activités de recherche

## II-A Préambule

La croissance et la reproduction d'un végétal en milieu naturel sont contraintes par un ensemble de paramètres biotiques et abiotiques (profil de distribution des ressources minérales et hydriques, luminosité, présence de ravageurs, d'agents phytopathogènes ou d'herbivores etc.), notamment dans le cadre d'une **compétition inter-plantes** pour l'accès aux ressources lumineuses et minérales. L'intégration de ces interactions contribue *in fine* à modeler la structure et la dynamique des communautés végétales (Crawley, 1997; Grubb, 1998). Cependant en 1904, Laurenz Hiltner fondait le concept de ***rhizosphère*** qui a permis d'étendre la gamme des **interactions des végétaux** avec leur **environnement** immédiat aux **microorganismes du sol**, notamment dans le cadre d'effets de **stimulation** de leur **croissance** ou de leur **résistance** à divers stress. Des travaux ponctuels récents ont d'avantage mis en perspective l'importance capitale de cette interface racines/microorganismes du sol dans la vie d'un végétal, en précisant que la réponse des microorganismes du sol aux fluctuations environnementales améliorait significativement la réponse adaptative des végétaux à ces mêmes fluctuations (Lau et Lennon, 2012), voire intervenait dans la sélection de traits adaptatifs (Lau et Lennon, 2011). Par ailleurs, l'existence de signaux moléculaires échangés entre les racines et divers champignons, bactéries et leur rôle dans les profils d'expression de gènes tant microbiens que végétaux (e.g. Steinkellner et al, 2007; Badri et al, 2009; Mathesius, 2009; Shenk et al, 2012) montrent à quel point ces interactions plantes/microorganismes du sol sont anciennes, c'est-à-dire intégrées dans les processus évolutifs, et finement régulées.

Aujourd'hui, face à l'accentuation des irrégularités climatiques, aux forçages anthropiques et leur cortège de déséquilibres écologiques, à la volonté politique et de l'opinion publique d'engager la réduction de l'usage intensif des intrants synthétiques (e.g. plan français EcoPhyto II), il devient de plus en plus stratégique de faire progresser une recherche destinée à intégrer et amplifier les interactions plantes/microorganismes du sol dans la préservation et la valorisation des ressources génétiques végétales, naturelles ou agricoles.

Depuis ma formation initiale en thèse, les différentes étapes de mon parcours m'ont conduit à étudier différentes facettes de l'écologie microbienne appliquée à la rhizosphère me permettant d'orienter à l'avenir mes efforts sur la possibilité d'accentuer et de prolonger l'action phytostimulatrice des communautés bactériennes du sol, sélectionnées par l'effet rhizosphère, principalement via une gestion de la composition de communautés végétales mixtes (i.e. interactions plante-plante positives).

Références citées :

**Badri** DV et al (**2009**) Rhizosphere chemical dialogues: plant-microbe interactions. *Current Opinion in Biotechnology* 20 : 642-650.

**Crawley** MJ (**1997**) The structure of plant communities. In: Crawley, M. J. (ed.), Plant ecology. Blackwell Science Ld, pp. 475-531.

**Grubb** PJ (**1998**) A reassessment of the strategies of plants which cope with shortages of ressources. *Perspectives in Plant Ecology, Evolution and Systematics* 1 : 3-31.

**Hiltner** (**1904**) Über neuer Erfahrungen und Probleme auf dem Gebiet der Bodenbakteriologie unter besonderer Nerücksichtingung der Gründüngung und Brache. *Arbeiten aus dem Deutschen Landwirtschafts Geselshaft* 98 : 59-78.

**Lau** JA, **Lennon** JT (**2011**) Evolutionary ecology of plant-microbe interactions: soil microbial structure alters selection on plant traits. *New Phytologist* 192 : 215-224.

**Lau** JA, **Lennon** JT (**2012**) Rapid responses of soil microorganisms improve plant fitness in novel environments. *Proceedings National Academy of Sciences* 109 : 14058-14062.

**Mathesius** U (**2009**) Comparative proteomic studies of root-microbe interactions. *Journal of Proteomics* 72 : 353-366.

**Steinkellner** S et al (**2007**) Flavonoids and strigolactones in root exudates as signals in symbiotic and pathogenic plant-fungus interactions. *Molecules* 12 : 1290-1306.

**Shenk** PM et al (**2012**) Unraveling plant-microbe interactions: can multi-species transcriptomics help ? *Trends in Biotechnology* 30 : 177-184.

## II-B Recherches doctorales (1998/2001)
## Influence de la rhizodéposition sur les communautés bactériennes du sol

***" Impact des rhizodépôts du maïs sur la structure des communautés bactériennes telluriques "***
***Doctorat de Sciences Agronomiques (ENSAIA-INPL, Nancy, sous la direction du Pr. Emile Benizri, Laboratoire Agronomie et Environnement)***

Bien qu'ayant été déjà évaluées au cours de la soutenance de thèse, ces activités sont brièvement reprises ici afin de mieux apprécier l'évolution de mon projet de recherche axé sur la rhizosphère et aussi d'en repréciser le principe général basé sur la rhizodéposition.

### Contexte :

La rhizosphère désigne le volume du sol soumis à l'influence directe des racines en croissance : ses caractéristiques physiques, chimiques et microbiennes se distinguent de celles d'un sol non planté (Hiltner, 1904). L'estimation de ses dimensions physiques n'excède pas la plus part du temps quelques millimètres d'épaisseur (e.g. Semenov et al, 1999). La modification de certaines propriétés physiques de ce compartiment du sol est d'ailleurs visuellement appréciable si l'on considère qu'il existe toujours une certaine quantité de sol adhérent aux surfaces racinaires résistant à une agitation modérée. Du point de vue microbien, ce sol rhizosphérique est le siège d'une prolifération d'ampleur variable selon les espèces végétales, leur stade phénologique et la nature du sol principalement (e.g. Wieland et al, 2001). Cet effet stimulant des racines sur les densités microbiennes (cultivables *in vitro*) désigne historiquement l'*effet rhizosphère*. Depuis, les travaux réalisés en écologie microbienne ont permis de mettre également en évidence des effets sur la diversité microbienne (réduite ou augmentée, e.g. Marschner et al, 2001) et leurs activités (très souvent intensifiées, e.g. Söderberg et Bååth, 1998). Certaines études ont mis en évidence que certaines de ces bactéries rhizosphériques libres (souches cultivables *in vitro*) pouvaient jouer un rôle stimulant dans le développement d'espèces annuelles ou pérennes, par divers modes d'action dont les principaux ciblent la nutrition minérale facilitée (e.g. phosphore, azote, fer), la stimulation hormonale de la croissance racinaire (e.g. auxines, gibbérellines, cytokinines) et l'antibiose face à des agents phytopathogènes. Ces bactéries sont regroupées sous le vocable fonctionnel de PGPR pour Plant Growth Promoting Rhizobacteria.

Il est apparu que cette stimulation des activités et des effectifs microbiens aux abords des racines vivantes était provoquée et entretenue par l'émission racinaire dans le sol de divers composés organiques dénommés rhizodépôts. Ceux-ci dérivent directement des photoassimilats alloués au système racinaire pour sa croissance et son fonctionnement (Curl et Truelove, 1986; Whipps, 1990). Ces flux de carbone représenteraient en moyenne sur une gamme variable de végétaux environ 17% des photosynthétats, avec des valeurs extrêmes pouvant atteindre 40% (Nguyen, 2003). La classification des rhizodépôts (Rovira, 1969, 1973; Rovira et al, 1979) tient compte de leur nature biochimique et de leur mode d'émission. On distingue principalement les exsudats (prioritairement des glucides, puis des acides organiques et en plus faibles proportions les acides aminés, tous solubles et

libérés par diffusion passive au travers des parois et jonctions cellulaires), les sécrétions (essentiellement des enzymes activement excrétées), le mucilage (produit surtout au niveau des apex mais aussi par les poils absorbants principalement composé de polysaccharides), les lysats (produits de l'autolyse des cellules rhizodermiques sénescentes et desquamées) et les gaz (e.g. éthylène, propylène, gaz carbonique). La quantité et la diversité des composés rhizodéposés dépend de la combinaison de nombreux facteurs : identité de la plante et son stade phénologique, substrat de culture, intensité lumineuse et température, ressources minérales et hydriques, mais aussi évolution des densités et des activités microbiennes (e.g. Rovira, 1969; Lespinat et Berlier, 1975).

Dans le cadre du sol rhizosphèrique où les flux de carbone organique végétal sont le moteur de la stimulation générale du compartiment microbien, l'objectif général de la thèse était de vérifier, avec le maïs (*Zea mays* L.) comme plante modèle et du sol agricole, que (1) la variation saptio-temporelle de la rhizodéposition (zones morphologiques racinaires et stades de développement) pouvait être corrélée à la modification de la structure des communautés bactériennes rhizosphèriques et que (2) la variation quantitative et qualitative de la disponibilité en exsudats pouvait à elle seule modifier cette structure.

**Hypothèses de travail :**

L'approche s'est déroulée en deux phases :

(1) constater que différentes régions du système racinaire (transect longitudinal : sol adhérent aux apex, aux zones d'élongation et pilifère, et aux ramifications secondaires) et différents compartiments de la rhizosphère (transect transversal : sol adhérent aux surfaces racinaires, rhizoplan), connus pour leurs disponibilité variable en rhizodépôts, hébergent des communautés bactériennes distinctes,

(2) vérifier que la seule variation qualitative et quantitative de la disponibilité en certains exsudats dans le sol, en particulier *in planta*, est suffisante pour induire des modifications de la structure des communautés bactériennes.

**Méthodologie :**

Les expérimentations ont toutes été conduites avec des cultures de maïs en pots dans des phytotrons. La communauté bactérienne totale a été caractérisée sous le seul angle de sa structure (sans étude de fonctions particulières) par détermination de son effectif (dénombrements cinétiques *in vitro* sur divers milieux gélosés), de sa structure génétique (empreintes moléculaires électrophorétiques de type RISA) et de ses potentialités cataboliques *in vitro* (système Biolog® GN2). L'exploration de l'hypothèse 1 a reposé sur une dissection des différents compartiments rhizosphériques (transect transversal) et zones morphologiques (transect longitudinal) de systèmes racinaires complets de plants de maïs à deux stades phénologiques (15 et 30 jours post-semis). La deuxième hypothèse a nécessité de modifier la disponibilité en exsudats directement à l'échelle des systèmes racinaires. Des solutions simplifiées d'exsudats ont été élaborées par mélanges de glucides (glucose, fructose, saccharose),

acides aminés (alanine, sérine, acide glutamique) et acides organiques (acides succinique, lactique, citrique) de synthèse répertoriés dans la littérature comme exsudats du maïs. Dans ce cas, les exsudats ont été appliqués quotidiennement pendant 15 jours à 20 et 100 µg C $g^{-1}$ sol selon deux ratios C/N (20 et 40, obtenus par modulation de la proportion relative des acides aminés) sur des plants de maïs initialement âgés de 15 jours.

**Résultats principaux :**

première hypothèse (transect longitudinal et transversal)

Une fluctuation longitudinale de l'effet rhizosphère (élévation des densités) au sein du système racinaire a été observée dès 15 jours notamment entre le sol rhizosphérique de la zone de ramifications et de la zone d'élongation/pilifère qui présentait des densités bactériennes supérieures de plus d'un Log à celles du sol des extrémités apicales. Cependant, seule la zone de ramification a manifesté une spécificité des aptitudes cataboliques par rapport aux autres zones morphologiques, indiquant que l'évolution des aptitudes cataboliques peut être découplée de l'effet sur les densités bactériennes. Ce constat a aussi été posé et prolongé avec l'approche transversale : en l'absence d'effet sur les densités cultivables à 15 jours, le degré de proximité au rhizoplan a cependant induit une augmentation significative de la diversité des aptitudes cataboliques s'accompagnant d'une modification de la structure génétique des communautés bactériennes. Le stade de développement du maïs est apparu avoir influencé plus fortement les communautés bactériennes du sol adhérent que la morphologie du système racinaire, notamment au niveau des densités et de la structure génétique.

Ces diverses observations apparaissent cohérentes avec une variabilité spatio-temporelle de la rhizodéposition, notamment une augmentation de la rhizodéposition avec le développement de l'appareil photosynthétique.

deuxième hypothèse (enrichissement du sol en exsudats)

L'apport journalier d'exsudats de synthèse directement dans la rhizosphère de maïs durant deux semaines (+ 1,4 mg C final $g^{-1}$ sol au maximum) a permis de conclure qu'une augmentation de la disponibilité de cette classe de rhizodépôts était significativement impliquée *in planta* dans la prolifération bactérienne, l'augmentation des aptitudes cataboliques et la modification de la structure génétique des communautés bactériennes du sol rhizosphérique (**Figure 1**).

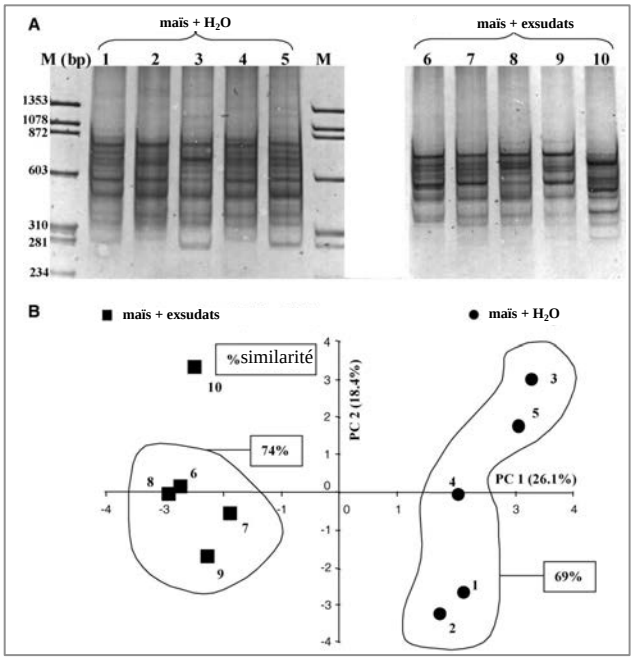


**Figure 1.** Effets de l'augmentation de la disponibilité en exsudats dans la rhizosphère de maïs durant 15 jours sur la structure génétique des communautés bactériennes décrites par les empreintes RISA (A) et comparées par analyse en composantes principales (B).

Leur influence pouvait être modulée par leur concentration (20 et 100 µg C $g^{-1}$ sol $j^{-1}$) et/ou leur teneur en azote (ratio C/N de 20 et 40). A titre d'exemple, deux apports identiques en concentration (100 µg C $g^{-1}$ sol $j^{-1}$) mais de diversité moléculaire contrastée ont induit des effets distincts, avec une prolifération bactérienne et une augmentation de la diversité catabolique supérieures lors de l'usage de la solution d'exsudats la plus diversifiée.

N.B. 4 articles publiés dont une synthèse bibliographique, référencés n° 1-2-3-4 dans la section V-A

Références citées :


**Curl** EA, **Truelove** B (**1986**) The rhizosphere. Curl EA, Truelove B (Eds.), Springer-Verlag, New York, 288 pp.

**Lespinat** PA, **Berlier** Y (**1975**) Les facteurs externes agissant sur l'excrétion racinaire. pp 21-30. Société Botanique de France, Collection Rhizosphère.

**Marschner** P et al (**2001**) Soil and plant specific effects on bacterial community composition in the rhizosphere. *Soil Biology and Biochemistry* 33 : 1437-1445.

**Nguyen** C (**2003**) Rhizodeposition of organic C by plants: mechanisms and controls. *Agronomie* 23 : 375-396.

**Rovira** AD (**1969**) Plant root exudates. *The Botanical Review* 35 : 35-55.

**Rovira** AD (**1973**) Zones of exsudation along plant roots and spatial distribution of micro-organisms in the rhizosphere. *Pesticide Science* 4 : 361-366.

**Rovira** AD et al (**1979**) Origin, nature and nomenclature of the organic materials in the rhizosphere, pp. 1-4. In: The soil root interface. Harley J.L. (Ed.), Academic Press.

**Semenov** AM et al (**1999**) Moving waves of bacterial populations and total organic carbon along roots of wheat. *Microbial Ecology* 37 : 116-128.

**Söderberg** KH, **Bååth** E (**1998**) Bacterial activity along a young barley root measured by the thymidine and leucine incorporation techniques. *Soil Biology and Biochemistry* 30 : 1259-1268.

**Wieland** G et al (**2001**) Variation of microbial communities in soil, rhizosphere, and rhizoplane in response to crop species, soil type, and crop development. *Applied and Environmental Microbiology* 67 : 5849-5854.

**Whipps** JM (**1990**) Carbon economy, pp. 59-97. In: The Rhizosphere. Lynch J.M., Wiley J. (Eds.), Chichester, U.K.

## II-C Recherches post-doctorales (2001/2003)
## Influence de l'inoculation de bactéries phytostimulatrices sur les communautés microbiennes du sol

| Thèse<br>1998/2001 | **Post-doctorat**<br>**2001/2003** |
|---|---|
| Dynamique spatio-temporelle du microbiote racinaire | Inoculation de bactéries phytostimulatrices |
| Rhizodéposition | Dynamique du microbiote racinaire |

***Activités de recherche réalisées dans le cadre du projet européen EcoSafe (n° QLK3-CT-2000-31759, Ecological and environmental biosafety assessment of novel plant and microbial biotechnology products) dans l'UMR CNRS-5557 Ecologie Microbienne (Université Claude Bernard Lyon1)***

### Contexte :

Depuis plusieurs décennies, les souches bactériennes PGPR sont inoculées dans le sol ou sur les semences pour améliorer la croissance ou l'état sanitaire des cultures (Okon et Labandera-Gonzalez, 1994). Les effets écologiques de ces inoculations sur la diversité des communautés microbiennes résidentes des sols agricoles ont souvent été étudiés mais la plus part du temps dans le cas des souches de lutte biologique (*i.e.* antibiose, composés antifongiques) (*e.g.* Bakker et al, 2002; Walsh et al, 2003). Cependant, le mode d'action principal de souches PGPR du genre *Azospirillum* (α-Protéobactérie) fait intervenir la synthèse de phytohormones telles que l'auxine acide indole-3-acétique (AIA) (Dobbelaere et al, 1999; Bashan et al, 2004; Spaepen et al, 2008). Bien que ce genre bactérien fasse partie des fixateurs libres d'azote (diazotrophes), la contribution de ce trait fonctionnel dans l'expression du potentiel PGPR semble très minimale. Ainsi, le bénéfice attendu de telles inoculations s'exprime par une prospection racinaire accrue du sol et une meilleure nutrition hydro-minérale (Lin et al, 1983; Okon et Kapulnik, 1986; Malhotra et Srivastava, 2008), permettant alors d'envisager une augmentation des rendements sur des sols contraints par la disponibilité en minéraux ou dans une perspective de réduction des fertilisants de synthèse. Si les effets agronomiques de souches *Azospirillum* sont bien documentés sous diverses conditions édaphiques et climatiques, leur impact écologique, notamment sur la microflore du sol, n'a été que très peu étudié (Okon et Labandera-Gonzalez, 1994; Dobbelaere et al, 2001). Or dans la mesure où l'AIA peut avoir un effet stimulant sur le développement racinaire (ramification, densité des zones pilifères) et sa rhizodéposition (Heulin et al, 1987; Lippmann et al, 1995), il est prévisible que le recours à ce type de souches PGPR provoque un remaniement des communautés microbiennes rhizosphériques (comme a pu le suggérer la deuxième partie de ma thèse via la manipulation de la disponibilité en exsudats). De plus, l'introduction à hautes densités d'une seule souche peut induire des phénomènes de compétition directe avec la microflore résidente. Cependant, les essais réalisés à l'époque avec diverses souches

d'*Azospirillum* n'avaient pas réussi à mettre en évidence un tel impact (Herschkovitz et al, 2005a-b; Lerner et al, 2006). Dans la mesure où ces essais ont été réalisés en serre dans des pots, la question se pose toujours pour un usage en plein champ.

Ainsi mes objectifs dans le projet EcoSafe consistaient à apprécier au champ (sur maïs) et en serre (sur blé) la perturbation des communautés bactériennes et fongiques à l'échelle du système racinaire suite à des inoculations contrôlées sur semences de bactéries phytostimulatrices du genre *Azospirillum*, notamment la souche commerciale *A. lipoferum* CRT1.

Ce questionnement m'a permis de complexifier celui initié en thèse en envisageant les interactions plante-microorganismes selon la séquence : (1) effets d'une population bactérienne fonctionnelle sur la plante et (2) effets de la plante traitée sur le compartiment bactérien et fongique de sa rhizosphère (tandis que seul l'effet unidirectionnel de la plante sur les communautés bactériennes était abordé dans la démarche de la thèse). Enfin, c'est à ce stade de mon parcours que j'ai commencé à travailler en conditions écologiques réelles au champ.

**Hypothèses de travail :**

Les inoculations de ce type de souches PGPR sont supposées agir sur l'architecture du système racinaire, et donc sur les flux de photosynthétats qui lui sont alloués ainsi que sur les flux hydriques et minéraux, impliquant un remaniement de l'habitat microbien. L'impact de telles modifications des flux de matière et des microhabitats racinaires sur la composition de la microflore indigène du sol peut être envisageable mais reste à préciser notamment en conditions réelles (amplitude, effet additif en cas d'inoculations répétées entre cycles de culture, rémanence en cas d'inoculation unique, interaction avec le niveau de fertilisation azotée).

Les souches du genre *Azospirillum* disponibles étant par ailleurs manipulables par génie génétique, il est possible d'augmenter leur potentiel de biosynthèse en hormones de croissance (i.e. acide indole-3-acétique) afin éventuellement d'amplifier leurs effets sur la plante (souches génétiquement modifiées fournies par l'équipe partenaire belge-Dr. J. Vanderleyden). Dans ce cas aussi, l'impact écologique des souches génétiquement modifiées (GM) reste à mesurer et à comparer à celui des souches parentales.

Enfin, il est possible que l'inoculation d'un représentant du genre *Azospirillum* induise un recrutement rhizosphérique d'autres souches indigènes du même genre. Afin de confirmer cette hypothèse, il est nécessaire de pouvoir apprécier les variations de composition des populations d'*Azospirillum*. A l'instar de la souche CRT1, cette vérification à l'échelle du genre peut être réalisée *in vitro* avec les techniques d'isolement sur milieux de culture spécifiques et hybridation de l'ADN des colonies bactériennes avec un panel de sondes moléculaires spécifiques. L'intérêt majeur de la définition d'un outil de détection PCR appliqué sur extraits d'ADN environnemental est que son emploi serait nettement moins fastidieux et qu'il s'affranchirait du biais de la « cultivabilité » des bactéries isolées de l'environnement, permettant *a priori* une sensibilité accrue. Ainsi, une mise au

point méthodologique d'amorces PCR destinées (1) à détecter voire quantifier la présence de la souche CRT1 dans le sol et donc de suivre son devenir *in planta* et (2) à caractériser la structure génétique des populations indigènes d'*Azospirillum* spp. a été élaborée sur la base du polymorphisme de séquence de l'espaceur intergénique 16S-23S de l'opéron ribosomal bactérien. Cette région a été choisie en raison de son haut degré de polymorphisme de séquence et de taille (*ca*. de 50 à 1500 pb) au sein des Eubactéries.

**Méthodologie :**

La caractérisation de l'impact microbien a été menée à l'échelle du cortège bactérien et/ou fongique par des méthodes électrophorétiques automatisées d'empreintes moléculaires (ARISA) appliquées sur des extraits d'ADN total isolés des tissus racinaires et de leur sol adhérent.

Essais au champ sur maïs : souche *A. lipoferum* CRT1

Les inoculations de la souche CRT1 ($3 \times 10^7$ UFC par graine) ont été réalisées au champ (Côte Saint André, Isère) sur semences de maïs sur deux années consécutives (effet additif) et au travers d'un gradient de fertilisation azotée (0-70-130 unités N-ammonitrate $ha^{-1}$) afin d'étudier la réponse du maïs et du compartiment microbien à ces inoculations le long d'un gradient de stress nutritionnel susceptible de moduler l'expression de l'effet phytostimulateur. Trois échantillonnages des systèmes racinaires ont été réalisés au cours de l'année 1 : stade 3 feuilles (*ca*. 7 jours post-inoculation), stade 10 feuilles (*ca*. 35 jours) et floraison femelle (*ca*. 65 jours). Deux échantillonnages au cours de l'année 2 : stade 3 feuilles (*ca*. 18 jours post-inoculation), stade 10 feuilles (*ca*. 57 jours). Un échantillonnage unique a été effectué en année 3 (uniquement plots 70 N) en l'absence d'inoculation (effet rémanent). Le dispositif statistique était un essai en 4 blocs de 6 placettes (6 × 15 m, 8 lignes de semis) combinant les 3 modalités de fertilisation (0-70-130 N) et les 2 modalités d'inoculation (présence/absence).

Essais en serre confinée sur blé : souches GM *A. brasilense* 245

Les inoculations des souches GM d'*A. brasilense* Sp245 ($1 \times 10^6$ UFC par graine) ont été réalisées avec le sol du champ expérimental (2,5 kg/pot, prélevé hors zone d'essai souche CRT1) sur semences de blé avec une période de croissance limitée à un mois et étude de l'effet rémanent (deuxième semis sur le sol de la première culture sans réinoculation). Trois souches GM pour le gène *ipdc* (phenylpyruvate decarboxylase) impliqué dans l'une des voies de synthèse de l'AIA ont été inoculées indépendamment (8 réplicats), en plus de la souche parentale non modifiée. La première souche GM contenait une copie surnuméraire plasmidique du gène *ipdc* sous le contrôle d'un promotteur inductible par les exsudats (PsbpA), la seconde sous le contrôle d'un promoteur constitutif (PnbpII), la troisième ne contenant que le plasmide vecteur vide (témoin plasmidique). La surproduction d'AIA par les deux souches contenant des copies surnuméraires du gène *ipdc* a été vérifiée *in vitro* sur milieu nutritif liquide (*ca*. 3 versus 2 µg AIA $ml^{-1}$ milieu de culture en phase plateau) par les partenaires belges du projet EcoSafe.

Dessin d'amorces PCR dans l'IGS ribosomal 16S-23S

Le séquençage de l'espaceur intergénique 16S-23S de l'opéron ribosomal a été réalisé sur les souches *A. lipoferum* CRT1 et *A. brasilense* Sp245. Après confrontation des séquences sur GenBank, l'identification de zones *a priori* spécifiques d'une longueur adéquate a permis la définition d'amorces PCR (1: amplification spécifique de l'ADN cible de la souche CRT1 ; 2: amplification spécifique de l'ADN cible de souches du genre *Azospirillum*). Leur spécificité et leur sensibilité ont alors été testées par application sur une collection d'ADN de souches du genre *Azospirillum* et d'autres genres bactériens, ainsi que sur les ADN environnementaux issus de l'inoculation de la souche CRT1 au champ et sur microcosmes de sol (de $10^8$ à 10 UFC $g^{-1}$ sol).

**Résultats principaux :**

Essais au champ sur maïs : souche *A. lipoferum* CRT1

La comparaison statistique des empreintes ARISA des plants de maïs témoins non inoculés a mis en évidence une modification significative de la structure génétique des communautés bactériennes rhizosphériques entre chacun des trois stades de prélèvement. Dans ce contexte de cet effet phénologique, l'inoculation de la souche CRT1 au champ sur maïs a provoqué en année 1 une modification significative de la composition du cortège bactérien, concomitante à une augmentation de la biomasse racinaire et/ou de la longueur du système racinaire. Cependant, cet effet n'a pu être observé au troisième stade (65 jours) pour lequel une décroissance d'environ 2 Log des densités cultivables de la souche CRT1 (de $2{,}1 \times 10^8$ au deuxième stade à $9{,}5 \times 10^5$ UFC $g^{-1}$ sol) a d'ailleurs été enregistrée. Les bandes ARISA discriminantes ne correspondaient pas à celles de la souche CRT1 et différaient d'un stade phénologique à l'autre, indiquant que des taxa bactériens différents étaient impactés au cours de la croissance végétale (**Figure 2**).

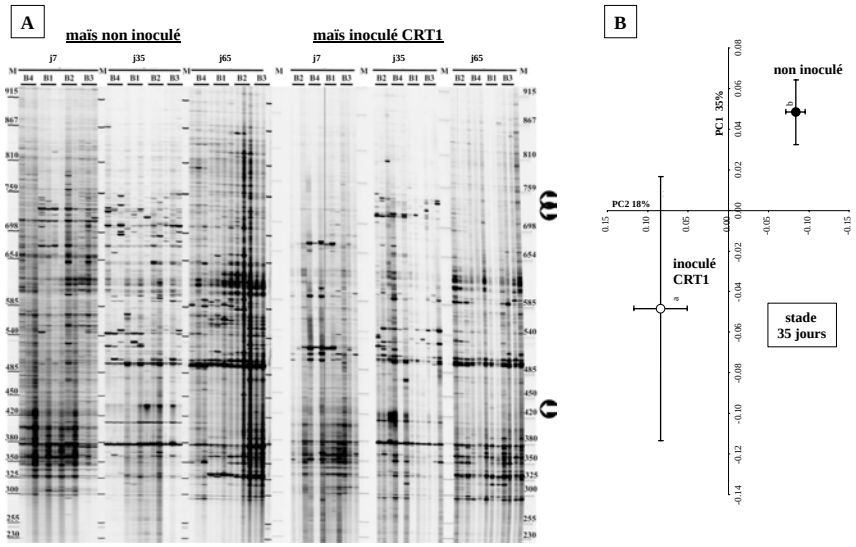


**Figure 2.** Effets de la première inoculation au champ de la souche CRT1 sur la structure génétique des communautés bactériennes rhizosphériques du maïs décrites par les empreintes ARISA à 7-35-56 jours (A) manifestant des divergences significatives avec celles des maïs non-inoculés jusqu'au stade 35 jours maximum (B). Les flèches indiquent la position des 3 bandes IGS de la souche CRT1.

L'inoculation en année 2 a provoqué un effet significatif uniquement pour les parcelles moyennement fertilisées, et surtout non fertilisées depuis deux ans, l'impact étant toujours circonscrit aux stades végétatifs précoces. Ce résultat indiquerait que l'impact écologique transitoire de la souche CRT1 serait accentué lorsque la disponibilité en azote minéral est limitée. Par ailleurs, l'inoculation en l'année 2 n'a pas conduit à des effets supérieurs par comparaison aux effets mesurés en année 1, ce qui plaide pour l'absence d'effet additif. Enfin, aucun effet rémanent n'a été décelé en année 3 lorsque le semis a été réalisé sur des parcelles 70N inoculées les deux années précédentes. Les rendements en grains ou leur qualité (poids de 1000 grains, teneur N) n'ont pas été affectés par cette pratique de l'inoculation.

Essais en serre confinée sur blé : souches GM *A. brasilense* 245

Les diverses inoculations de souches *A. brasilense* Sp245 ont provoqué par rapport au blé non inoculé une augmentation significative de la biomasse aérienne de +25% avec la souche surproductrice d'AIA sous promoteur constitutif contre +11% avec le système de promoteur inductible mais une diminution significative de l'épaisseur des racines avec toutes les souches (parentale -19%, témoin plasmidique -25%, promoteur constitutif -22% et inductible -23%). Ainsi, l'effet de la surpoduction d'AIA et de sa régulation par deux promoteurs différents a stimulé la croissance aérienne mais sans affecter la biomasse racinaire ou sa ramification. Ces effets variables entre souches ont été obtenus avec des densités rhizosphériques finales toutes similaires (*ca*. 4,0 $10^5$ UFC $g^{-1}$ racine), indiquant par ailleurs que l'introduction des plasmides vecteurs du gène *ipdc* et celle du plasmide témoin n'ont pas modifié l'aptitude colonisatrice des souches modifiées.

Quelle que soit la souche inoculée, la composition des communautés bactériennes n'a pas été statistiquement modifiée par comparaison avec le traitement témoin non inoculé. Cependant, l'effet du témoin plasmidique différait de celui de la souche portant le système de surexpression inductible. Les effets des 2 souches portant les constructions *ipdc* n'ont pu être significativement distingués. Les communautés fongiques quant à elles ont été significativement impactées par le témoin plasmidique, et dans une moindre mesure par la souche portant le système de surexpression inductible, par rapport à celles du blé non inoculé. Les communautés bactériennes et fongiques ont donc manifesté des réponses spécifiques aux diverses inoculations dont l'ampleur n'était corrélée ni avec le potentiel de production d'AIA des souches, ni avec leurs effets sur le blé. Enfin, aucun effet rémanent n'a pu être observé sur la biométrie du blé ou les communautés microbiennes après reprise de la culture sur les sols du premier cycle. Les différentes souches survivantes ont pu recoloniser les nouveaux systèmes racinaires mais à des taux 10 fois inférieurs à ceux mesurés à l'issue du premier cycle, suggérant un classique effet PGPR densité dépendant même avec les souches surproductrices.

Dessin d'amorces PCR dans l'IGS ribosomal 16S-23S

Les souches CRT1 et Sp245 possédent 2 bandes « longues » (*ca*. 750 pb) hébergeant en tandem les séquences des ARNt de l'alanine et de l'isoleucine, et une bande « courte » (*ca*. 430 pb).

C'est dans la bande longue que les amorces spécifiques de la souche CRT1 (fCRT1/rCRT1) ont été dessinées, permettant l'amplification d'un fragment de 249 pb. Mais la spécificité des amorces fCRT1/rCRT1 n'a pas pu être validée du fait de la réponse positive d'autres souches *Azospirillum lipoferum*. Cependant, l'usage de fCRT1/rCRT1 sur des ADN de sol inoculé avec la souche CRT1 a permis de détecter par PCR gigogne la souche CRT1 dans le sol de $10^8$ à quasiment $10^2$ UFC $g^{-1}$ sol (**Figure 3**). De plus, avec les ADN issus de l'essai d'inoculation au champ en année 2, un signal PCR spécifique n'a été obtenu qu'avec les ADN correspondants aux plants de maïs inoculés au semis et échantillonnés aux stades 18 et 57 jours (**Figure 3**).

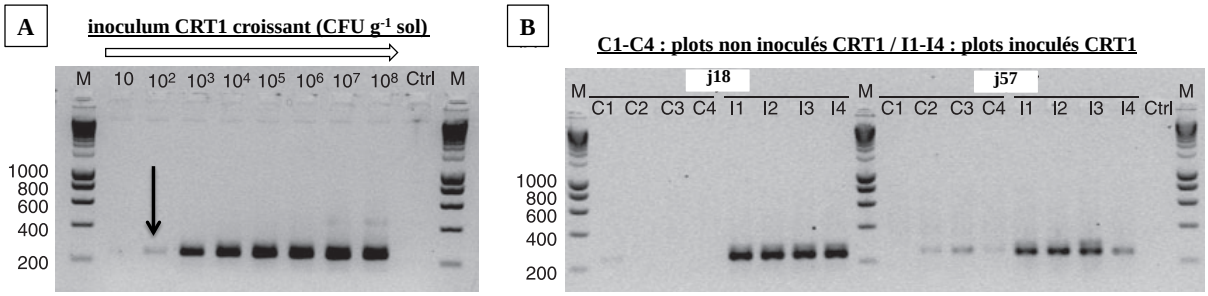


**Figure 3.** Application du couple d'amorces fCRT1/rCRT1 définies dans l'intergène ribosomal 16S-23S pour la détection PCR sur ADN de sol de la souche CRT1 inoculée sur microcosmes de sol de 10 à $10^8$ UFC $g^{-1}$ (A) ou au champ sur maïs (B) échantillonné 18 et 57 jours après semis.

Pour le dessin des amorces spécifiques du genre *Azospirillum* (fAZO/rAZO), les domaines des ARNt ont dû être écartés du fait de leurs séquences hautement conservées au niveau des Protéobactéries. Deux domaines répondant aux exigences ont été trouvés aux extrémités des bandes longues. Cependant, la spécificité des amorces fAZO/rAZO n'a pas pu être validée du fait de la réponse positive de souches non-*Azospirillum* (e.g. *Nitrobacter winogradskyi*, *Rhizobium etli*, *Bacillus pumilus*, *Microbacterium esteraromaticum*…). Cependant, l'usage de fAZO/rAZO sur les ADN issus de l'essai d'inoculation au champ (année 2) a permis d'obtenir des amplifiats de taille unique (identique à celle des amplifiats de la souche CRT1) uniquement avec les ADN issus de plants de maïs inoculés avec CRT1 et échantillonnés aux stades 18 et 57 jours.

Ainsi, les deux couples d'amorces apparaissent efficaces pour la détection PCR de la souche CRT1 dans le sol mais le couple fAZO/rAZO ne permet pas de caractériser la structure génétique des populations indigènes d'*Azospirillum* à partir d'ADN environnemental.

N.B. 6 articles publiés, référencés n° 7-8-10-12-13-16 dans la section V-A

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## II-D Chargé de recherche 2ème classe – IRD (2004/2008)
## Fertilisation organique et dénitrification en milieu sub-tropical

| Thèse<br>1998/2001 | Post-doctorat<br>2001/2003 | **CR2-IRD<br>2004/2008** |
|---|---|---|
| Dynamique spatio-temporelle du microbiote racinaire | Inoculation de bactéries phytostimulatrices | Bactéries dénitrifiantes |
| Rhizodéposition | Dynamique du microbiote racinaire | Matières organiques |

***Activités de recherche réalisées dans le Laboratoire d'Ecologie Microbienne des Sols et Agrosystèmes Tropicaux – campus IRD-ISRA à Dakar (Sénégal)***

### Contexte :

En mai 2008, j'ai été recruté CR2 à l'IRD en tant que microbiologiste (ex-UR 83 IBIS et 179 SeqBio, aujourd'hui UMR Eco&Sols) pour développer au Sénégal une approche de la diversité fonctionnelle des communautés bactériennes dénitrifiantes dans les sols arables en rapport avec les modes locaux d'usage des terres et de fertilisation organique (résidus de culture, déchets urbains), avec pour cadre général d'étude la réduction des émissions agricoles d'oxyde nitreux issu de la dénitrification.

L'oxyde nitreux ($N_2O$) est un gaz à effet de serre qui expliquerait 8% du réchauffement climatique. A l'instar du $CO_2$ (80% du réchauffement) et du $CH_4$ (12%), les teneurs atmosphériques en $N_2O$ sont en constante progression (+0,3% annuel) (GIEC, 2007). Il est par ailleurs impliqué dans la destruction de l'ozone stratosphérique et son potentiel de réchauffement est 300 fois supérieur à celui du $CO_2$. Les écosystèmes terrestres seraient à l'origine de plus de 50% des émissions mondiales de $N_2O$ estimées à 18 millions de tonnes de N-$N_2O$ par an (GIEC, 2007). Les sols tropicaux en particulier représenteraient plus de 25% de ces émissions mondiales (Bouwman, 1998; GIEC, 2001). La contribution agricole aux émissions globales est aussi très large : plus de 6 millions de tonnes de N-$N_2O$ par an, soit près de 60% des rejets issus des sols (Mosier et al, 1998; Kroeze et al, 1999). Cette émission de $N_2O$ à partir des surfaces cultivées peut également constituer une perte dommageable d'azote minéral (nitrate) pour la fertilisation des cultures et donc l'élaboration des rendements. Les origines agricoles du $N_2O$ sont très majoritairement associées aux processus bactériens couplés de la nitrification et de la dénitrification dans le sol. Le processus de dénitrification est connu pour être favorisé par la matière organique (fertilisation organique, résidus végétaux), le nitrate (fertilisation minérale) ainsi qu'une faible aération du sol (compaction et/ou humidité élevée consécutives aux précipitations ou à l'irrigation) (e.g. Philippot et al, 2007). En particulier, il est connu que la qualité des résidus de culture constitue un déterminant significatif des rejets en $N_2O$ (e.g. Baggs et al, 2001; Millar et al, 2004). En règle générale, des émissions plus importantes sont enregistrées avec des résidus végétaux ayant des ratios C/N faibles (e.g. légumineuses) (Baggs et al, 2001; Huang et al, 2004) tandis que ces rejets sont négativement corrélés à la teneur en polyphénols et en lignine (Millar

et Baggs, 2004). Dans la mesure où les intrants azotés et organiques ainsi que la gestion de l'eau sont des paramètres agronomiques majeurs dans la conduite des agrosystèmes, en particulier sub-sahéliens, une meilleure compréhension de l'écologie de la dénitrification dans ces systèmes apparaît opportune pour évaluer les effets stimulants/limitatifs de certaines pratiques paysannes sur ce processus bactérien et les pertes azotées qu'il occasionne, dommageables pour les rendements et l'environnement.

La dénitrification hétérotrophe est un processus respiratoire bactérien facultatif et séquentiel qui réduit le nitrate en diazote gazeux uniquement en conditions d'anoxie/microaérobie. La minéralisation de la matière organique crée le pouvoir réducteur (électrons, protons) servant à réduire les oxydes d'azote qui jouent ainsi le rôle d'accepteurs terminaux de protons et d'électrons dans la chaîne respiratoire membranaire. Durant ce processus, les formes ioniques de l'azote (nitrate $NO_3^-$ puis nitrite $NO_2^-$) sont séquentiellement réduites en formes gazeuses (oxyde nitrique NO, puis oxyde nitreux $N_2O$, puis $N_2$) selon : $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ (Tiedje, 1988).

La dénitrification est une propriété distribuée irrégulièrement au travers de plus de 55 genres bactériens (Zumft, 1997; Philippot et al, 2007). De plus, des taxons très proches, voire des souches de la même espèce, peuvent manifester des potentialités dénitrifiantes contrastées (Cavigelli et Robertson, 2000-2001). Par ailleurs, les gènes codant pour toutes les étapes de la chaîne de dénitrification sont rarement tous présents dans une même bactérie. Ces constats rendent l'étude des communautés dénitrifiantes par des outils taxonomiques inopérante. Dans ce contexte, l'étude de l'écologie de ces communautés (densité-diversité) ne peut s'effectuer que par le suivi des gènes fonctionnels de la dénitrification (*narG-napA*, *nirK-nirS*, *norB*, *nosZ*) (Philippot, 2002). Ces gènes codent chacun pour la sous-unité catalytique des complexes enzymatiques assurant la réduction des espèces azotées à chacune des étapes de la chaîne de dénitrification (Philippot et Hallin, 2006; Philippot et al, 2007).

**Ainsi, l'objectif directeur lié à mon affectation sur le Sénégal était de définir (1) dans quelle mesure l'activité de la communauté bactérienne dénitrifiante, notamment l'émission réelle ou potentielle de $N_2O$, était influencée par les modalités locales de la fertilisation organique (principalement paillis et déchets urbains), et (2) dans quelle mesure cette fluctuation de la dénitrification était corrélée à la densité et/ou la diversité des gènes fonctionnels de ce processus.** A cette fin, il m'a fallu transférer des méthodes moléculaires d'étude de la diversité et de la densité des bactéries dénitrifiantes dans les sols (qPCR-DGGE-RFLP).

Du point de vue de mon parcours, cette thématique m'a fait étudier l'impact de facteurs environnementaux (travail du sol, fertilisation organique) sur une nouvelle communauté bactérienne fonctionnelle pouvant influencer négativement la nutrition azotée des plantes, les bactéries dénitrifiantes étant par ailleurs souvent favorisées dans la rhizosphère en raison de leur aptitude à consommer la matière organique en anoxie ou en microaérobie. Ce passage du modèle PGPR au modèle dénitrification m'a permis d'intégrer une approche microbiologique fonctionnelle (mesures de

métabolites et de gènes fonctionnels) tandis que seule une approche structurale/taxonomique était appliquée précédemment. Par ailleurs, c'est à ce niveau de mon parcours que j'ai commencé à connecter mes activités de recherche à l'encadrement de stagiaires M2.

**Hypothèses de travail :**

L'hypothèse principale était relative à l'effet de la variation de la qualité biochimique des intrants organiques sur le couplage entre (1) activité dénitrifiante (émissions de $N_2O$), (2) densité et (3) diversité des communautés dénitrifiantes. Dans certaines expérimentations, les rôles spécifiques de la matière organique soluble du sol (issue en partie de la minéralisation des intrants organiques, modèle détritusphère) et du travail du sol (modifiant entre autre la surface de contact entre intrants organiques et biomasse microbienne) ont été abordés.

L'hypothèse secondaire était que la dénitrification pouvait être modulée au travers des réseaux trophiques impliquant les nématodes bactérivores.

Ces hypothèses ont été abordées au travers de 2 projets principaux liés à des essais en milieu agricole (prélèvements *in situ* et expérimentations en microcosmes le cas échéant) : projet MUTEN-Madagascar (paillis de résidus végétaux de qualités contrastées, gérés en labour versus semis-direct), et projet JEA-APN-Sénégal (irrigation maraîchère par les eaux usées).

**Méthodologie :**

La manipulation du paramètre matière organique au champ ou *in vitro* (microcosmes de sol) a donné lieu à des prélèvements de sol et de gaz lesquels ont été analysés pour leur teneur en $N_2O$ (chromatographie gazeuse avec détecteur soit à capture d'électrons, soit à ionisateur de flamme). Les sols ont fait l'objet (1) d'analyses de l'activité dénitrifiante potentielle ou semi-potentielle, (2) d'analyses moléculaires (qPCR, DGGE, RFLP de gènes marqueurs de la dénitrification) basées sur des extraits d'ADN de sol.

**Résultats principaux :**

**projet ECCO-PNBC-MUTEN-Madagascar « Modes d'utilisation des terres et flux de $N_2O$ : caractérisation des déterminants du fonctionnement des communautés dénitrifiantes » (porteur Dr. A. Brauman, DR IRD) (2004/2007)**

Mise au point méthodologique d'un protocole de PCR quantitative du gène dénitrifiant *nirK* (nitrite réductase à cuivre) dirigée par Dr. L. Philippot-DR INRA (Dijon)

Le gène marqueur *nirK* est associé à une séquence fondamentale de la chaîne de dénitrification, celle qui opére le passage irréversible de la dernière forme ionique de l'azote (nitrite) à lapremière forme gazeuse (oxyde nitrique). Le but de ce travail était de définir un couple d'amorces PCR ciblant

spécifiquement ce gène afin de le valoriser dans des estimations de la densité des bactéries dénitrifiantes par PCR quantitative.

L'alignement multiple de séquences *nirK* de GenBank et amplifiées à partir de souches cultivables a permis d'identifier 2 zones à forte homologie entre toutes ces séquences. Elles ont permis de dessiner 2 amorces dégénérées nirK876 et nirK1040, permettant d'amplifier un fragment de 165 pb. Ces amorces ont été validées de façon cohérente sur une collection d'ADN génomique de souches dénitrifiantes (possèdant ou non ce gène) et sur des ADN de souches non dénitrifiantes. Leur usage en PCR quantitative sur 6 sols différents (sols agricoles, sol de forêt pluvial, sol de termitière) a permet d'obtenir un amplifiat de taille unique et spécifique pour tous les échantillons pour lequel la courbe de fusion a révélé un pic unique correspondant à celui du standard. Les densités environnementales étaient comprises entre $9,7 \times 10^4$ et $3,9 \times 10^6$ copies $g^{-1}$ sol (**Figure 4**). Le séquençage des amplicons a permis de conclure à une similarité de 60 à 80% avec des séquences environnementales de la GenBank, notamment appartenant aux Alpha-Protéobactéries (**Figure 4**). Ce couple d'amorces était le premier disponible permettant une étude environnementale par PCR quantitative des dénitrifiants à nitrite réductase à cuivre.

A

Nombre de copies du gène *nirK* dans divers sols

| Soils | *nirK* gene copy number per nanogram of DNA | *nirK* gene copy number per gram of soil |
|---|---|---|
| Bouzule amended with water | $9.7\times10^1$ $(1.1\times10^1)^a$ | $8.9\times10^5$ $(1.0\times10^5)^a$ |
| Bouzule amended with C | $4.2\times10^2$ $(1.1\times10^2)^c$ | $3.9\times10^6$ $(9.8\times10^5)^c$ |
| Kenya | $8.9\times10^1$ $(2.2\times10^1)^a$ | $2.1\times10^5$ $(5.3\times10^4)^a$ |
| Paris | $1.9\times10^2$ $(4.4\times10^1)^b$ | $9.7\times10^4$ $(2.5\times10^4)^a$ |
| Rennes | $7.7\times10^1$ $(4.0\times10^1)^a$ | $4.2\times10^5$ $(5.3\times10^4)^a$ |
| Termite mound Burkina Faso | $5.1\times10^1$ $(2.5\times10^1)^a$ | $2.2\times10^5$ $(2.2\times10^5)^a$ |
| Vannecourt | $3.0\times10^1$ $(1.2\times10^1)^a$ | $1.4\times10^6$ $(5.1\times10^5)^b$ |

Values followed by the same letter within columns do not significantly differ according to LSD test ( $P<0.05$).
Standard errors indicated in parenthesis.

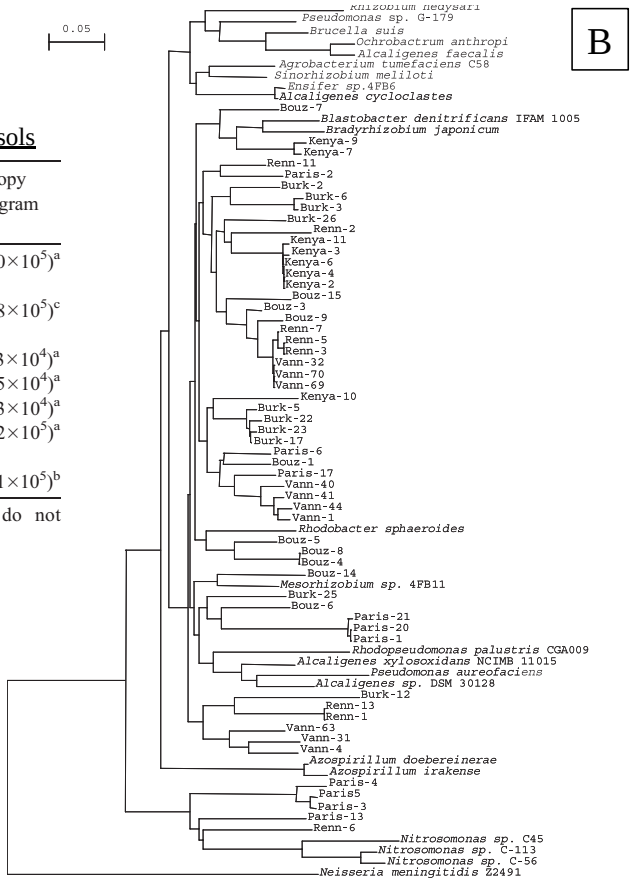


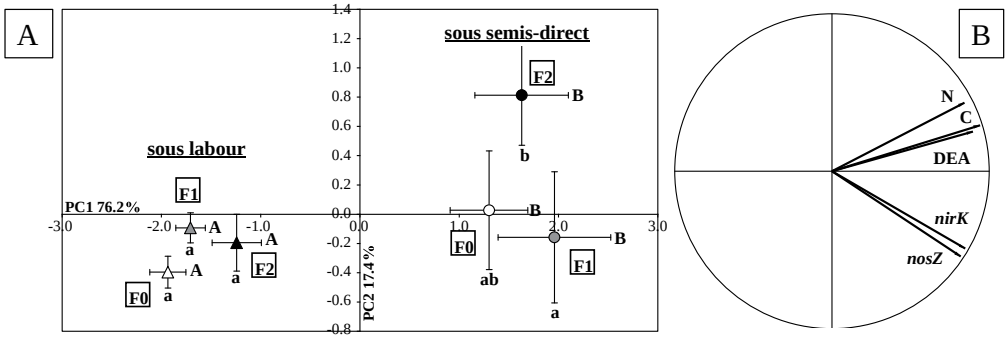

**Figure 4.** Estimation par qPCR des densités du gène *nirK* dans divers sols (A) et comparaison phylogénétique des séquences ainsi obtenues avec des séquences référencées dans GenBank (B).

N.B. 1 article publié, référencé n°6 dans la section V-A

Impact des systèmes alternatifs, semis-direct et litières permanentes (chercheur FOFIFA associé Dr. R. Bodovololona, Madagascar)

L'impact (1) du travail du sol, (2) de paillis de résidus de culture (labour sans conservation des résidus / semis-direct sous paillis des résidus) et (3) d'un gradient de fertilisation (sans fertilisant / fumier 5 t $ha^{-1}$ / fumier + NPK 70-30-40) sur les communautés de bactéries dénitrifiantes (densité des gènes *nirK* et *nosZ*, activité potentielle de dénitrification) a été envisagé au travers d'un échantillonnage réalisé sur un site agronomique des Hautes Terres malgaches (région d'Antsirabé) en février 2005 (soja cultivé sous litière de riz-2004) et 2006 (riz cultivé sous litière de soja-2005).

Il est apparu que le facteur agronomique influençant majoritairement les dénitrifiants sur ces deux années consécutives était le travail du sol. Le labour a été à l'origine d'un effet significatif dépressif sur les teneurs du sol en C-N, ainsi que sur la taille et l'activité de la communauté dénitrifiante par rapport au semis-direct (**Figure 5**).




gradient de fertilisation : F0 (aucune) – F1 (NPK) – F2 (NPK+fumier)

N-C: teneurs du sol en N-C
DEA : potentiel de dénitrification
*nirK-nosZ* : densités des gènes dansle sol

**Figure 5.** Analyse en composantes principales révélant l'effet prédominant du travail du sol sur celui de la fertilisation lors de la culture de soja (A) et précisant l'effet stimulateur du semis-direct sur les différentes variables, notamment les densités des gènes *nirK-nosZ* et le potentiel de dénitrification (B).

En particulier, l'activité dénitrifiante oscillait entre 17 et 26 ng $N\text{-}N_2O$ $g^{-1}$ sol $h^{-1}$ pour le traitement labour et entre 44 et 60 pour le traitement semis-direct. Les densités moyennes du gène *nosZ* étaient comprises entre $8{,}0 \times 10^1$ et $7{,}0 \times 10^2$ copies $ng^{-1}$ ADN de sol pour le traitement labour et entre $7{,}0 \times 10^2$ et $1{,}0 \times 10^3$ pour le traitement semis-direct. Pour le gène *nirK*, les effectifs moyens fluctuaient entre $2{,}0 \times 10^3$ et $2{,}0 \times 10^4$ (labour) et entre $1{,}0 \times 10^4$ et $3{,}0 \times 10^4$ (semis-direct). Le niveau de corrélation entre la densité des dénitrifiants et leur activité potentielle s'est révélé très faible, suggérant que l'activité dénitrifiante n'était pas majoritairement contrôlée par les effectifs estimés des dénitrifiants. Curieusement, le gradient de fertilisation n'a pas eu d'effet notable sur les paramètres mesurés (**Figure 5**), pas plus que la qualité biochimique du paillis (riz/soja) et la rotation culturale. Enfin, ce site agricole n'a pas manifesté de capacité de réduction du $N_2O$ en $N_2$, quel que soit le mode d'usage des terres appliqué. Cette absence caractéristique de réduction du $N_2O$ permet d'envisager que

les dégagements *in situ* de $N_2O$ seraient plus intenses pour les sols gérés en semis-direct qui favorise la communauté dénitrifiante (activité-densité) et les teneurs du sol en C-N.

N.B. 1 article publié, référencé n°11 dans la section V-A

Impact de la prédation des nématodes bactérivores sur la dénitrification (co-encadrement stage post-doctoral Dr. D. Djigal, Sénégal avec Dr. C. Villenave-DR IRD)

L'effet de la prédation par une unique espèce de nématode bactérivore (*Cephalobus pseudoparvus*) sur la communauté dénitrifiante d'un sol maraîcher sahélien sableux a été mesuré par inoculation directe de ces nématodes dans des microcosmes.

Cette inoculation a induit une baisse significative du potentiel de dénitrification (environ -8%) par rapport au témoin. Les densités des gènes *nirK-nirS* et *nosZ* ont également été diminuées en présence des nématodes mais sans effet significatif malgré l'ampleur de l'effet dépressif (entre -63 et -79% selon le gène et l'âge des microcosmes), indiquant une forte variabilité de la réponse des dénitrifiants à ce type de prédation. Seule la structure génétique de la communauté nitrite-réductase-*nirK* a été modifiée par l'inoculation, suggérant que les communautés *nirK* et *nirS* occuperaient des niches écologiques différentes.

En l'absence de fertilisation organique pouvant modifier la biomasse et la diversité bactérienne et donc le niveau de prédation sur les bactéries dénitrifiantes, cette expérimentation indique que l'effet de la prédation par une espèce unique de nématode sur les dénitrifiants peut être décelable et de type dépressif, suggérant un possible ralentissement du cycle de l'azote. La portée de cette présente étude reste aussi à nuancer par les interactions avec d'autres types trophiques de nématodes bactérivores.

N.B. 1 article publié, référencé n°14 dans la section V-A

Infuence de la qualité biochimique de résidus végétaux sur la structure génétique des communautés bactériennes actives (stage post-doctoral Dr. M. Diouf, Sénégal)

L'incorporation (+2%) dans un sol agricole sénégalais de résidus végétaux de compositions contrastées (soja C/N=62 ; maïs C/N=91) a permis de révéler après une incubation courte de 3 jours que ces différences biochimiques avaient activé des communautés bactériennes de strutures génétiques distinctes (RT-PCR du gène 16S et empreintes moléculaires DGGE).

N.B. 1 article publié, référencé n°15 dans la section V-A

Impact de la qualité biochimique des paillis de résidus en décomposition sur l'activité et la composition des communautés dénitrifiantes (stage M2-A. Dieng, Sénégal)

L'objectif ici était de savoir si des différences biochimiques initiales marquées entre résidus végétaux appliqués en surface (paillis) pouvaient spécifiquement modifier : (1) le profil des émissions $N_2O$ *in situ*, (2) l'aptitude de la matière organique soluble du sol à stimuler la dénitrification, et (3) la communauté dénitrifiante (activité potentielle / structure génétique par empreintes moléculaires DGGE-*nirK* et RFLP-*nosZ*). A cette fin, la décomposition de deux types de résidus (arachide C/N=17, maïs C/N=117) appliqués en paillis (equ. *ca*. 1 t $ha^{-1}$) sur microcosmes de sol agricole fertilisé en nitrate (+ 0,018 mmol N $g^{-1}$ sol) a été suivie hebdomadairement en cinétique sur 7 semaines.

La décomposition des deux types de paillis n'a pas occasionné une augmentation significative des émissions de $N_2O$ dans l'atmosphère des microcosmes. Néanmoins, le potentiel de dénitrification des sols a été spécifiquement et significativement augmenté par les deux types de résidus, particulièrement avec ceux de maïs au cours des trois premières semaines. Ainsi, l'activité dénitrifiante de la couche de résidus elle-même pourrait avoir intercepté les flux sortants de $N_2O$ issus du sol sous-jacent. Les paillis sortis des microcosmes ont d'ailleurs manifesté une forte activité potentielle de réduction du $N_2O$ en $N_2$ (conversion intégrale en $N_2$ de 0,3% de $N_2O$).

Seule la matière organique soluble issue des sols sous paillis d'arachide lors des 3 premières semaines de décomposition a permis de stimuler des émissions potentielles de $N_2O$ du sol d'origine.

La composition des communautés dénitrifiantes *nirK* et *nosZ* a été spécifiquement influencée par le type de paillis dès la première semaine d'incubation. De plus, elle a régulièrement évolué au cours de la cinétique, sauf pour la communauté *nosZ*. Néanmoins, aucune corrélation entre le niveau d'activité dénitrifiante potentielle et la diversité des deux communautés dénitrifiantes n'a été observée.

Ainsi, l'application en surface de résidus végétaux est potentiellement un déterminant important de l'activité et de la diversité des dénitrifiants du sol, sous réserve que le nitrate et l'humidité ne soient pas limitants. Ce placement en surface de matière organique végétale induit dans le sol une fraction soluble pouvant elle-même stimuler la dénitrification. Par ailleurs, l'amplitude et l'orientation des effets du paillis sur les dénitrifiants apparaissent dépendre significativement de la qualité biochimique du résidu. Ces résultats suggèrent que la pratique du paillis devrait tenir compte des niveaux d'intrants azotés dans le sol et du type de résidu au risque d'accélérer les sorties d'azote du système. Enfin, l'activité de dénitrification au sein même des paillis devrait être prise en compte dans l'évaluation de leur effet sur les dégagements de $N_2O$ au champ.

N.B. 1 article publié, référencé n°30 dans la section V-A

**projet JEA-APN « Jeune Equipe Associée-Agriculture Périurbaine des Niayes », dirigé par Dr. A. Guissé-UCAD (chercheur ISRA associé Dr. Y. Ndour, Sénégal) (2004/2007)**

L'irrigation de certaines zones maraîchères en banlieue dakaroise (zone de Niayes) s'effectue avec des eaux usées non traitées, pratique assez répandue en milieu urbain d'Afrique de l'Ouest. Ces effluents, riches en minéraux tels que N-P et en matières organiques, assurent aussi une forme de fertilisation complémentaire. Dans les Niayes, la nappe phréatique, dont l'eau est aussi utilisée pour l'irrigation, est affleurante. L'objectif était de caractériser l'impact chimique et microbien de cette pratique, notamment à l'échelle de la communauté nitrifiante.

Les eaux usées ont présenté des concentrations élevées en ammonium (108 mg N $l^{-1}$) mais quasiment pas de nitrate (2 mg N $l^{-1}$), à l'inverse des eaux de nappe (1,5 et 350 mg N $l^{-1}$, respectivement). La teneur en ammonium du sol était environ 4 fois supérieure dans les parcelles irriguées avec les effluents par comparaison avec les parcelles irriguées avec l'eau de nappe. De plus, les teneurs en nitrate dans ces sols sous eaux usées étaient cinquante fois plus élevées que celle mesurée dans ces mêmes effluents, et similaires à celles mesurées dans les sols sous eau de nappe. La structure du compartiment bactérien total (DGGE-ADNr 16S) a été spécifiquement impactée par les deux types d'eau, surtout en saison humide. La communauté fonctionnelle des nitrifiants (DGGE-*amoA*) s'est révélée très sensible au mode d'irrigation et aux variations saisonnières. Ce groupe bactérien a été systématiquement favorisé dans les parcelles irriguées par les eaux usées (apport d'ammonium, métabolite de la nitrification) mais il est resté très difficilement détectable par PCR dans les sols irrigués par les eaux de nappe, notamment en saison sèche.

Au final, ce régime d'irrigation induit de fortes modifications du fonctionnement microbiologique du sol, notamment à l'échelle des communautés nitrifiantes. Ce site présente ainsi une double et forte pollution azotée, initiée par l'apport continuel d'ammonium via les eaux usées. L'ammonium est alors nitrifié au cours de son transit au travers de la couche de sable et enrichit en nitrate la nappe phréatique sous jacente laquelle à son tour induit une pollution du sol en nitrate lors de son emploi pour l'irrigation.

N.B. 1 article publié, référencé n°9 dans la section V-A

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## II-E Chargé de recherche 1ère classe – IRD (depuis 2009)
## Etude de l'effet mycorhizosphère en milieu méditerranéen et sub-tropical

| Thèse<br>1998/2001 | Post-doctorat<br>2001/2003 | CR2-IRD<br>2004/2008 | **CR1-IRD<br>2009/2016** |
|---|---|---|---|
| Dynamique spatio-temporelle du microbiote racinaire<br><br>Rhizodéposition | Inoculation de bactéries phytostimulatrices<br><br>Dynamique du microbiote racinaire | Bactéries dénitrifiantes<br><br>Matières organiques | Symbiose mycorhizienne<br><br>Effet mycorhizosphère |

***Activités de recherche réalisées dans l'UMR 113 - Laboratoire des Symbioses Tropicales et Méditerranéennes dépendant de l'UR-IRD 040-Montpellier***

### Contexte :

Les activités de l'UMR-LSTM (IRD-CIRAD-INRA-SupAgro-UM) sont centrées sur l'étude des associations symbiotiques rhizobiennes et mycorhiziennes dans les milieux méditerranéens et tropicaux où ces interactions plantes/microorganismes peuvent être instrumentalisées dans le but d'améliorer les productions agricoles et forestières ou de restaurer des formations végétales dégradées ou en cours de dégradation. Mon programme dans cette Unité est développé au niveau de l'équipe RISA « Réseaux et Interactions Symbiotiques dans les Agro-écosystèmes » dont les priorités thématiques adressent : (1) le rôle tenu par la diversité des acteurs des symbioses plantes-microorganismes et leur interaction dans la stabilité des écosystèmes, (2) l'impact de perturbations d'origine naturelle ou anthropique sur ces processus symbiotiques, et (3) la valorisation des acquis obtenus sur l'écologie des symbioses en milieu naturel pour proposer des scénarios d'ingénierie écologique visant à réamorcer la dynamique des successions végétales de zones dégradées.

Plus particulièrement, les **conditions stressantes** telles que celles qui prévalent dans des agro-écosystèmes méditerranéens et sub-sahéliens (ressource hydrique restreinte, carences minérales/organiques) peuvent favoriser l'émergence de **relations de facilitation** entre certains végétaux (**interactions plantes-plantes positives**) au détriment de la compétition, ce qui leur permet d'accroître mutuellement leur résistance face aux contraintes environnementales (Pugnaire et Luque, 2001; Callaway et al, 2002; Tirado et Pugnaire, 2005; Maestre et al, 2009). Un mécanisme microbien essentiel identifié dans le phénomène de facilitation repose sur l'augmentation du potentiel infectieux mycorhizogène du sol et sur le partage de symbiotes mycorhiziens entre plantes. Ce réseau mycélien en croissance, alimenté en carbone organique par les végétaux partenaires de la symbiose, peut alors stimuler via des émissions d'exsudats organiques le développement des communautés microbiennes éloignées des systèmes racinaires (effet hyphospère) et participer ainsi à la reprise des activités microbiennes dont certaines peuvent être phytostimulatrices. Ce **réseau mycélien extra-matriciel partagé** peut aussi autoriser des **échanges nutritionnels** entre les partenaires végétaux dont les légumineuses, créant ainsi une circulation facilitée de matière organique et de nutriments (C-N-P) (Ingleby et al, 1997; Simard et al, 1997; He et al, 2004). Ces interactions positives plantes/symbiotes

mycorhiziens/bactéries libres participent ainsi à la structuration progressive d'**ilôts de fertilité chimique et microbienne** capables d'accueillir plus favorablement de nouvelles espèces végétales. Le sol s'enrichit graduellement en matière organique (nécromasse racinaire, litière) dont la minéralisation par la biomasse microbienne assure la mise en disponibilité de nouveaux éléments minéraux prélevés dans des horizons plus profonds par les racines des végétaux pionniers. Le développement de divers systèmes racinaires et de leurs réseaux mycéliens, l'augmentation du taux de matière organique améliorent la résistance structurale du sol (notamment face à l'érosion), sa capacité de rétention en eau et en nutriments, sa richesse en microhabitats microbiens et sa diversité microbienne.

J'ai cherché à inscrire mes activités au sein de l'équipe RISA dans le cadre du fonctionnement de communautés végétales soumises à **diverses contraintes abiotiques et biotiques** en explorant dans quelle mesure la **diversité mycorhizienne et microbienne** était modifiée par ces contraintes et les espèces végétales en présence. J'ai ainsi pu commencer à développer ce questionnement au niveau de **trois projets principaux** présentant des associations végétales très variables, deux relatifs à des contraintes biotiques (ANR-Systruf 2010-2014, projet impact microbiologique d'espèces exotiques-modèle majeur *Jatropha curcas* 2010/2014) et un relatif à des contraintes abiotiques fortes (ANR-SyMetal 2011-2015).

Du point de vue de la méthodologie générale, toutes ces activités étaient basées sur des échantillonnages de sol et de racines menés au niveau de sites expérimentaux, agronomiques ou encore naturels. Du point de vue microbiologique, les analyses ont porté sur la diversité microbienne (empreintes moléculaires type DGGE-RISA pour la structure génétique, clonage/séquençage voire pyroséquençage de gènes taxonomiques), l'activité (microrespirométrie, activités enzymatiques, traits PGPR *in vitro*), et la densité (milieux de culture *in vitro* de compositions diverses).

**Projet ANR Systruf (2010/2014)**

**« Bases d'une gestion écologique durable des écosystèmes truffiers producteurs de *Tuber melanosporum* », coordonné par le Pr. M.-A. Selosse (à l'époque à l'UM2, actuellement au Muséum d'Histoire Naturelle)**

Ce projet a porté sur les interactions entre les cortèges floristique et microbien, et la production de truffe noire (*Tuber melanosporum*) dans le cadre écologique de truffières à chênes verts en Région Languedoc-Roussillon. Ce type d'écosystème s'insère dans mes hypothèses de travail dans la mesure où le fonctionnement de l'ectomycorhizosphère liée à *T. melanosporum-Quercus ilex* perturbe considérablement le développement de la strate herbacée et, par voie de conséquence, probablement la vie microbienne du sol truffier (i.e. modification des effets rhizosphère et mycorhizosphère associés à la flore compagne). C'est l'effet du « brûlé » caractérisé par une forte inhibition de croissance de nombreux végétaux aux pieds des chênes mycorhizés par ce champignon ectomycorhizien, dès lors qu'il entame son cycle de reproduction sexuée pour plusieurs années consécutives (**Figure 6**).

**Figure 6.** Matérialisation du "brûlé" autour d'un chêne vert producteur de *Tuber melanosporum*. Certaines espèces végétales (plantes compagnes) parviennent à se maintenir dans la zone influencée par la mycorhizosphère du tandem *T. melanosporum-Quercus ilex*. Leur diversité et leurs cortèges de champignons mycorhiziens à arbuscules ont été étudiés.

Ces phénomènes sont à raccorder, dans notre classification des facteurs environnementaux affectant le fonctionnement de la mycorhizosphère, aux contraintes biotiques en tant qu'éléments perturbateurs des équilibres natifs flore/microflore. Dans ce projet, j'ai été responsable de trois sous-tâches : 3b (Diversité moléculaire des champignons mycorhiziens à arbuscules de la flore compagne), 4a (Analyse microbiologique des sols de brûlés et hors-brûlés) et 7a (Saprophytisme de l'ascocarpe : influence de la continuité mycorhize / ascocarpe ). La sous-tâche 7a, trop éloignée des effets sur la microflore, n'est pas abordée ici. Les échantillonnages de sol et de racines des sous-tâches 3b et 4a ont été réalisés sur 2 années consécutives, 4 truffières (Uzès et Beaulieu - truffières plantées en vergers/ Tourbes et Pézilla - truffières naturelles sur marges forestières, avec 9 chênes sélectionnés par site) et 2 saisons (stade « truffette » printanier et stade maturité hivernale). Les chênes truffiers ont été répartis en 3 statuts figurant le "gradient biotique" induit par le développement de *T. melanosporum* : non producteurs et sans brûlé, non producteurs avec brûlé, producteurs avec brulé.

**Sous-tâches 3b-4a.** Les données de pyroséquençage (diversité bactérienne du sol et mycorhizienne des racines) ont montré de fortes disparités entre échantillons (nombre de séquences brutes et exploitables) induisant des difficultés de robustesse statistique dans les comparaisons. Une approche plus globale est en cours basé sur un redécoupage du cortège statistique (composite de certains statuts, truffières et saisons). En revanche, l'analyse de la structure génétique des communautés fongiques et mycorhiziennes par la technique d'empreinte DGGE a permis de mettre en évidence certaines spécificités de la composition de ces deux communautés microbiennes surtout en fonction de la saison, et dans une moindre mesure selon le statut des chênes (effet site et type communauté microbienne dépendant). L'effet saison a été identifié pour la plus part des arbres

indépendamment de leur statut et pour chacune des deux communautés microbiennes. Seules les communautés mycorhiziennes des sites d'Uzès et de Tourbes n'ont pas pu être distinguées en fonction de la saison, soulignant une dynamique saisonnière des Gloméromycètes site dépendante. De plus, dans le cas du site de Tourbes uniquement, la composition des communautés mycorhiziennes des sols de brûlés producteurs s'est nettement différenciée de celle des sols hors-brûlés, aux deux saisons. L'analyse des communautés bactériennes par DGGE est en cours.

Les analyses comparatives des densités bactériennes cultivables, des profils de diversité catabolique ou encore de la qualité de la matière organique (spectres proche infra-rouge) n'ont pas révélé de disparités systématiques entre les 3 statuts « hors-brûlé, brûlé non-producteur et brûlé producteur », de même que les analyses chimiques classiques du sol (e.g. teneurs C-N-P). Néanmoins, des divergences transitoires entre arbres producteurs (avec et sans brûlés) et arbres non producteurs ont été identifiées sur certaines truffières au cours de la période estivale au niveau de la qualité de la matière organique (**Figure 7**) et de la diversité catabolique (**Figure 8**). Au final avec notre approche statistique et technique, il est apparu que les sols de truffière hors-brûlés, de brûlés non producteurs et producteurs ne présentent de spécificités microbiologiques et chimiques que ponctuelles, site et saison dépendantes.

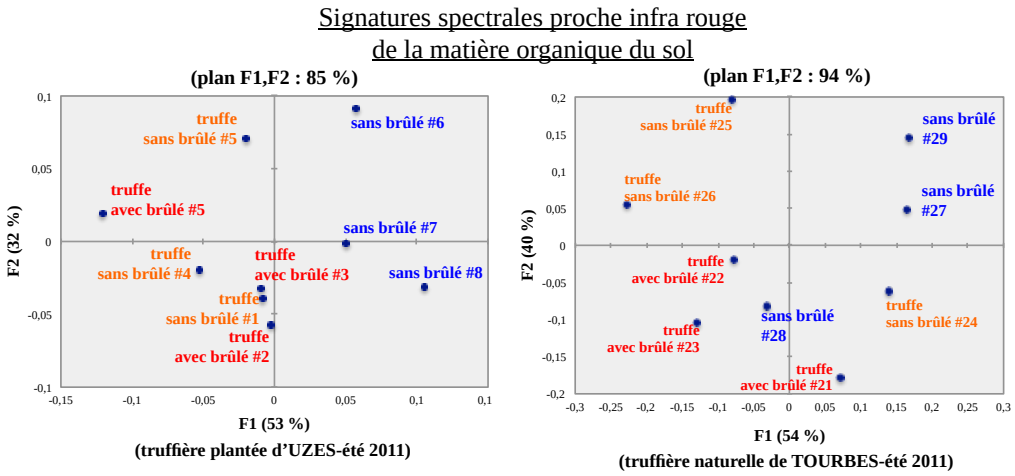


**Figure 7.** Analyses en composantes principales des spectres proche infra rouge de la matière organique des sols truffiers permettant d'opérer une distinction entre les sols des chênes producteurs (rouge et orange) et ceux des chênes non producteurs (bleu) des truffières d'Uzès et de Tourbes en période estivale. #numéro : identification des chênes.

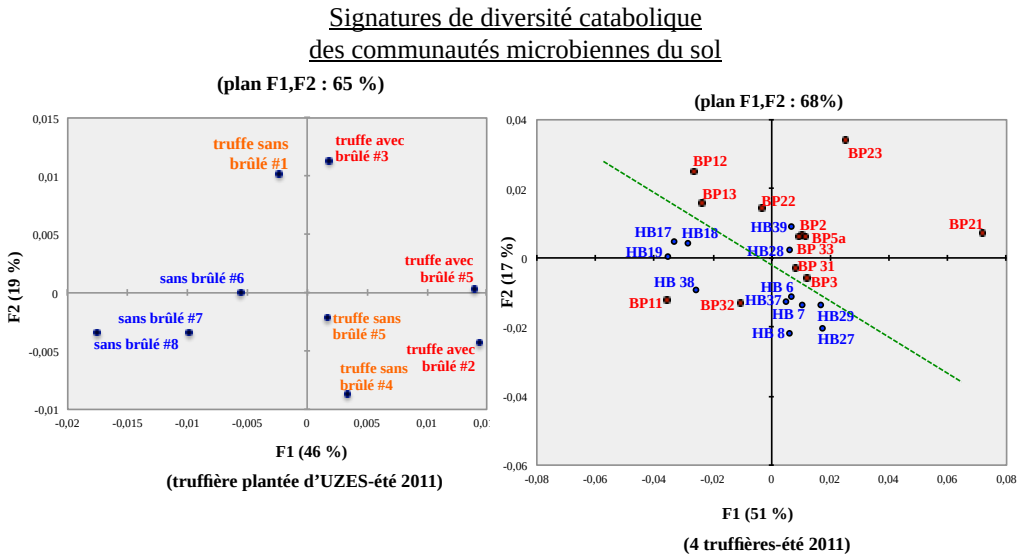


**Figure 8.** Analyses en composantes principales des signatures de diversité catabolique des sols truffiers permettant d'opérer une distinction entre les sols des chênes producteurs (rouge et orange) et ceux des chênes non producteurs (bleu) de la truffière d'Uzès ainsi que des 4 truffières en période estivale. #numéro : identification des chênes ; HB : sans truffe et sans brûlé ; BP : avec truffes et brûlé.

N.B. 1 article en cours de rédaction, voir la section V-A

## Projet ANR CES-SyMetal (2011/2015)

### « Rhizostabilisation de déblais miniers à fortes teneurs en métaux par des plantes métallicoles associées à leurs microorganismes symbiotiques », coordonné par le Dr. J.-C. Cleyet-Marel (DR-INRA au LSTM)

Ce projet a porté sur l'évaluation chimique et microbiologique des interactions entre espèces métallicoles associées, notamment une légumineuse, dans le but de définir le meilleur scénario de phytostabilisation d'un bassin de décantation de déblais miniers en Région Languedoc-Roussillon (Saint Laurent-le-Minier) présentant un profil exceptionnel de contraintes abiotiques (contamination polymétallique Zn-Pb-Cd particulièrement élevée 170-34-1,2 g $kg^{-1}$ respectivement avec pH neutre, et carences minérales/organiques sévères e.g. 1 mg $P_{tot}$ et 0.1 mg $N_{tot}$ $kg^{-1}$). La finalité de la phytostabilisation est de limiter la dispersion des particules solides chargées en métaux lourds dans l'environnement (inflitration, ruissellement, érosion éolienne) par divers mécanismes chimiques liés à la production et à l'accumulation de matières organiques végétales et microbiennes (adsorption, chélation, précipitation, modification de valence des métaux lourds). Dans ce projet, j'ai été co-responsable de 2 livrables : (1) identification du cortège mycorhizien d'écotypes végétaux inféodés aux sites pollués, (2) infuence des scénarios de végétalisation sur le développement du compartiment microbien.

Les scénarios de phytostabilisation ont consisté à transplanter dans le bassin de décantation de jeunes plants de métallophytes en établissant toutes les combinaisons de 1 à 3 espèces, avec deux écotypes

locaux de graminées (*Festuca arvernensis*, *Koeleria vallesiana*) et une légumineuse locale fixatrice d'azote (*Anthyllis vulneraria*) associée à son symbiote autochtone *Mesorhizobium metallidurans*. Les placettes étaient par ailleurs inoculées ou pas avec un mélange de propagules mycorhiziennes allochtones de 5 souches résistantes au stress polymétallique (Symbiom Symbivit REMED®, origine minière). Les témoins ont consisté en des zones non végétalisées. Les analyses ont été menées après un an de croissance sans fertilisation (hormis l'apport initial de compost).

Les couverts les plus productifs étaient ceux composés de 2-3 espèces, notamment l'association des 3 métallophytes (**Figure 9**).


(A)

(B)

**Figure 9.** Vue générale de l'essai de phytostabilisation après un an (A) et placette végétalisée associant *Anthyllis-Koeleria-Festuca* (B).

La réponse de chaque espèce végétale s'est révélée dépendre de la composition floristique du couvert et de la mycorhization. Par exemple, la biomasse aérienne de la fétuque co-cultivée avec *Anthyllis* a été significativement augmentée par rapport à sa monoculture (+32%) lorsque les 2 types de végétaux avaient reçu l'inoculum mycorhizien. En revanche, la biomasse foliaire de l'*Anthyllis* n'a pas été modifiée.

Les variables chimiques des déblais rhizosphériques ont cependant peu évolué sous l'effet du développement végétal, ou de la mycorhization, par rapport au déblai non végétalisé malgré une production de biomasse aérienne conséquente. Les points significatifs ont concerné : une augmentation du pH de 0,2 points (pH 7,6-7,7) quelle que soit la modalité d'implantation végétale, une augmentation du taux de matière organique (+54%) uniquement dans le cas de la fétuque co-cultivée avec la légumineuse.

Du point de vue microbien, les densités totales de bactéries cultivables ont été significativement augmentées d'un point Log, quel que soit le traitement (max. = 6 Log CFU $g^{-1}$ sol) mais la biomasse microbienne (fumigation au chloroforme) n'a été significativement augmentée (+30%) qu'avec la légumineuse mycorhizée (100 vs. 60 mg C/kg sol sec). Les racines de la légumineuse (indépendamment de sa mycorhization) ont été plus aptes à héberger diverses espèces cultivables de *Pseudomonas* fluorescents (**Figure 10**) manifestant des traits PGPR *in vitro* d'ampleur variable (AIA, sidérophores, solubilisation $P_{min}$, activité ACC-déaminase) dont 12 ont été testées *in vitro* sur déblai minier afin d'évaluer leur effet PGPR sur l'*Anthyllis* et une autre légumineuse métallicole, le lotier (*Lotus corniculatus*). L'effet le plus manifeste a concerné la stimulation significative de la biomasse nodulaire du lotier (×2) par certaines souches *Pseudomonas* en mélange.

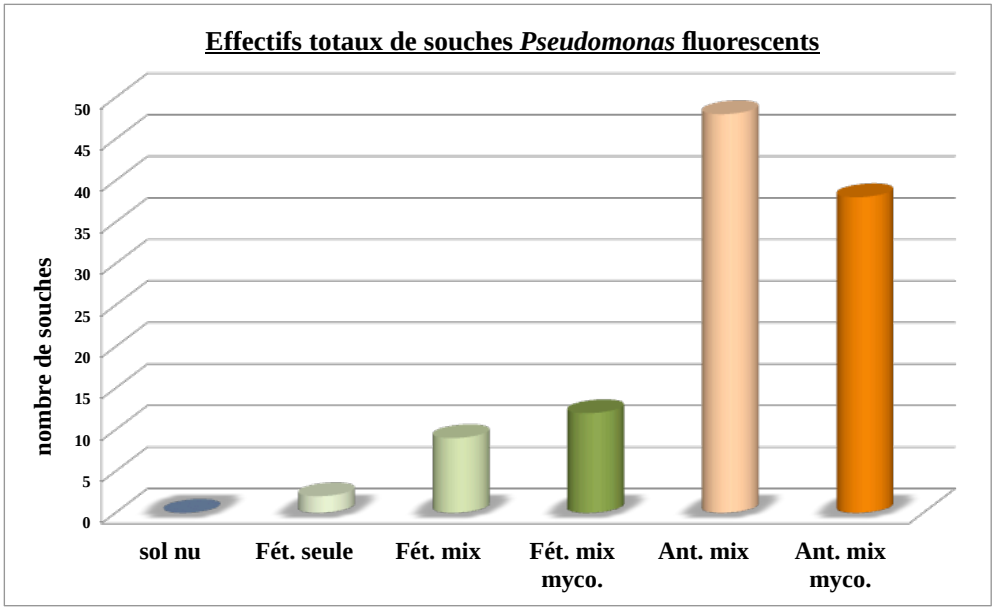


**Figure 10.** Influence de l'espèce de métallophyte sur les effectifs rhizosphériques de *Pseudomonas* producteurs de sidérophores fluorescents. Fét. : fétuque ; Ant. : *Anthyllis* ; mix : association fétuque-*Anthyllis* ; myco. : mycorhization contrôlée REMED.

Ce résultat renforce l'intérêt de recourir à des couverts plurispécifiques intégrant des espèces végétales capables de stimuler des populations fonctionnelles PGPR résidentes. A cet égard, les traits "sidérophores" (carence en fer) et "ACC-déaminase" (stress hormonal éthylène) sont particulièrement intéressants à approfondir en rapport avec la tolérance des métallophytes au stress polymétallique. Par ailleurs, la minéralisation cumulée sur 28 jours de l'azote du déblai rhizosphérique a été significativement augmentée sous la légumineuse (indépendamment de la mycorhization), et dans une moindre mesure sous la fétuque en co-culture uniquement, réaffirmant le rôle majeur des légumineuses dans la dynamique du cycle de l'azote dans les communautés végétales. En revanche, la diversité des aptitudes cataboliques et la minéralisation cumulée du carbone sur 28 jours n'ont pas été affectées. L'analyse des empreintes moléculaires DGGE sur ADN rhizosphériques est en cours (gène *nifH* marqueur de la fixation d'azote, gènes ribosomaux 16S et 18S marqueurs de la taxonomie des bactéries et des fonges).

Du point de vue suivi moléculaire de l'inoculum mycorhizien REMED (5 souches) au niveau des cortèges symbiotiques racinaires (résultats partenaire INRA-Dijon, Dr. D. van Tuinen), une des deux souches de *Rhizophagus* ainsi la souche de *Septoglomus constrictum* n'ont été détectées que dans un seul échantillon. Deux des trois autres souches n’ont pu être détectées un an après leur inoculation. Cependant, la cinquième et dernière souche de l’inoculum de genre *Rhizophagus* a été systématiquement identifiée dans les racines des différentes métallophytes. Ce résultat suggère l'existence d'un réseau mycorhizien partagé par les métallophytes au sein des placettes quelle que soit leur structure. Or, l'expression de la facilitation inter-plantes peut reposer sur le partage entre partenaires végétaux de certains symbiotes mycorhiziens. Ce résultat conforte la possibilité de manipuler par inoculation la composante mycorhizienne des couverts mixtes de métallophytes.

Globalement, la stimulation du compartiment microbien par l'effet (myco)rhizosphère des métallophytes s'est révélée de faible amplitude en dépit d'une production de biomasse aérienne conséquente seulement un an après la mise en place du dispositif. Néanmoins, certains de ces indicateurs microbiens indiquent que la structure du couvert végétal et sa mycorhization peuvent significativement influencer le compartiment microbien. Les interactions entre ces deux paramètres doivent être mieux comprises à l'avenir afin d'optimiser la composition des couverts végétaux et leur implantation microbiologiquement assistée (inoculation PGPR-souches mycorhiziennes).

N.B. 1 article soumis et 1 article en cours de rédaction, voir la section V-A

**Projet «  impact microbiologique d'espèces végétales exotiques » (2010/2014)**

Ce projet développé au travers de la formation doctorale d'Amadou Dieng financée par l'IRD s'est proposé d'établir le diagnostic des modifications microbiologiques du sol, notamment à l'échelle des symbioses mycorhiziennes et rhizobiennes, associées à l'introduction récente de *Jatropha curcas* sur des sols arables au Sénégal dans une optique de production d'agrocarburant (huile des graines). En effet, cette espèce d'euphorbiacée oléifère ligneuse originaire d'Amérique Centrale produit dans tous ses organes (feuilles, racines, écorce, sève...) toute une gamme de composés plus ou moins toxiques à la fois pour les eucaryotes (Homme, bétail, larves d'insectes) que pour les procaryotes *in vitro*. A cet égard, cette espèce est valorisée depuis longtemps dans la pharmacopée traditionnelle et aussi comme haie-vive pour contrôler la divagation des animaux autour des parcelles cultivées. Plus récemment, ses extraits sont étudiés dans d'élaboration d'insecticides. Cependant, cette espèce demeure très peu connue comme en témoigne en premier lieu l'absence de maîtrise de son germplasme. Ce type d'agrosystème répond à mes hypothèses de travail (contrainte biotique) dans la mesure où le développement racinaire de *J. curcas* et de sa litière pourrait perturber la microflore du sol et donc compromettre ses interactions avec le développement de cultures vivrières ultérieures. Des questions d'ordre écotoxicologique se posent face au déploiement de sa monoculture, notamment au détriment de terres arables destinées aux cultures vivrières. Ainsi dans la perspective d'un arrachage des vergers de *J. curcas* une fois leur exploitation terminée, il apparaît légitime d'anticiper si les modifications des communautés microbiennes du sol induites par plusieurs années de monoculture seraient en mesure d'affecter les cultures vivrières qui réintègreront ces surfaces agricoles.

Le projet s'est d'abord attaché à caractériser le compartiment microbien du sol de 3 plantations de jatropha d'âge croissant (1-2-15 ans) et celui de leur parcelle adjacente en jachère (témoins). Dans un deuxième temps, les effets induits sur la croissance de diverses cultures locales (mil, niébé, acacia sénégalais) et sur leur statut symbiotique (cortèges de rhizobiums nodulants et de champignons mycorhiziens à arbuscules) ont été étudiés en faisant croître en serre ces trois espèces dans des sols transférés des plantations de jatropha et de leur parcelle témoin.

La monoculture de *J. curcas* a significativement modifié la structure génétique de la communauté des bactéries fixatrices d'azote du sol, et surtout celle de la communauté fongique totale et davantage encore celle de la communauté des champignons mycorhiziens à arbuscules, par comparaison aux sols témoins (**Figure 11**).

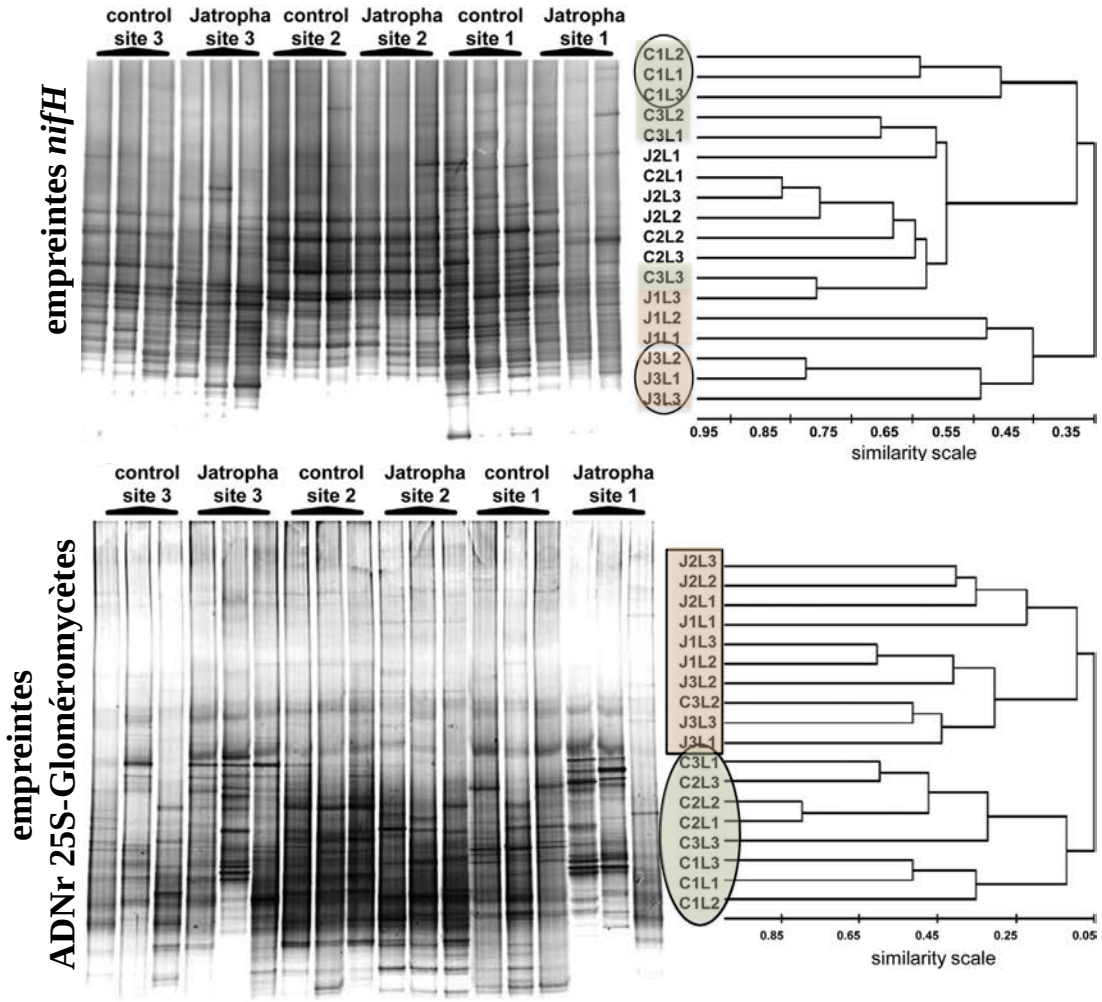


**Figure 11.** Empreintes moléculaires type DGGE, associées à leur analyse par classification hiérarchique, des communautés bactériennes fixatrices d'azote et des communautés de champignons mycorhiziens à arbuscules dans les sols des parcelles agricoles *J. curcas* (surlignage orange) et témoins (surlignage vert).
J1-J3 : parcelles *J. curcas* site1-site3 ; C1-C3 : parcelles témoin site1-site3 ; L1-L3 : lignes intra-parcelles 1-3.

L'ampleur de ces effets ne semble pas corrélée à la durée de la monoculture. La communauté bactérienne totale du sol en revanche n'a pas été particulièrement affectée par cette culture, quelle que soit son ancienneté. La composition taxonomique des cortèges mycorhiziens (Glomeraceae, Gigasporaceae) des 3 espèces végétales cultivées sur les sols issus des parcelles jatropha/témoin (**Figure 12**) a reflété les clivages précédemment observés à l'échelle des empreintes moléculaires de ces sols.

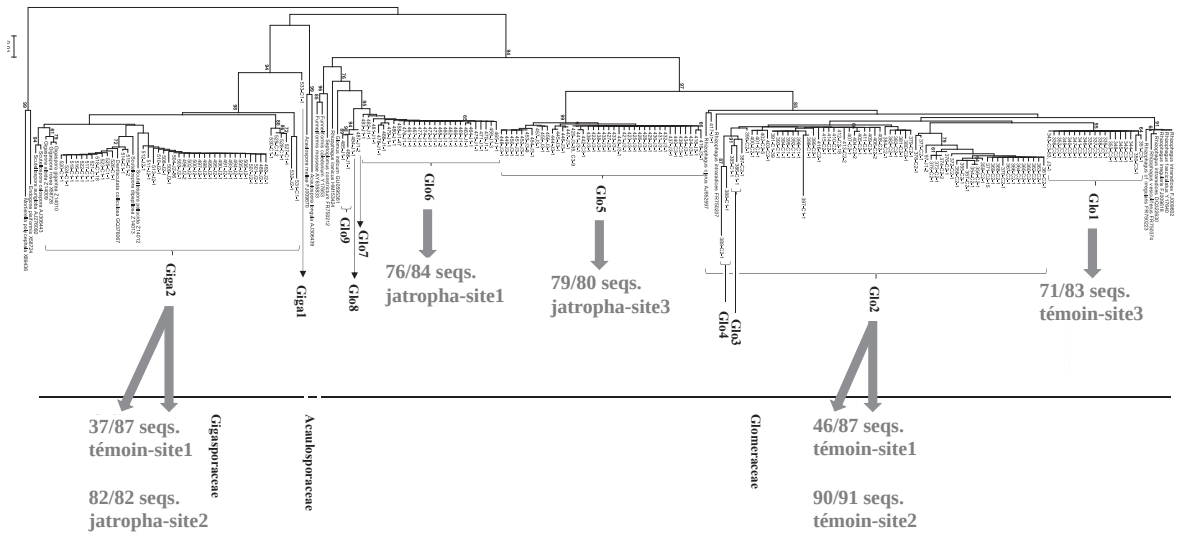


**Figure 12.** Comparaison phylogénétique des séquences d'ADNr 18S représentatives des compositions contrastées des cortèges mycorhiziens du mil (*Pennisetum glaucum*) ayant poussé dans les sols transférés des trois parcelles jatropha et des trois parcelles témoins associées (site1-3). Seqs. : nombre de séquences associées à une origine de sol donnée, dans un phylotype donné.

L'intensité de mycorhization a été augmentée dans de nombreux cas, en particulier avec le mil et le niébé. Les modifications des empreintes *nifH* entre les sols jatropha/témoin ont été sans conséquence sur la diversité taxonomique des rhizobiums nodulant l'acacia et le niébé. Cependant, ces derniers produisaient des nodules significativement moins nombreux et plus gros lorsqu'ils étaient cultivés sur les sols des parcelles jatropha. L'ensemble de ces modifications n'a pas eu d'effet négatif sur la croissance ou les teneurs foliaires N-P des plantes testées, lesquelles étaient plutôt stimulées. Ainsi, les effets négatifs supposés de jatropha sur la microflore du sol et l'établissement des symbioses n'ont pas pu être confirmés. Cependant, le développement de jatropha peut manifestement provoquer des modifications drastiques de la composition des communautés fongiques, en particulier celle des champignons mycorhiziens dont les conséquences fonctionnelles restent à approfondir particulièrement sous des conditions de stress abiotique telle que la disponibilité en eau (non limitante dans ces expérimentations).

N.B. 3 articles publiés, référencés n°28-29-31 et 1 article soumis, voir la section V-A

Références citées :

**Callaway** RM et al (**2002**) Positive interactions among alpine plants increase with stress. *Nature* 417 : 844-847.

**He** X et al (**2004**) Reciprocal N ($^{15}NH_4^+$ or $^{15}NO_3^-$) transfer between non $N_2$-fixing *Eucalyptus maculata* and $N_2$-fixing *Casuarina cunninghamiana* linked by the ectomycorrhizal fungus *Pisolithus* sp. *New Phytologist* 163 : 629-640.

**Ingleby** K et al (**1997**) Mycorrhizas in agroforestry: spread and sharing of arbuscular mycorrhizal fungi between trees and crops: complementary use of molecular and microscopic approaches. *Plant Soil* 294 : 125-136.

**Maestre** FT et al (**2009**) Refining the stress-gradient hypothesis for competition and facilitation in plant communities. *Journal of Ecology* 97 : 199-205.

**Pugnaire** FI, **Luque** MT (**2001**) Changes in plant interactions along a gradient of environmental stress. *Oikos* 93 : 42-49.

**Simard** SW et al (**1997**) Net transfer of carbon between ectomycorrhizal tree species in the field. *Nature* 388 : 579-582.

**Tirado** R, **Pugnaire** FI (**2005**) Community structure and positive interactions in constraining environments. *Oikos* 111 : 437-444.

# III - Progression du projet de recherche

## III-A Trajectoire du projet de recherche

## Etude de l'effet rhizosphère le long du continuum plante individuelle - couvert végétal sous contraintes abiotiques

Au fil de mon parcours (**Figure 13**), j'ai introduit dans le premier modèle d'étude adopté au cours de ma formation doctorale des niveaux supplémentaires d'interaction plantes/microorganismes m'ayant amené à dépasser le cadre de l'effet unilatéral « plante individuelle » sur « bactéries du sol » pour accéder aux interactions entre « communauté de plantes » et « microorganismes libres et symbiotiques » dans divers contextes environnementaux. Les différents niveaux de complexité qui se sont combinés concernent (1) la diversité végétale (de mono à pluri-spécifique), (2) la diversité microbienne (communautés bactériennes puis fongiques totales, puis symbiotes mycorhiziens et rhizobiens et populations bactériennes fonctionnelles, *e.g.* nutrition en azote, en phosphore, en fer, dénitrification) et enfin (3) la contrainte environnementale (nutrition hydro-minérale, essence végétale exotique, contaminants métalliques).

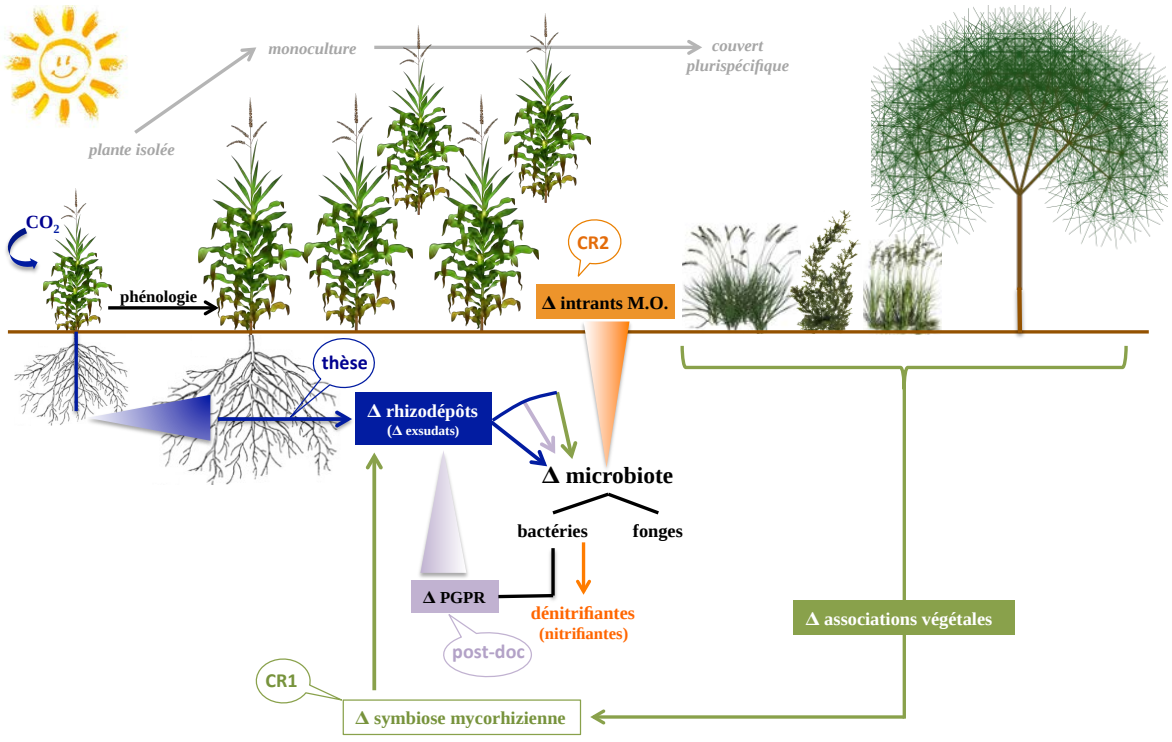


**Figure 13.** Synthèse de mes activités de recherche sur les interactions plantes/communautés microbiennes du sol.

C'est ainsi qu'en thèse j'étudiais, en conditions contrôlées et non stressantes, l'effet unilatéral d'une plante isolée (maïs) sur la structure de la communauté bactérienne totale, notamment via la variation contrôlée de la disponibilité en exsudats.

Au niveau du post-doctorat, j'ai pu introduire un premier niveau d'interaction plante/bactéries en étudiant les effets produits sur la diversité microbienne du sol (bactéries et fonges totaux) par l'inoculation contrôlée d'une souche PGPR unique sur un végétal donné, en conditions contrôlées et non stressantes (blé) mais aussi à l'échelle de la parcelle agricole (monoculture de maïs et stress azoté induit par gradient de fertilisation minérale).

Mes premières activités de chargé de recherche à l'IRD (CR2) m'ont ensuite permis d'aborder la communauté microbienne (étudiée jusqu'alors sans référence à des fonctions) sous l'angle de la diversité fonctionnelle (dénitrification) dans le cadre de pratiques paysannes de fertilisation organique sur diverses monocultures. J'ai pu en particulier caractériser les communautés bactériennes dénitrifiantes en termes d'activité potentielle et de densité/diversité de gènes fonctionnels dénitrifiants.

Depuis le passage CR1, j'ai réorienté l'approche de la diversité fonctionnelle bactérienne vers la mise en disponibilité des minéraux (fixation libre et symbiotique de l'azote, solubilisation du phosphore minéral, séquestration du fer) et l'activité minéralisatrice (respirométrie $CO_2$). Enfin, j'ai introduit la mise en réseau des acteurs végétaux et microbiens par une ouverture sur la diversité des symbiotes mycorhiziens dans des formations végétales naturelles, agroforestières ou encore recomposées, soit pour la première fois dans mon parcours, plurispécifiques.

## III-B Perspectives

### III-B-1 Valoriser le microbiote rhizosphérique par la gestion du couvert végétal sous contraintes abiotiques

#### Contexte et objectifs

Mes travaux depuis la thèse ont largement abordé l'effet rhizosphère et mycorhizosphère dans différents contextes agronomiques et environnementaux, avec dans la dernière période de mes activités des projets incluant plus fréquemment des couverts végétaux plurispécifiques. Je souhaiterais placer mes prochaines études des communautés microbiennes du sol dans le prolongement de cette évolution vers les **communautés végétales mixtes raisonnées,** incluant le groupe des **légumineuses**, sous **contraintes abiotiques fortes**, en focalisant la méthodologie d'observation de la **diversité microbienne fonctionnelle** sur ses conséquences en termes de **phytostimulation**. Le but est dans un premier temps d'identifier pour un écosystème et une contrainte donnés, une/des combinaison(s) floristique(s) permettant d'**améliorer la productivité du couvert** et de déterminer par comparaison avec les combinaisons moins performantes quelles sont les **attributs spécifiques de l'effet mycorhizosphère** associé au(x) couvert(s) le(s) plus performant(s). Dans une deuxième **phase mécanistique**, les **microbiotes rhizosphériques** (ou certaines de leurs composantes) issus de ces différents scénarios seront comparés pour leur **capacité à stimuler le développement végétal**. Cette phase mécanistique pourra être connectée à des **études appliquées** (**inoculums** bactériens). C'est donc

globalement une perspective d'**ingénierie du microbiote racinaire** par raisonnement de la **diversité végétale**.

L'accroissement de la richesse spécifique végétale est généralement reconnu pour conditionner l'augmentation de la productivité primaire (e.g. écosystèmes prairiaux), voire des rendements (systèmes de polycultures et "overyields") jusqu'à un certain point d'inflexion au delà duquel la tendance voire s'inverse (e.g. Schnitzer et al, 2011). Dans le cas du projet SyMetal (Rhizostabilisation de déblais miniers à fortes teneurs en métaux par des plantes métallicoles associées à leurs microorganismes symbiotiques), les placettes végétalisées par 2 graminées (*Festuca-Koeleria*) et une légumineuse (*Anthyllis*) étaient à ce titre exemplaires en terme d'augmentation de la productivité par comparaison aux autres combinaisons floristiques. Divers mécanismes non mutuellement exclusifs sont à l'oeuvre dans des communautés végétales mixtes. Ils incluent la complémentarité de niches entre systèmes racinaires, éventuellement la présence de rares espèces très productives (prairies) mais aussi la facilitation inter-plantes. Comme déjà évoqué, cette facilitation peut impliquer les réseaux mycorhiziens et leurs ramifications entre diverses composantes du couvert végétal, justifiant ici le recours aux communautés végétales mixtes pour stimuler la symbiose mycorhizienne et ses effets sur le microbiote. A cet égard, le fait d'implanter ces communautés végétales mixtes sous des contraintes environnementales fortes peut favoriser le développement du processus de facilitation.

Le choix des légumineuses comme composante majeure des peuplements végétaux repose ici essentiellement sur leur aptitude à introduire de l'azote combiné dans le sol et sur la stimulation du potentiel infectieux mycorhizogène du sol généralement plus élevé avec ce groupe végétal fonctionnel. Ces deux propriétés sont irréductiblement liées à la fertilité du sol, notamment celle de systèmes à faibles niveaux d'intrants ou naturellement contraints par la faible disponibilité en ressources hydro-minérales. Depuis quelques années, les avantages des légumineuses sont d'ailleurs re-examinés au travers des pratiques agroécologiques, des systèmes de cultures associées et de l'intensification écologique. Ces avantages peuvent aussi inclure des aspects liés à l'amélioration de l'état sanitaire global des communautés végétales mixtes. Par ailleurs, l'azote demeure un élément minéral essentiel pour le fonctionnement de la biomasse microbienne.

Au delà de l'inscription de cette démarche dans le périmètre scientifique du LSTM pour le projet quinquennal en cours (voir ci-dessous), il convient aussi de l'inscrire dans le contexte écologique des interactions plantes/microorganismes au sens le plus large. La littérature récente confirme que la **structure des communautés végétales** (richesse spécifique et abondance relative, distribution spatiale), leur **productivité** et leur stabilité sont conditionnées de manière déterminante par les **interactions** qui s'établissent **entre les végétaux et les communautés microbiennes du sol**, notamment les **champignons mycorhiziens** (van der Heijden et al, 1998, 2008; Johansson et al, 2004; Maherali et Klironomos 2007; Gianinazzi et al, 2010; Schnitzer et al, 2011). Les types de microorganismes interagissant avec les plantes dépassent d'ailleurs l'opposition classique entre agents

pathogènes et symbiotiques. Ainsi tous les végétaux hébergent dans leurs tissus une certaine diversité d'endophytes fongiques et bactériens dont les rôle restent ambigus (Koide et Mosse, 2004; Vandenkoornhuyse et al, 2007; Rodriguez et al, 2009; Reinhold-Hurek et Hurek, 2011). Les végétaux conditionnent par ailleurs fortement le développement du compartiment microbien du sol dont la biomasse peut s'évaluer à plusieurs centaines de kg de carbone par hectare, ce qui souligne son rôle stratégique dans les cycles biogéochimiques (Eisenhauer et al, 2010). **Il apparaît donc écologiquement illégitime d'agir sur les écosystèmes terrestres sans prendre en compte et essayer d'orienter les principales interactions établies entre la flore et la microflore du sol.**

## Schématisation de la démarche expérimentale (**Figure 14**)

La démarche consistera à étudier dans quelle mesure la manipulation de la structure du couvert végétal (nombre et identité des espèces en présence dont les légumineuses, densités relatives entre espèces, agencement spatial) soumis à un ou deux facteurs limitants pour sa productivité peut : (1) améliorer cette productivité, (2) modifier le microbiote du sol et notamment (3) augmenter son potentiel phytostimulateur.

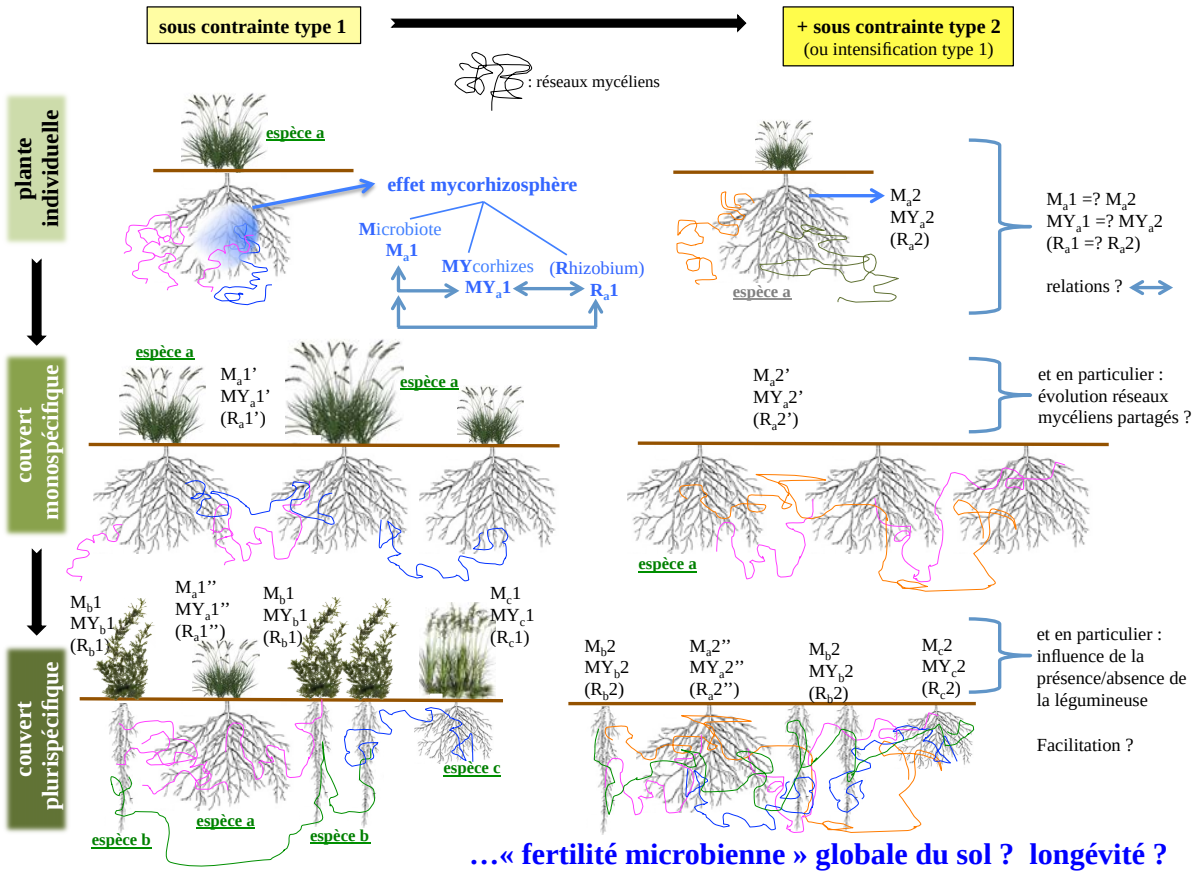


**Figure 14.** Effets de la structure du couvert végétal et de la contrainte environnementale sur le microbiote rhizosphérique et sa potentialité de phytostimulation.

Ce type d'approche peut permettre de répondre à toute une série de questions génériques dans l'orientation des interactions plantes/microorganismes du sol parmi lesquelles :

- dans quelles proportions varie l'effet mycorhizosphère sous l'effet de l'intensification d'une contrainte (ex. **Fig. 14** : $M_a1$ =? $M_a2$) ? et dans quelle mesure le rapport entre diversité du microbiote racinaire et diversité du cortège mycorhizien est-elle impactée (relations $M_a \Leftrightarrow MY_a$ sous contraintes 1 et 2) ?
- à contrainte constante, la composition de la communauté végétale elle-même peut-elle influencer l'effet mycorhizosphère de l'espèce "a" (ex. $M_a1$ =? $M_a1'$ =? $M_a1''$) ? et la variation de l'effet mycorhizosphère associé à une espèce "a" suite à l'augmentation d'une contrainte peut-elle différer selon la structure du peuplement végétal dans lequel elle est incluse ($M_a2$ =? $M_a2'$ =? $M_a2''$) ?
- après récolte et exportation des parties aériennes, dans quel cas de figure le sol est-il le plus bénéfique pour la croissance de la plante, en particulier du point de vue de ses caractéristiques microbiologiques ? comment évolue cette fertilité microbienne selon que le couvert végétal est "artificiellement" invariant (ex. monoculture ou polyculture répétée) ou s'il est modifié (ex. rotation) ?
- la résistance et la résilience de certaines fonctions assurées par la communauté microbienne du sol sont-elles influençables par la diversité floristique ? quel que soit le niveau de contrainte ?
- si l'augmentation d'une contrainte affecte le microbiote racinaire d'une espèce "a", l'augmentation de la diversité du couvert végétal peut-elle contrer cet impact négatif à l'échelle de la même espèce ($M_a2''$ >? $M_a2'$ >? $M_a2$) ?
- si des relations de facilitation sont opérantes sous une diversité végétale donnée, peuvent-elles être maintenues, ou augmentées, ou amoindries si la diversité végétale est augmentée ?...

En cas d'augmentation des propriétés phytostimulatrices du microbiote et au-delà de cette démarche, toute une série d'autres questions relatives à la stabilité du phénomène s'imposeront immédiatement : quelle est la longévité ou l'héritabilité de cette phytostimulation accrue (à l'instar des sols résistants au phénomène de la fatigue des sols) ? en cas d'héritabilité fonctionnelle, celle-ci est-elle associée à une stabilité structurale de la communauté microbienne du sol dans son ensemble ou d'une partie (association stable de divers motifs taxonomiques) ? cette phytostimulation accrue peut-elle s'exprimer avec d'autres associations végétales que celle qui a présidé à son émergence (niveau de spécificité des interactions plantes/microorganismes) ?...

### Ecosystèmes ciblés dans le projet de recherche

Ces communautés végétales mixtes seront mises en place dans **deux contextes écologiques distincts** ayant **en commun** la **contrainte minérale et hydrique** en milieu méditerranéen ou sub-tropical :

**(1)** les **systèmes agricoles** à bas intrants ou sur terres peu fertiles, type **polyculture**

**(2)** les **friches minières** éligibles à des opérations de phytomanagement telles que la **phytostabilisation**

Dans le premier cas, le choix des espèces végétales mises en présence sera naturellement lié aux possibilités ou impératifs agronomiques locaux. La diversité pourra aussi se définir au sein d'une espèce entre cultivars anciens ou récents. Dans le second cas, la disponibilité en semences d'espèces métallicoles endémiques ou allochtones décidera des associations envisageables, tout en favorisant si possible les espèces à phénotype d'exclusion des métaux lourds dans les parties aériennes. Par ailleurs, le choix des légumineuses métallophytes sera aussi dépendant de la maîtrise de leur nodulation par des rhizobiums compatibles et tolérants au stress polymétallique envisagé. Le cas des friches minières implique *de facto* une contrainte abiotique supplémentaire en l'espèce des concentrations élevées en métaux lourds et métalloïdes (souvent : zinc, cadmium, plomb, aluminium). C'est avec ce type de système où la contrainte abiotique globale est la plus forte que je favoriserai mes interventions, avec cet *a priori* que la résultante des interactions plantes/microorganismes et les relations de facilitation inter-plantes seraient sinon exacerbées, du moins plus évidentes à observer par rapport à un milieu moins hostile pour la flore et la microflore. Du point de vue des métaux lourds, ces études ne viseront pas à "phytoremédier" des sols contaminés ou à en "phytoextraire" des métaux lourds mais à fixer ces polluants par phytostabilisation microbiologiquement assistée. Néanmoins, d'autres compétences externes pourront se rattacher à cette démarche, comme dans le cas du projet SyMetal, afin de caractériser l'ampleur des modifications physico-chimiques induites par les diverses communautés végétales et leur cortège microbien, en particulier en termes de disponibilité/biodisponibilité et de mobilité des métaux lourds.

### Méthodologie d'étude du microbiote rhizosphérique et de son potentiel phytostimulateur

L'évaluation spécifique de la "fertilité microbienne" du sol peut s'avérer complexe à isoler rigoureusement d'autres phénomènes induits par le développement d'un couvert végétal, notamment les aspects structuraux et chimiques qui entrent en interaction directe avec le fonctionnement de la microflore et la croissance végétale.

Ce potentiel phytostimulateur sera appréhendé **indirectement** par le suivi de divers indicateurs microbiologiques classiques liés par la plus part aux cycles de la matière, sous le postulat qu'une augmentation de ces indicateurs microbiologiques traduit un meilleur fonctionnement des cycles biogéochimiques et donc à terme une meilleure disponibilité en minéraux pour le végétal. A titre d'exemples :

- traits PGPR sur la fraction cultivable (solubilisation $P_{min.}$, sidérophores, et signalisation hormonale type auxines et ACC-déaminase),
- développement d'un volet exploratoire très récent relatif aux effets phytostimulateurs des composés organiques volatiles bactériens (fraction cultivable) en qualité de trait PGPR innovant,
- densité (qPCR)/diversité (clonage, NGS) de gènes bactériens marqueurs de fonctions impliquées dans les cycles C-N-P tels que *nifH*-fixation $N_2$ et *chiA*-dégradation chitine, ou encore *amoA* (nitrification)...,
- mesures métaboliques et enzymatiques directes sur sol liées aux cycles C-N-P (minéralisation matière organique végétale ou cellulose-$CO_2$, diversité catabolique-$CO_2$, fixation $N_2$ par ARA, activités uréase, chitinase...) ; ce volet analytique peut aussi être réalisé en imposant un stress abiotique au sol (ex. pH-température-humidité) afin de mesurer en plus la résistance et la résilience fonctionnelle des communautés microbiennes,

et **directement (approche mécanistique)** par inoculation du microbiote échantillonné dans les placettes de végétalisation sur le (s) même(s) végétal(aux) hôte(s), en utilisant préférentiellement comme substrat de culture le sol témoin non modifié par l'installation des couverts végétaux (sol stérilisé/non stérilisé) et/ou en prenant un substrat neutre stérile (e.g. vermiculite, attapulgite, sable). Ici, le microbiote est assimilé au sol rhizosphérique qui l'héberge. Il peut donc comporter des éléments nutritionnels et des propagules fongiques et mycorhiziennes. Le microbiote pourra être celui d'une espèce végétale au sein du couvert (échantillon composite) ou le microbiote "moyen" du couvert (mélange des sols rhizosphériques représentatifs des proportions des différentes espèces végétales en présence). Un couplage à la stérilisation du sol-microbiote sera aussi nécessaire pour essayer de dégager l'effet microbiote de l'effet chimique du sol qui le contient[*]. Le but final de ces opérations d'isolement du microbiote à des fins d'inoculation est d'observer si les microbiotes issus des communautés végétales performantes induisent une phytostimulation des végétaux inoculés (biomasse, teneurs N-P-chlorophylle, statut symbiotique) supérieure à celle observée avec les microbiotes issus des communautés végétales moins vigoureuses. D'autres modalités d'inoculation peuvent être envisagées en se basant sur l'extraction d'une partie de la microflore du sol rhizosphérique par exemple par suspension dans l'eau (centrifugation du surnageant, lavages du culot, remise en suspension) ce qui pourrait permettre de réduire considérablement l'interférence avec les nutriments du sol. Ces deux approches d'inoculation globale se prêtent également à des effets de dilution du microbiote initial et donc de sa diversité fonctionnelle et de son potentiel phytostimulateur. Pour être pleinement informatives, ces expérimentations pourront être prolongées par une caractérisation des microbiotes rhizosphériques initiés par les inoculations, notamment dans le cas des dilutions. En effet, la comparaison taxonomique des microbiotes racinaires le long du gradient de dilution, en association

avec la biométrie végétale, pourrait permettre au minimum d'identifier (1) des associations de motifs taxonomiques dominants (fortes dilutions) ne soutenant pas la phytostimulation, (2) les proportions relatives entre groupes taxonomiques compatibles avec un certain niveau de phytostimulation. Dans cette perspective, il est supposé que la dilution du microbiote induit à partir d'un certain degré un clivage net des performances végétales.

*l'effet de la stérilisation du sol pour séparer les effets chimiques des effets microbiens peut être envisagé. Cependant, les biais pouvant être anticipés sont liés à l'altération d'une partie de la matière organique par le processus de stérilisation (autoclave) mais aussi à l'élimination des éventuels microorganismes phytopathogènes. Ainsi, une matière organique en partie hydrolysée par autoclave peut augmenter la disponibilité en nutriments qui bénéficieront à la nutrition d'un végétal n'ayant plus par ailleurs à lutter contre d'éventuels agents phytopathogènes ou autres parasites. Aussi avec un sol stérilisé, la croissance végétale pourrait s'avérer supérieure à celle mesurée avec un sol non stérilisé, conduisant à penser que l'effet de la manipulation du couvert végétal a surtout produit des effets de fertilité chimique.

En parallèle à ces mesures fonctionnelles directes et indirectes, il sera aussi envisageable de recourir à l'utilisation de puces ADN (e.g. Geo Chip 3.0, 150 fonctions ciblées) pour caractériser génétiquement le microbiote au niveau de gènes fonctionnels sans *a priori*, ce qui pourrait permettre d'identifier des fonctions significativement et spécifiquement exprimées selon le contexte floristique.

### Etudes appliquées associées au projet de recherche

Quel que soit l'écosystème envisagé, des études appliquées pourront être inscrites en aval de la démarche scientifique. Elles concerneront la sélection de souches bactériennes cultivables en tant qu'inoculums destinés au semis. Ces études seront donc destinées à valoriser certains résultats de caractérisation des propriétés phytostimulatrices du microbiote rhizosphérique. A cet égard, le LSTM dispose d'une plateforme *Transferts et Innovations* d'acceuil des activités en lien contractuel avec les entreprises privées (thèses Cifre, post-doctorat, CDD) qui pourra permettre d'associer des acteurs économiques au développement d'inoculants microbiens ou de certains de leurs métabolites.

## III-B-2 Interdisciplinarité du projet

En plus des compétences techniques et méthodologiques acquises au fil de mon parcours, l'étude des fonctions et propriétés du microbiote rhizosphérique fera appel à des compétences externes liées à l'agronomie et l'agro-écologie (systèmes de polyculture, interculture, évolution physico-chimique du sol) et à la botanique (espèces métallicoles). D'autres compétences déjà présentes au LSTM, voire dans l'équipe RISA, mais aussi mobilisables au travers de collaborations externes, seront requises dans le domaine des technologies d'écologie moléculaire (métatranscriptomique, métagénomique, puce ADN) afin d'obtenir les données relatives à la diversité taxonomique et fonctionnelle du microbiote et des symbiotes mycorhiziens. Le recours aux techniques de mesure d'abondance isotopique naturelle ($^{15}N$) pourra être ponctuellement nécessaire dans le cas de l'étude des communautés diazotrophes.

### III-B-3 Positionnement du projet dans l'UMR-LSTM

Mes perspectives participent à la logique du projet quinquennal de mon équipe actuelle RISA (Réseaux et Interactions Symbiotiques dans les éco-Agrosystèmes) qui concerne l'étude du rôle des communautés symbiotiques (hôtes végétaux et leurs microsymbiotes) dans la restauration d'agro-écosystèmes dégradés (résilience) ou dans la recherche d'une durabilité/résistance accrue face à l'augmentation de contraintes abiotique (e.g. stress hydrominéral) ou biotique (e.g. espèce végétale invasive) induite par les changements climatiques en s'appuyant sur l'identification et la gestion des ressources végétales et microbiennes locales.

" *Le projet de l'équipe s'appuie sur le constat que les symbioses et leurs interactions avec la microflore associée constituent des liens essentiels entre unités fonctionnelles*[*] *et que la diversité et la spécificité de ces liens assurent la productivité et la stabilité des éco-agrosystèmes.* "

[*]Unité fonctionnelle : plante en association avec sa communauté microsymbiotique et son cortège microbien.

Mes perspectives s'inscrivent donc précisément au niveau de l'effet mycorhizosphère de communautés végétales mixtes sur la communauté microbienne du sol (microbiote) qui fait partie des processus identifiés par l'équipe. C'est dans ce cadre que j'étudierai la dynamique du potentiel phytostimulateur des communautés bactériennes rhizosphériques. Ces activités seront à raisonner dès que possible en lien avec le suivi de la diversité taxonomique du cortège mycorhizien afin d'observer la nature du lien existant entre diversité mycorhizienne d'un couvert végétal soumis à diverses contraintes d'une part, et certains attributs du microbiote racinaire d'autre part. Cette connexion des deux jeux de données pourrait contribuer à compléter la définition de la diversité fonctionnelle mycorhizienne et des réseaux mycorhiziens inter-plantes. L'analyse de cette diversité mycorhizienne par les outils NGS se développant au sein de l'équipe, avec en particulier 2 chercheurs ressources dans ce domaine, cette opportunité de mieux analyser le lien communautés bactériennes/mycorhiziennes dans des communautés végétales mixtes sous contraintes est réaliste et souhaitable et devrait permettre d'ajouter une pertinence supplémentaire aux futures réponses aux appels d'offre. Enfin, la valorisation de cette approche dans le cadre particulier de sites miniers à "phytostabiliser" permettra de renforcer le lien avec l'axe 2 de l'équipe MASCE actuelle initié depuis le projet ANR-SyMetal et toujours actif (cf. projet ADEME).

Du point de vue de la stratégie au Sud, je favoriserai pour la zone tropicale/sub-tropicale les études en lien avec l'Afrique de l'Ouest, et avec le Sénégal en particulier, du fait de liens historiques forts et productifs et de l'intérêt d'une communauté scientifique locale bien structurée pour les légumineuses et

pour la durabilité de la production agricole face à des contraintes édapho-climatiques permanentes (Unité LCM associée au LSTM, 2 LMI-IRD LAPSE et IESOL, ISRA).

Du point de vue des écosystèmes méditerranéens, je n'envisage pas de déploiement particulier et systématique sur la zone Maghreb, surtout du point de vue des dispositifs expérimentaux. A cet égard, je souhaite favoriser des sites locaux (Région Languedoc Roussillon) pour des raisons logistiques. Ainsi, le Maghreb sera concerné dans mon projet davantage par le volet formation.

J'envisage également d'ouvrir une réflexion en direction de l'Asie (missions longues durées ou expatriation envisageables) où l'aspect PGPR et donc la phytostimulation sont identifiés comme perspectives à court terme. Cette ouverture sur l'Asie est par ailleurs en cours de maturation au sein du LSTM, principalement au travers des activités de l'équipe MSLT (Mécanismes Symbiotiques chez les Légumineuses Tropicales, animation Eric Giraud-DR-IRD) avec le Vietnam, l'Inde et la Thaïlande. Plus particulièrement, les activités du LMI Rice-2 basé au Vietnam dont le LSTM est partenaire (co-dir. Pr. M. Lebrun), pourraient offrir un cadre d'étude à ma démarche à l'échelle d'agrosystèmes de riziculture. En effet, l'étude de la variation des déterminants génétiques de l'architecture des systèmes racinaires du riz (comparaison inter-variétés) en lien avec les stress abiotiques (eau, salinité), pourrait être prolongée à l'échelle des microbiotes racinaires en vue d'évaluer leur contribution dans la résistance aux contraintes abiotiques. Ainsi, une partie de mon projet pourrait alimenter la réflexion collective sur l'implication du LSTM en Asie.

Les principales UMR françaises avec lesquelles collaborer pour consolider ce projet (antériorité des relations, convergence thématique partielle ou partage d'objets d'étude) sont l'UMR Agroécologie de Dijon, l'UMR Ecologie Microbienne de Lyon et l'UMR Eco&Sols de Montpellier.

### III-B-4 Positionnement du projet en rapport avec les orientations générales de la tutelle IRD

L'un des piliers des actions de l'IRD est " de préserver l'environnement et les ressources dans la perspective d'atteindre les Objectifs du millénaire pour le développement [...] en traitant de questions cruciales pour les Suds telles que les changements climatiques, les écosystèmes tropicaux et méditerranéens. "

Ainsi :

" Au cours des prochaines années, les grands enjeux de la recherche pour le développement vont se concentrer sur trois domaines interdépendants : les conséquences du changement climatique, les dynamiques démographiques et la mondialisation. "

" Il s'agira aussi d'orienter la politique scientifique de l'Institut sur des thématiques cibles, considérées comme prioritaires par l'ensemble des communautés scientifiques et les institutions internationales au Nord comme au Sud. Ces thématiques s'inscrivent dans les grands domaines que sont la société, la santé, l'environnement et les ressources. "

Enfin :

" Cette politique s'accompagnera d'une réaffirmation des priorités géographiques au profit de l'Afrique subsaharienne et des pays du pourtour méditerranéen. Les événements majeurs et les instabilités récentes dans ces zones appellent l'attention et nécessitent des adaptations évidentes des modes d'action. "

et

" Les caractéristiques du Sud sont diverses. Quatre catégories principales peuvent être identifiées et à chacune d'entre elles peuvent être associés des mécanismes d'intervention différents :

**1** Les pays les moins avancés (Afrique subsaharienne), où les efforts devront se concentrer pour soutenir la formation diplômante et la recherche en favorisant une meilleure coordination avec les autres acteurs français, européens et du Sud

**2** Les pays à revenus intermédiaires (Méditerranée et Asie du Sud-Est), auxquels seront associés des modes d'intervention adaptés à leur niveau de développement et la qualité de leurs institutions scientifiques "

Mes perspectives sont en accord avec l'orientation stratégique générale de l'IRD au niveau de la préservation des ressources, de la sécurité alimentaire et de l'environnement. Elles devront cependant tenir compte des contrastes existant entre les Suds, notamment dans le partage (volets expérimentation et sites, formation, expatriation) et l'orientation de mes activités entre l'Afrique Occidentale (Sénégal), le Maghreb, et l'Asie (Thaïlande *a priori*).

Références citées :


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**Duponnois** R (**2006**) Mycorrhiza helper bacteria: their ecological impact in mycorrhizal symbiosis. In: « Handbook of Microbial Biofertilizers » ; Rai M.K. (ed), pp 117-136, Haworth Press, USA.

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**Nara** K (**2006a**) Ectomycorrhizal networks and seedling establishment during early primary succession. *New Phytologist* 169 : 169-178.
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**Requena** N et al (**2001**) Management of indigenous plant-microbes symbioses aids restoration of desertified ecosystems. *Applied Environnmental Microbiology* 67 : 495-498.
**Rodriguez** et al (**2009**) Fungal endophytes: diversity and functional roles. *New Phytologist* 182 : 314-330.
**Schnitzer** SA et al (**2011**) Soil microbes drive the classic plant diversity-productivity pattern. *Ecology* 92 : 296-303.
**Simard** SW et al (**1997**) Net transfer of carbon between ectomycorrhizal tree species in the field. *Nature* 388 : 579-582.
**Tirado** R, **Pugnaire** FI (**2005**) Community structure and positive interactions in constraining environments. *Oikos* 111 : 437-444.
**Vandenkoornhuyse** P et al (**2007**) Active root-inhabiting microbes identified by rapid incorporation of plant-derived carbon into RNA. *Proceedings of the National Academy of Sciences* 104 : 16970-16975.
**van der Heijden** MGA et al (**1998**) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature* 396 : 69-72.
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# IV - Activités de formation universitaire et collaborations

## IV-A Formations universitaires

### IV-A-1 niveaux L3-M1-M2

**Niveau collège (classe de 3ème)** : séquence d'observation en milieu professionnel (5 jours)
Antonin **Berger** (**2014**), Collège François Villon-Saint Gély-du-Fesc
Léo **Nouvelle** (**2012**), Collège Pic Saint Loup-Saint Clément-de-Rivière

**Niveau L3** :
Damien **Cecillon** (**2013**), Univ. Montpellier II, projet européen LegumeFutures « Incidence de l'interculture *Crotalaria juncea* sur la structure génétique des communautés bactériennes et fongiques totales du sol »
Thibaud **Darre** (**2013**), Univ. Montpellier II, projet européen LegumeFutures «« Incidence de l'interculture *Crotalaria juncea* sur la structure génétique des communautés bactériennes et fongiques totales du sol »
Natalja **Kulagina** (**2012**), Univ. Montpellier II, projet européen LegumeFutures « Incidence de l'interculture *Crotalaria juncea* sur la diversité catabolique du compartiment microbien et le potentiel infectieux mycorhizogène du sol »

**Assistante Ingénieur IRD** :
Julie Bourillon : (fév. **2012**-fév. **2013**) responsable de sa formation en interne à la microrespirométrie et à l'écologie moléculaire (extraction ADN) et membre du jury de recrutement dans le cadre de son année de stage fonctionnaire pour un recrutement en qualité d'assistante ingénieur-IRD (agent titularisé)

**Niveau M1** :
Antoine Floury (**2012**), Univ. Montpellier II, projet impact environnemental de *Jatropha* « Impact de la culture de l'agrocarburant *Jatropha curcas* sur les populations de rhizobium du sol : effet sur la diversité du cortège nodulant l'acacia (*Acacia seyal*) et le niébé (*Vigna unguiculata*) »
Rana Haidar (**2011**), Univ. Montpellier II, projet ANR Systruf « Incidence de la mycorhization du chêne vert *Quercus ilex* par la truffe noire *Tuber melanosporum* sur les densités cultivables de pseudomonades du sol »

**Niveau ingénieur (stage 3ème année)** :
Arnaud Chamoin (**2006**), Univ. Technique de Compiègne, projet MUTEN « Impact de la fraction organique soluble de résidus de culture sur la dynamique des dénitrifiants d'un sol agricole »

**Niveau M2** :
Rima Benguesmia Chadly (en tant qu'appui) (Univ. d'Oran Es-Senia), Algérie (**2010/2011**) projet TASSILI « Etude de la diversité moléculaire des mycorhizes à arbuscules le long d'une séquence de dégradation d'une formation végétale dunaire »
Kaoutar Chaafi (en tant qu'appui) (Univ. Cadi Ayyad de Marrakech), Maroc (**2009**) projet Aires-Sud « Diversité moléculaire du cortège racinaire mycorhizien de plants d'*Acacia raddiana* planté sur des sols issus de la strate herbacée et arbustive pionnière »
Amadou Dieng (en tant qu'encadrant) (Univ. Cheikh Anta Diop de Dakar, Dpt. Biologie Végétale), Sénégal (**2007/2008**) projet MUTEN « Influence de la qualité biochimique des paillis sur les émissions de $N_2O$ et la diversité des dénitrifiants du sol »
N.B. 1 article publié, référencé n°30 dans la section V-A

### IV-A-2 niveau doctorat

Camille Gutzwiller (en tant que co-directeur et en attente de l'obtention HDR, directeur de thèse J.-C. Cleyet-Marel-DR INRA) (Univ. Montpellier, filière BDI), France (**nov. 2015/2018**) " Intensification écologique de la nutrition minérale de cultures céréalières par inoculation de bactéries phytostimulatrices et fixatrices d'azote ", financement ANRT-bourse CIFRE, entreprise Xurian-Béziers (Dr. Benoît de Sarrau)
Nouria Derkaoui (en tant qu'appui) (Univ. d'Oran Es-Senia), Algérie (**2013/2014**) projet TASSILI «Suivi du potentiel mycorhizogène de sols de carrière en cours de revégétalisation avec la légumineuse ligneuse *Acacia saligna* »
Khadidja Bouazza Marouf (en tant qu'appui) (Univ. d'Oran Es-Senia), Algérie (**2012/2013**) projet TASSILI «Suivi du niveau de stimulation de la diversité des activités cataboliques bactériennes dans des sols de carrière en cours de revégétalisation avec la légumineuse ligneuse *Acacia saligna* »
Raya Abdedaiem (en tant qu'appui) (Univ. de Gabes), Tunisie (**2011/2013**) projet UTIQUE « Diversité moléculaire du cortège racinaire mycorhizien des légumineuses *Retama raetam* et *Acacia raddiana* »
Amadou Dieng (**en tant que co-directeur**) (Univ. Cheikh Anta Diop de Dakar), Sénégal (**2010/2013**) « Culture de *Jatropha curcas* au Sénégal : perturbation microbiologique des sols et mitigation par co-culture avec *Crotalaria*, légumineuse hypermycotrophe »
NB : 3 articles publiés référencés n°28-29-31 (voir la section V-A)
Imène Boudiaf (en tant qu'appui) (Univ. Badji Mokhtar d'Annaba), Algérie (**2009/2012**) « Analyse des facteurs microbiens régissant le caractère invasif d'*Acacia decurrens* (willd) dans la subéraie du Parc National d'El-Kala (Nord- Est Algérien) »
NB : 2 articles publiés référencés n°23-26 (voir la section V-A)
Fatou Ndoye (en tant qu'appui) (Lab. Commun de Microbiologie de Dakar), Sénégal (**2011**) « Influence d'*Acacia senegal* en plantations versus formations naturelles sur la diversité bactérienne et mycorhizienne »
Nathalie Diagne
Sanâa Wahbi (en tant qu'appui) (Univ. Cadi Ayyad de Marrakech), Maroc (**2011**) projet Agropolis Fondation Fabatropimed « Influence des cultures mixtes fève/blé sur le potentiel mycorhizien des sols et la structure de la microflore mycorhizophérique au Maroc »

### IV-A-3 niveau post-doctorat

Dr. Souhir Soussou (**2013/2014**, 12 mois), projet ANR-SyMetal « Evaluation comparée de l'effet rhizosphère (diversité-densité-activité microbiennes) de différents scénarios de végétalisation de déblais miniers hautement contaminés par des métaux lourds (Zn, Pb, Cd) avec sélection et application de souches *Pseudomonas*-PGPR résistantes aux métaux »
Dr. Alessandra Pontiroli (**2011-2012**, 17 mois), projet ANR-Systruf « Analyse par pyroséquençage de la diversité bactérienne et endomycorhizienne sur sol truffier à *Tuber melanosporum* et chêne vert »
Dr. Hanane Hamdali (en tant qu'appui, 10% ETP) (Univ. Cadi Ayyad de Marrakech), Maroc (**2009**) projet Aires-Sud « Diversité catabolique bactérienne et diversité moléculaire des spores de champignons mycorhiziens sous la strate herbacée et arbustive pionnière d'*Acacia raddiana* »
Dr. Djibril Djigal (Lab. Ecol. Microb. Sol Agrosyst. Trop.), Sénégal (**2006/2007**) projet MUTEN « Impact de la prédation d'une espèce de nématode bactérivore sur la communauté dénitrifiante d'un sol maraîcher »
Dr. Michel Diouf (Lab. Ecol. Microb. Sol Agrosyst. Trop.), Sénégal (**2007**) projet MUTEN « (i) Impact des systèmes à semis direct et litière permanente sur la structure génétique de la communauté bactérienne totale du sol, (ii) caractérisation des communautés bactériennes activées par des résidus de culture de qualités biochimiques contrastées »
Dr. Ablassé Bilgo (INERA), Burkina Faso (**2006**) projet DUSSOL « Etude des communautés nitrifiantes et dénitrifiantes dans des parcelles agricoles péri-urbaines de Ouagadougou amendées en déchets domestiques »

**Niveau chercheur statutaire** :
Dr. Salaheddine Bakkali (5% ETP) (chercheur - Centre de Recherche Forestière de Rabat), Maroc (**2010/2012**) projet PRAD « Influence des cistes en tant que plantes facilitatrices du chêne-liège sur la diversité catabolique bactérienne du sol »
Dr. Mosbah Mahdi (5% ETP) (chercheur - Univ. Gabès), Tunisie (**2010/2011**) projet UTIQUE « Détermination de la diversité moléculaire du cortège mycorhizien de racines de *Retama raetam*»

### IV-A-4 formation collective-enseignement

**Formations collectives** :

Participation aux travaux pratiques de deux écoles thématiques d'écologie microbienne à Dakar/Mbour-**Sénégal** :

1-MICROTROP « Ecologie microbienne des milieux tropicaux dégradés » (28 avril-28 mai **2005**)

2-ETET « Ecole Thématique en Ecologie Tropicale – Réseaux trophiques : de la théorie à la gestion rationnelle des ressources biologiques » (17 septembre-1er octobre **2006**), co-animation des travaux de recherche en groupe sur les interactions faune du sol/microorganismes à la réserve de Bandia

Formation théorique et pratique aux techniques moléculaires de la caractérisation de la diversité des champignons mycorhiziens à arbuscules pour les étudiants de niveau M2 et doctorat du Laboratoire de Biotechnologie des Rhizobiums et Amélioration des Plantes, Faculté des Sciences Es-Senia, Oran, **Algérie** (12-16 février **2012**)

**Enseignement** :

M**onitorat** à l'IUT du Montet, 2ème année agronomie (Nancy) ; 3×96 heures de Travaux Pratiques (clefs de détermination insectes-champignons, essais comparatif *in vitro* de fongicides, essais de fertilisation en serre, sorties et observations sur parcelles agricoles…).

**Cours** au Master2 spécialité Interactions Microorganismes, Hôtes et Environnements-Montpellier (23 novembre 2015) de 1H30 sur le thème : les bactéries rhizosphériques phytostimulatrices.

## IV-B Principaux contrats de recherche et collaborations

### Co-animation de projets de recherche (responsable et co-responsable de tâches et sous-tâches)

**projet ADEME** (**2016/2018**) - 64 k€ dont 29 pour le LSTM
"Faisabilité du phytomanagement de l'ancienne mine de Carnoulès à Saint Sébastien d'Aigrefeuille" - coord. J.C. Cleyet Marel DR INRA
Co-responsable analyses microbiennes des sols sous métallophytes.
en collaboration avec UMR Biochimie et Physiologie Moléculaire des Plantes (Montpellier)

**projet ANR Systruf** (2010/2014) - 600 k€ dont 232 pour le LSTM
"Bases d'une gestion écologique durable des écosystèmes truffiers producteurs de *Tuber melanosporum*" - coord. Pr. M.A. Selosse ex-Univ. Montpellier2
Responsable de 3 sous-tâches (voir II-E p26).
en collaboration avec : Centre d'Ecologie Fonctionnelle et Evolutive (Montpellier) ; UMR Interactions Arbres/Microorganismes + UMR Ecologie et Ecophysiologie Forestière + Laboratoire Agronomie et Environnement-ENSAIA + UR Biogéochimie des Ecosystèmes Forestiers (Nancy) ; Fédération

Française des Trufficulteurs (Paris) ; Centre d'Etudes Techniques et Economiques Forestières (Montpellier)

**projet ANR SyMetal** (2011/2015) - 600 k€ dont 118 pour le LSTM
"Rhizostabilisation de déblais miniers à fortes teneurs en métaux par des plantes métallicoles associées à leurs microorganismes symbiotiques" - coord. J.C. Cleyet-Marel DR INRA
Co-responsable de 2 tâches (voir II-E p29).
en collaboration avec : UMR Agroécologie (Dijon) ; Laboratoire d'Etude des Transferts en Hydrologie et Environnement (Grenoble) ; Institut des Sciences Analytiques et de Physico-chimie pour l'Envrionnement et les Matériaux (Pau)

### Association à des projets de recherche

**projet ACI ECCO MUTEN** (2004/2007)
" Modes d'utilisation des terres et flux de $N_2O$ : caractérisation des déterminants du fonctionnement des communautés dénitrifiantes " - coord. A. Brauman DR IRD
en collaboration avec : Centre d'Ecologie Fonctionnelle et Evolutive (Montpellier) ; UMR Agroécologie (Dijon) ; FOFIFA Système de Culture et Riziculture Durable d'Antsirabe + Laboratoire des Radio Isotopes d'Antananarivo (Madagascar)

**Jeune Equipe Associée** "Agriculture Périurbaine des Niayes" (2004/2007)
en collaboration avec : Institut Sénégalais de Recherches Agricoles + Université Cheikh Anta Diop (Dakar)

**projet européen EcoSafe** (2001/2003)
"Ecological and environmental biosafety assessment of novel plant and microbial biotechnology products" - coord. Pr. Y. Moënne-Loccoz Univ. Lyon1
en collaboration avec : UMR Ecologie Microbienne (Lyon) ; UMR Microbiologie du Sol et de l'Environnement (Dijon) ; Faculté d'Agriculture-département de science du sol de Shibin El Kom (Egyte)

## IV-C Expertises

Expertise interne IRD
Evaluation d'un projet de bourse de thèse IRD-ARTS (**2014**, France-Vietnam)
Evaluation d'un projet AIRD-Action Thématique Structurante (**2013**, France-Sénégal)
2 évaluations d'un projet AIRD-Jeunes Equipes Associées Internationales (**2012/2013**, France-Cuba)

Expertise externe
Expert consultable par le Conseil d'Orientation Stratégique-Recherche et Innovation du plan EcoPhyto2 **2016** (France)
ANR blanc international **2011** (Roumanie)
IFCPAR-CEFIPRA **2011** (Inde)

Membre de jury M2-Université de Montpellier 2
Marie Le Crollet (**2014**-Ecosystèmes) "Evaluation des capacités de phytostabilisation des éléments traces métalliques et métalloïdes par Globularia alypum et ses symbiotes racinaires dans le Parc National des Calanques" ; encadrant Dr. Isabelle Laffont-Schwob, IMBE Marseille
Sylvain Monteux (**2013**-Ecosystèmes) "Microbial diversity and biomineralization processes in Asperge Cave, France" ; encadrants Dr. Tomaso Bontognali et Stefano Torriani, ETH Zürich

Relecteur occasionnel pour des revues de rang A
Soil and Tillage Research, Environmental Science and Pollution Research, Research in Microbiology, Pedosphere, Chemosphere, FEMS Microbiology Ecology, Microbial Ecology, Plos One, Soil Science

Society of America Journal, Applied Soil Ecology, Annals of Microbiology, African Journal of Biotechnology, African Journal of Microbiology Research, African Journal of Agricultural Research.

# V - Valorisation de la recherche

## V-A Publications de rang A avec CL

Le nom des étudiants co-encadrés en thèse apparaît en ***gras-italique-surligné***.

IF= : impact factor des revues (2014)

**[1] Baudoin E.**, Benizri E., Guckert A. Metabolic fingerprint of microbial communities from distinct maize rhizosphere compartments. *European Journal of Soil Biology*, **2001**, 37, p. 85-93. IF=1,7

**[2]** Benizri E., **Baudoin E.**, Guckert A. Root colonization by inoculated Plant Growth Promoting Rhizobacteria. *Biocontrol Science and Technology*, **2001**, 11, p. 557-574. IF=0,9

**[3] Baudoin E.**, Benizri E., Guckert A. Impact of growth stage on the bacterial community structure along maize roots, as determined by metabolic and genetic fingerprinting. *Applied Soil Ecology*, **2002**, 19, p. 135-145. IF=2,6

**[4] Baudoin E.**, Benizri E., Guckert A. Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. *Soil Biology and Biochemistry*, **2003**, 35, p. 1183-1192. IF=3,9

**[5]** Selosse M.-A., **Baudoin E.**, Vandenkoornhuyse P. Symbiotic microorganisms, a key for ecological success and protection of plants. *Comptes Rendus Biologies (Académie des Sciences de l'Institut de France)*, **2004**, 327, p. 639-648. IF=0,9

**[6]** Henry S., **Baudoin E.**, Lopez-Gutierrez J.C., Martin-Laurent F., Brauman A., Philippot L. Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR. *Journal of Microbiological Methods*, **2004**, 59, p. 327-335. IF=2

**[7]** El Zemrany H., Cortet J., Lutz M.P., Chabert A., **Baudoin E.**, Haurat J., Maughan N., Félix D., Défago G., Bally R., Moënne-Loccoz Y. Field survival of the phytostimulator *Azospirillum lipoferum* CRT1 and functional impact on maize crop, biodegradation of crop residues, and soil faunal indicators in a context of decreasing nitrogen fertilisation. *Soil Biology and Biochemistry*, **2006**, 38, p. 1712-1726. IF=3,9

**[8]** Lerner A., Herschkovitz Y., **Baudoin E.**, Nazaret S., Moënne-Loccoz Y., Okon Y., Jurkevitch E. Effect of *Azospirillum brasilense* inoculation on rhizobacterial communities analyzed by denaturing gradient gel electrophoresis and automated ribosomal intergenic spacer analysis. *Soil Biology and Biochemistry*, **2006**, 38, p. 1212-1218. IF=3,9

**[9]** Ndour Y., **Baudoin E.**, Guissé A., Seck M., Khouma M., Brauman A. Impact of irrigation water quality on soil nitrifying and total bacterial communities. *Biology Fertility of Soils*, **2008**, 44, p. 797-803. IF=3,3

**[10] Baudoin E.**, Nazaret S., Mougel C., Ranjard L., Moënne-Loccoz Y. Impact of inoculation with the phytostimulatory PGPR *Azospirillum lipoferum* CRT1 on the genetic structure of the rhizobacterial community of field-grown maize. *Soil Biology and Biochemistry*, **2009**, 41, p. 409-413. IF=3,9

**[11] Baudoin E**., Philippot L., Chèneby D., Chapuis-Lardy L., Fromin N., Bru D., Rabary B., Brauman A. Direct seeding mulch-based cropping system increases both the activity and the abundance of denitrifier communities in a tropical soil. *Soil Biology and Biochemistry*, **2009**, 41, p.1703-1709. IF=3,9

**[12] Baudoin E**., Couillerot O., Spaepen S., Moënne-Loccoz Y., Nazaret S. Applicability of the 16S-23S rDNA internal spacer for PCR detection of the phytostimulatory PGPR inoculant *Azospirillum lipoferum* CRT1 in field soil. *Journal of Applied Microbiology*, 108, **2010**, p. 25-38. IF=2,4

**[13] Baudoin E.**, Lerner A., Mirza M.S., El Zemrany H., Prigent-Combaret C., Jurkevich E., Spaepen S., Vanderleyden J., Nazaret S., Okon Y., Moënne-Loccoz Y. Effects of *Azospirillum brasilense* with genetically modified auxin biosynthesis gene *ipdC* upon the diversity of the indigenous microbiota of the wheat rhizosphere. *Research in Microbiology*, **2010**, 161, p. 219-226. IF=2,7

**[14]** Djigal D., **Baudoin E.**, Philippot L., Brauman A., Villenave C. Shifts in size, genetic structure and activity of the soil denitrifier community by nematode grazing. *European Journal of Soil Biology*, **2010**, 46, p. 112-118. IF=1,7

**[15]** Diouf M., **Baudoin E.**, Dieng L., Assigbetsé K., Brauman A. Legume and gramineous crop residues stimulate distinct soil bacterial populations during early decomposition stages. *Canadian Journal of Soil Science*, **2010**, 90, p. 289-293. IF=1,3

**[16]** Couillerot O., Bouffaud M.-L., **Baudoin E.**, Muller D., Caballero-Mellado J., Moënne-Loccoz Y. Development of a real-time PCR method to quantify the PGPR strain *Azospirillum lipoferum* CRT1 on maize seedlings. *Soil Biology and Biochemistry*, **2010**, 42, p. 2298-2305. IF=3,9

**[17]** Duponnois R., Ouahmane L., Kane A., Thioulouse J., Hafidi M., Boumezzough A., Prin Y., **Baudoin E.**, Galiana A., Dreyfus B. Nurse shrubs increased the early growth of *Cupressus* seedlings by enhancing belowground mutualism and soil microbial activity. *Soil Biology and Biochemistry*, **2011**, 43, p. 2160-2168. IF=3,9

**[18]** Bilgo A., Sangare S.K., Thioulouse J., Prin Y., Hien V., Galiana A., **Baudoin E.**, Hafidi M., Bâ A.M., Duponnois R. Response of native soil microbial functions to the controlled mycorrhization of an exotic tree legume, *Acacia holosericea* in a Sahelian ecosystem. *Mycorrhiza*, **2012**, 22, p. 175-187. IF=3,4

**[19]** Baohanta R., Thioulouse J., Ramanankierana H., Prin Y., Rasolomampianina R., **Baudoin E.**, Rakotoarimanga N., Galiana A., Randriambanona H., Lebrun M., Duponnois R. Restoring native forest ecosystems after exotic tree plantation in Madagascar: combination of the local ectotrophic species *Leptolena bojeriana* and *Uapaca bojeri* mitigates the negative influence of the exotic species *Eucalyptus camaldulensis* and *Pinus patula*. *Biological Invasions*, **2012**, 14, p. 2407-2421. IF=2,5

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1 article projet ANR-SyMetal, titre provisoire : « Chemical and microbial features of mine spoils vegetalized by multispecific metallophyte covers »

1 article ANR-Systruf, titre provisoire : « Soil chemical and microbial features as influenced by the production of black truffle in association with *Quercus ilex* »

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Wahbi S., **Baudoin E.**, Maghraoui T., Tournier E., Hafidi M., Thioulouse J., Sanguin H., Oufdou K., Prin Y., Galiana A., Duponnois R., **2014**. The Faba bean / wheat association creates new soil microbial habitats that improved soil microbial functions and wheat growth in a P-deficient soil. *International Congress on Mycorrhizae*, 15-17 octobre, Marrakesh (Maroc), **oral**.

Derkaoui N., Bouchiba Z., **Baudoin E.**, Dupponois R., Bekki A., **2014**. Effet de la flore locale sur la mycorhization d'*Acacia saligna* utilisé dans la revégétalisation de la sablière de Terga. *International Congress on Mycorrhizae*, 15-17 octobre, Marrakesh (Maroc), **affiche**.

Abdedaiem R., **Baudoin E.**, Mahdhi M., De Lajudie P. Mars M., **2014**. Caractérisation de la diversité génétique des champignons mycorhiziens à arbuscules associés aux racines de deux Fabacées dans quatre sites de l'aride tunisien. *International Congress on Mycorrhizae*, 15-17 octobre, Marrakesh (Maroc), **oral**.

Soussou S., Brunel B., Pervent M., van Tuinen D., Cleyet-Marel J.C., Baudoin E., **2015**. Nodulation and growth of *Fabaceae* in multi-metal-contaminated mine spoils as influenced by inoculation of resident rhizobacteria displaying multi-trait phytostimulation properties. *Rhizosphere 4, stretching the interface of life*, 21-25 juin, Maastricht (Pays-Bas), **affiche**.

## V-D Chapitres d'ouvrage

Sanon A., Ndoye F., **Baudoin E.**, Prin Y., Galiana A., Duponnois R. "Management of the mycorrhizal soil infectivity to improve reforestation program achievements in Sahelian ecosystems", dans : *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*. Ed. Antonio Méndez Vilas, Formatex Research Center, vol. 1, **2010**, p. 230-238 (ISBN 978-84-614-6194-3)

Sanon A., Beguiristain T., Cébron A., Berthelin J., Ndoye I., Leyval C., Prin Y., Galiana A., **Baudoin E.**, Duponnois R. « Towards the influence of plant-soil-microbes feedbacks on plant biodiversity, grassland variability and productivity », dans : *Grassland biodiversity: habitat types, ecological processes and environmental impacts*. Eds. Runas Johan, Dahlgren Theodor, Nova Science Publishers, Inc., New York, **2010**, p. 267-301 (ISBN 978-1-60876-542-3)

Duponnois R., Bâ A.M., Prin Y., **Baudoin E.**, Galiana A., Dreyfus B. « Les champignons mycorhiziens: une composante majeure dans les processus biologiques régissant la stabilité et la productivité des écosystèmes forestiers tropicaux », dans : *Le projet majeur africain de la Grande Muraille Verte : concepts et mise en œuvre*. IRD Editions, p. 421-440, **2010** (ISBN 978-2-7099-1696-7)

Sanon A., **Baudoin E.**, Prin Y., Galiana A., Duponnois R., Ndoye F. « Plant coexistence and diversity mediated belowground: the importance of mycorrhizal networks », dans: *Botanical Research and Practices*, Nova Science Publishers, Inc., New York, **2011**, 43 p. (ISBN 978-1-61209-152-5)

Manaut N., Chaffii K., Ouhammou A., Ouahmane L., Thioulouse J., Hafidi M., Boumezzough A., Prin Y., Galiana A., **Baudoin E.**, Lebrun M., Duponnois R. « Shrub plants and associated mycorrhizal fungi sustainably improve the native tree establishment in Mediterranean ecosystems », dans : *Mediterranean Ecosystems*. Ed. Gina S. Williams, Nova Science Publishers, Inc., New York, **2011**, 11 p. (ISBN 978-1-61209-146-4)

Duponnois R., Bâ A., Mousain D., Galiana A., **Baudoin E.**, Dreyfus B., Prin Y. « Biotechnological processes used in controlled ectomycorrhizal practices », dans : *Diversity and Biotechnology of Ectomycorrhizae*. Soil Biology vol. 25 part 2, Eds. M. Rai, A. Varma, Spinger-Verlag, Berlin Heidelberg, **2011**, p. 143-155 (DOI 10.1007/978-3-642-15196-5_7)

Duponnois R., Hafidi M., Wahbi S., Sanon A., Galiana A., **Baudoin E.**, Sanguin H., Bâ A.M., Prin Y., Bally R. « La symbiose mycorhizienne et la fertilité des sols dans les zones arides: un outil biologique sous-exploité dans la gestion des terres de la zone sahélo-saharienne », dans : *La Grande Muraille Verte: capitalisation des recherches et valorisation des savoirs locaux*. Eds. Dia A., Duponnois R., IRD Editions, **2012**, p. 351-369 (ISBN 978-2-7099-1738-4)

Duponnois R., **Baudoin E.**, Thioulouse J., Galiana A., Lebrun M., Prin Y. « The impact of mycorrhizosphere bacterial communities on soil biofunctioning in tropical and Mediterranean forest ecosystems », dans : *Bacteria in Agrobiology: Plant Probiotics*. Ed. Maheshwari D.K., Nova Science Publishers, Inc., Springer, New York, **2012**, p. 79-95 (ISBN 978-3-642-27514-2)

Baohanta R., Ramanankierana H., Thioulouse J., Prin Y., Rasolomampianina R., **Baudoin E.**, Rakotoarimanga N., Galiana A., Randriambanona H., Lebrun M., Duponnois R. « Mycorrhizal fungi diversity and their importance on the establishment of native species seedlings within Madagascarian degraded sclerophyllous forest », dans : *Ectomycorrhizal Symbioses in Tropical and Neotropical forests*. Eds. Bâ A., McGuire K.L., Diédhiou A.G., CRC Press-Taylor & Francis Group **2013** (ISBN 1466594683, 9781466594685)

Duponnois R., Bâ A., Galiana A., **Baudoin E.**, Sanguin H., Lebrun M., Prin Y. « Biotechnologie et mycorhization contrôlée en milieu tropical », dans : *Des champignons symbiotiques contre la désertification dans des écosystèmes mediterranéens, tropicaux et insulaires*. Eds. Duponnois R., Hafidi M., Ndoye I., Ramanankierana H., Bâ A., IRD Editions, **2013**, p. 262-279 (ISBN 978-2-7099-1827-5)

Manaut N., Hafidi M., Ouahmmou A., **Baudoin E.**, Chafii K., Prin Y., Ouahmane L., Sanguin H., Galiana A., Boumezzough A., Duponnois R. « Plante nurse : vecteur de propagation de champignons mycorhiziens pour optimiser les performances des opérations de reboisement au Maroc », dans : *Des champignons symbiotiques contre la désertification dans des écosystèmes méditerranéens, tropicaux et insulaires*. Eds. Duponnois R., Hafidi M., Ndoye I., Ramanankierana H., Bâ A., IRD Editions, **2013**, p. 391-409 (ISBN 978-2-7099-1827-5)

Baohanta R., Ramanankierana H., Thioulouse J., Prin Y., Rasolomampianina R., **Baudoin E.**, Rakotoarimanga N., Galiana A., Randriambanona H., Lebrun M., Duponnois R. « Improvement of the early growth of endemic tree species by soil mycorrhizal management in Madagascar », dans :

*Seedlings: Ecology, Growth and Environmental Influences*. Nova Science Publishers, Inc. (Eds). New York. USA. **2013**

## V-E Autres communications

Benizri E., **Baudoin E**., Di Battista-Leboeuf C., Guckert A., **2001**. Des bactéries pour la santé des plantes. *Biofutur*, n°210, p. 52-55.

**Baudoin E**., Guissé A., Ndour Y., Masse D., **2006**. Déchets des villes, engrais aux champs. *Sciences au Sud – Le journal de l'IRD*, n°37, p. 9.

Mousain D., Coste J.-M., Galiana A., Ducousso M., LeRoux C., Domergue O., Duponnois R., Bourillon J., **Baudoin E.**, Pontiroli A., Sanguin H., Prin Y., **2013**. La flore des sites truffiers : les apports du projet Systruf. *Le Trufficulteur*, n°82, p. 14-16.

**Baudoin E.**, Pontiroli A., Sanguin H., Bourillon J., Galiana A., Ducousso M., LeRoux C., Domergue O., Mousain D., Duponnois R., Prin Y., **2013**. Le monde microbien du brûlé producteur de *Tuber melanosporum*. *Le Trufficulteur*, n°82, p. 17.

## V-F Diffusions auprès du Grand Public

Désertif'actions (samedi 13 juin 2015, Montpellier)
Animation de la "tente Terres en vie" autour de posters et de visuels (racines nodulées et mycorhizées sous loupe binoculaire) afin d'illustrer (1) les interactions qui s'établissent entre les plantes cultivées et les microorganismes du sol, (2) les moyens techniques pour orienter ou favoriser ces interactions positives pour la croissance végétale.

Fascination of Plants Day (18-22 mai 2015, Montpellier)
Co-organisation (E. Guiderdoni-CIRAD) et co-animation (rédaction de 3 posters, accueil du public autour des posters et des visuels-rhizotrons, racines nodulées...) de la manifestation organisée sous l'égide de l'EPSO et accueillie par Agropolis International. Thème de la manifestation montpelliéraine: " les racines : la face cachée des plantes " destinée à démontrer l'importance agronomique et environnementale des racines (gestion des intrants dont l'eau et les engrais, atténuation de certaines pollutions du sol).

Exposition itinérante Sciences au Sud-IRD (décembre 2006-mars 2007, Dakar-Ziguinchor, Sénégal)
Animation de la discussion autour de la diffusion du film "Bacteriland" (Mona Lisa prod.-France5-IRD, Alain Brauman) et des bactéries dans la vie quotidienne.

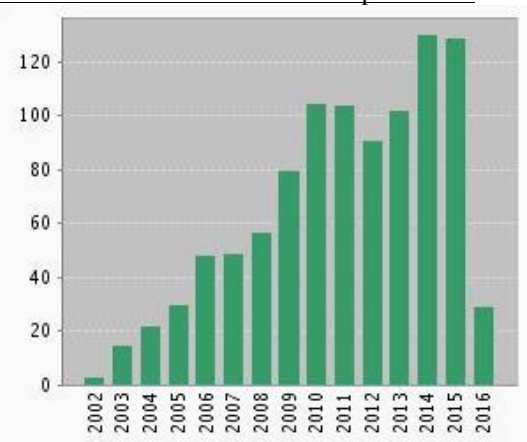
# ANNEXE 1
# Analyse bibliométrique

Colloques nationaux et internationaux (auteur principal ou en association) :
**28** affiches
**31** communications orales

**12** associations à des chapitres d'ouvrage

**33** publications répertoriées dans le Web of Science (2001-2016)
**H index = 13** (avril 2016)
nombre total de citations : 980
nombre moyen de citations par article : 29,7

Cumul du nombre de citations par année




| | 2012 ◄ | 2013 | 2014 | 2015 | 2016 ► | Total | Average Citations per Year |
|---|---|---|---|---|---|---|---|
| Use the checkboxes to remove individual items from this Citation Report<br>or restrict to items published between 2001 and 2016 Go | 90 | 102 | 128 | 128 | 29 | 980 | 65.33 |
| 1. **Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR**<br>By: Henry, S; Baudoin, E; Lopez-Gutierrez, JC; et al.<br>JOURNAL OF MICROBIOLOGICAL METHODS Volume: 59 Issue: 3 Pages: 327-335 Published: DEC 2004 | 20 | 25 | 30 | 34 | 11 | 217 | 16.69 |
| 2. **Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere**<br>By: Baudoin, E; Benizri, E; Guckert, A<br>SOIL BIOLOGY & BIOCHEMISTRY Volume: 35 Issue: 9 Pages: 1183-1192 Published: SEP 2003 | 16 | 15 | 22 | 17 | 4 | 177 | 12.64 |
| 3. **Root colonization by inoculated plant growth-promoting rhizobacteria**<br>By: Benizri, E; Baudoin, E; Guckert, A<br>BIOCONTROL SCIENCE AND TECHNOLOGY Volume: 11 Issue: 5 Pages: 557-574 Published: SEP 2001 | 12 | 13 | 15 | 13 | 2 | 125 | 7.81 |
| 4. **Impact of growth stage on the bacterial community structure along maize roots, as determined by metabolic and genetic fingerprinting**<br>By: Baudoin, E; Benizri, E; Guckert, A<br>APPLIED SOIL ECOLOGY Volume: 19 Issue: 2 Pages: 135-145 Article Number: PII S0929-1393(01)00185-8 Published: FEB 2002 | 7 | 5 | 13 | 3 | 0 | 101 | 6.73 |
| 5. **Symbiotic microorganisms, a key for ecological success and protection of plants**<br>By: Selosse, MA; Baudoin, E; Vandenkoornhuyse, P<br>COMPTES RENDUS BIOLOGIES Volume: 327 Issue: 7 Pages: 639-648 Published: JUL 2004 | 13 | 5 | 8 | 6 | 0 | 84 | 6.46 |
| 6. **Metabolic fingerprint of microbial communities from distinct maize rhizosphere compartments**<br>By: Baudoin, E; Benizri, E; Guckert, A<br>EUROPEAN JOURNAL OF SOIL BIOLOGY Volume: 37 Issue: 2 Pages: 85-93 Published: APR-JUN 2001 | 2 | 4 | 1 | 3 | 1 | 52 | 3.25 |
| 7. **Direct seeding mulch-based cropping increases both the activity and the abundance of denitrifier communities in a tropical soil**<br>By: Baudoin, Ezekiel; Philippot, Laurent; Cheneby, Dominique; et al.<br>SOIL BIOLOGY & BIOCHEMISTRY Volume: 41 Issue: 8 Pages: 1703-1709 Published: AUG 2009 | 2 | 3 | 8 | 4 | 1 | 31 | 3.88 |
| 8. **Impact of inoculation with the phytostimulatory PGPR Azospirillum lipoferum CRT1 on the genetic structure of the rhizobacterial community of field-grown maize**<br>By: Baudoin, Ezekiel; Nazaret, Sylvie; Mougel, Christophe; et al.<br>SOIL BIOLOGY & BIOCHEMISTRY Volume: 41 Issue: 2 Pages: 409-413 Published: FEB 2009 | 5 | 4 | 3 | 4 | 3 | 29 | 3.62 |
| 9. **Field survival of the phytostimulator Azospirillum lipoferum CRT1 and functional impact on maize crop, biodegradation of crop residues, and soil faunal indicators in a context of decreasing nitrogen fertilisation**<br>By: El Zemrany, Hamdy; Cortet, Jerome; Lutz, Matthias Peter; et al.<br>SOIL BIOLOGY & BIOCHEMISTRY Volume: 38 Issue: 7 Pages: 1712-1726 Published: JUL 2006 | 2 | 5 | 0 | 1 | 1 | 26 | 2.36 |
| 10. **Impact of irrigation water quality on soil nitrifying and total bacterial communities**<br>By: Ndour, Ndeye Yacine Badiane; Baudoin, Ezekiel; Guisse, Aliou; et al.<br>BIOLOGY AND FERTILITY OF SOILS Volume: 44 Issue: 5 Pages: 797-803 Published: MAY 2008 | 3 | 2 | 4 | 7 | 0 | 22 | 2.44 |

| | | 2012 ◄ | 2013 | 2014 | 2015 | 2016 ► | Total | Average Citations per Year |
|---|---|---|---|---|---|---|---|---|
| Use the checkboxes to remove individual items from this Citation Report<br>or restrict to items published between 2001 and 2016 Go | | 90 | 102 | 128 | 128 | 29 | 980 | 65.33 |
| ☐ 11. | **Effect of Azospirillum brasilense inoculation on rhizobacterial communities analyzed by denaturing gradient gel electrophoresis and automated ribosomal intergenic spacer analysis**<br>By: Lerner, A; Herschkovitz, Y; Baudoin, E; et al.<br>SOIL BIOLOGY & BIOCHEMISTRY Volume: 38 Issue: 6 Pages: 1212-1218 Published: JUN 2006 | 1 | 3 | 1 | 3 | 1 | 22 | 2.00 |
| ☐ 12. | **Shifts in size, genetic structure and activity of the soil denitrifier community by nematode grazing**<br>By: Djigal, Djibril; Baudoin, Ezekiel; Philippot, Laurent; et al.<br>EUROPEAN JOURNAL OF SOIL BIOLOGY Volume: 46 Issue: 2 Pages: 112-118 Published: MAR-APR 2010 | 2 | 2 | 5 | 4 | 2 | 18 | 2.57 |
| ☐ 13. | **Effects of Azospirillum brasilense with genetically modified auxin biosynthesis gene ipdC upon the diversity of the indigenous microbiota of the wheat rhizosphere**<br>By: Baudoin, Ezekiel; Lerner, Anat; Mirza, M. Sajjad; et al.<br>RESEARCH IN MICROBIOLOGY Volume: 161 Issue: 3 Pages: 219-226 Published: APR 2010 | 2 | 2 | 5 | 4 | 0 | 14 | 2.00 |
| ☐ 14. | **Nurse shrubs increased the early growth of Cupressus seedlings by enhancing belowground mutualism and soil microbial activity**<br>By: Duponnois, R.; Ouahmane, L.; Kane, A.; et al.<br>SOIL BIOLOGY & BIOCHEMISTRY Volume: 43 Issue: 10 Pages: 2160-2168 Published: OCT 2011 | 0 | 4 | 1 | 6 | 1 | 12 | 2.00 |
| ☐ 15. | **Development of a real-time PCR method to quantify the PGPR strain Azospirillum lipoferum CRT1 on maize seedlings**<br>By: Couillerot, Olivier; Bouffaud, Marie-Lara; Baudoin, Ezekiel; et al.<br>SOIL BIOLOGY & BIOCHEMISTRY Volume: 42 Issue: 12 Pages: 2298-2305 Published: DEC 2010 | 2 | 4 | 1 | 1 | 1 | 10 | 1.43 |
| ☐ 16. | **Ectomycorrhizal diversity enhances growth and nitrogen fixation of Acacia mangium seedlings**<br>By: Diagne, N.; Thioulouse, J.; Sanguin, H.; et al.<br>SOIL BIOLOGY & BIOCHEMISTRY Volume: 57 Pages: 468-476 Published: FEB 2013 | 0 | 1 | 4 | 4 | 0 | 9 | 2.25 |
| ☐ 17. | **Applicability of the 16S-23S rDNA internal spacer for PCR detection of the phytostimulatory PGPR inoculant Azospirillum lipoferum CRT1 in field soil**<br>By: Baudoin, E.; Couillerot, O.; Spaepen, S.; et al.<br>JOURNAL OF APPLIED MICROBIOLOGY Volume: 108 Issue: 1 Pages: 25-38 Published: JAN 2010 | 0 | 0 | 2 | 2 | 0 | 7 | 1.00 |
| ☐ 18. | **The exotic legume tree species, Acacia mearnsii, alters microbial soil functionalities and the early development of a native tree species, Quercus suber, in North Africa**<br>By: Boudiaf, I.; Baudoin, E.; Sanguin, H.; et al.<br>SOIL BIOLOGY & BIOCHEMISTRY Volume: 65 Pages: 172-179 Published: OCT 2013 | 0 | 1 | 2 | 2 | 1 | 6 | 1.50 |
| ☐ 19. | **Effects of Jatopha curcas L. plantation on soil bacterial and fungal communities**<br>By: Dieng, Amadou; Ndoye, Ibrahima; Duponnois, Robin; et al.<br>SOIL BIOLOGY & BIOCHEMISTRY Volume: 72 Pages: 105-115 Published: MAY 2014 | 0 | 0 | 0 | 5 | 0 | 5 | 1.67 |

# ANNEXE 2
# Sélection de publications les plus représentatives

Je propose 8 publications représentatives des principaux thémes et méthodologies abordés :

**[A]** Baudoin E., Nazaret S., Mougel C., Ranjard L., Moënne-Loccoz Y. Impact of inoculation with the phytostimulatory PGPR *Azospirillum lipoferum* CRT1 on the genetic structure of the rhizobacterial community of field-grown maize. *Soil Biology and Biochemistry*, 2009, 41, p. 409-413
afin d'illustrer l'effet de l'inoculation d'une souche PGPR sur la composition du microbiote racinaire

**[A]** Baudoin E., Couillerot O., Spaepen S., Moënne-Loccoz Y., Nazaret S. Applicability of the 16S-23S rDNA internal spacer for PCR detection of the phytostimulatory PGPR inoculant *Azospirillum lipoferum* CRT1 in field soil. *Journal of Applied Microbiology*, 108, 2010, p. 25-38
afin d'illustrer la nécessité de développer des outils moléculaires taxonomiques pour le suivi simplifié par PCR de souches bactériennes inoculées dans la rhizosphère

**[A]** Henry S., Baudoin E., Lopez-Gutierrez J.C., Martin-Laurent F., Brauman A., Philippot L. Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR. *Journal of Microbiological Methods*, 2004, 59, p. 327-335
afin d'illustrer la nécessité de développer des outils moléculaires sur gènes fonctionnels pour le suivi simplifié par PCR de populations bactériennes fonctionnelles dans la rhizosphère

**[A]** Baudoin E., Philippot L., Chèneby D., Chapuis-Lardy L., Fromin N., Bru D., Rabary B., Brauman A. Direct seeding mulch-based cropping system increases both the activity and the abundance of denitrifier communities in a tropical soil. *Soil Biology and Biochemistry*, 2009, 41, p.1703-1709
afin d'illustrer le suivi par PCR quantitative des densités de populations bactériennes fonctionnelles dans la rhizosphère

**[N]** Diouf M., Baudoin E., Dieng L., Assigbetsé K., Brauman A. Legume and gramineous crop residues stimulate distinct soil bacterial populations during early decomposition stages. *Canadian Journal of Soil Science*, 2010, 90, p. 289-293
afin d'illustrer l'accès à la diversité de la fraction bactérienne métaboliquement active face à la ressource organique

**[A]** Dieng A., Ndoye I., Duponnois R., Baudoin E. Effects of *Jatropha curcas* L. plantation on soil bacterial and fungal communities. *Soil Biology and Biochemistry*, 2014, 72, p. 105-115
+
**[A]** Dieng A., Duponnois R., Floury A., Laguerre G., Ndoye I., Baudoin E. Impact of the energy crop *Jatropha curcas* L. on the composition of rhizobial populations nodulating cowpea (*Vigna unguiculata* L.) and acacia (*Acacia seyal* L.) *Systematic and Applied Microbiology*, 2015, 38, p. 128-134
+
**[A]** Dieng A., Duponnois R., Ndoye I., Baudoin E. Cultivation of *Jatropha curcas* L. leads to pronounced mycorrhizal community differences. *Soil Biology and Biochemistry*, 2015, 89, p. 1-11
afin d'illustrer l'impact microbien global et celui sur l'établissement des symbioses rhizobiennes et mycorhiziennes de l'effet mycorhizosphère d'un végétal exotique

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journal homepage: www.elsevier.com/locate/soilbio

Short communication

# Impact of inoculation with the phytostimulatory PGPR *Azospirillum lipoferum* CRT1 on the genetic structure of the rhizobacterial community of field-grown maize

Ezékiel Baudoin [a,b,c,1], Sylvie Nazaret [a,b,c], Christophe Mougel [d], Lionel Ranjard [d], Yvan Moënne-Loccoz [a,b,c,*]

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[b] *Université Lyon 1, Villeurbanne, France*
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Impact

ABSTRACT

The phytostimulatory PGPR *Azospirillum lipoferum* CRT1 was inoculated to maize seeds and the impact on the genetic structure of the rhizobacterial community in the field was determined during maize growth by Automated Ribosomal Intergenic Spacer Analysis (ARISA) of rhizosphere DNA extracts. ARISA fingerprints could differ from one plant to the next as well as from one sampling to the next. Inoculation with strain CRT1 enhanced plant-to-plant variability of the ARISA fingerprints and caused a statistically significant shift in the composition of the indigenous rhizobacterial community at the first two samplings. This is the first study on the ecological impact of *Azospirillum* inoculation on resident bacteria done in the field and showing that this impact can last at least one month.



For decades, plant growth-promoting rhizobacteria (PGPR) have been added to soil or seeds to improve plant growth and/or health (Okon and Labandera-Gonzalez, 1994; Bashan et al., 2004). The ecological impact of PGPR inoculation on the diversity of the resident rhizosphere microbial community has been assessed in many occasions, but most studies were performed with biocontrol PGPR (e.g. Girlanda et al., 2001; Moënne-Loccoz et al., 2001; Bakker et al., 2002). In the case of phytostimulatory PGPR, such as the α-Proteobacterium *Azospirillum*, several modes of action on the plant are implicated (Bashan et al., 2004), especially the synthesis of phytohormones such as indole-3-acetic acid (Spaepen et al., 2007; Malhotra and Srivastava, 2008). The agronomic benefit of *Azospirillum* inoculation is well documented (Okon and Labandera-Gonzalez, 1994; Dobbelaere et al., 2001), but its ecological impact has only been studied on a few occasions (Basaglia et al., 2003; Herschkovitz et al., 2005a, b; Russo et al., 2005; Lerner et al., 2006).

Since *Azospirillum* inoculation can have a strong effect on root development and exudation (Heulin et al., 1987; Jacoud et al., 1998; Dobbelaere et al., 2001), it may be anticipated that the use of these phytostimulatory PGPR will also modify the composition of the resident bacterial community of the rhizosphere. Unexpectedly, such an impact has not been evidenced so far with *Azospirillum* inoculants (Herschkovitz et al., 2005a, b; Lerner et al., 2006), but these studies were done under greenhouse conditions and whether this would also be the case under field conditions is unknown. Therefore, the objective of this work was to assess the impact of seed inoculation with a phytostimulatory PGPR (*A. lipoferum* CRT1) on the dynamics of the genetic structure of the rhizobacterial community of field-grown maize. This was done using Automated Ribosomal Intergenic Spacer Analysis (ARISA), which is based on length polymorphism analysis of PCR-amplified 16S–23S ribosomal intergenic sequences.

The field experiment was described in detail by El Zemrany et al. (2006). Briefly, it was carried out in 2001 at La Côte Saint-André

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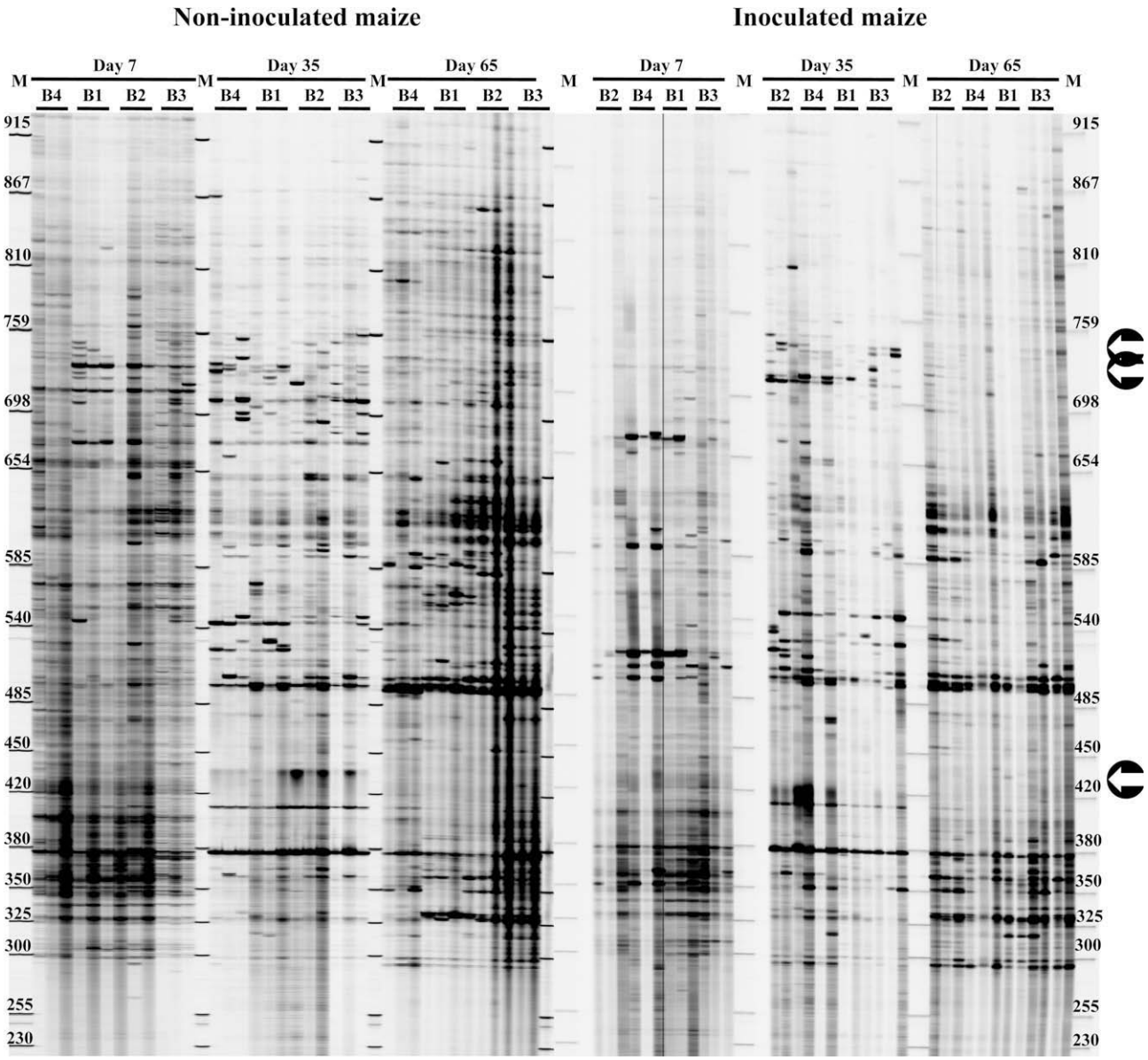


**Fig. 1.** ARISA fingerprints of the rhizobacterial community from non-inoculated maize and maize inoculated with *A. lipoferum* CRT1, at three samplings (7, 35 and 65 days after sowing). The fingerprint from each of the three individual plants studied per treatment in each of the four blocks (B1, B2, B3 and B4) corresponds to the digitally rebuilt picture scan derived from time-course measurement of fluorescence at the bottom of the running gel device. M, molecular marker (bp) corresponding to phage M13 mp18 (displayed from 230 to 915 bp; Promega, Charbonnières, France). The positions corresponding to the three bands of strain CRT1 are indicated using arrows.

(near Lyon, France). The soil at the site was a luvisol and the surface horizon a loam. In each of four blocks, one plot with non-inoculated maize and one plot with inoculated maize were studied. In February, bovine–ovine compost was applied (providing an estimated 55 kg mineral N $ha^{-1}$ during the year; El Zemrany et al., 2006). The maize isolate *A. lipoferum* CRT1 (Fages and Mulard, 1988) was prepared commercially using a sterile peat formulation (Azo-Green™; Lipha/Nitragin, Meyzieu, France) and used to inoculate seeds ($3 \times 10^7$ CFU and 20 mg peat carrier added per seed) of maize cv. Eurostar (Rustica, Mondonville, France). Inoculant purity was checked based on physiological and molecular tests (El Zemrany et al., 2006). Sowing took place on 29 May (80,000 seeds $ha^{-1}$). Mineral N (half nitrate and half ammonium) was applied on 5 June (50 kg N $ha^{-1}$) and 26 June (20 kg N $ha^{-1}$).

Samplings were done at 7 (5 June; at 2–3 leaves), 35 (3 July; at 9–10 leaves) and 65 days after sowing (2 August; at female flowering) and *A. lipoferum* CRT1 was recovered respectively at $5.0 \times 10^7$, $2.1 \times 10^8$ and $9.5 \times 10^5$ CFU $g^{-1}$ root (El Zemrany et al., 2006). Compared with the control, inoculated plants displayed enhanced rooting depth and/or root biomass (El Zemrany et al., 2006). At each sampling, three plants were taken in each of the eight plots. Each root system (minus the primary axis of nodal roots, which was hard to grind and gave poor DNA yield) and adhering soil was ground in liquid nitrogen using a pestle and mortar. Total DNA was extracted using FastDNA® SPIN® Kit (For Soil) (BIO 101, Inc., Carlsbad, CA). Triplicate DNA extracts (from three 500 mg crushed samples) were pooled for each root system and DNA concentration was adjusted to 2 ng DNA $\mu l^{-1}$.

The bacterial 16S–23S ribosomal intergenic spacer was amplified using primers FGPS1490-72 (IRD-800 labelled) and FGPL132-38a, and the GeneAmp PCR System 2400 (Perkin-Elmer, Paris, France), as specified in Ranjard et al. (2003). PCR was performed in 50-µl volumes containing 5 µl of 10× dilution buffer (supplemented with 1.5 mM $MgCl_2$), 200 µM of each dNTP, 0.5 µM of each

primer, 1.5 μg of T4 gene 32 protein (Roche, Meylan, France), 2.5 units of *Taq* polymerase (Q-BIOgene, Illkirch, France) and 10 ng of template DNA. After purification (MinElute; Qiagen, Courtaboeuf, France), 0.5–1.0 μl of PCR product was processed on a LiCor® DNA sequencer (ScienceTec, Les Ulis, France) and peak data (i.e. ARISA bands) were analyzed using the 1D-Scan software (ScienceTec) (Ranjard et al., 2003).

Principal component analysis (PCA) was performed on the covariance matrix based on the 100 most dominant bands/peaks (data matrix with samples as rows and peaks as columns), using ADE-4 software (Thioulouse et al., 1997). Treatment comparison on PCA was performed using a Monte Carlo permutation test ($n = 12$; $P < 0.05$), as done by Ranjard et al. (2003).

Within each plot, the three ARISA fingerprints obtained at each sampling were not identical to one another (Fig. 1). This means that the genetic structure of the bacterial community in the rhizosphere differed from one plant to the next, regardless of whether inoculated or non-inoculated maize was considered. PCA showed that individual plant samples obtained at 7, 35 and 65 days formed three groups, which were statistically distinct from each other (illustrated for non-inoculated maize in Fig. 2). This phenology effect on the rhizobacterial community has already been documented, but often with other plant species and at a larger (seasonal) time scale (Smalla et al., 2001; Heuer et al., 2002; Mougel et al., 2006). It has often been attributed to a modification of rhizodeposition patterns in time (Folman et al., 2001; Marschner et al., 2004).

In this context, inoculation was assessed at each sampling date (Fig. 1). PCA of ARISA data showed a significant effect of inoculation with *A. lipoferum* CRT1 on days 7 and 35 (Fig. 3), but not on day 65. Analysis of the fingerprints indicated that these differences did not correspond to the contribution of strain CRT1's own bands (i.e. three bands of approximately 430, 730 and 750 bp; Fig. 1) to the global ARISA profile, in accordance with the fact that the main bands obtained in PCR fingerprinting methods are only contributed by the most dominant taxa (Wintzingerode et al., 1997). This indicates that resident bacteria had been affected, unlike in previous work (Herschkovitz et al., 2005a, b; Lerner et al., 2006). In comparison with the latter studies, however, there were several ecologically important differences, including inoculant identity (*A. lipoferum* CRT1 vs *A. brasilense* strains) and formulation, maize cultivar, soil type and growing conditions (field vs greenhouse experiments), and a different molecular method (ARISA vs mainly DGGE).

Assessment of PCA results indicated that the impact of inoculation was not due to emergence/disappearance of particular bands, but rather to multiple changes in the relative intensity of the bands studied (especially for the 5–10 most discriminant bands), meaning that broad community shifts took place. In addition, the ARISA bands enabling discrimination between the two treatments differed from one sampling to the next, suggesting that different bacterial taxa were implicated at different stages of maize growth.

The impact of inoculation on resident bacteria may have resulted from both direct and indirect effects. Direct competitive effects of strain CRT1 were expected, especially at the first sampling, as (i) the number of CRT1 cells was high (more than $10^7$ CFU $g^{-1}$ root), (ii) the effect on root growth was not very important then (El Zemrany et al., 2006), and (iii) the amount of exudates released by seedlings is known to be relatively low (Quian and Doran, 1996).

Indirect effects were also expected, especially at the second sampling, since inoculation resulted in a bigger, more ramified root system (El Zemrany et al., 2006), which implies (i) an increase and a modified distribution of root colonization sites for bacteria and (ii) root exploration of a larger volume of soil. Unexpectedly, the scatter in the ARISA coordinates at 7 and 35 days was higher in the case of inoculated maize (Fig. 3), meaning that inoculation with *A. lipoferum* enhanced the variability of the bacterial community between individual plants. This effect was not documented before, even with phytohormone-producing inoculants (Lippmann et al., 1995). *Azospirillum* inoculation can also change root physiology and exudation patterns (Heulin et al., 1987; Volpin et al., 1996), which in turn was likely to result in microbial selection (Savka and Farrand, 1997), in accordance with findings derived from diversity analysis of soil microbial communities exposed to opine-producing transgenic plants (Mansouri et al., 2002) or artificial root exudates (Baudoin et al., 2003). Such indirect effects of CRT1 inoculation took place without modifying the total number of root bacteria (in the order of $10^{8-9}$ cells per g of root at 7 and 35 days), as determined for selected samples by acridine orange direct microscopic counts.

In conclusion, inoculation of maize seeds with *A. lipoferum* CRT1 caused a shift in the structure of the indigenous rhizobacterial community at 7 and 35 days. This is the first assessment of *Azospirillum* inoculation on resident bacteria done in the field, and documenting the occurrence of a statistically significant ecological impact.

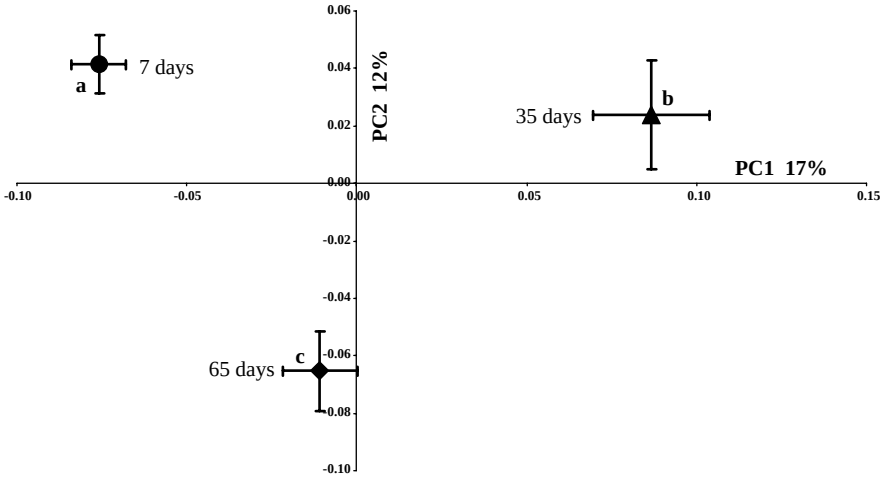


**Fig. 2.** Dynamics of the genetic structure of the rhizobacterial community of maize in non-inoculated plots, as indicated by PCA ordination of ARISA fingerprints obtained at three samplings i.e. at 7, 35 and 65 days after sowing. For each sampling, the means and standard errors are shown (3 plants from each of the 4 blocks; $n = 12$ plants). Each sampling was statistically distinct from the others according to Monte Carlo test (shown with letters a, b and c).

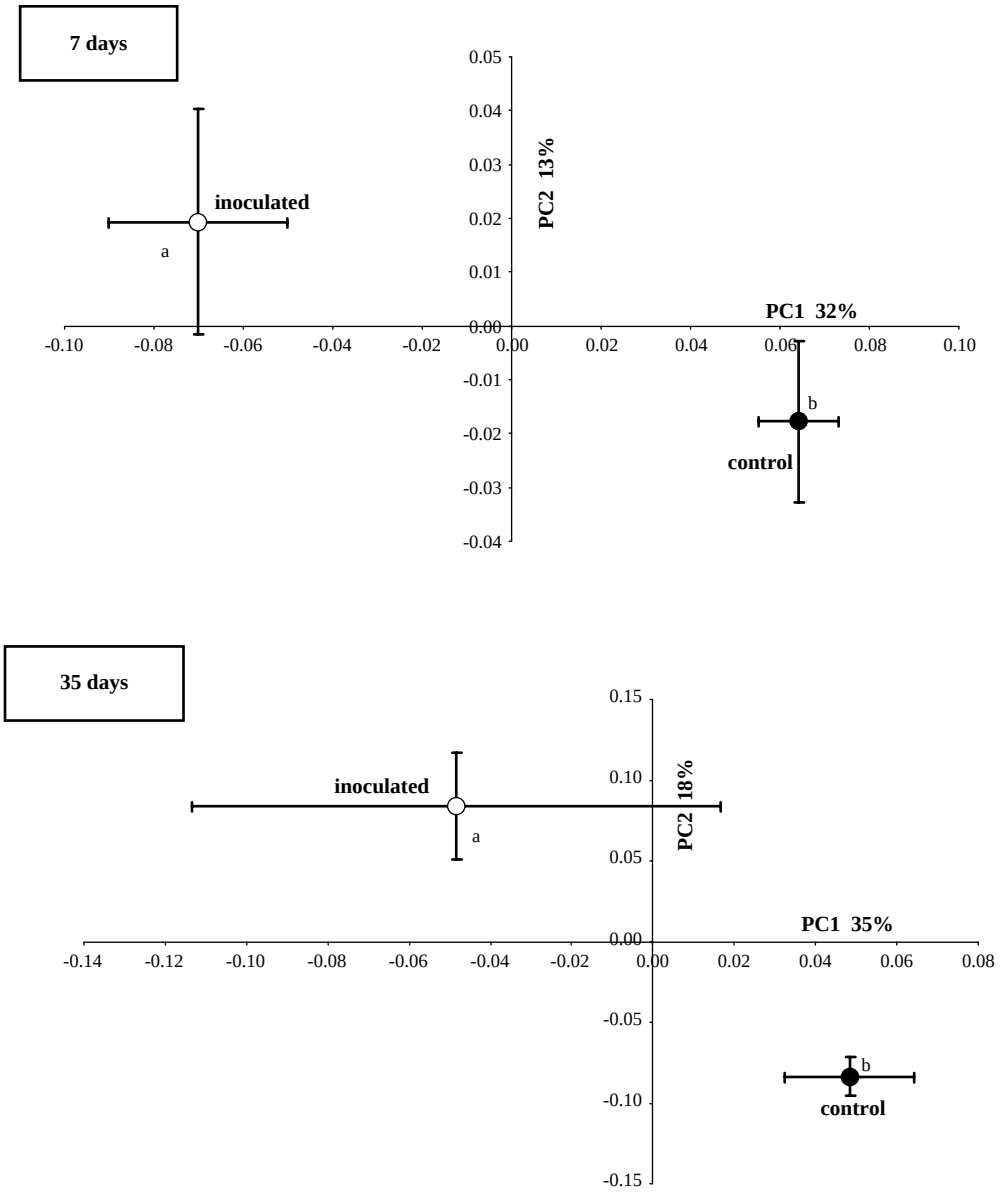


**Fig. 3.** Effect of seed inoculation with *A. lipoferum* CRT1 on the genetic structure of the rhizobacterial community of maize plants, as indicated by PCA ordination of ARISA fingerprints from non-inoculated plots and plots inoculated with CRT1 at 7 and 35 days after sowing. PCA results at 65 days could not be exploited since the weights of axes PC1 and PC2 were insufficient. At 7 and 35 days, the means and standard errors are shown (3 plants from each of the 4 blocks for each treatment; $n = 12$ plants). The impact of inoculation was statistically significant at each of the two samplings according to Monte Carlo test (shown with letters a and b).

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ORIGINAL ARTICLE

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

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**Keywords**
*Azospirillum*, plant growth-promoting rhizobacteria, specific primers, soil detection, 16S–23S rDNA intergenic spacer.

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## Abstract

**Aims:** To assess the applicability of the 16S–23S rDNA internal spacer regions (ISR) as targets for PCR detection of *Azospirillum* ssp. and the phytostimulatory plant growth-promoting rhizobacteria seed inoculant *Azospirillum lipoferum* CRT1 in soil.

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

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

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

## Introduction

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

Four main strategies are available to achieve this goal. The first relies on the introduction of one or several genetic markers (often conferring antibiotic resistance or encoding a fluorescent protein) in the bacterial strain prior to inoculation (Rothballer *et al.* 2003; Jäderlund *et al.* 2008). However, such genetically modified strains are primarily designed for experimental purposes. Their release is regulated (Morrissey *et al.* 2002) and is seldom implemented in the field (Viebahn *et al.* 2003). This

marker gene strategy presents other disadvantages as (i) the exogenous genes can represent a metabolic burden and may affect ecological fitness and/or phytostimulatory properties and (ii) cell enumeration following selective plating or microscopic observations is cumbersome and (with selective plating) does not enable monitoring of viable nonculturable cells. The second strategy, which is based on the use of spontaneous antibiotic-resistant mutants (Moënne-Loccoz *et al.* 2001; Mascher *et al.* 2003), requires careful screening of candidate mutants (to ensure that other cell properties are not affected; Mahaffee *et al.* 1997) and displays the drawbacks associated with colony counts. A third strategy based on natural antigenic properties of the cell may be followed if a specific antibody is available (Mascher *et al.* 2003). This strategy does not require any genetic modification, but monitoring is tedious and the detection limit is rather high.

Research might also focus on a fourth strategy that relies on the identification of strain-specific DNA sequences and the edition of probes/primers. This approach has been implemented on *Azospirillum* with randomly selected sequences (Fancelli *et al.* 1998; Jacoud *et al.* 1998), but can also target selected genes. The ribosomal intergenic spacer region (ISR) located between the 16S rRNA and 23S rRNA genes shows a high degree of variation in length and sequence and holds potential for intraspecies discrimination (Gürtler and Stanisich 1996; Buchan *et al.* 2001; Sadeghifard *et al.* 2006). Indeed, its sequence variability has been successfully exploited to edit probes and primers allowing species or subspecies discrimination when applied on clinical or environmental isolates (Glennon *et al.* 1996; Rachman *et al.* 2004; Valcheva *et al.* 2007). To date, the usefulness of the ISR to design strain-specific PCR primers and develop tools for environmental detection of bacteria has been successfully applied only for rhizobia inoculants (Tan *et al.* 2001). This would be of particular interest in the case of PGPR inoculants, where strain monitoring after large-scale environmental release is an important issue, because these bacteria interact in various ways with different microbial components of the soil/plant microbiota (Kabir *et al.* 1996; Moënne-Loccoz *et al.* 2001; Viebahn *et al.* 2003).

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

## Materials and methods

### Bacterial strains

The bacterial strains used are listed in Table 1. They were grown overnight at 28°C with shaking in liquid Luria–Bertani (Sambrook *et al.* 1989) medium supplemented with 2·5 mmol $l^{-1}$ $CaCl_2$ and 2·5 mmol $l^{-1}$ $MgSO_4$ (i.e. LB–$CaCl_2$–$MgSO_4$). Analysis with *Nitrobacter hamburgensis* X14 and *Nitrobacter winogradskyi* AG was carried out using total DNA kindly provided by V. Degrange (Université Lyon 1).

### Microcosm set-up and field inoculation

The soil used for the microcosms was collected at the experimental farm of La Côte Saint André (near Lyon, France). It was taken in the loamy surface horizon [clay 16%, silt 44%, sand 40%, organic matter 2·1%, pH (water) 7·0] of a luvisol (FAO), syn. alfisol (typic hapludalf; US Soil Taxonomy) cultivated with maize (El Zemrany *et al.* 2006).

Fresh soil was sieved (2 mm) and transferred to eight microcosms (14-cm diameter Petri dishes), each holding 103 g soil (equivalent to 90 g dry soil). Cells of *A. lipoferum* CRT1 in late log phase were collected from LB–$CaCl_2$–$MgSO_4$ plates and suspended in pure sterile water prior to inoculation into soil at $10^1$–$10^8$ culturable CRT1 cells per gram of soil (eight inoculation levels), as follows. For each inoculation level, volume of the cell suspension was adjusted in reference to the calibration curve between OD at 580 nm and density of culturable CRT1 cells grown on modified Luria–Bertani plates. The suspension was centrifuged (5000 ***g*** for 15 min), and the pellet was resuspended in sterilized pure water (total volume 10·8 ml). Each inoculum was evenly spread onto the soil surface of a microcosm using a pipette, and the soil was mixed thoroughly with a spatula. Soil was at water holding capacity (26% w/w as determined by gravimetry; Ranjard *et al.* 1997) after inoculation. The microcosms were incubated 24 h at 28°C and immediately processed for DNA extraction as described.

For field inoculation in La Côte Saint André, *A. lipoferum* CRT1 was prepared commercially in a peat formulation (Azo-Green™; Lipha/Nitragin, Meyzieu, France) and used to inoculate seeds ($3 \times 10^7$ CFU added per seed) of maize (cultivar PR38a24; Pioneer, Aussonne, France) immediately prior to sowing (80 000

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

| Species | Strain | Reference | PCR band using fAZO/rAZO | PCR band using fCRT1/rCRT1 |
|---|---|---|---|---|
| *Azospirillum* ssp. | | | | |
| *Azospirillum brasilense* | Sp245 | Baldani *et al.* 1986 | + (500)* | – |
| | PH1 | Rinaudo 1982 | + (480) | – |
| | Sp7 | Tarrand *et al.* 1978 | + (500) | – |
| | L4 | Kabir *et al.* 1996 | + (500) | – |
| | CFN-535 | Dobbelaere *et al.* 2001 | + (520) | – |
| | UAP-154 | Dobbelaere *et al.* 2001 | + (520) | – |
| *Azospirillum irakense* | KBC1 | Khammas *et al.* 1989 | + (450) | – |
| *Azospirillum lipoferum* | CRT1 | Fages and Mulard 1988 | + (520)† | + (250)‡ |
| | 4B | Bally *et al.* 1983 | + (520) | – |
| | $4V_I$ | Alexandre *et al.* 1999 | + (520) | – |
| | B506 | Elbeltagy *et al.* 2001 | + (520) | + (280) |
| | B510 | Elbeltagy *et al.* 2001 | + (520) | + (260) |
| | B518 | Elbeltagy *et al.* 2001 | + (520) | + (260) |
| | Br10 | Tarrand *et al.* 1978 | + (520) | + (280) |
| | Br17 | Tarrand *et al.* 1978 | + (520) | + (250) |
| | TVV3 | Trân Van *et al.* 1997 | + (520) | + (280) |
| Other $\alpha$-Proteobacteria | | | | |
| *Agrobacterium tumefaciens* | C58 | van Larebeke *et al.* 1974 | – | – |
| *Nitrobacter hamburgensis* | X14 | Bock *et al.* 1983 | – | – |
| *Nitrobacter winogradskyi (agilis)* | AG | Degrange and Bardin 1995 | + (1000) | – |
| *Rhizobium etli* | CFN42 | Quinto *et al.* 1985 | + (500) | – |
| *Rhizobium tropici* | CFN299 | Martínez-Romero *et al.* 1991 | – | – |
| $\beta$-Proteobacteria | | | | |
| *Ralstonia solanacearum* | GMI 1000 | Boucher *et al.* 1985 | + (700) | – |
| *Burkholderia vietnamiensis* | TVV75 | Trân Van *et al.* 2000 | – | – |
| *Alcaligenes xylosoxidans* | Cm4 | Belimov *et al.* 2001 | + (500) | – |
| $\gamma$-Proteobacteria | | | | |
| *Enterobacter agglomerans* | Cka5 | This study | + (500) | – |
| *Stenotrophomonas maltophilia* | Cy2 | This study | + (500) | – |
| Firmicutes | | | | |
| *Bacillus pumilus* | Fp1 | Belimov *et al.* 2001 | + (500) | – |
| *Bacillus* sp. | Cb17 | This study | – | – |
| *Microbacterium esteraromaticum* | Cr59 | This study | + (600) | – |
| *Rhodococcus* sp. | Fp2 | Belimov *et al.* 2001 | – | – |

*PCR product was made of 503- and 504-bp fragments as defined by sequencing.
†PCR product was made of 517- and 529-bp fragments as defined by sequencing.
‡PCR product was made of 249-bp fragments as defined by sequencing.

seeds per ha), as described (El Zemrany *et al.* 2006). Current commercial farming practices were followed for chemical control of pests and weeds. The four non-inoculated and four inoculated plots (each 6 m wide × 15 m long) received 70 kg mineral N per ha (half nitrate and half ammonium). The two samplings (three plants per plot) were performed when maize reached two to three leaves (18 days after seed inoculation) and nine to ten leaves (57 days after seed inoculation). These samples were used for CRT1 enumeration based on colony hybridization (El Zemrany *et al.* 2006) and for the present study.

### DNA extraction

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

Environmental DNA was extracted in triplicate from 500 mg bulk soil (microcosm experiment) or crushed maize root system and adhering soil (field experiment; Baudoin *et al.* 2009) with the FastDNA® SPIN® kit (for soil) (BIO 101, Inc., Carlsbad, CA, USA). DNA samples

were resolved by electrophoresis in a 0·8% agarose gel, stained with ethidium bromide and photographed using a Gel Doc 1000 camera (Bio-Rad, Ivry sur Seine, France). Dilutions of calf thymus DNA (Boehringer Mannheim, Meylan, France) were included in each gel, and a standard curve of DNA concentration (25, 50, 100, 200, 400 and 800 ng) versus integrated pixel ($R^2 = 0{\cdot}99$) was used to estimate the final DNA concentration in the extracts. The ethidium bromide staining intensities were analysed using Molecular Analyst software (Bio-Rad).

### PCR analyses

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

Primers fAZO and rAZO were designed to amplify target sequences of the genus *Azospirillum*, and primers fCRT1 and rCRT1 for detection of strain CRT1 (Table 2). These primers were used in a nested configuration: a PCR was performed first on strain genomic DNA or environmental DNA (10 ng) using the universal primers FGPS1490-72/FGPL132-38. Then, 0·5 $\mu$l of these PCR products was used as target DNA in a second PCR round, using either fAZO/rAZO or fCRT1/rCRT1. In the case of environmental DNA, the intensities of the banding patterns produced in a 2% agarose electrophoretic gel by the first-round PCR products were quantified by image analysis, and amplicon concentrations were adjusted to a same level (when necessary) to perform the second PCR round on equivalent amounts of target DNA. This second PCR was performed in a 50-$\mu$l volume containing 5 $\mu$l of 10× dilution buffer (supplemented with 1·5 mmol $l^{-1}$ $MgCl_2$), 200 $\mu$mol $l^{-1}$ of each dNTP, 0·5 $\mu$mol $l^{-1}$ of each primer, 1·5 $\mu$g of T4 gene 32 protein (Roche, Meylan, France), 2·5 units of Expand® High Fidelity *Taq* polymerase (Boehringer Mannheim) and 0·5 $\mu$l of ISR amplicon. Amplifications were performed using a GeneAmp PCR System 2400 (Perkin-Elmer, Courtaboeuf, France), with an initial denaturation for 3 min at 94°C, 25 PCR cycles (1 min at 94°C, 1 min at 59°C for fAZO/rAZO or 58°C for fCRT1/rCRT1, 1 min at 72°C), a final elongation for 5 min at 72°C, followed by a cooling step at 5°C. Nested PCR amplicons were resolved in 2% agarose gels with Smart ladder (Eurogentec, Seraing, Belgium), stained with ethidium bromide and photographed using Gel Doc 1000.

Selected *A. lipoferum* strains were compared based on the repetitive sequence-based PCR protocols BOX-PCR (using the Box-A1R primer, Versalovic *et al.* 1998) and ERIC-PCR (enterobacterial repetitive intergenic consensus-PCR) as described by Rademaker *et al.* (1998), and also RAPD analysis using primer 1253 (5′-GTTTCCGC-CC-3′; Fancelli *et al.* 1998) as described by Vial *et al.* (2006). Electrophoretic profiles were compared using GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium) and were combined for the three methods. Similarity analysis of the resulting composite profiles was performed based on presence/absence of bands (Jaccard coefficient) and clustering using the unweighted pair group method with arithmetic means (UPGMA).

### 16S–23S rDNA ISR cloning and sequencing

Clone libraries of the PCR-amplified rDNA of *A. lipoferum* CRT1 and *A. brasilense* Sp245 obtained with primers FGPS1490-72/FGPL132-38 were constructed using the pGEM®–T Easy Vector (Promega, Charbonnières, France) and chemically competent *Escherichia coli* DH5α™ cells (Invitrogen, Cergy Pontoise, France). Briefly, for each strain the two main bands, as identified on agarose gel, were excised and purified with MinElute (Qiagen). In both cases, the agarose band of the highest length harboured indeed two sequences of similar sizes as revealed by an acrylamide gel migration (see Fig. 1). The PCR products were then ligated with pGEM®–T Easy Vector, according to manufacturer's instructions. *E. coli* DH5α™ cells (Invitrogen) were transformed with the constructs, as specified by the supplier, and grown overnight at 37°C on LB–$CaCl_2$–$MgSO_4$ medium supplemented with 100 $\mu$g $ml^{-1}$ ampicillin, 0·5 mmol $l^{-1}$ IPTG and 80 $\mu$g $ml^{-1}$ X-Gal.

**Table 2** Primers used in the study

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

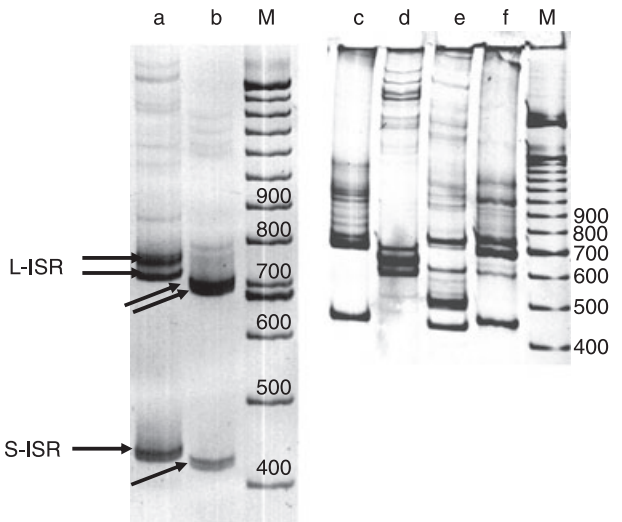



**Figure 1** 16S–23S rDNA intergenic spacer fingerprints of *Azospirillum lipoferum* CRT1 (a), *Azospirillum brasilense* Sp245 (b), *A. lipoferum* $4V_I$ (c), *A. brasilense* Sp7 (d), *A. brasilense* PH1 (e) and *A. brasilense* L4 (f) on a 5% polyacrylamide gel. M, 100-bp ladder. Three main bands were obtained for each strain; in lane b, band visualization is hampered by the proximity between two of the main bands. Arrows indicates large and small ISR bands that were excised from agarose gels for sequencing.

From 20 to 100 colonies were selected for each type of 16S–23S ISR identified in *Azospirillum* strains. Cloned DNA was extracted by boiling in 100 $\mu$l ultrapure water for 15 min. The bacterial lysates were centrifuged (10 000 ***g***, 10 min), and 2 $\mu$l supernatant was used as template DNA in subsequent PCR steps. Plasmid inserts from the positive clones were amplified with primers M13f and M13r (Promega) and digested with *Alu*I and *Hae*III (Boehringer Mannheim) for *A. lipoferum* CRT1 and *Alu*I for *A. brasilense* Sp245. Individual clones were placed into restriction groups based on a 100% identity threshold of the restriction patterns. A total of 16 and 7 clones were sequenced from the two libraries of *A. lipoferum* CRT1, respectively, and ten and five clones from those of *A. brasilense* Sp245. The clones selected for sequencing were amplified with primers M13f/M13r. Purified PCR products were then sequenced in both directions with primers FGPS1490-72/FGPL132-38. Sequencing was performed by Genome Express (Meylan, France) on an ABI377 sequencer FS (Perkin-Elmer) using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with Ampli*Taq* DNA polymerase (Perkin-Elmer).

### Sequence analysis

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

### Nucleotide sequence accession numbers

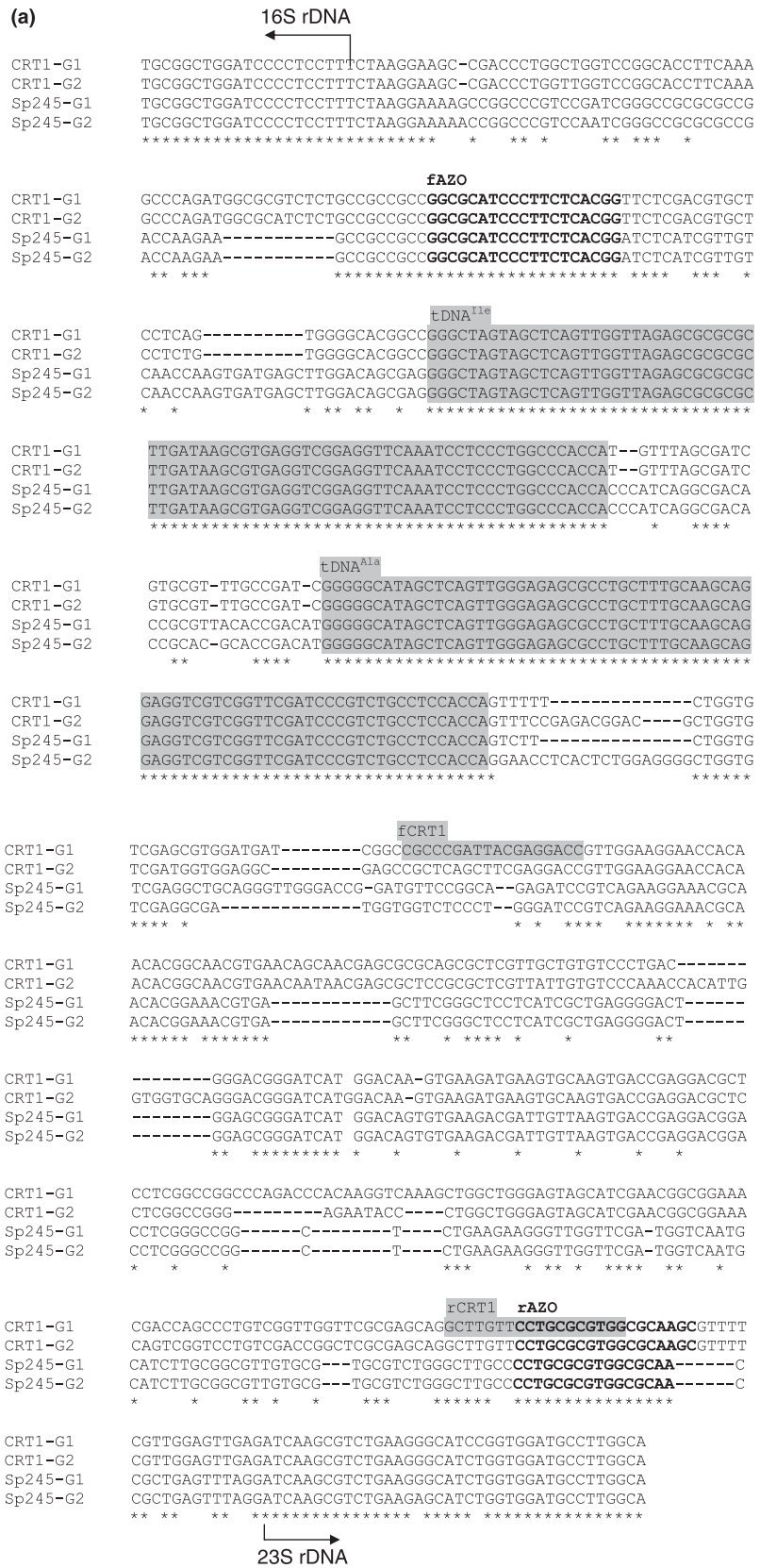
The sequences of the small and large 16S–23S ISR DNA of *A. lipoferum* CRT1 and *A. brasilense* Sp245 were deposited in the GenBank nucleotide database under the numbers AY685928, AY685927, AY685926, AY685925, AY685924 and AY685923.

## Results

### Size and sequence of the 16S–23S ISR

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

Restriction analysis of the L-ISR revealed two restriction groups within each strain. Alignment of L-ISR sequences obtained for *A. lipoferum* CRT1 yielded a 613-bp sequence (60·8% GC) and a 601-bp sequence (61·6% GC), which corresponded to the two restriction groups (Fig. 2a). These two sequences showed 87·8% identity with one another. Similarly, a 572-bp sequence (61·1% GC) and a 571-bp sequence (61·4% GC) identical at 92% were obtained for *A. brasilense* Sp245. For each strain, alignments of the S-ISR sequences depicted a single sequence (Fig. 2b) of 281 bp for *A. lipoferum* CRT1 (56·6% GC) and 269 bp for *A. brasilense* Sp245 (58·7% GC). Each of the two L-ISR and the S-ISR of *A. lipoferum* CRT1 shared respectively 46% and 62% homology with those of *A. brasilense* Sp245. Multiple alignments of L-ISR





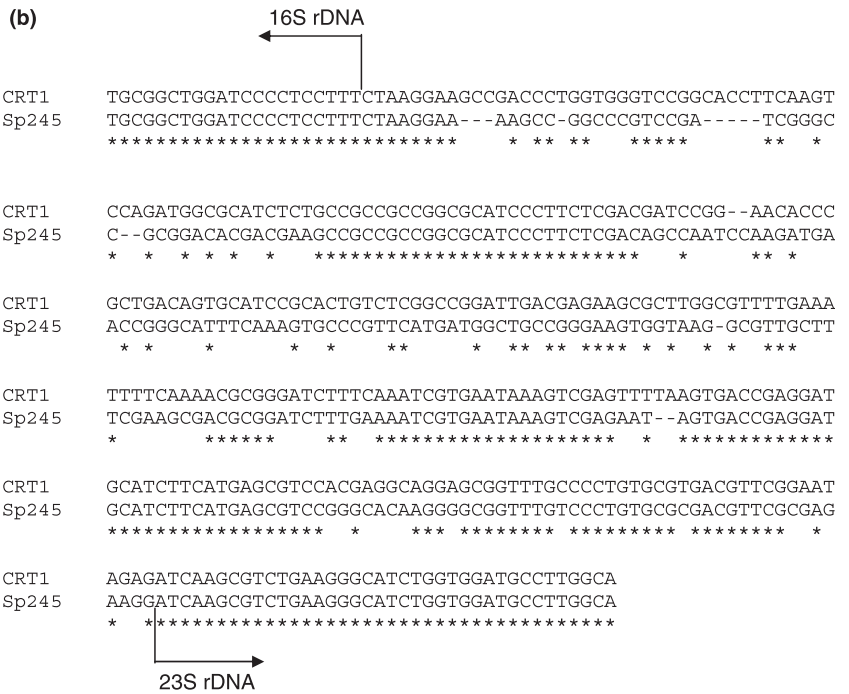



**Figure 2** Multiple sequence alignment from representative sequences of the two L-ISR groups (a) and the S-ISR group (b) for *Azospirillum lipoferum* CRT1 and *Azospirillum brasilense* Sp245. In (a), the L-ISR are termed CRT1-G1 and CRT1-G2 (for CRT1), and Sp245-G1 and Sp245-G2 (for Sp245). tRNA sequences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

and S-ISR sequences representative of the different restriction groups also highlighted several domains identical in strains CRT1 and Sp245 (Fig. 2). The two largest domains conserved (76–77 bp), which were located in tandem within the L-ISR sequences, were identified as the genes encoding $tRNA^{Ile}$ and $tRNA^{Ala}$. BlastN analysis revealed that the latter genes were highly conserved when considering the Proteobacteria (more than 96% homology among the first 100 sequences producing significant alignments). The S-ISR elements were lacking tRNA genes. The other domains identical in CRT1 and Sp245 were much smaller (at the most 28 and 31 bp in L-ISR and S-ISR, respectively).

BlastN of the L-ISR and S-ISR of *A. brasilense* Sp245 with preliminary data from the Sp245 sequencing project gave 96% and 91% homology, respectively. The observed partial homologies could then be related to the presence of several copies of the *rrn* operons in the genome of *A. brasilense* Sp245 (Martin-Didonet *et al.* 2000) as well as the incomplete sequencing of the genome and partial screening of ISR regions in our study.

### Design of genus- and strain-specific PCR primers

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

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

### Specificity of primers targeting the *Azospirillum* genus

The primers fAZO/rAZO, which target the genus *Azospirillum*, were first used on bacterial DNA extracted from *Azospirillum* and non-*Azospirillum* strains. All 16 *Azospirillum* strains were successfully amplified, yielding mainly one strong band (*c.* 450–520 bp; Table 1). In addition, 8 of 14 non-*Azospirillum* strains also yielded a unique band (Table 1), but of lower intensity, and whose size

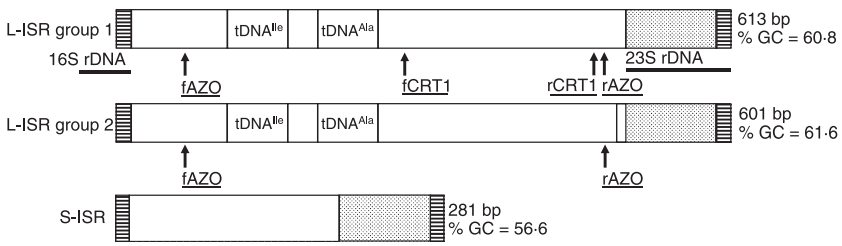



**Figure 3** 16S–23S rDNA intergenic spacer structure for the two L-ISR groups and the single S-ISR group of *Azospirillum lipoferum* CRT1. Length and %GC are given for ISR sequences (without the 16S and 23S rDNA tails). Locations of primers fCRT1/rCRT1 and fAZO/rAZO are indicated. Primer rCRT1 has an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

(500–1000 bp) was in some cases similar to that of the dual band visible for *A. lipoferum* CRT1 (at about 520 bp). Among them, the phylogenetically-closest bacteria were the α-Proteobacteria *Rhizobium etli* CFN42 and *Nit. winogradskyi* AG. Increasing the annealing temperature from 59 to 60°C increased specificity, but when tested on seven representative *Azospirillum* strains the amplification was only successful with three of them (i.e. CRT1, Sp245 and PH1; not shown). Nevertheless, these primers were applied in the next evaluation steps (at 59°C) to assess their usefulness with complex environmental DNA extracts.

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

### Specificity and detection level of primers targeting *Azospirillum lipoferum* strain CRT1

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

To assess whether these primers could detect *A. lipoferum* CRT1 in environmental samples, they were directly

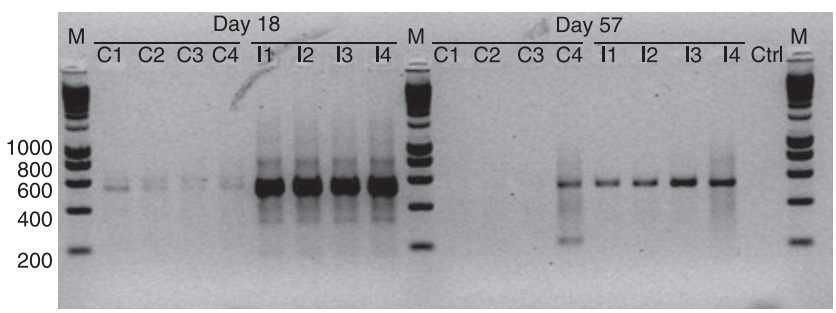



**Figure 4** Two percent agarose migration profiles of amplicons obtained by nested PCR with universal primers FGPS1490-72/FGPL132-38 and then primers fAZO/rAZO on DNA extracts from field maize rhizosphere, at 18 and 57 days after sowing. C1–C4, noninoculated control (one sample per plot); I1–I4, seed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

applied in a PCR assay using total soil DNA extracted from microcosms inoculated with various levels of CRT1 cells. Strain CRT1 could be detected after refinement of initial PCR conditions (i.e. primer concentration doubled and annealing temperature raised from 55 to 58°C), provided it was at least at $10^6$ CFU $g^{-1}$ soil (not shown). However, the detection level was increased $10^4$-fold when using a nested PCR protocol starting with the universal primers FGPS1490-72/FGPL132-38 (Fig. 6a). Signal intensity decreased progressively (yet in a nonlinear way) between $10^8$ and $10^3$ CFU $g^{-1}$ soil and dropped abruptly between $10^3$ and $10^2$ CFU $g^{-1}$ soil.

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

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

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

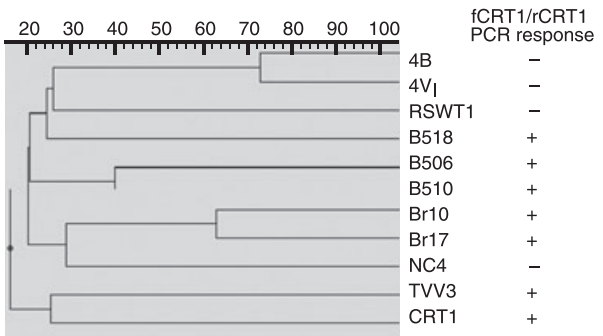



**Figure 5** Genetic similarity based on combined analysis of BOX-A1R, ERIC and RAPD markers of *Azospirillum lipoferum* strains responding or not to amplification with primers fCRT1/rCRT1. The UPGMA clustering method was applied to a similarity matrix calculated with the Jaccard coefficient.

## Discussion

Despite the use of *A. lipoferum* CRT1 as phytostimulatory inoculant of crops in different countries (Okon and Labandera-Gonzalez 1994), no rapid method was available to monitor the fate of the strain once released into the field. So far, detection of CRT1 in soil is based on the use of a semiselective medium followed by colony hybridization to a DNA probe obtained randomly from the CRT1

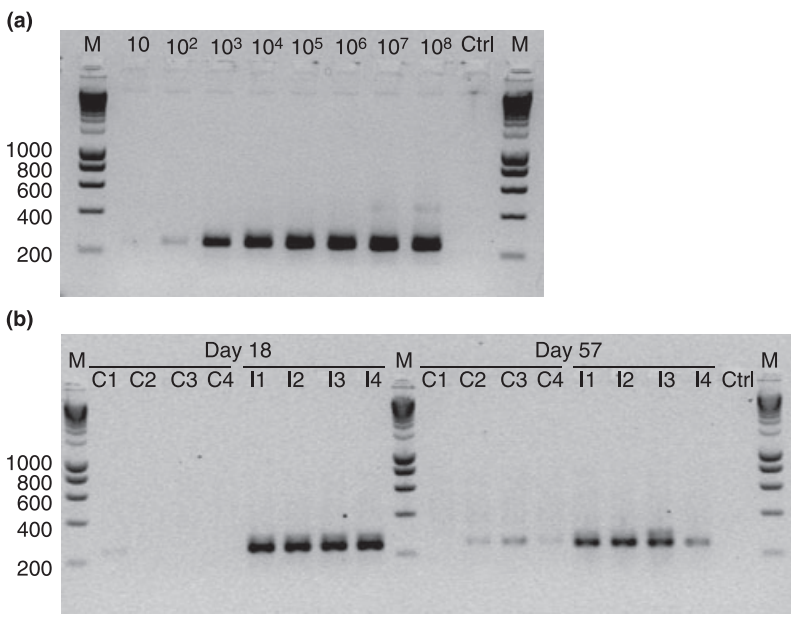



**Figure 6** Two percent agarose migration profiles of amplicons obtained by nested PCR with universal primers FGPS1490-72/FGPL132-38 and then primers fCRT1/rCRT1 on DNA extracts from microcosm soil (a) or on DNA extracts from field maize rhizosphere, at 18 and 57 days after sowing (b). Top lane numbers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

genome (Jacoud *et al.* 1998; El Zemrany *et al.* 2006). However, this approach is time-consuming because it relies on two cultivation steps (5 days of incubation on a semiselective medium followed by 2 days of growth on a generalist medium) and hybridization procedure. In this study, the sequences of the 16S–23S ISR of two strains from different *Azospirillum* species were determined to assess their applicability as priming sites for a PCR detection tool targeting the *Azospirillum* genus and the crop inoculant *A. lipoferum* CRT1 in environmental samples.

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

ISR polymorphism has been widely used to identify or differentiate isolates belonging to various clinical and environmental genera, such as *Legionella* (Riffard *et al.* 1998), *Escherichia* (Buchan *et al.* 2001), *Pseudomonas* (Moënne-Loccoz *et al.* 2001) or *Streptococcus* (Chen *et al.* 2005), either by edition of probes or by comparison of RFLP profiles. Even though the ribosomal spacer element is thought to be highly variable among eubacteria (Gürtler and Stanisich 1996), the level of this heterogeneity in a given bacterial species is not always so high, and the usefulness of this element in defining species- or subspecies-specific primers cannot be taken for granted. Successful primer design was reported for screening isolates at the species level, such as in several *Lactobacillus* (Berthier and Ehrlich 1998) and *Carnobacterium* (Rachman *et al.* 2004) species, *Mycoplasma pulmonis* (Takahashi-Omoe *et al.* 2004) or *Lactobacillus sanfranciscensis* (Valcheva *et al.* 2007). In contrast, the minor differences in ISR sequences from *Bacillus thuringiensis* strains were insufficient to design species-specific probes (Bourque *et al.* 1995). In our study, we observed sufficient sequence variability to suggest that the ISR could be useful to design PCR-based detection protocols. Thus, two couples of primers, one targeting the genus *Azospirillum* and the other the strain *A. lipoferum* CRT1, were designed from the L-ISR sequences.

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

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

Detection limit was $10^3$ and $10^2$ CFU of the inoculant with the primers targeting the *Azospirillum* genus and strain CRT1 respectively. However, it must be kept in mind that this sensitivity could not be reached without a nested PCR, using first universal primers FGPS1490-72/FGPL132-38. Some authors succeeded in applying group- (Smart *et al.* 1996), species- (Grote *et al.* 2002;

Wang *et al.* 2003) and even strain-specific primers (Tan *et al.* 2001) to environmental DNA extracts. Detection limits of the assay were not always reported, but it appears that with such a complex DNA mix, the nested PCR approach gave the best detection limit, enhanced by several ten folds in comparison with direct PCR. For instance, as little as 60 fg of *Phytophthora nicotianae* DNA was detected in DNA extracts obtained from artificially inoculated healthy roots, which was 1000 times more sensitive than conventional PCR (Grote *et al.* 2002). Likewise, our data indicate a $10^4$-fold lowering of the detection limit. Authors working with other target genes also improved their threshold detection in soil with a nested protocol (Tsushima *et al.* 1995; Rosado *et al.* 1998), but here this was perhaps facilitated by the fact that rDNA sequences are present in several copies in bacterial genomes, which is likely to have a positive effect on PCR efficiency. A comparison of the detection sensitivity of our approach to the published methods based on culture and colony hybridization (Jacoud *et al.* 1998; El Zemrany *et al.* 2006) showed that our method is very efficient. Detection limit was $10^2$ CFU g soil using fCRT1/rCRT1 primers in a culture-independent approach whereas other methods could not detect *A. lipoferum* CRT1 below $10^3$ CFU, which corresponded to the culture detection limit. Furthermore, our approach is less time-consuming allowing the treatment of a large number of samples. The use of fAZO/rAZO or fCRT1/rCRT1 primers for monitoring *A. lipoferum* CRT1 in field plots where inoculated seeds were used gave a stronger signal at the first sampling (18 days after inoculation) than at the second one. This could suggest a decline of the population size of strain CRT1 in maize rhizosphere between the two samplings, as often found with bacterial inoculants in field release studies (Tsushima *et al.* 1995; Moënne-Loccoz *et al.* 1998), but here such a decline was not observed when monitoring the inoculant by colony counts (El Zemrany *et al.* 2006). This discrepancy might result from a decrease in the efficacy of the current protocol between the two samplings as (i) the efficiency of DNA extraction from rhizosphere samples decreases as plant ages (E. Baudoin, O. Couillerot, S. Spaepen, Y. Moënne-Loccoz, S. Nazaret, unpublished data), (ii) plant metabolites and/or rhizodeposits inhibiting PCR are perhaps more prevalent in rhizosphere extracts from older plants, and (iii) exposure to environmental stress reduces the effectiveness of PCR protocols in bacteria (Rezzonico *et al.* 2003). Putative CRT1 cells were also detected in certain control plots, perhaps as a result of transport of soil and crop residues across neighbouring plots during tillage. However, it cannot be totally discounted that CRT1 or related *A. lipoferum* strains are naturally present in low numbers in this soil and managed to colonize the rhizosphere of certain maize plants, as the results of El Zemrany *et al.* (2006) suggest.

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

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# Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR

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**Abstract**

Denitrification, the reduction of nitrate to nitrous oxide or dinitrogen, is the major biological mechanism by which fixed nitrogen returns to the atmosphere from soil and water. Microorganisms capable of denitrification are widely distributed in the environment but little is known about their abundance since quantification is performed using fastidious and time-consuming MPN-based approaches. We used real-time PCR to quantify the denitrifying nitrite reductase gene (*nirK*), a key enzyme of the denitrifying pathway catalyzing the reduction of soluble nitrogen oxide to gaseous form. The real-time PCR assay was linear over 7 orders of magnitude and sensitive down to $10^2$ copies by assay. Real-time PCR analysis of different soil samples showed *nirK* densities of $9.7\times10^4$ to $3.9\times10^6$ copies per gram of soil. Soil real-time PCR products were cloned and sequenced. Analysis of 56 clone sequences revealed that all cloned real-time PCR products exhibited high similarities to previously described *nirK*. However, phylogenetic analysis showed that most of environmental sequences are not related to *nirK* from cultivated denitrifiers.


*Keywords:* Nitrite reductase; *NirK*; Real-time PCR; Soil; Denitrification

## 1. Introduction

Denitrification is a respiratory process in which oxidized nitrogen compounds are used as alternative electron acceptors for energy production when oxygen is limited. It is the major mechanism by which fixed nitrogen returns to the atmosphere from soil and water, thus completing the N-cycle. This removal of soluble nitrogen oxide from the biosphere is of great importance in agriculture, where it can account for significant losses of nitrogen fertilizer from soil, and also in wastewater treatment.

Denitrification has also received considerable interest recently because it leads to $N_2O$ emissions, it is an

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important greenhouse gas (Lashof and Ahuja, 1995) and a natural catalyst of stratospheric ozone degradation (Bange, 2000). Bacteria capable of denitrification are widely distributed in the environment and exhibit a high taxonomic diversity (Tiedje, 1988).

Denitrification consists of four reaction steps by which nitrate is reduced into dinitrogen gas by the metalloenzymes nitrate reductases, nitrite reductases, nitric oxide reductases and nitrous oxide reductase. The nitrite reductase is the key enzyme of this respiratory process since it catalyzes the reduction soluble nitrite into gas. Thus, previous studies have used probes or antibodies against this enzyme to identify denitrifying isolates (Coyne et al., 1989; Ward, 1995; Ward and Cockcroft, 1993). Two types of nitrite reductase that are different in terms of structure and prosthetic metal have been characterized: a copper nitrite reductase encoded by the *nirK* gene and a cytochrome $cd_1$-nitrite reductase encoded by the *nirS* gene (Zumft, 1997). Since 1998, several studies have reported the use of *nirK* or *nirS* as molecular markers of the denitrifying bacteria to study their diversity in various environments (Avrahami et al., 2003; Braker et al., 1998, 2000, 2001; Hallin and Lindgren, 1999; Liu et al., 2003; Prieme et al., 2002; Yoshie et al., 2004). However, abundance of denitrifiers in the environment is still determined by MPN and only the *nirS* gene has been used to quantify this functional community using competitive PCR and real-time PCR as cultivation-independent approaches (Michotey et al., 2000; Gruntzig et al., 2001). Since denitrification is not restricted to cytochrome $cd_1$-nitrite reductase denitrifiers, we developed a real-time PCR assay targeting the *nirK* gene in order to quantify in soils the denitrifiers having the copper nitrite reductase.

## 2. Materials and methods

### 2.1. Environmental samples and bacterial strains

Six different soils were selected for their contrasting physicochemical characteristics (Table 1). All samples were obtained from the first 20-cm top layer. La Bouzule soil was collected from a wheat planted plot in an experimental field of the ENSAIA domain of La Bouzule (Meurthe et Moselle, North East of France). This soil was amended either with C 150 μg C g soil$^{-1}$ day$^{-1}$ or water. Vannecourt soil was collected from a winter wheat agricultural field (Moselle, North East of France). Paris soil was obtained from garden soils near Paris. Soil from Kenya was collected in a glade of the Kakamega rainforest located in the highlands of western Kenya. Termite nests (*Cubitermes* sp) from Burkina Faso were collected in a savannah landscape located in Tiogo in the west part of Burkina Faso. Rennes soil was collected in a maize planted field amended with ammo-nitrate (110 kg N ha$^{-1}$ year$^{-1}$). The strains used in this study are listed in Table 2.

Table 1
Properties of the soils analysed

| Soils | % of: | | | | | pH |
|---|---|---|---|---|---|---|
| | Clay | Sand | Silt | N | C org | |
| Bouzule | 33.3 | 15.4 | 51.3 | N.D. | 15.3 | 5.8 |
| Kenya | 35.6 | 33.8 | 32.3 | 0.27 | 3.77 | N.D. |
| Paris | 13 | 77 | 10 | 0.009 | 1.1 | 7.7 |
| Rennes | 14 | 19.3 | 66.6 | 1.04 | 9.41 | 5.89 |
| Termite mound Burkina | 25.9 | 22.5 | 51.2 | 0.29 | 3.46 | N.D. |
| Vannecourt | 22.5 | 33.2 | 44.3 | 0.2 | 1.6 | 6 |

### 2.2. DNA extraction

DNA was extracted from three 250-mg aliquots of soil samples (Martin-Laurent et al., 2001). Briefly, samples were homogenized in 1 ml of extraction buffer during 30 s at 1600 rpm in a minibead beater cell disrupter (Mikro-DismembratorS; B. Braun Biotech International). Soil and cells debris were eliminated by centrifugation (14,000×*g* for 5 min at 4 °C). Afterwards, 5 M sodium acetate was used to remove the proteins and nucleic acids were precipitated using cold ethanol. DNA was washed with 70% ethanol and purified through a sepharose 4 B spin column. At the end, DNA was quantified by spectrophotometry at 260 nm using a BioPhotometer (Eppendorf, Hamburg, Germany) and by comparison to DNA standard in 1% (wt/v) agarose gel electrophoresis.

### 2.3. nirK primers development and testing

To design the *nirK* primers, *nirK* sequences from cultivated strains, from complete and unfin-

Table 2
Bacterial strains used in this study and test of the *nirK* primer sets to amplify the copper nitrite reductase

| Strains | Nir type | Result of PCR[a] |
|---|---|---|
| *Alcaligenes faecalis* ATCC8750 | Cu (2,0) | + |
| *Achromobacter cycloclastes* ATCC21921 | Cu (0,0) | + |
| *Bradyrhizobium japonicum* 526 | Cu (0,2) | + |
| *Escherichia coli* JM109 | None | − |
| *Rhizobium meliloti* | Cu (1,0) | + |
| *Rhodobacter sphaeroides* DSM158 | Cu (2,0) | + |
| *Pseudomonas fluorescens* C7R12 | $cd_1$ | 0 |

Numbers of mismatches of the *nirK* sequences from reference strains with forward and reverse primers are indicated in parenthesis.

[a] +, visible band of the expected size; −, no visible band; 0, non-specific bands.

ished bacterial genomes and from environmental *nirK* libraries, were aligned using the ClustalX software V.101 (Thompson et al., 1997) and compared to select conserved regions by eye. Two degenerated primers (5′–3′) nirK876 (ATYGGCGGVAYGGCGA) and nirK1040 (GCCTCGATCAGRTTRTGGTT) were designed to amplify a 165-bp fragment (*nirK* from *Sinorhizobium meliloti* 1021 was used as reference sequence for numbering). The nirK1040 primer is based on the sequence of the *nirK* primer designed by Braker et al. (1998) and Hallin and Lindgren (1999). Several copper nitrite reductase and cytochrome $cd_1$ denitrifiers and non-denitrifying strains were used as positive and negative control to test the specificity of the primer set (see Table 2).

### 2.4. Real-time PCR assay

Amplification of real-time PCR products was carried out with a Smart Cycler (Cypheid®, USA) using SYBR Green as detection system in a reaction mixture of 25 μl containing: 0.5 μM of each primer for *nirK*, 12.5 μl of SYBR Green PCR master mix, including HotStar Taq™ DNA polymerase, Quanti Tec SYBR Green PCR Buffer, dNTP mix with dUTP, SYBR Green I, ROX and 5 mM $MgCl_2$ (QuantiTect™ SYBR® Green PCR Kit, QIAGEN, France), 1.25 μl of DNA diluted template corresponding to 12.5 ng of total DNA, and Rnase-free water to complete the 25-μl volume.

The conditions for nirK real-time PCR were 120 s at 50 °C, 900 s at 95 °C for enzyme activation as recommended by the manufacturer (QuantiTect™ SYBR® Green PCR Kit, QIAGEN); afterwards six touchdown cycles were performed: 15 s at 95 °C for denaturation, 30 s at 63 °C for annealing, 30 s at 72 °C for extension and 15 s at 80 °C for a final data acquisition step. The annealing temperature was progressively decreased by 1 °C down to 58 °C. Finally, a last cycle with an annealing temperature of 58 °C was repeated 40 times. One last step from 60 to 95 °C with an increase of 0.2 deg/s was added to obtain a specific denaturation curve. Purity of amplified products was checked by the observation of a single melting peak and the presence of a unique band of the expected size in a 3% agarose gel stained with ethidium bromide.

### 2.5. Quantification of nirK from soil samples

Two independent real-time PCR assays were performed on each of the three replicate soil DNA extracts. The standard curve was created using 10-fold dilution series of three linearized plasmids containing the different *nirK* genes from environmental samples. The detection limit of our assay in soils was determined using 10-fold dilutions of soil DNA. Soil DNA was also tested for inhibitory effect of coextracted substance by determining the *nirK* copy number in 10-fold dilutions of soil DNA and by adding $10^6$ copies of the target gene in the lowest dilution of soil DNA.

To check specificity, real-time PCR products from one replicate of each environment were cloned into the pGEM-T Easy Vector System (Promega, France) according to the instructions of the manufacturer. Approximately eight clones from each soil were then randomly chosen for sequencing using DTCS-1 kit (Beckman, Coulter) with universal primer T7 in a Ceq 2000 XL sequencer. The resulting sequences were deposited in GenBank under accession numbers AY675449-AY675504.

### 2.6. Calculation and statistical analysis

A one-way analysis of variance was performed to compare the *nirK* abundance between the different

soil samples. Means were compared using the least significant difference (LSD) test at $P<0.05$.

## 3. Results

### 3.1. nirK primers specificity

DNA from denitrifying strains containing either the copper or the cytochrome $cd_1$ nitrite reductase gene and from a non-denitrifying strain was used to test the specificity of the designed primers. No non-copper nitrite reductase strain gave a PCR amplification (Table 2). Application of the designed primers to real-time PCR assay using DNA extracted from various soil environments as template results in a single band of the expected size of approximately 160 bp except in the Kenya soil exhibiting two non-specific faint bands (Fig. 1). Analysis of data from RT-PCR showed that a single melting peak corresponding to the standard DNA was observed for all soil samples (data not shown).

### 3.2. Performance of standard curves and detection limit

Plasmids containing cloned *nirK* genes were used to draw a standard curve relating Ct to the added mass of linearized plasmid DNA and the number of gene copies. The same linear response was observed with the three tested plasmids for 7 orders of magnitude, ranging from $10^2$ to $10^8$ *nirK* gene copies ($r^2$=0.999) (Fig. 2).

The sensitivity of the assay was determined using a dilution series of extracted soil DNA. The overall sensitivity in soil samples was $10^2$ targets per assay, corresponding to the same order of magnitude when compared to DNA standard curve. After addition of $10^6$ copies of the standard DNA to soil samples diluted below the detection limit, $1.9\times10^6$ (Standard Error: $1.8\times10^4$) copies were obtained out of the $1.3\times10^6$ (Standard Error: $3.2\times10^5$) expected. The absence of inhibitory substance at the dilution used was also confirmed by the similar amplification efficiencies obtained with the 10-fold dilution of soil DNA extracts.

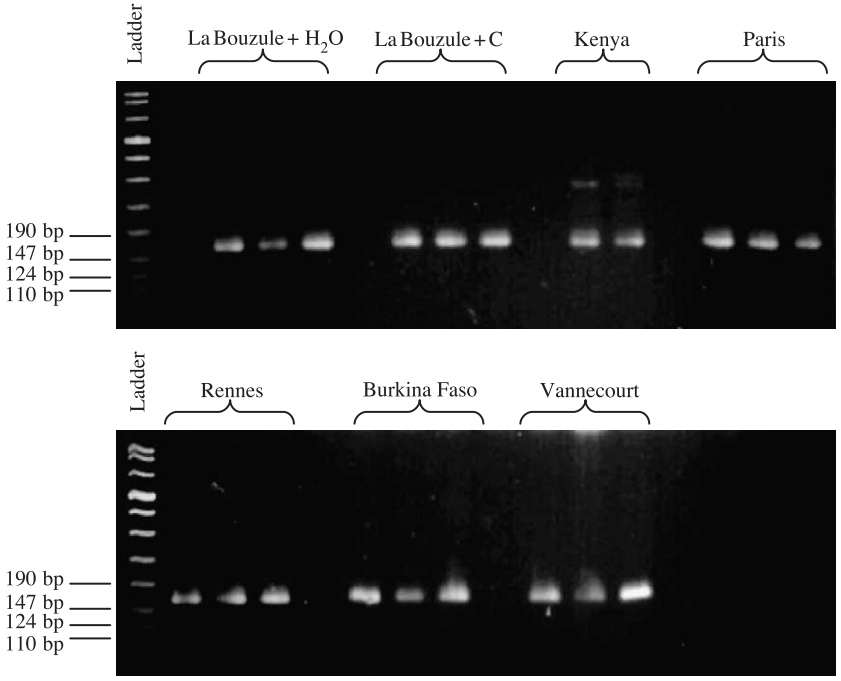


Fig. 1. Agarose gel (3%) electrophoresis of the real-time PCR products using DNA extracted from the different soil samples as template. Molecular size marker VIII from Boehringer Mannheim was used as ladder.

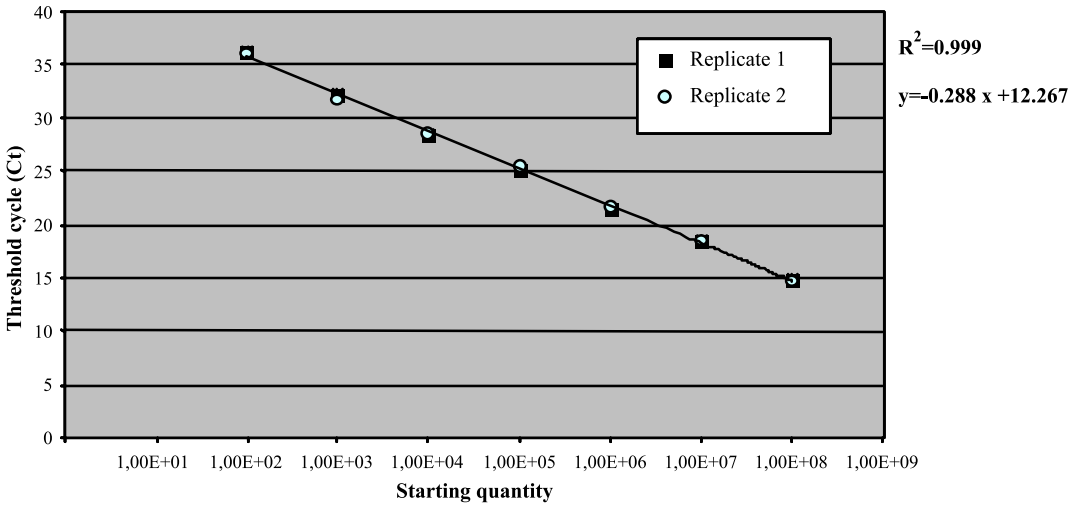


Fig. 2. Calibration curve plotting log starting *nirK* copy numbers versus threshold cycle. Data point represent duplicate measurement performed during two independent real-time PCR using dilutions of one of the plasmid containing *nirK* as template.

### 3.3. Quantification of nirK in soil samples

For evaluation of the method, five soils exhibiting contrasted physicochemical characteristics in terms of soil structure and organic content and a soil amended either with water or 150 μg C $g^{-1}$ soil $day^{-1}$ during 3 weeks were analysed. Two independent real-time PCR measurements were performed on triplicate DNA extraction for each soil. The number of *nirK* target molecules ranged between $9.7\times10^4$ and $3.9\times10^6$ copies per gram of soil (Table 3). A higher *nirK* abundance (approximately $3.9\times10^6$ copies per gram of soil) was observed in the agricultural soil from La Bouzule amended with C. Comparison of the soil amended with C or $H_2O$ revealed a significant four fold increase of the number of *nirK* copies in the studied soil.

Table 3
*nirK* copy number in the different soil samples

| Soils | *nirK* gene copy number per nanogram of DNA | *nirK* gene copy number per gram of soil |
|---|---|---|
| Bouzule amended with water | $9.7\times10^1$ $(1.1\times10^1)^a$ | $8.9\times10^5$ $(1.0\times10^5)^a$ |
| Bouzule amended with C | $4.2\times10^2$ $(1.1\times10^2)^c$ | $3.9\times10^6$ $(9.8\times10^5)^c$ |
| Kenya | $8.9\times10^1$ $(2.2\times10^1)^a$ | $2.1\times10^5$ $(5.3\times10^4)^a$ |
| Paris | $1.9\times10^2$ $(4.4\times10^1)^b$ | $9.7\times10^4$ $(2.5\times10^4)^a$ |
| Rennes | $7.7\times10^1$ $(4.0\times10^1)^a$ | $4.2\times10^5$ $(5.3\times10^4)^a$ |
| Termite mound Burkina Faso | $5.1\times10^1$ $(2.5\times10^1)^a$ | $2.2\times10^5$ $(2.2\times10^5)^a$ |
| Vannecourt | $3.0\times10^1$ $(1.2\times10^1)^a$ | $1.4\times10^6$ $(5.1\times10^5)^b$ |

Values followed by the same letter within columns do not significantly differ according to LSD test ($P<0.05$).
Standard errors indicated in parenthesis.

### 3.4. Phylogenetic diversity of the nirK real-time PCR products

A total of 56 clones from five libraries obtained by cloning the real-time PCR products from the analysed soil samples were randomly chosen and sequenced. Comparison of the obtained sequences with the NCBI database by using a BLAST search revealed that all the sequences exhibited similarities ranging between 60% and 80% with the closest known *nirK* sequence. The copper nitrite reductase from *Neisseria meningitidis* was used as an outgroup for phylogenetic analysis. Neighbor-joining tree showed that the majority of the cloned real-time PCR products are distributed in a major cluster containing mainly *nirK* sequences from the α-Proteobacteria (Fig. 3). However, some sequences from the soil of Paris are related to *nirK* from the β-Proteobacteria *Nitrosomonas*. Most of the NirK sequences from cultivated denitrifying bacteria are present in a cluster, which did not contain environmental clones.

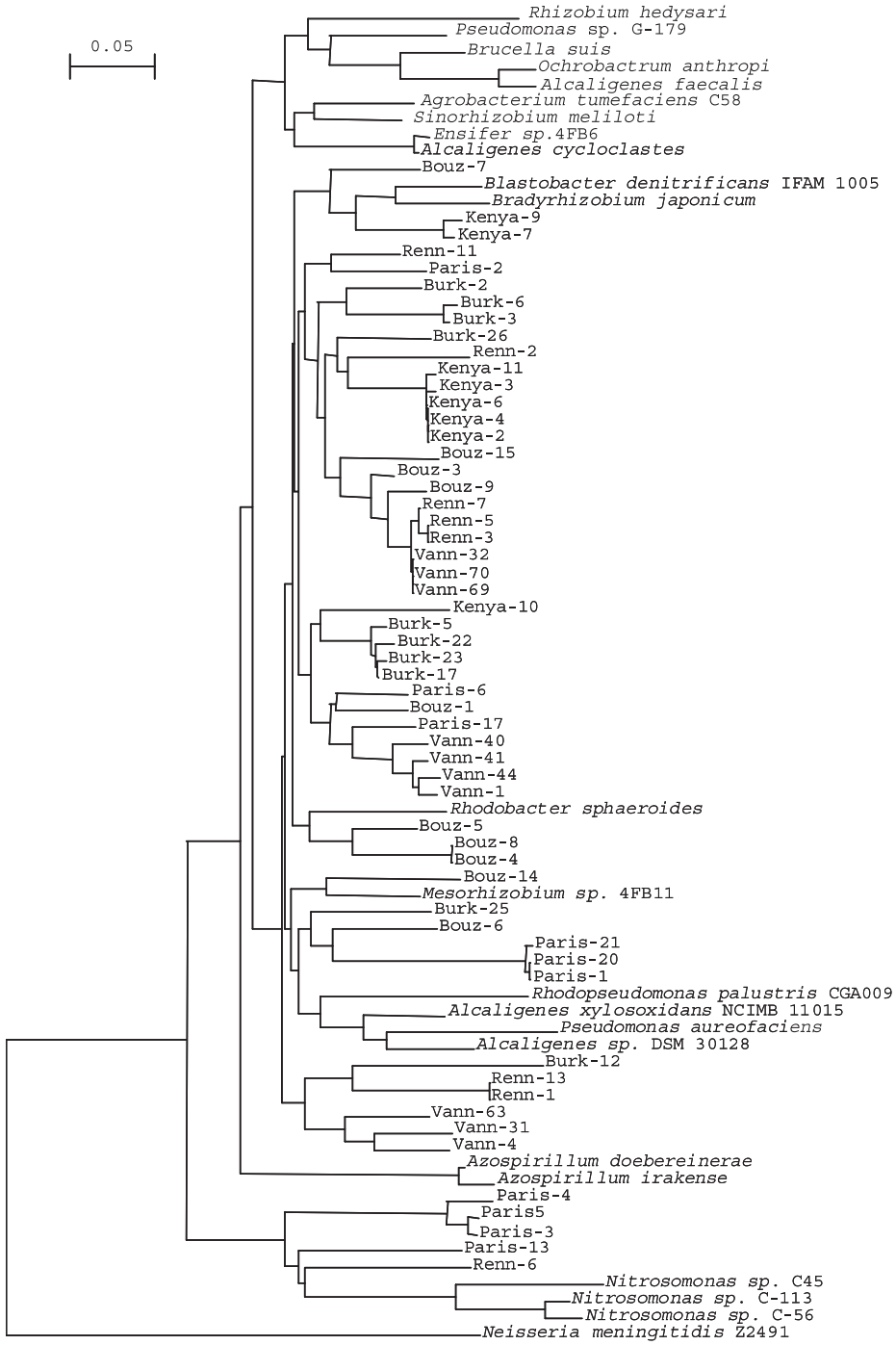


Fig. 3. Phylogenetic neighbor-joining (NJ) tree of *nirK* genes (partial, around 165 bp) from environmental clones obtained in this study and from known bacteria.

## 4. Discussion

Quantification of bacteria capable of denitrification is important for a better understanding of denitrifying activity and $N_2O$ fluxes in the environment. Commonly used methods such as MPN are biased by unculturability of many microorganisms. Therefore, in this study, a real-time PCR assay was developed to quantify the denitrifying bacteria using the *nirK* gene encoding the copper nitrite reductase, a key enzyme of the denitrifying pathway.

In addition to strains from culture collections and genome sequences, we selected cloned *nirK* sequences from various environmental libraries to design *nirK* primers more accurate for application in the environment. In order to be able to amplify most of the Cu nitrite reductase denitrifiers, it was necessary to design degenerated primers, increasing the risk of non-specific amplification. However, a good specificity of our set of primers was observed with the cultured strains (Table 1).

Application of the *nirK* primers to environmental samples was performed using SyberGreen as detection system as discussed by Stubner (2002). In contrast to the TaqMan™ detection system, SyberGreen detection does not need the development of additional probes which is unrealistic for the *nirK* gene due to its high polymorphism between the different taxonomic group of denitrifiers (Philippot, 2002). Our real-time PCR assay was linear over 7 orders of magnitude and sensitive down to $10^2$ copies by assay, similar to the results obtained in other studies (Bach et al., 2002; Gruntzig et al., 2001; Kolb et al., 2003; Lopez-Gutierrez et al., 2004; Stubner, 2002).

Environmental soil samples analysed by real-time PCR displayed a range of 2 orders of magnitude of *nirK* abundance between the different soil samples (Table 3). The higher density was observed in the agricultural soil from La Bouzule amended with C. Interestingly, the real-time PCR assay developed in this study is sensitive enough to detect a significant increase ($P<0.05$) in the density of the denitrifying community between a soil amended with a mix of different carbon substrates compared to a soil amended with water (Table 3).

Microorganisms capable of denitrification are widely distributed in the environment with densities estimated using MPN-method ranging between $10^4$ and $10^6$ bacteria $g^{-1}$ soil (Cheneby et al., 2000; Gamble et al., 1977; Vinther et al., 1982; Weier and MacRae, 1992). In contrast to 16S rDNA, the *nirK* gene copy number can be directly correlated to cell numbers since only one copy of the *nirK* gene has been identified in denitrifying bacteria up to now (Philippot, 2002). Therefore, we can assumed that densities of copper nitrite denitrifiers reported in this study are in the range of $10^4$–$10^6$ bacteria $g^{-1}$ soil (Table. 3). Considering that only a part of the denitrifying community has been taken into account in this study—the copper nitrite reductase containing denitrifiers—while MPN count both types of denitrifiers, our results confirmed that MPN underestimated number of denitrifiers as previously observed (Michotey et al., 2000). Unfortunately, the proportion of copper nitrite reductase denitrifiers among the total denitrifying community in nature is still unknown. However, previous study based on the analysis of a collection of isolated denitrifiers reported that while cytochrome $cd_1$ nitrite reductase dominated (between 64% and 92%), regardless of soil type or geographic origin, the Cu type was found in more taxonomically unrelated strains (Coyne et al., 1989).

Besides verifying that application of our real-time PCR assay results in a single band, specificity of the assay was also evaluated by cloning and sequencing of the real-time PCR products obtained from the different soil samples. Phylogenetic analysis revealed that the clone sequences are distributed over the whole *nirK* tree confirming the validity of our primers (Fig. 3). While all the clone sequences exhibited similarities to *nirK*, most of them are not closely related to *nirK* from cultivated bacteria as previously observed (Prieme et al., 2002). No strong soil-specificity among the environmental clones was observed.

Previous studies have developed PCR-based assay to quantify denitrifying bacteria using the genes encoding the cytochrome $cd_1$ nitrite reductase as molecular marker. Thus, a real-time PCR study targeting the *nirS* gene has been published by Gruntzig et al. (2001). However, the designed primers were specific to *Pseudomonas stutzeri* and therefore cannot be used to quantify this taxonomically diverse functional community. More recently, Michotey et al. (2000) developed a competitive PCR assay to also quantify *nirS*. The designed primers were more universal but competitive PCR is fastidious and cannot be used for rapid analysis of multiple samples.

In summary, to our knowledge, this is first PCR-based approach enabling a rapid quantification of the copper nitrite reductase denitrifiers in the environment. In the future, combination of quantitative PCR-based approaches targeting the *nirK* and *nirS* genes would be useful to determine both the total number denitrifying bacteria using cultivation-independent method and the proportion of Cu and cytochrome $cd_1$ denitrifiers among the total denitrifying community. Thanks to the quantitative PCR approaches, the effect of agricultural practices or of other factors on the size of the denitrifying community can now be studied using a rapid cultivation-independent technique.

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# Direct seeding mulch-based cropping increases both the activity and the abundance of denitrifier communities in a tropical soil

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Gene abundances
*nirK* gene
*nosZ* gene
16S rDNA gene

ABSTRACT

This study evaluated the impact of direct seeding mulch-based cropping (DMC), as an alternative to conventional tilling (CT), on a functional community involved in N cycling and emission of greenhouse gas nitrous oxide ($N_2O$). The study was carried out for annual soybean/rice crop rotation in the Highlands of Madagascar. The differences between the two soil management strategies (direct seeding with mulched crop residues versus tillage without incorporation of crop residues) were studied along a fertilization gradient (no fertilizer, organic fertilizer, organic plus mineral fertilizers). The activity and size of the denitrifier community were determined by denitrification enzyme activity assays and by real-time PCR quantification of the denitrification genes. Denitrification activity and total C and N content in the soil were significantly increased by DMC both years, whereas the fertilization regime and sampling year (crop and mulch types, climatic conditions) had very little effect. Similar results were also observed for denitrification gene densities. Denitrification enzyme activity was more closely correlated with C content than with N content in the soil and denitrification gene densities. Principal component analysis confirmed that soil management had the strongest impact on the soil denitrifier community and total C and N content for both years and further indicated that changes in microbial and chemical soil parameters induced by the use of fertilizer were favored in DMC plots. Overall, the alternative DMC system had a significant positive effect on denitrifier densities and potential activities, which was not altered by crop rotation and the level of fertilization. These data also suggest that in these clayey soils, the DMC system simultaneously increased the size of the soil N pool and accelerated the N cycle, by stimulating the denitrifier community. Complementary investigations should further determine in greater detail the influence of DMC on *in situ* N-fluxes caused by denitrification.



## 1. Introduction

Direct seeding mulch-based cropping (DMC) is a conservation agriculture technique devoted to establishing agricultural sustainability to ensure the perennial productivity of the soil through a reduction in soil erosion and mineral fertilizer inputs and an increase in soil nutrients (Smart and Bradford, 1999). DMC is being increasingly adopted worldwide (about 90 million hectares, Derpsch, 2003), especially in tropical and semi-arid tropical agroecosystems, to cope with soil degradation induced by combinations of arbitrary agricultural practices (e.g. no intercropping, non-optimal rotation, systematic intensive cultivation) and adverse climatic conditions. A large body of literature has reported that alternative practices can favor cascades of beneficial changes to chemical, structural and biological soil properties. Soil structure is an important regulator in soil functioning that can be improved in DMC managed fields through increased aggregation (Paustian et al., 2000) often associated with increased organic matter content (Doran, 1980) and soil moisture (Steiner, 1989). DMC management has been reported as increasing the diversity and abundance of faunal communities (Brévault et al., 2007; Blanchart et al., 2007), as well as several other microbial characteristics (Govaerts et al., 2007;

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Cookson et al., 2008). For instance, increased microbial biomass and various enzyme activities (e.g. β-glucosidase, phosphatase, urease) have often been reported as being enhanced in no-till soils, especially in the upper surface soil layer and in fine textured soils (Doran, 1980; Bergstrom et al., 1998; Rabary et al., 2008).

While conservation agricultural practices are globally beneficial to soil quality, reduced tillage and residue conservation may trigger negative environmental side effects, such as increased $N_2O$ emissions (Baggs et al., 2003; Rochette, 2008). Reduced availability of oxygen and air space in DMC soils, together with the decomposition of mulched crop residues in the superficial soil layers, are likely to favor anaerobic processes such as denitrification (Baggs et al., 2003; Sarkodie-Addo et al., 2003). Increased denitrification rates are also likely to be mediated by an increased denitrifier biomass as suggested by early studies documenting a significant increase in denitrifier counts in no-till fields (Doran, 1980; Broder et al., 1984). Against this background, the denitrification rates in such systems are worth attention since this microbial process can represent a significant source of $N_2O$ (Mosier et al., 1998) contributing to global warming and destruction of the ozone layer (Tabazadeh et al., 2000).

Although a large body of literature describes the significant effects of tillage intensity, fertilizer types or loading rates on potential denitrification activity (review in Philippot et al., 2007) or *in situ* losses through $N_2O$ or $N_2$ releases (Baggs et al., 2006; Liu et al., 2007), few studies have analyzed the effects of agricultural practices on both denitrification activity and size of the denitrifier community. However, measuring the size and activity of denitrifier community and analyzing the relationship between them are of great interest as the regulation of biogeochemical cycles by the size of the microbial community is still in dispute (Coleman and Whitman, 2005; Philippot and Hallin, 2005; Röling, 2007).

This study was based on the hypothesis that the size and activity of the denitrifier community would be increased under DMC management and that combined mineral and organic fertilizers would strengthen this effect. This hypothesis was studied in the Highlands of Madagascar where DMC systems were initially implemented to deal with extensive soil erosion (Rabary et al., 2008). The study was carried out in an agronomic field trial set up eight years ago to study the long-term effects of DMC management on soil functioning. The impact of DMC was evaluated by comparison with tilled plots in two consecutive years, for soybean/rice crop rotation, along a fertilization gradient including mineral and manure inputs. The activity and size of the denitrifier community were determined by monitoring denitrification enzyme activity and by real-time PCR quantification of the denitrification genes, respectively.

## 2. Materials and methods

### 2.1. Field experimental design

The experimental station was located near Antsirabe, in Bemasoandro (19°46′S, 47°06′E), Madagascar. This area has a tropical altitude climate, with around 10–20 days of frost annually (Oldeman, 1990) and a mean annual temperature of 17 °C. The site was 1600 m above sea level with an average rainfall of 1665 and 1203 mm during the 2004/2005 and 2005/2006 seasons, respectively. Rainfall during the rainy season was particularly low in 2006 (223 mm through January and February compared with an average of 556 mm for the same period in the previous 5 years). This soil is andic Dystrustept (Soil Survey Staff, 2003). In 2003 the main characteristics of the 0–25 cm soil layer in a soybean/rice rotation under DMC systems were: pH(water) 5.1, clay 79%, fine silt 10%, coarse silt 2%, fine sand 4%, coarse sand 5%, carbon 2.1%, nitrogen 0.16%, and CEC 17 cmol $kg^{-1}$ (Razafimbelo, 2005). This field experiment was set up in 1997 and consisted of two soil management strategies (conventional tillage (CT) without crop residue conservation and direct seeding (DMC) with mulched crop residue conservation) combined with three fertilization regimes: F0-no fertilizer, F1-organic fertilizer (5 t zebu manure $ha^{-1}$ $y^{-1}$), F2-organic and mineral fertilizers (5 t zebu manure $ha^{-1}$ $y^{-1}$, 70 kg N, 30 kg P and 40 kg K $ha^{-1}$), which resulted in a total of six different treatments. Manure was applied at the beginning of December while seeding, and mineral fertilizers were usually spread a couple of weeks later. Plots (13.5 $m^2$) were completely randomized with three plots for each combination of treatment. This study focused on a soybean (*Glycine maxima* L.)/rice (*Oryza sativa* L.) annual rotation using rainwater only.

The soil was sampled in two consecutive years during the rainy season on January 24 for the soybean crop (2005) and February 13 for the rice crop (2006) to characterize the denitrifier communities during the period most favorable to denitrification (i.e. high soil moisture, recent fertilizer inputs, plant growth) and to take account of possible seasonal and crop type effects (e.g. residue quality, quantity and quality of root exudates). Soil cores were taken along three parallel lines located between rows (40 cm wide for soybean and 30 cm wide for rice). No samples were taken from the soil between the first two rows of crops on either side of each plot to avoid possible edge effects. For each sampling line, five elementary soil cores (5 cm depth, 5 cm diameter) were collected at three separate locations along the line and mixed to give a total of 3 composite samples per plot (54 composite soil samples each year). Soil samples were immediately air-dried, sieved at 2 mm and stored at room temperature.

### 2.2. Chemical analyses

The total soil C and N contents were determined by dry combustion using a CHN analyzer (Thermo-Finnigan EA 1112NC Soil Analyzer). Measurements of nitrate-ammonium soil contents were performed by ISO 9001 LAMA Laboratory (Dakar, US Imago, IRD), but only on 2005 samples.

### 2.3. Activity measurements

Denitrification enzyme activity (DEA) was measured according to the method described by Smith and Tiedje (1979). 20 g (dry weight) sub-sets of soil samples were made anoxic by flushing the flask headspace with helium. 2 mg C $g^{-1}$ dry soil (added as a 50/50 w/w glucose and glutamic acid) and 0.2 mg N $g^{-1}$ soil (added as $KNO_3$) were added to each sample. The flask contents were incubated with 10% (v/v) acetylene to allow the accumulation of denitrified nitrogen as $N_2O$. DEA was calculated as the rate of N accumulated as $N_2O$ in the headspace in the presence of acetylene between 2 and 6 h in the dark at 100% water holding capacity and at 25 °C, and analyzed using a gas chromatograph (Varian Star 3900, Varian, Walnut Creek, CA, USA). The same protocol was used to quantify potential $N_2O$ emissions but without acetylene to determine the proportion of N denitrified as $N_2O$ during the assay.

### 2.4. DNA extraction

DNA was extracted from 0.25 to 1 g of composite soil samples with the Ultra Clean Soil DNA kit according to the manufacturer's instructions (Ozyme, Mo Bio, France). DNA extracts were quantified by spectrophotometry at 260 nm using a BioPhotometer (Eppendorf, Hamburg, Germany). For each plot, three independent soil DNA extractions were performed, corresponding to the three sampling lines per plot, giving a total of 54 DNA extracts, used as PCR templates, for each year.

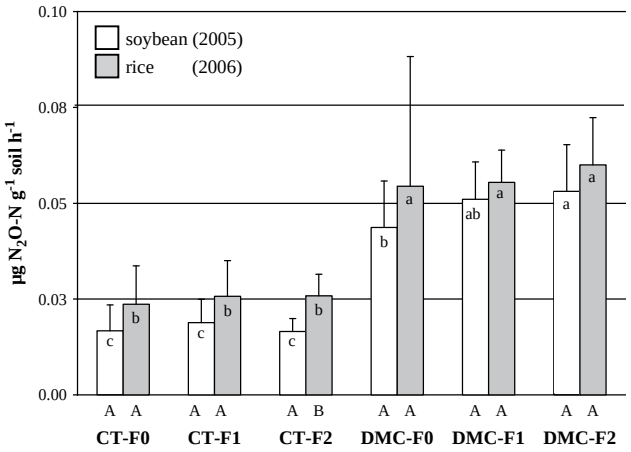


**Fig. 1.** Denitrification enzyme activity (DEA) (mean and standard deviation, $n = 9$) measured in the presence of acetylene with field samples collected in two consecutive cropping seasons (soybean in 2005 and rice in 2006, respectively). CT: conventional tillage, DMC: direct seeding mulch-based cropping system, F0: no fertilizer, F1: manure, F2: manure + mineral fertilizer. Different capital letters below columns indicate significant differences between sampling years ($P < 0.05$). Different lowercase letters on top of columns indicate significant differences between fertilization/cultivation technique modalities for a given year.

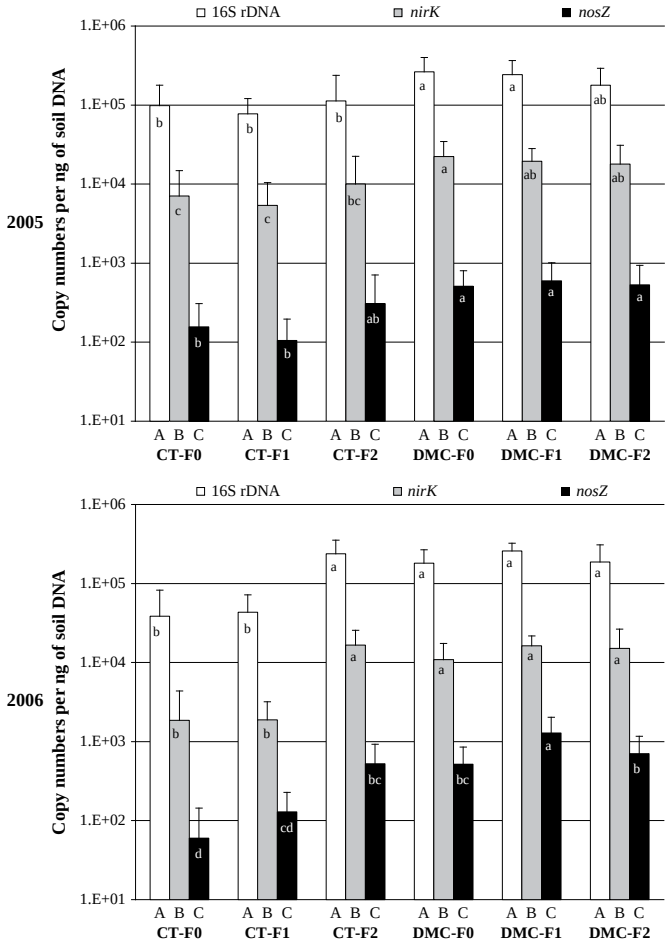


**Fig. 2.** 16S rRNA, *nirK* and *nosZ* gene densities (mean and standard deviation, $n = 9$) measured with field soil samples collected in two consecutive years (2005: soybean crop and 2006: rice crop). CT: conventional tillage, DMC: direct seeding mulch-based cropping system, F0: no fertilizer, F1: manure, F2: manure + mineral fertilizer. Different capital letters below columns indicate significant differences between the three types of gene densities ($P < 0.05$). Different lowercase letters on top of columns indicate significant differences between fertilization/cultivation technique modalities for a given gene.

### 2.5. Quantification of the denitrifier community size

The size of the denitrifier community was estimated by quantitative PCR (qPCR) of the genes encoding the catalytic subunit of the key enzymes of the denitrification pathway. Fragments of the *nirK*, *nirS* and *nosZ* genes encoding the copper and $cd_1$ nitrite reductases and the nitrous oxide reductase, respectively, were amplified in a 20 µl reaction volume containing SYBR Green PCR Master Mix (Absolute QPCR SYBR Green Rox ABgene, France), 1 µM of each primer, 100 ng of T4 gene 32 (QBiogene, France) and 6.5 ng of template DNA. The primers and PCR conditions are described in López-Gutiérrez et al. (2004) for 16S rDNA, Henry et al. (2004) for *nirK*, Kandeler et al. (2006) for *nirS* and Henry et al. (2006) for *nosZ*. Thermal cycling, fluorescence measurements and data analysis were carried out using an ABI Prism® 7900HT sequence detection system according to the manufacturer's instructions. Two or three no-template controls were run for each quantitative PCR assay. All assays were run using genomic DNA from either *Bradyrhizobium japonicum* USDA110, *Pseudomonas aeruginosa* PAO1, *Agrobacterium tumefaciens* C58 or *Sinorhizobium meliloti* 1021, containing known copy numbers of targeted genes as external standards (Henry et al., 2006). The potential presence of PCR inhibitors in soil DNA extracts was tested by running a real-time PCR assay on serial dilution of soil DNA extracts. No inhibition was detected in any case. To eliminate bias related to the DNA extraction efficiency, gene copy numbers were calculated both by nanogram of DNA and gram of dry soil.

### 2.6. Statistics

The results were analyzed using Fisher's LSD test with XLSTAT software (2007.8.03 version, Addinsoft, Paris, France) on raw data

**Table 1**
Influence of cultivation technique and fertilization on total C and N, $NH_4^+$ and $NO_3^-$ content in the soil.

| | | Conventional tillage | | | Direct seeding mulch-based system | | |
|---|---|---|---|---|---|---|---|
| | | F0[a] | F1[a] | F2[a] | F0 | F1 | F2 |
| Carbon (%) | soybean (2005) | 2.3 (0.3) a | 2.4 (0.2) ab | 2.6 (0.3) b | 3.5 (0.3) c | 3.7 (0.4) cd | 3.9 (0.5) d |
| | rice (2006) | 2.5 (0.3) a | 2.7 (0.2) a | 2.9 (0.2) b | 3.5 (0.3) c | 3.9 (0.3) d | 4.5 (0.5) e |
| Nitrogen (%) | soybean (2005) | 0.16 (0.02) a | 0.17 (0.02) ab | 0.19 (0.03) b | 0.25 (0.03) c | 0.25 (0.03) c | 0.25 (0.05) c |
| | rice (2006) | 0.18 (0.03) a | 0.20 (0.02) a | 0.23 (0.02) b | 0.28 (0.03) c | 0.31 (0.03) c | 0.37 (0.05) d |
| Ammonium[b] | soybean (2005) | 6.2 (2.0) a | 8.5 (2.4) a | 15.2 (2.7) b | 14.2 (2.5) b | 13.1 (2.6) b | 15.3 (3.4) b |
| Nitrate[b] | soybean (2005) | 6.8 (1.0) b | 4.2 (1.0) a | 4.0 (1.4) a | 9.9 (1.9) c | 6.3 (2.5) b | 5.2 (3.0) ab |

Numbers represent means ($n = 9$) followed by their standard deviations in parenthesis. Different letters within a row indicate significant differences ($P < 0.05$) between all six treatments for a given crop.

[a] Fertilization levels: F0 no fertilizer, F1 manure, F2 manure + mineral fertilizer.

[b] Expressed in µg $NH_4^+/NO_3^-$ – N $g^{-1}$ soil.

or log-transformed data for total soil C and N content. Principal component analyses (PCA) were done by using normalized data with ADE4 software (Thioulouse et al., 1997).

## 3. Results and discussion

This study set out to characterize the impact of zero tillage and residue conservation on the size and activity of the denitrifier community. DMC management significantly stimulated denitrification enzyme activity with respect to conventional tillage (CT) (Fig. 1). Average DEA values for soil collected from tilled plots ranged from 17 to 26 ng $N_2O$–N $g^{-1}$ dry soil $h^{-1}$ for both years and across the whole fertilization range, while significantly higher rates of 44–60 ng $N_2O$–N $g^{-1}$ dry soil $h^{-1}$ were observed for DMC (Fig. 1). Tillage has been reported either to stimulate (Calderón et al., 2001) or decrease denitrification (Liu et al., 2006, 2007). The absence of a clear effect of tillage on denitrification can be attributed to the association of tillage with other agricultural practices that can also potentially affect denitrifying communities. Moreover, the effect of tillage depends on the timing, frequency and depth which determine the extent of aggregate disruption, organic matter protection and dissolved organic matter availability, which in turn affect soil microbial functioning (Cookson et al., 2008). Total C and N, together with $NH_4^+$ and $NO_3^-$ concentrations, also increased with the DMC (Table 1). Previous studies have reported that, in no-till systems, part of the soil organic matter can be physically protected by inclusion in stable aggregates (Six et al., 2000; Oorts et al., 2007). However, this study showed that DEA was significantly correlated with soil C and N contents in both years ($R^2$ from 0.53 to 0.83 for C and from 0.53 to 0.59 for N, $P < 0.001$). As already reported (Burford and Bremner, 1975), soil C content appears to be a good predictor of DEA across very different soil types.

Denitrifying enzyme activity did not depend on the sampling year (i.e. mulch and crop types, climatic conditions) or fertilization rate (Fig. 1). Organic matter and/or mineral N fertilizers inputs have usually been reported to stimulate denitrification (Mulvaney et al., 1997; review in Philippot et al., 2007), especially under direct seeding (Baggs et al., 2003; Liu et al., 2006, 2007) and it is possible that there may have been a transient fertilizer effect before the samples were taken. No significant potential reduction of $N_2O$ into $N_2$ was noticed in the absence of acetylene for any of the combinations of treatment (data not shown), suggesting that $N_2O$ is the end product of denitrification in the soil studied. Incomplete denitrification processes have been reported in agricultural soils elsewhere (Hénault et al., 2001). However since our results are based on only two sampling dates, a thorough investigation of *in situ* $N_2O$ emissions is necessary before concluding that DMC also increased emissions of $N_2O$ from the field. A recent study has shown low $N_2O$ fluxes for the whole growing season in no-till plots in the same area (Chapuis-Lardy et al., 2009).

For both sampling dates, the number of 16S rRNA copies per nanogram of soil DNA ranged from $2.32 \times 10^2$ to $4.76 \times 10^5$ (Fig. 2), which corresponded to $8.12 \times 10^5$ and $6.75 \times 10^9$ gene copies per gram of dry soil (data not shown). Lower values were observed for *nirK* and *nosZ* gene abundances, with values ranging from $1.16 \times 10^2$ to $3.77 \times 10^4$ copies and from $1.31 \times 10^3$ to $2.86 \times 10^3$ copies per ng DNA, respectively (from $3.44 \times 10^5$ to $4.79 \times 10^8$ and from $2.47 \times 10^3$ to $5.16 \times 10^7$ copies per gram dry soil, respectively). In this study, the *nirS* gene density was below the detection limit as for the study by Dandie et al. (2008). The density of *nirK* and *nosZ* denitrification genes estimated in this study varied within the ranges previously reported: *nirK* and *nosZ* densities of $2 \times 10^6$ to $2 \times 10^8$ and $3 \times 10^6$ to $8 \times 10^7$ copies per gram of soil were reported for various arable soils (Henry et al., 2006; Dandie et al., 2008; Hallin et al., 2009). The effect of DMC on denitrification gene abundances (Fig. 2) was similar to that on denitrification enzyme activity (Fig. 1) with significantly higher denitrification gene copy numbers in DMC than in conventional tillage plots, with the exception of the plots receiving the highest fertilizer inputs. Both *nirK* and *nosZ* gene densities were significantly correlated with N content (data not shown) and more strongly with C content (Fig. 3A). This confirmed the heterotrophic nature of the denitrifying community (Tiedje, 1988), which was stimulated by the increased soil organic matter content (DMC) and by manure inputs. In the field studied, the

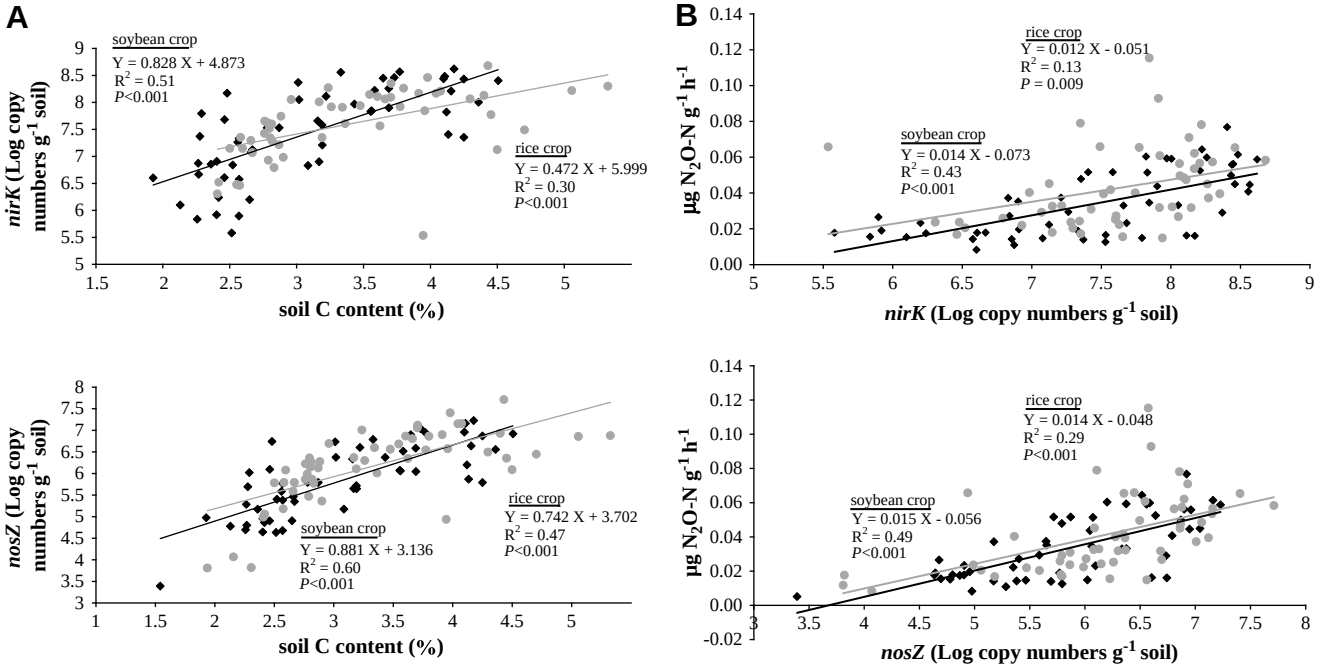


**Fig. 3.** Linear regressions established between (A) *nirK* or *nosZ* gene densities and soil C contents of samples, (B) denitrification enzyme activity (DEA) and *nirK* or *nosZ* gene densities, collected under soybean (black diamonds) and rice crops (grey circles) on the base of the whole data set ($n = 54$, all treatments).

**Table 2**
Influence of cultivation technique and fertilization on the ratios of *nirK* and *nosZ* genes to 16S rDNA gene (*nirK*/16S, *nosZ*/16S) and ratio of *nosZ* gene to *nirK* gene (*nosZ*/*nirK*).

| | | Conventional tillage | | | Direct seeding mulch-based system | | |
|---|---|---|---|---|---|---|---|
| | | F0[a] | F1[a] | F2[a] | F0 | F1 | F2 |
| *nirK*/16S | soybean (2005) | 5.23 (3.47) a | 6.10 (2.72) ab | 8.67 (3.65) bc | 9.50 (3.44) c | 8.83 (2.69) bc | 9.36 (2.06) c |
| | rice (2006) | 3.23 (2.25) a | 4.06 (2.32) ab | 6.99 (1.09) c | 5.79 (1.64) bc | 6.38 (1.35) c | 7.48 (3.41) c |
| *nosZ*/16S | soybean (2005) | 0.18 (0.08) a | 0.14 (0.06) a | 0.39 (0.34) b | 0.28 (0.29) ab | 0.28 (0.20) ab | 0.27 (0.08) ab |
| | rice (2006) | 0.21 (0.18) a | 0.42 (0.40) bc | 0.20 (0.06) a | 0.27 (0.06) ab | 0.48 (0.18) c | 0.42 (0.16) bc |
| *nosZ*/*nirK* | soybean (2005) | 3.10 (2.22) ab | 2.70 (1.63) ab | 4.35 (2.98) b | 2.61 (1.19) a | 2.95 (1.41) ab | 2.85 (0.49) ab |
| | rice (2006) | 2.35 (1.91) a | 5.96 (2.93) bc | 2.99 (0.97) ab | 4.77 (0.93) abc | 7.54 (2.37) c | 7.83 (6.93) c |

Numbers represent means ($n = 9$) followed by their standard deviations in parenthesis. Different letters within a row indicate significant differences ($P < 0.05$) between all six treatments for a given crop.
[a] Fertilization levels: F0 no fertilizer, F1 manure, F2 manure + mineral fertilizer.

proportion of *nirK* and *nosZ* genes to 16S rRNA gene ranged from 3.2 to 9.5% and from 0.14 to 0.48% respectively (Table 2). These results are in agreement with culture-based studies that have found that the proportion of denitrifiers to total bacteria is less than 5% (Tiedje, 1988; Chèneby et al., 2000, 2004). In this study, *nosZ* gene abundance was less than 5% of that of the *nirK* gene (Table 2). The higher abundance of *nirK* genes compared to *nosZ* genes has already been observed in a temperate agricultural soil (Henry et al., 2006), and suggests that, in the fields studied, many of the denitrifiers may lack the *nosZ* gene and, therefore, are genetically unable to reduce $N_2O$ into $N_2$. However, the *nosZ* primers used may not be as universal as the *nirK* primers, which could account for the high *nirK* to *nosZ* gene ratio reported here. On the other hand, the low proportion of *nosZ* denitrifiers could explain the absence of a detectable potential reduction of $N_2O$ in the DEA assay.

PCA of the chemical and microbial data, all expressed per gram of soil, confirmed that soil management had a greater influence than the fertilization regime. There was a significant segregation of the soil management strategies along the first axis, explaining 76 and 71% of the variance for soybean and rice crops, respectively (only shown for the soybean crop in Fig. 4). PCA ordination of the variables indicated that they were all associated with the first axis, showing that the soil C and N content, gene densities and DEA were all stimulated by DMC (Fig. 4). A higher variability in PCA scores was observed for DMC plots which could reflect the higher structural heterogeneity of the soil after several years of zero tillage. PCA also showed that there was a weak effect between the F1 and F2 fertilization treatments but only with DMC soil management, as indicated by a significant segregation of points along the second axis explaining 17.4% of the total variance. Linear regressions between DEA and denitrification gene densities (Fig. 3B) were significant in all cases suggesting that potential denitrification activity is partly regulated by the size of the denitrifier community (average $R^2 = 0.33$, $P < 0.001$). However, regression coefficients and slopes were rather low indicating a limited predictive value of such regressions. For instance, some soil samples having up to two log differences in denitrifier density exhibited similar denitrification levels. Such weak correlation between denitrifier biomass and denitrification activity has already been reported (Martin et al., 1988; Dandie et al., 2008). Several hypotheses can explain the low correlation between size and activity observed in this study. Firstly, the number of denitrification genes was quantified to estimate the size of the community genetically capable of denitrifying but this did not provide information on the size of the active fraction of this community. Moreover, although *nirS* gene densities were too low to be reliably estimated, it is possible that cytochrome $cd_1$ nitrite reductase activity significantly influenced the measured potential denitrification activity. Another hypothesis is that agronomic treatments may have modified the composition of the denitrifier community by selecting denitrifier populations with different specific activities or having denitrification enzymes that were susceptible to abiotic factors (Cavigelli and Robertson, 2000). Chèneby et al. (2009) observed, at the same experimental site, significant differences in the nitrate reducer community structure between treatments. Such compositional shifts are likely to loosen the link between denitrification activity and the size of the denitrifier community.

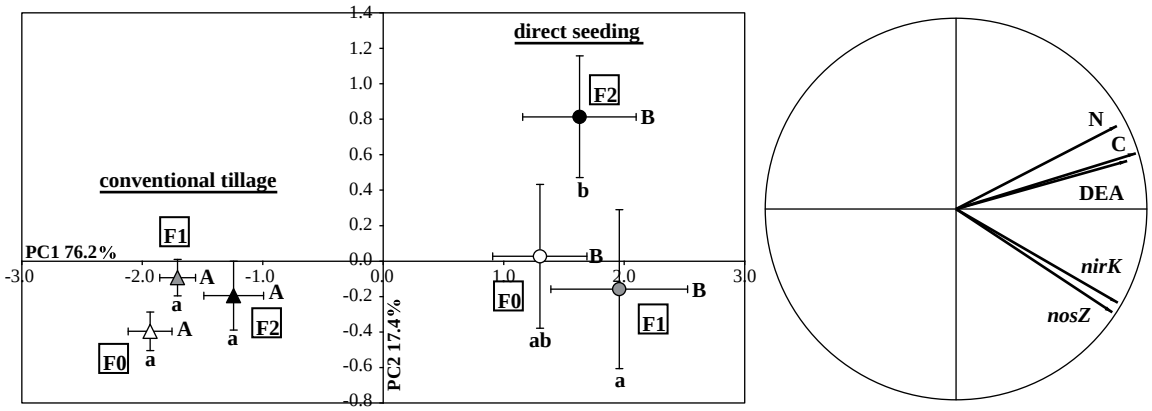


**Fig. 4.** Principal component analysis and associated correlation circle carried out on the whole data set (total soil C and N contents, *nirK*–*nosZ* gene densities, denitrification enzyme activity (DEA), $n = 54$) of samples collected under the soybean crop. Symbols represent mean coordinates of triplicate samples scores, with associated standard errors. Different capital and lowercase letters indicate significant differences ($P < 0.05$) between mean abscises and ordinates, respectively. Triangle and full circle symbols refer to conventional tillage and direct seeding mulch-based cropping systems, respectively. White, grey and black colors of symbols refer to the fertilization gradient (F0: no fertilizer, F1: manure, F2: manure + mineral fertilizer, respectively).

## 4. Conclusions

This study showed that DMC systems may favor soil denitrification potential with the size and potential activity of the denitrifier community increased by direct seeding and crop residue retention compared to conventional tillage. However, different levels of fertilization had no significant impact on the denitrifier community in the tropical clayey soil studied. These results suggest that the tillage system (determining the soil physical status and residue retention) modifies several soil properties, making it the dominant driver of the denitrifier community over fertilization practices in these arable soils. It also seems that even on such clayey soils, the DMC system can simultaneously favor soil N content and N-cycling microbial communities. However, data on *in situ* N-fluxes caused by denitrification are needed to confirm the impact of DMC on nitrogen loss. In addition, the lack of a clear-cut correlation between denitrification gene abundances and potential denitrifying activity highlights the complexity of the mechanisms determining N-fluxes by denitrification. Nevertheless, focusing on the active fraction of the denitrifier community that contributes to the $N_2O$ and $N_2$ emissions would help clarifying the role of denitrifier community size and diversity in regulating N-fluxes by denitrification.

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SHORT COMMUNICATION

# Legume and gramineous crop residues stimulate distinct soil bacterial populations during early decomposition stages

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[1]IRD-UMR 210 Eco&Sols, LEMSAT, BP 1386, Dakar, Sénégal; and [2]IRD-UMR 210 Eco&Sols, Montpellier SupAgro, 2 place Viala, bâtiment 12, 34060 Montpellier cedex 1, France (e-mail: Ezekiel.Baudoin@ird.fr).
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Diouf, M., Baudoin, E., Dieng, L., Assigbetsé, K. and Brauman A. 2010. **Legume and gramineous crop residues stimulate distinct soil bacterial populations during early decomposition stages.** Can. J. Soil Sci. **90**: 289–293. This study characterized the genetic structure of the active soil bacterial populations involved in the decomposition of maize and soybean residues over 3 d. Significant compositional differences between the total bacterial community and its active component were observed that were residue specific, suggesting that residue management should be further evaluated as a driver of soil C cycle through selection of bacterial populations.

**Key words:** 16S rRNA DGGE, active bacterial community, organic matter, crop residue

Diouf, M., Baudoin, E., Dieng, L., Assigbetsé, K. et Brauman A. 2010. **Des résidus de culture de légumineuse et de graminée stimulent des populations bactériennes distinctes du sol au cours des stades précoces de décomposition.** Can. J. Soil Sci. **90**: 289–293. Cette étude a permis de caractériser la structure génétique des populations bactériennes actives du sol impliquées dans la décomposition de résidus de maïs et de soja sur trois jours. Des différences significatives dans la composition de la communauté bactérienne totale et de sa fraction active ont été observées et se sont avérées spécifiques du résidu appliqué, suggérant que la gestion des résidus devrait être évaluée en tant qu'effecteur du cycle du carbone dans le sol opérant par sélection de populations bactériennes.

**Mots clés:** 16S rRNA DGGE, communauté bactérienne active, matière organique, résidus de culture

The mineralization of organic matter is of great interest given its central role in soil energy fluxes, nutrient cycling and carbon sequestration (Trumbore 2006). Some studies have already noted changes in microbial community composition during plant material decomposition (Yang et al. 2003; Dilly et al. 2004). Yet, these studies did not specifically target the active microbial populations. Several molecular technologies enable a direct insight into the active components of the soil microbial community (Gray and Head 2001; McMahon et al. 2005). Among nucleic acid-based methodologies, the ribosomal RNA-based technique proved to be sensitive enough to investigate the impact of root physiology (Jossi et al. 2006) on soil microbial communities, but examples of its usefulness to characterize the plant-litter- degrading bacterial communities are still scarce (Aneja et al. 2004). In this context, our goal was to determine whether soil enrichment with crop residues of distinct biochemical qualities could trigger within days the activity of distinct subsets of the total bacterial community. Structural differences between the whole bacterial community and its active component were revealed by comparing the denaturing gradient gel electrophoresis (DGGE) profiles obtained from amplified 16S rDNA and reverse transcribed 16S rRNA fragments.

Arable soil (Lixisol, FAO classification) was collected in Senegal (Thyssé Kaymor). Its main characteristics were: sand 55.3%, silt 34.3%, clay 10.4%, pH (water) 5.8, CEC 5.4 meq $g^{-1}$, carbon 1.3%, nitrogen 0.1%. Soil was sieved (2 mm) and air-dried. Mature maize (*Zea mays* L.) and soybean (*Glycine maxima* L.) shoots were dried (65°C), ground and sieved (2 mm) but not autoclaved so as not to alter their biochemical attributes. It was assumed that the integrity of the bacterial RNA content of the residues was altered by their drying at 65°C (e.g., Ruwaida and Schlegel 1976). Crop residues displayed distinct biochemical attributes as evidenced by their C:N ratios (91 and 62, respectively) and near-infra red spectral signatures (data not shown).

Nine plastic flasks received 10 g dry soil and gravimetric soil water content was brought to 50%. Microcosms were incubated for 3 wk at 27°C and at 50% humidity in order to stabilize the microbial activity. Soil moisture was then increased to 70% in order to ease further residue moistening and diffusion of dissolved organic matter. Residues (200 mg, $n = 3$ each) were then incorporated into the soil and thoroughly mixed. Soil

contained in unamended microcosms was also mixed. Units were incubated for 3 d at 27°C in the dark and then frozen at −20°C.

Total soil DNA and RNA were simultaneously extracted from four-replicate 500-mg samples per microcosm (Griffiths et al. 2000). For each microcosm, the pooled nucleic acid extract was quantified in a 2% agarose gel electrophoresis (representative bands of total DNA and total 16S rRNA) along with calibrated dilutions of calf thymus DNA using TL120 software (Nonlinear Dynamics, Newcastle upon Tyne, UK). The purified total RNA pool was immediately prepared from total nucleic extracts with RQI RNase-free DNase I (Promega, Charbonnières, France). The reaction was carried out in a total volume of 5 μL containing 0.5 U of the enzyme and 10 ng of 16S rRNA. The presence of residual DNA in DNase-treated extracts was routinely checked by PCR amplification using the universal eubacterial primers EUB338 and UNIV907R (see below for amplification conditions), and no contamination was evident in any sample. Purified RNA extracts were then reverse-transcribed (see below) and stored at −40°C before use.

cDNA was synthesised from purified RNA templates using the ThermoScript™ RT-PCR system kit (Invitrogen, Cergy Pontoise, France) and a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Courtaboeuf, France). RNA template (5 ng) was mixed with random hexamers primers (50 ng) and dNTP (10 mM) in a final volume adjusted to 12 μL with DEPC-treated water. The mixture was then incubated for 5 min at 65°C and immediately stored on ice. Subsequently, reverse transcription was initiated by adding 8 μL of Thermoscript™ RT reaction mix with primer annealing at 25°C for 5 min, followed by cDNA synthesis at 50°C for 50 min. The reaction was stopped by incubation at 85°C for 5 min. Residual RNA was eliminated by adding 1 μL of RNase H (Invitrogen) to the resulting cDNA mixture incubated at 37°C for 20 min.

16S rDNA genes were amplified from total DNA and cDNA with the universal eubacterial primers EUB338 and UNIV907-R (Amann et al. 1992) using PureTaq™ Ready-To-Go PCR Beads (GE Healthcare, Orsay, France) as previously described (Assigbetsé et al. 2005). A 40-pb GC-clamp was added on the forward primer EUB338 for DGGE analysis (Muyzer et al. 1993). 16S rDNA amplicons were resolved by DGGE using 6% acrylamide gels and a gradient of 45–70% denaturant accordingly to Ndour et al. (2008). Staining and scanning of gels are described elsewhere (Assigbetsé et al. 2005).

DGGE patterns were encoded into matrices related to band presence and relative intensity by using TL120 software. Similarity level between DGGE patterns was assessed by principal component analysis of matrices (covariance matrix) with ADE4 software (Thioulouse et al. 1997). Significant differences between mean ordination scores of representative points in the principal plan were analysed by Fisher's LSD tests (Statview, 4.55 version, Abacus Concept, CA). As the PCA principal plans absorbed at least 80% of the total inertia of the data set, such statistical differences between mean ordination scores were indicative of salient dissimilarities between genetic structures.

The hypothesis underlying this work is that organic matter pools of contrasted composition are likely to stimulate within days the activity of distinct populations of the soil bacterial community. Microcosm enrichment with crop residues induced a significant increase of soil DNA and 16S rRNA contents (2.5- and 6-fold, respectively) (Table 1). Soil RNA contents were not driven by compositional differences between maize and soybean residues, but DNA yields were significantly higher under soybean residues. While the larger soil DNA pool size can partly be attributed to the DNA content of residues, higher soil RNA contents should be strictly correlated to real-time enhanced bacterial metabolic activities and cell division as RNAs are highly labile and reactive molecules that were undoubtedly degraded in original plant materials owing to their physical treatment before incorporation into soil. Thus, this fast increase in RNA contents strongly suggests an immediate stimulation of microbial activity following residues application, and is in agreement with studies focusing on the evolution of various bacterial features following soil enrichment with plant material, such as clover (Zelenev et al. 2005), rye (Lundquist et al. 1999), ryegrass (McMahon et al. 2005) or pea (Ha et al. 2008), and including a first sampling at early stages similar to our timescale (3–5 d).

DGGE patterns from 16S rDNA and reverse transcribed 16S rRNA were obtained for all experimental units (Fig. 1). Total DGGE bands with different mobilities amounted to 33 across all patterns. Bands in DNA-based patterns were more numerous (30) than in RNA-based ones (19). Fourteen DNA bands were not detected in RNA lanes and three of them were identified in all DNA fingerprints. Three bands were specifically detected in RNA lanes and one of them was present in all RNA patterns. Overall, 17 DGGE bands held a discriminatory value, in terms of band presence/absence, and nine bands were shared by all profiles. The most conspicuous ones among the last are highlighted in Fig. 1 (coded A to H). Fifteen bands (out of 33) and 13 bands (out of 19) were observed in all DNA and RNA patterns,

**Table 1. Soil nucleic acids extraction rates**

| Treatments | DNA yield | 16S rRNA yield |
|---|---|---|
| Unamended | 697 (186)*a* | 265 (62)*a* |
| Maize | 1683 (71)*b* | 1571 (51)*b* |
| Soybean | 1730 (352)*c* | 1701 (148)*b* |

Extraction rates expressed in ng $g^{-1}$ dry weight soil. Means ($n = 3$) and associated standard errors are in parentheses.
*a–c* Different letters within a column indicate significant differences ($P < 0.05$).

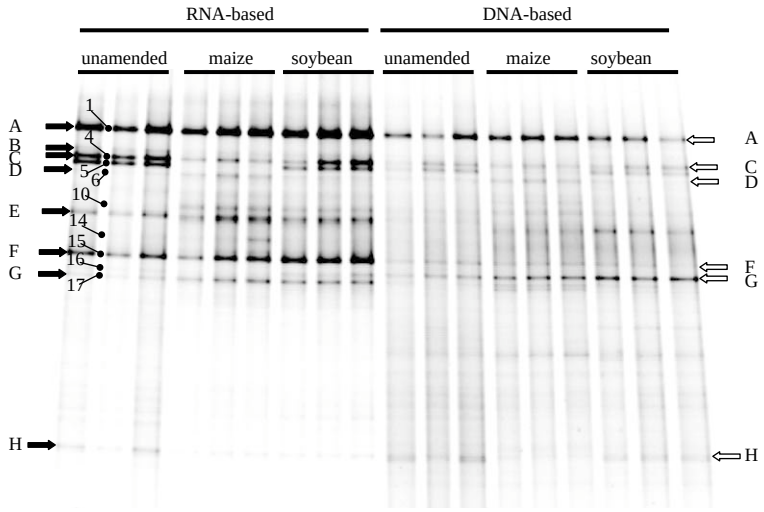


**Fig. 1.** 16S rDNA DGGE fingerprints based on soil rRNA for the first nine patterns, and soil rDNA for the last nine. Full arrows highlight conspicuous bands that are common to all RNA-based fingerprints (coded A to H), while open arrows indicate a subset of these bands that are also detected in all DNA-based fingerprints. Spots and associated numbers between the first two lanes indicate positions of the bands identified as discriminant in the PCA ordination of RNA-based profiles (see Fig. 2b).

respectively. This last result suggests that a majority of similar bacterial taxa were activated in response to plant materials of distinct moieties.

About 65% of the bacteria detected in unamended soil, as identified by band positions in DNA profiles, were active as deduced from the identification of RNA bands at identical positions. The presence of active bacterial populations within unamended microcosms could probably stem from mineralization of autochthonous soil organic matter that was less protected from microbial activity following the increase of soil moisture from 40 to 70% and partial disruption of aggregates during soil mixing. The proportions of metabolically active bacteria in maize and soybean soils were 57 and 52%, respectively. The influence of organic inputs on structural diversity was further evidenced by computing a PCA ordination of DGGE fingerprints (Fig. 2a). More than 80% of the total inertia was absorbed by the principal plan, conferring on it a high discriminatory value. As expected, total bacterial communities were unambiguously segregated from their corresponding active subsets along the first principal component whatever the treatment. Compositional shifts between total and metabolically active bacterial communities have already been reported within rhizosphere soils from chrysanthemum and grassland turfs (Duineveld et al. 2001; Griffiths et al. 2004). In this background, maize and soybean residues clearly sustained different total and active bacterial communities as indicated by plot discrimination along the first and second axes, respectively. Such an effect of residue quality on the composition of the total bacterial community was already observed with beech and spruce leaves (Aneja et al. 2004) or pea shoots of distinct growth stages (Ha et al. 2008). Treatment ordination was confirmed when RNA-based fingerprints were analysed separately (Fig. 2b). As suggested above on the basis of the high number of bands common to all RNA lanes, structural shifts between active populations evidenced by PCA were mainly driven by changes in the relative abundance of active members (i.e., relative band intensity) in response to distinct organic inputs. This observation could translate the existence of a particular level of functional redundancy in this soil, which is frequently observed in broad bacterial functions such as plant material decomposition.

While the strongest structural shift at the scale of total bacterial communities was induced by soybean residues as compared with control treatment, the active bacterial populations were more responsive to maize inputs (Fig. 2a). It could have been anticipated that shoot nitrogen content would trigger the strongest shift within the active populations since (i) organic matter mineralization is often limited by nitrogen availability (Trinsoutrot et al. 2000) and (ii) N availability is known to be implied in the formation of recalcitrant compounds during plant materials decay (Fog 2008). However, it should not be ruled out that original biochemical attributes of plant material such as polyphenolic compounds could also have impacted bacterial activities and community composition. Importantly, such effects could mostly have been mediated through the organic soluble fraction released from decaying residues, as illustrated with differential $^{13}C$ incorporation in microbial biomass from leached/unleached ryegrass residue (McMahon et al. 2005).

This study indicates that the structural diversity of active bacterial assemblages is responsive to distinct

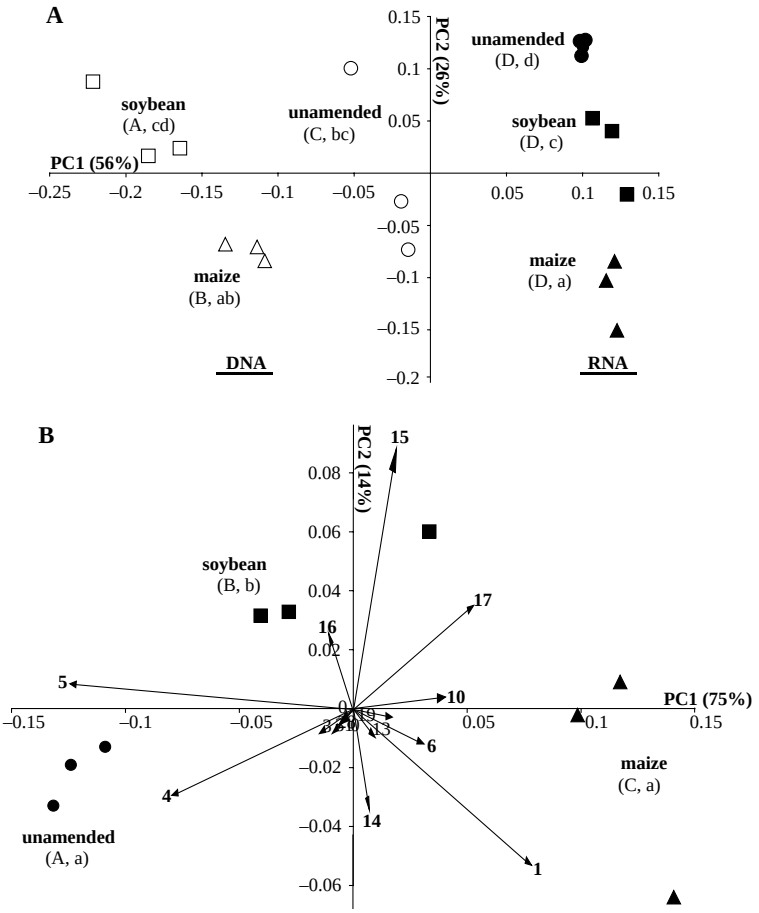


**Fig. 2.** (A) PCA ordination of DNA-based (white symbols) and RNA-based (black symbols) 16S rDNA DGGE fingerprints. Circle, unamended; triangle, maize; square, soybean. Different capital and lowercase letters in parentheses indicate significant differences ($P < 0.05$) between mean abscisses and ordinates, respectively. (B) PCA ordination of 16S rRNA-based DGGE fingerprints. Symbols and associated letters have the same meaning as in Fig. 2 (A). Annotated arrows represent the most discriminating DGGE bands and their numbers refer to the identification ranks of all detected bands between 1 and 33 along the DGGE gel (see spots in Fig. 1).

biochemical attributes of decaying residues. Future research should determine to which extent this early and specific elicitation of active bacteria affects in turn the C sequestration versus mineralization ratio from decaying crop residues and how managing the biochemical quality of crop residues could favour C sequestration in arable soils.

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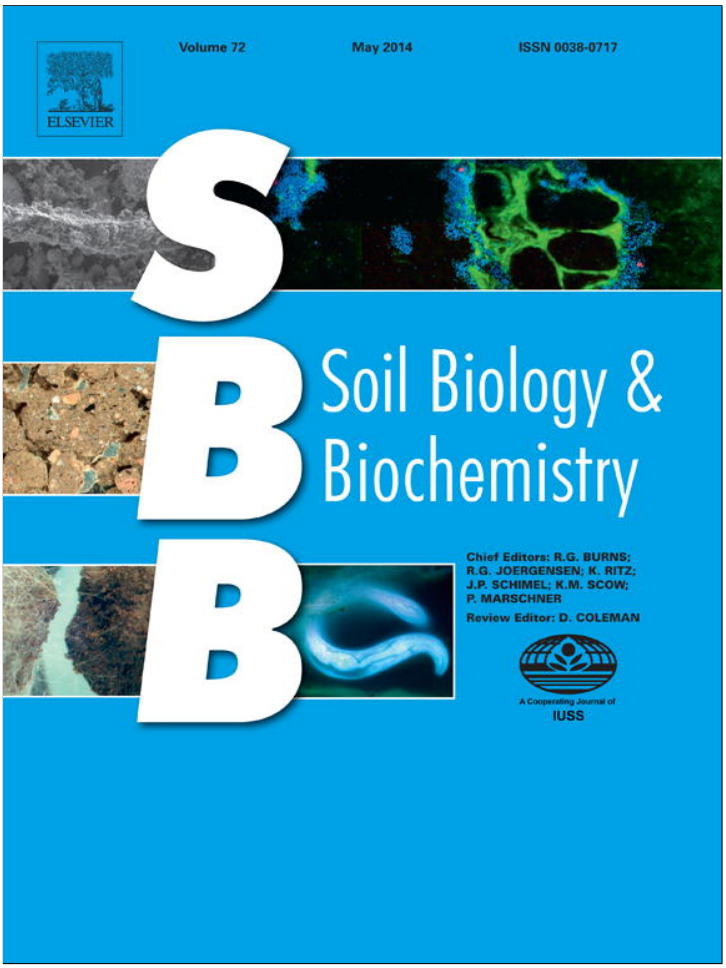

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# Effects of *Jatopha curcas* L. plantation on soil bacterial and fungal communities

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ABSTRACT

The use of *Jatropha curcas* L. (Jatropha) as agrofuel is currently increasing in tropical and sub-tropical regions. This plant and other Jatropha species are well known for synthetising various toxicants. However, the effects of Jatropha plantation on soil microbiota have barely been investigated. We sampled three sites planted with Jatropha for 1, 2 and 15 years in Senegal, together with their adjacent fallow plots, to test whether Jatropha could have detrimental effects on microbial activity and diversity. We further hypothetised that the extent of the effects of Jatropha would be correlated to plantation age. We observed that the genetic structure of the fungal community, and especially its Glomeromycota component, was strongly affected by Jatropha in all sites. The composition of the total bacterial community, especially of the nitrogen-fixing community, was also impacted but only in 2 sites out of 3. Besides, *in situ* catabolic potentials shifted similarly in response to Jatropha growth. Despite these microbial shifts, we cannot conclude to a negative impact as diversity indices (catabolic potentials, genetic profiles) were not lowered. Additionally, no cumulative effect was evidenced between the youngest and the oldest Jatropha plantations, indicating that microbial shifts occurred rapidly and lasted over the long term. Further studies dedicated to the functional implications of such structural shifts are needed since the genetic structure of essential microbial communities such as mycorrhizal fungi and nitrogen-fixing bacteria were proved sensitive to Jatropha.



## 1. Introduction

Over the past decades, a large body of literature has demonstrated that all plants locally modify physical, chemical and especially microbial soil features (diversity, density, activity) in the vicinity of their roots. This is referred to as the rhizosphere effect, a phenomenon mainly fuelled by rhizodeposition (Rovira, 1959; Sanguin et al., 2006; Hannula et al., 2012). The magnitude of this phenomenon is influenced by the integration of several factors among which soil type (*e.g.* pH, texture, nutritional status) (Marschner et al., 2004), plant species (Berg and Smalla, 2009), plant developmental stage (Gomes et al., 2003) or even mycorrhizal status (Andrade et al., 1997; Phillips and Fahey, 2006). Mineralisation of the root necromass and of the litter also contributes to the dynamics of soil microbial communities (Chapman and Newman, 2010; Baumann et al., 2011). The fundamental question raised beyond these changes in microbial community composition and activity patterns is the degree to which these shifts, driven by the plant community structure, affect soil processes such as organic matter mineralisation, C sequestration, nutrient cycling and ultimately the plant cover itself (Harris, 2009; Strickland et al., 2009; Schweitzer et al., 2011).

This long-lasting quest in microbial ecology meets a specific application framework in the case of exotic tree species introduced as monospecific stands such as pine, eucalyptus and acacia species in the last decades to revegetalize areas that face land degradation (Evans, 1982). Such non-indigenous species, along with diverse invasive plants, have recently been evidenced to trigger negative impacts on the soil microbial compartment and disrupt the native interactions between plant cover and soil microbiota (Reinhart and Callaway, 2006; van der Putten et al., 2007). For instance, the growth of *Gmelina arborea*, a fast-growing tree originating from India, in a sandy soil from Burkina Faso triggered a displacement of the herbaceous plant community and a concurrent modification of the catabolic potentialities of the soil microbial community (Sanon et al., 2006). After 7 years of plantation in a Senegalese soil, the Australian *Acacia holosericea* had reduced the resistance of the soil bacterial community to diverse abiotic stresses and strongly altered

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the composition of the arbuscular mycorrhizal community (Remigi et al., 2008). *Eucalyptus camaldulensis*, another Australian fast-growing tree, negatively impacted the bacterial community and the herbaceous plant community in a Sahelian soil (Kisa et al., 2007). In some cases, exotic species invasiveness strategy consists in targeting their detrimental effects to specific soil microbiota: *Alliaria petiolata*, a herbaceous species originating from Europe, presently invades North American forests by synthesising anti-fungal compounds that disrupt associations between native tree seedlings and their arbuscular mycorrhizal fungi (Stinson et al., 2006).

Against this background, the issue of ecosystem resilience is of central importance whenever areas previously devoted to the monoculture of exotic trees have to be converted back into stands of autochtonous plant species or agrosystems. The major concern is to predict whether the amount of ecological disturbance induced by years of Jatropha monoculture could impair the soil adaptative capacity to reach a new stable state compatible with crop production (Gunderson, 2000).

The oil-producing Euphorbiaceae Jatropha (*Jatropha curcas* L.), originally from Central America, was identified a few years ago as suitable for agrofuel production in tropical and subtropical areas, thanks to its adaptability to various pedoclimatic conditions (including semi-arid climates) and its low nutrient requirements (Heller, 1996). That is why it has been increasingly introduced over the last five years in West African (*e.g.* Senegal, Burkina Faso, Benin), Asian (*e.g.* India) and Central American (*e.g.* Mexico) countries. By 2015, estimates are that approximately 13 million hectares of land will be under Jatropha plant cultivation worldwide, while 1 million hectares were planted in 2008 (Renner et al., 2008). Indeed, this woody shrub is known to contain a cocktail of toxicants and anti-nutritional compounds in almost all of its parts (*e.g.* alcaloids, terpenoids, tannins, trypsin inhibitors) with phorbol esters and curcin the foremost toxic phytochemicals. Different kinds of its extracts proved to be toxic on *in vitro* and *in vivo* models on a range of organisms and microorganisms (Devappa et al., 2010). For instance, leaf extracts proved to be toxic against pathogenic bacterial species (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*) (Chunhakant et al., 2007) and latex displayed inhibitory effects on the growth of diverse fungal and bacterial strains (Oyi et al., 2002). Jatropha can also exhibit allelopathic potential on corn and tobacco cultures mainly *via* azelaic acid (Ma et al., 2011). Despite the well-known toxicity of the plant, hardly any information is available in Senegal or in other countries about the fate of Jatropha bioactive compounds, or about their environmental and ecotoxicological effects on soil microbiota that are essential to plant community diversity and productivity, such as the microbial guild engaged in nutrient acquisition by the plants (van der Heijden et al., 1998). Their abundance and/or functional diversity could be lowered owing to their putative sensitivity towards some of these chemicals. Moreover, it is plausible that some of these phytotoxic compounds could display a long residence time in the soil and thus accumulate along the years while their increased levels could strengthen their toxicity. Alternatively in the absence of such accumulation, continuous release of toxicants from roots and litter decay over the years could induce a progressive erosion of sensitive microbial populations. This concern is all the more legitimate that many exotic trees and invasive plants have been shown to exert negative effects on different soil microbiota, even in the absence of such a panel of phytotoxic compounds. Indeed, the abundance and/or diversity, activity of nitrogen-fixing bacteria (Xu et al., 2012) including the symbiotic rhizobiums (Faye et al., 2009; Sanon et al., 2009), of the arbuscular mycorrhizas (Mummey and Rillig, 2006; Stinson et al., 2006; Remigi et al., 2008) of the fungal (Broz et al., 2007) and bacterial (Kisa et al., 2007; Marchante et al., 2008) communities were proved to be altered by the development of exotic plant species.

Against this background, our objective was to describe the chemical and microbial impacts of the *J. curcas* monoculture on arable soils in Senegal. We hypothetised that (i) the extent of these effects would be correlated to the age of the *J. curcas* plantation, and (ii) the activity and/or diversity of different microbial communities would be decreased. This hypothesis was investigated in three *J. curcas* fields: a one-year old one, a two-year old one and a 15-year old living fence. Soil parameters were measured in each of the three *J. curcas* stands as well as in their adjacent fallow plots used as control plots.

## 2. Material and methods

### 2.1. Soil sampling

Smallholders who grew *J. curcas* were located in West Senegal, in the rural community of Ourour, Ouadiour district-Gossas county (N 14° 30′, W 16° 04′), Fatick region. The basic climate in that part of Senegal is Sudano-Sahelian (400–600 mm annual rainfall, 28 °C mean annual temperature) and the soil is classified as an Alfisol (F.A.O., 1998). Three semi-arid sites were selected that had grown *J. curcas* for 1 (site 1) and 2 (site 2) years as a monoculture and for 15 years as living fences (site 3). The three sites were separated from each other by ca. 2 km. Jatropha orchards (sites 1 and 2) were planted in fields previously managed for local crops. As such these arable soils had been submitted to usual agronomic treatments but none of the *J. curcas* plots received any mineral or organic fertilisers or pesticides. In each site, a fallow zone adjacent to the *J. curcas* plot was selected as a control plot that supported a spontaneous plant cover composed of dominant grass species (*Guiera senegalensis*, *Cenchrus biflorus*, *Zornia glochidata*). Control and *J. curcas* soil samples (0–20 cm depth) were taken in April 2009 at the end of the dry season. The sampling scheme consisted of 3 rows *per* plot with one composite soil *per* row, resulting in 18 composite soil samples. Along each row (20 m length and 10 m distance between two sampled rows), 3 soil samples were taken either around 3 *J. curcas* shrubs (within the plant shade area) or every 5 m in control plots, and bulked. Soil samples were sieved (2-mm) and homogenised. A subsample was stored at −20 °C prior to DNA extraction and the remaining soil was stored at +5 °C for the remaining analyses.

### 2.2. Soil chemical analysis

Analyses were performed by the IRD Laboratory LAMA-US 191 (Dakar, Senegal) certified ISO 9001 : 2008 by Euro Quality System (http://www.lama.ird.sn). Total P (1 g soil) was extracted with nitric and chlorhydric acids (Murphy and Riley's method, 1962). Assimilable P or Olsen phosphorus (4 g soil) was extracted with sodium bicarbonate. Extracted forms of P were quantified colorimetrically (phosphomolybdate complex reducted with ascorbic acid) on an auto-analyzer AxFlow-Technicon. Total C and N (0.5 g soil) were quantified by full combustion (1800 °C) on a CHN Thermo Finnigan EA 1112 Series Flash Elemental Analyzer. pH values were measured in water.

### 2.3. Bacterial density

The total bacterial community was described in terms of culturable densities. A 2-g soil aliquot was suspended in 18 mL of 0.9% NaCl and mixed for 30 min with an orbital shaker (120 rpm). The soil suspension was used in a decimal serial dilution and 100 μL of diluted suspensions were spread-plated onto 10%-TSA medium in triplicate. The plates were incubated in the dark at 28 °C and

counted for 9 days. Bacterial counts obtained within the first two days and between the second and ninth day were assigned to *r*- and *K*-strategists, respectively and according to the definition of De Leij et al. (1993). The three counts *per* plot were averaged and bacterial density values were expressed as log CFU $g^{-1}$ dry soil.

### 2.4. Glomeromycota spore density

The arbuscular mycorrhizal community was described in terms of soil spore density. Spores were extracted three times from 100 g of soil by wet sieving with 1 L of tap water and 500/100/25-μm sieves (Gerdemann and Nicolson, 1963). Spores and debris collected at 100 and 25 μm were pooled and centrifuged (2000 rpm 1 min, model 5810R, Eppendorf) through a sucrose gradient (20–60%, w/v). The supernatant was poured onto a 25-μm sieve, spores were rinsed with tap water and suspended in a volume that was precisely determined *a posteriori*. The spore suspension was homogenised and three 100-μL aliquots were individually counted under a stereo microscope (×40). The three counts *per* plot were averaged and spore density was expressed as spore numbers *per* 100 g of dry soil.

### 2.5. Soil DNA extraction

The DNA from triplicated soil aliquots (500 mg) was extracted by bead-beating according to Porteous et al. (1997), further purified on a PVPP column (Edel-Hermann et al., 2004) and quantified by fluorescence with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, St. Aubin, France) and a Tecan infinite M200 microtiterplate reader (Tecan, Lyon, France).

### 2.6. PCR amplifications

The marker genes chosen to study the genetic structure of (i) the total bacterial and *Pseudomonas* communities, (ii) the nitrogen-fixing bacterial community, (iii) the total fungal community and (iv) the arbuscular mycorrhizal community were the 16S rDNA, *nifH*, 18S rDNA and 25S rDNA genes, respectively. All PCR amplifications were performed using an Applied Biosystems Veriti 96-well thermocycler, with GoTaq® DNA Polymerase (Promega, Lyon, France) on 10 ng of template DNA for a final volume of 25 μL. The PCR conditions related to the total bacterial community are described in Ndour et al. (2008). The *Pseudomonas* and the nitrogen-fixing bacteria communities were studied by a nested PCR approach according to Costa et al. (2007) and Diallo et al. (2004), respectively. The total fungal community was studied according to Okubo and Sugiyama (2009). The arbuscular mycorrhizal community was also studied by a nested PCR approach. The eukaryote-specific primers LR1/NDL22 were used in the first step (van Tuinen et al., 1998) and the Glomeromycetes specific primers FLR3/FLR4 in the second step (Gollotte et al., 2004).

### 2.7. Molecular fingerprints

Denaturing gradient gel electrophoresis fingerprints were obtained with the Ingeny phorU device (Ingeny International, Goes, The Netherlands) at 60 °C and 50 mA-100 V for 17 h in TAE 1×. Amplicons were resolved in 8% acrylamide gels (acrylamide–bisacrylamide 40%, 37.5:1, Sigma) with a linear gradient of denaturing agents from 45 to 70% for the 16S rDNA and the *nifH* genes, from 25 to 55% for the 25S rDNA gene and from 35 to 60% for the 18 rDNA gene. Gels were stained in SYBR Gold 1× solution (Invitrogen). Banding patterns were encoded in matrices related to band position and intensity (*i.e.* peak height) using Phoretix 1D (v10) software according to the default parameters (TotalLab, Newcastle upon Tyne, UK). DGGE matrices were then standardised by replacing the absolute intensity of each band on a *per* lane basis by its relative intensity ($p_i$) calculated as the absolute intensity of the band divided by the sum of the intensities of all bands.

### 2.8. In situ catabolic potentials

The diversity of the catabolic potentials of the total soil bacterial community was assessed according to Campbell et al. (2003) by a microrespirometry method performed in 96-well microtiter plates. In order to ensure the resumption of microbial activity, sterile distilled water was added to reach 30% of the water-holding capacity and plates were incubated 3 days in the dark at 28 °C. On the fourth day, soil wells were spiked with 29 organic substrates (3 replicated wells *per* substrate). Stock solutions for 10 carbohydrates (D-mannitol, D-mannose, D-trehalose, L-arabinose, D-xylose, D-sucrose, meso-inositol, D-galactose, L-rhamnose monohydrate, D-sorbitol), 6 carboxylic acids (L-ascorbic acid, citric acid, maleic acid, Na-gluconate, α-ketoglutaric acid, D,L-malic acid) and 12 amino acids (L-asparagine, D,L-histidine, D,L-valine, L-methionine, L-glutamine, D,L-alanine, D,L-serine, L-proline, L-leucine, L-lysine, Na-glutamic acid, L-arginine) were prepared with distilled water and their concentrations were calculated to bring respectively 0.03, 0.04 and 0.004 mmol $g^{-1}$ soil. Basal respiratory activity was determined in triplicate with distilled water. The colorimetric detection plates were assembled and used according to MicroResp™ (Aberdeen, UK) recommendations. Absorbance was measured at 572 nm with a Tecan infinite M200 reader before substrate spiking ($t_0$) and after 6 h of incubation at 28 °C ($t_6$). For each well, absolute respiratory activity was calculated by subtracting the absorbance value at $t_0$ from the value at $t_6$. The average basal respiration value was then subtracted from all the individual substrate respiration values. For each carbon source, this substrate-specific respiratory activity was averaged and the value was finally divided by the sum of all the mean substrate-specific respiratory activities ($p_i$ value).

### 2.9. Statistics

All the statistical analyses were performed with XLSTAT (v2010.5.04) software. The percentage and the density values were arcsin(sqrt)- and log-transformed, respectively, prior to statistical analysis. Significant differences between quantitative values were determined by analysis of variance using ANOVA followed by REQWQ tests for pairwise multiple comparisons ($\alpha = 0.05$). The standardised DGGE matrices and respiratory profiles were compared by hierarchical cluster analysis using Ward's method and Euclidean distance. In addition, the respiratory profiles were compared by principal component analyses (covariance matrix) in order to identify the discriminating substrates. In parallel to multivariate analyses, the Simpson–Yule index of diversity was computed on the basis of the $p_i$ values as (1):

$$D = \sum_i p_i^2 \quad (1)$$

with $i$ varying from 1 to S (total DGGE bands or numbers of catabolised substrates).

## 3. Results

### 3.1. Chemical soil analyses

The soil pH was acid in all plots (from 5.51 to 6.26) (Table 1). pH value was not affected by Jatropha growth in site 2 (two-year old field) but was more acid in site 1 (one-year old field) and less acid in

**Table 1**
Soil chemical characteristics of the experimental plots.

| Soil origin | pH ($H_2O$) | Total C (%) | Total N (%) | Total P | Assimilable P |
|---|---|---|---|---|---|
| *Jatropha*-site 1 | 5.59 ± 0.06 b | 0.57 ± 0.06 a | 0.043 ± 0.005 a | 67.3 ± 4.7 a | 4.3 ± 0.7 a |
| Control-site 1 | 6.14 ± 0.15 a | 0.19 ± 0.05 b | 0.013 ± 0.001 b | 36.0 ± 3.1 bc | 1.7 ± 0.3 b |
| *Jatropha*-site 2 | 5.67 ± 0.13 b | 0.19 ± 0.01 b | 0.013 ± 0.002 b | 28.7 ± 1.3 c | 3.3 ± 0.9 ab |
| Control-site 2 | 5.51 ± 0.06 b | 0.24 ± 0.01 b | 0.019 ± 0.003 b | 32.7 ± 1.8 bc | 1.7 ± 0.3 b |
| *Jatropha*-site 3 | 6.26 ± 0.07 a | 0.29 ± 0.05 b | 0.021 ± 0.004 b | 46.7 ± 4.1 b | 2.3 ± 0.3 ab |
| Control-site 3 | 5.52 ± 0.15 b | 0.21 ± 0.01 b | 0.015 ± 0.001 b | 36.7 ± 3.3 bc | 2.0 ± 0.6 ab |

Phosphorus contents are expressed in mg $kg^{-1}$ dry soil. Different letters in a column indicate significant differences between soil origins (mean ± standard deviation, $n = 3$, $P < 0.05$).

site 3 (15-year old living fence). Soil C (from 0.19 to 0.57%) and N (from 0.013 to 0.043%) contents were significantly increased only in site 1 in the presence of Jatropha. A similar trend was noticed with total (from 28.7 to 67.3 mg P $kg^{-1}$) and assimilable (from 1.7 to 4.3 mg P $kg^{-1}$) phosphorus contents.

### 3.2. Culturable bacterial and Glomeromycota spore densities

Average data counts performed two days after plate inoculations ranged from 6.07 to 6.39 log CFU *per* gram of soil. For each site, soil from the Jatropha plot displayed similar bacterial density to that of the corresponding control plot (Table 2). Similar trends were observed from data counts obtained nine days after plate inoculations (from 6.30 to 6.64 log CFU). Average spore densities ranged between 203 and 134 spores *per* 100 g of soil (Table 2). The extent of the differences between control and Jatropha plots was low but significant in site 3 with fewer spores in the Jatropha plot.

### 3.3. In situ catabolic potentials (total bacterial community)

The catabolic signatures of the entire set of soil samples were 86% similar (Fig. 1). Cluster analysis indicated that the extent of Jatropha impact was site-dependent and strongest in site 1, as evidenced by the two monospecific clusters that opposed the 3 control plot samples to the 3 Jatropha plot samples. The effect of Jatropha was still obvious in site 3 (despite higher intraplot variability in both fields) but no longer in site 2 owing to (i) an overall similarity of *c.* 96% among the 6 samples and (ii) the grouping of a Jatropha sample and a control one at *c.* 97% similarity.

Individual PCAs carried out on a site basis and coupled to ANOVA tests on relative oxidation rates allowed for the identification of the most discriminating substrates (Fig. 2). In site 1, Jatropha soil bacterial communities were mainly characterised by preferential oxidation of ascorbate (and trehalose, sucrose, galactose and gluconate to a lesser extent). In contrast, control soils displayed higher relative oxidation of ketoglutarate. In site 2, ketoglutarate and xylose (and inositol to a lesser extent) were the only catabolic markers associated to Jatropha and control soils, respectively. In site 3, Jatropha soils were mostly characterised by a higher relative utilization rate of citrate, maleate and malate while control soils were associated to trehalose and galactose (and xylose to a lesser extent). There was no significant difference at levels of $P < 0.05$ in diversity indices between Jatropha and control plots for any site (Table 3).

**Table 2**
Densities of culturable bacteria (TSA 10% medium) and Glomeromycetes spores.

| Soil origin | Bacterial counts (day 2) | Spores numbers |
|---|---|---|
| *Jatropha*-site 1 | 6.39 ± 0.07 ab | 203 ± 5 a |
| Control-site 1 | 6.09 ± 0.08 ab | 187 ± 12 a |
| *Jatropha*-site 2 | 5.96 ± 0.06 b | 169 ± 10 ab |
| Control-site 2 | 6.07 ± 0.04 ab | 194 ± 7 b |
| *Jatropha*-site 3 | 6.39 ± 0.08 a | 134 ± 12 b |
| Control-site 3 | 6.26 ± 0.15 ab | 183 ± 12 a |

Bacterial counts are expressed in log CFU $g^{-1}$ dry soil and spores numbers *per* 100 g soil. Different letters in a column indicate significant differences between soil origins (mean ± standard deviation, $n = 3$, $P < 0.05$).

### 3.4. 16S rDNA-DGGE fingerprints (total bacterial community)

Cluster analysis of the 16S rDNA-DGGE fingerprints (Fig. 3A) indicated a similarity level of 40% across all lanes, but failed to reveal any specific and systematic effect of Jatropha culture on the genetic structure of the soil bacterial community (Fig. 3B). The strongest differences in banding patterns occurred in sites 1 and 3, for which the three control plot (site 1) or Jatropha plot (site 3) profiles clustered separately from the profiles related to the corresponding adjacent plot. In the case of site 2, the impact of Jatropha was blurred by composite clusters that gathered two profiles of a given plot with one profile of the adjacent plot. There was no significant difference at levels of $P < 0.05$ in the diversity indices between the Jatropha and control plots for any site (Table 3).

### 3.5. 16S rDNA-DGGE fingerprints (Pseudomonas community)

For almost all plots, the conspicuous variability of the three 16S rDNA banding patterns (Fig. 3C) did not yield any clearcut clustering among soil origins (Fig. 3D), suggesting that the *Pseudomonas* community was not sensitive to the soil modifications induced by Jatropha growth. Diversity indices did not reveal any further information (Table 3).

### 3.6. nifH-DGGE fingerprints (bacterial nitrogen-fixing community)

In contrast to the *Pseudomonas* community, intra-plot variability was far less extended within the functional guild of the nitrogen-fixing bacteria, especially in site 2 (Fig. 3E). Moreover, obvious

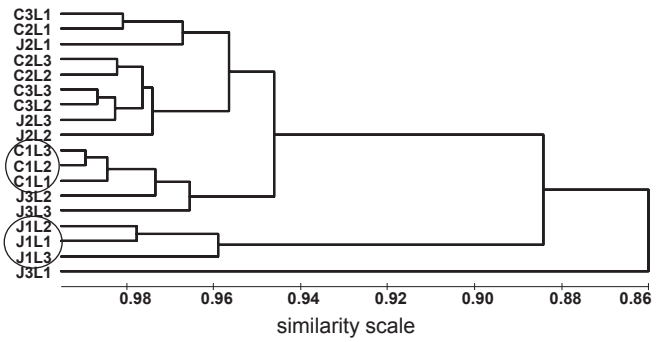


**Fig. 1.** Cluster analysis of the soil catabolic profiles. J1–J3: Jatropha plantation site 1–site 3; C1–C3: control field (fallow plot) site 1–site 3; L1–L3: within field sampling lines. Circled codes point out relevant groupings.

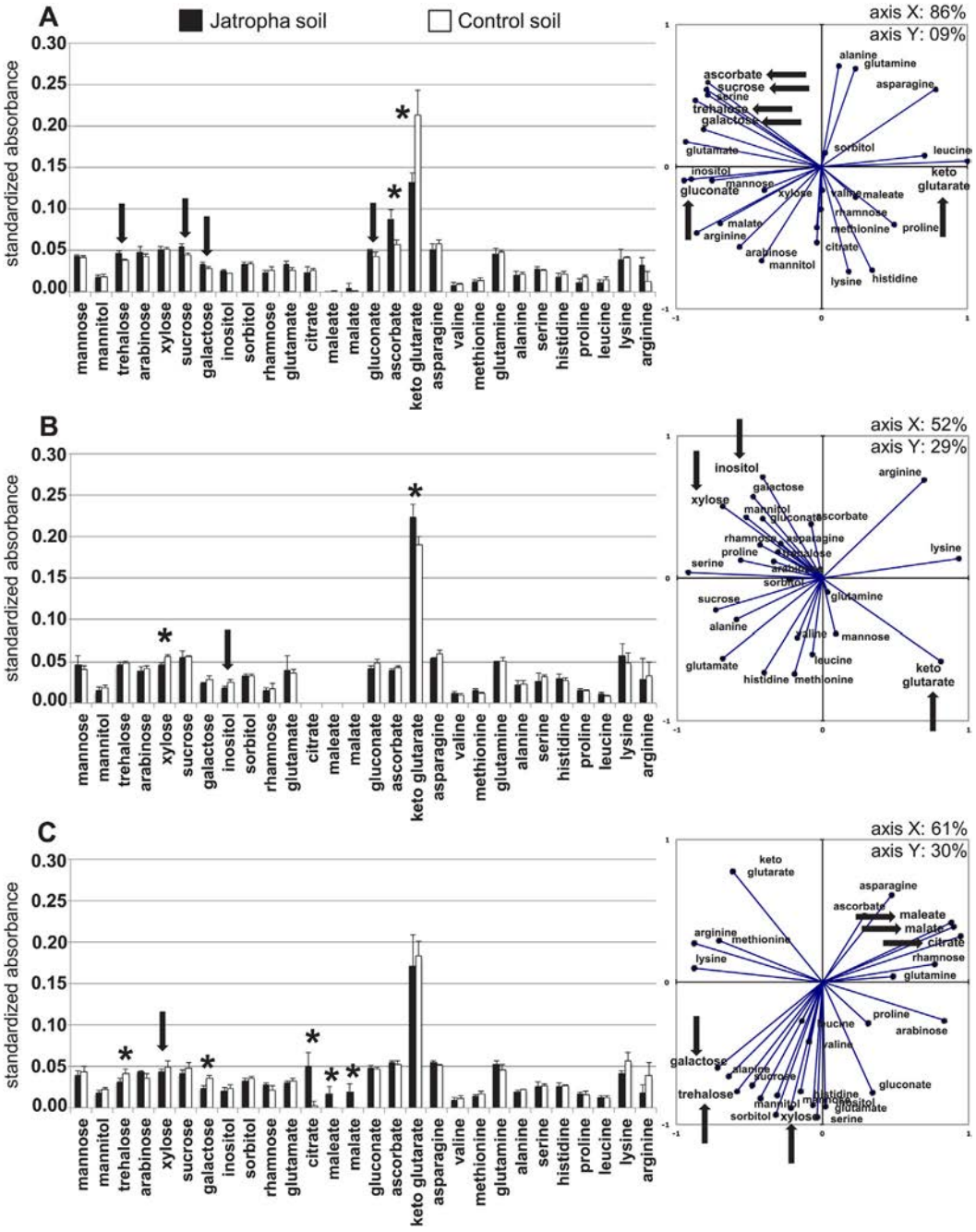


**Fig. 2.** Catabolic profiles and associated correlation circles of their principal component analyses of Jatropha and control soil samples from site 1 (A), site 2 (B) and site 3 (C). Asterisks indicate significant differences (mean ± standard deviation, $n = 3$, $P < 0.05$) while arrows point out substrates for which significant differences were identified with the less stringent Newman–Keuls test. All these discriminating substrates are pointed out by arrows in the adjacent correlation circles that also display the inertia values of the first two principal components.

differences were observed between sites. Visual observation of the migration profiles allowed us to predict that the genetic structure of this functional community was obviously affected by Jatropha monoculture in sites 1 and 3 but not in site 2. Cluster analysis (Fig. 3F) confirmed this interpretation, as evidenced by divergent branchings between control and Jatropha profiles from sites 1 and 3, some of them monospecific, *e.g.* site 1 control profiles. As anticipated, it was not possible to delineate specific clusters composed of control or Jatropha profiles in site 2. There was no significant difference in the diversity indices between the Jatropha and control plots for any site (Table 3).

### 3.7. 18S rDNA-DGGE fingerprints (total fungal community)

The banding patterns revealed limited intra-plot variability for all plots and an obvious effect of Jatropha in site 3 (Fig. 4A). Cluster analysis (Fig. 4B) evidenced 6 monospecific clusters defined at similarity levels ranging from 73 to 86%, each exclusively composed of the three DGGE profiles representative of a given plot. Moreover, for a given site except site 2, the Jatropha cluster was not directly connected to the corresponding control cluster. Thus, the fungal community was highly susceptible to the effect of Jatropha in all three sites and the amplitude was the strongest in sites 1 and 3. No

**Table 3**
Simpson diversity indices calculated for the different fingerprinting methods.

| Soil origin | Catabolic profiles | Total bacteria-DGGE | *Pseudomonas*-DGGE | *nifH*-DGGE | Fungi-DGGE | AMF-DGGE |
|---|---|---|---|---|---|---|
| *Jatropha*-site 1 | 0.056 ± 0.004 b | 0.037 ± 0.004 a | 0.042 ± 0.003 a | 0.051 ± 0.005 ab | 0.039 ± 0.004 b | 0.092 ± 0.038 a |
| Control-site 1 | 0.076 ± 0.013 ab | 0.030 ± 0.003 ab | 0.054 ± 0.010 a | 0.035 ± 0.004 b | 0.045 ± 0.008 b | 0.099 ± 0.021 a |
| *Jatropha*-site 2 | 0.081 ± 0.006 a | 0.029 ± 0.002 b | 0.044 ± 0.004 a | 0.055 ± 0.014 a | 0.051 ± 0.002 ab | 0.096 ± 0.035 a |
| Control-site 2 | 0.070 ± 0.004 ab | 0.031 ± 0.000 ab | 0.042 ± 0.002 a | 0.055 ± 0.005 a | 0.047 ± 0.001 ab | 0.085 ± 0.018 a |
| *Jatropha*-site 3 | 0.061 ± 0.011 ab | 0.031 ± 0.003 ab | 0.058 ± 0.005 a | 0.043 ± 0.004 ab | 0.059 ± 0.004 a | 0.077 ± 0.005 a |
| Control-site 3 | 0.066 ± 0.006 ab | 0.031 ± 0.004 ab | 0.054 ± 0.018 a | 0.054 ± 0.006 a | 0.048 ± 0.004 ab | 0.101 ± 0.005 a |

Different letters in a column indicate significant differences between soil origins (mean ± standard deviation, $n = 3$, $P < 0.05$).

significant change in diversity indices was noticed amongst the different soil origins (Table 3).

### 3.8. 25S rDNA-DGGE fingerprints (arbuscular mycorrhizal community)

The intra-plot variability of the banding patterns was particularly obvious in sites 1 and 3 (Fig. 4C). Despite this site variability, the cluster analysis dendrogram (Fig. 4D) was structured in two early divergent branchings (at *c.* 0.1% similarity) each one containing all the control and Jatropha profiles, respectively (except a single control profile identified in the Jatropha branching). The different sub-clusters within these two main branchings were defined at variable similarity levels (most of them between 30 and 50%) claiming for a heterogeneous mycorrhizal community both at the site and plot scales, as previously anticipated. Thus, as part of the total fungal community, the functional guild of the arbuscular mycorrhizal fungi was also highly responsive to the soil modifications induced by Jatropha development. Comparison of the diversity indices did not reveal any further information (Table 3).

## 4. Discussion

This field study clearly shows that Jatropha monoculture had a significant effect, over a relatively short time-period (one-year old plantation in site 1) on the composition of all targeted microbial groups but the *Pseudomonas* community. As Jatropha plantations were not fertilised and grown on the same arable soil as control plots, the differences evidenced between each fallow plot and its adjacent Jatropha plot were solely attributable to Jatropha development. Besides, the extent of these differences and the correlated intra-plot variability appeared site as well as parameter dependent. This field heterogeneity is inherent to such cropping systems based on low input levels and mechanical disturbance that highlight local disparities. This natural heterogeneity may have obscured the impact of Jatropha on some chemical or microbial features. Thus, the between sites comparison remains indicative of possible cumulative effects that need a different experimental design to be unambiguously addressed. The sampling of Jatropha plantations of identical age over the years together with that of plantations initiated each year would help solving this issue.

### 4.1. Impact of Jatropha on fungal communities

The strongest effects were recorded with the fungal microbiota in all three sites. The genetic structure of the total fungal community in control soils differed systematically from that of the Jatropha soils. As Jatropha impact on soil chemical properties was only significant in site 1 (Table 1), the compositional shifts affecting the fungal community in all sites seem not to have involved the soil C–N–P levels. The extent of these differences was highest in the oldest plantation in site 3 (Fig. 4B) : control and Jatropha profiles shared 41% similarity while those of site 1 shared 65%. In contrast, the comparison of bacterial fingerprints (Fig. 3B) did not result in such a dichotomic classification since the migration profiles of a given site rarely formed such monospecific and segregated clusters. Thus, it is tempting to infer that fungi are more sensitive to Jatropha than bacteria, and that a somewhat cumulative effect was observed over a decade. In a microcosm experiment implying the decomposition of diverse Jatropha residues, higher fungal-to-bacterial FAME ratios were found in residue-amended soils as compared to the control (Chaudhary et al., 2011). Their result also claims for a differential impact on fungi and bacteria at the scale of their abundance in response to soil amendment with Jatropha material that appears more favourable to fungal biomass. It remains that direct contact with diluted Jatropha extracts often triggers phytotoxic effects on the *in vitro* growth of fungal colonies (Devappa et al., 2010). Here, the sudden, unambiguous and long-term shift of the fungal community structure requires further investigations linked to key soil processes that involve fungal activities (*e.g.* organic matter mineralisation and aggregation dynamics).

The shift within the arbuscular mycorrhizal community was even more pronounced, as indicated by the dichotomic topology of the cluster analysis dendrogram (Fig. 4D). However, it was not possible to sort sites according to modification strength, even though the remaining amount of soil spores at that sampling time was significantly lower in the Jatropha field of site 3 (Table 2). These elements give no clue about a potential cumulative effect of Jatropha on Glomeromycota. A few studies have also observed that Jatropha can influence this particular fungal phylum. Bean seedlings mycorrhization decreased when soil was amended with *Jatropha gaumeri* leaves (2%). However, plant dry biomass was not altered (del Carmen et al., 2008). Two years and a half after plantation in a tropical system, cultivation of *J. curcas* cut by half soil AMF diversity (Shannon index = 1.20) as compared to the native vegetation soil (Shannon index = 2.42) (Alguacil et al., 2012). Nonetheless, this decrease in soil AMF diversity was correlated to significant improvements in soil organic C, total N and microbial biomass, like in the present study for C, N and P levels (site 1). Despite these reported negative effects, *J. curcas* is a species that can be highly mycorrhized (Charoenpakdee et al., 2010; Kumar et al., 2010). Thus the mechanisms that underly the modification of the AMF community composition in these studies and in our case cannot be attributed to a low mycorrhizal dependency of Jatropha. Whatever the mechanisms involved, it should be kept in mind that a consistent modification of the AMF community is a trait that is frequently associated to an invasive behaviour of exotic species (Stampe and Daehler, 2003; Mummey and Rillig, 2006; Stinson et al., 2006).

### 4.2. Impact of Jatropha on functional bacterial communities

Among the studied bacterial groups, nitrogen-fixing bacteria turned out to be the most sensitive to the presence of Jatropha (Fig. 3E–F). This community comprises the free (associative) nitrogen fixers as well as the symbiotic Rhizobia (and possibly some

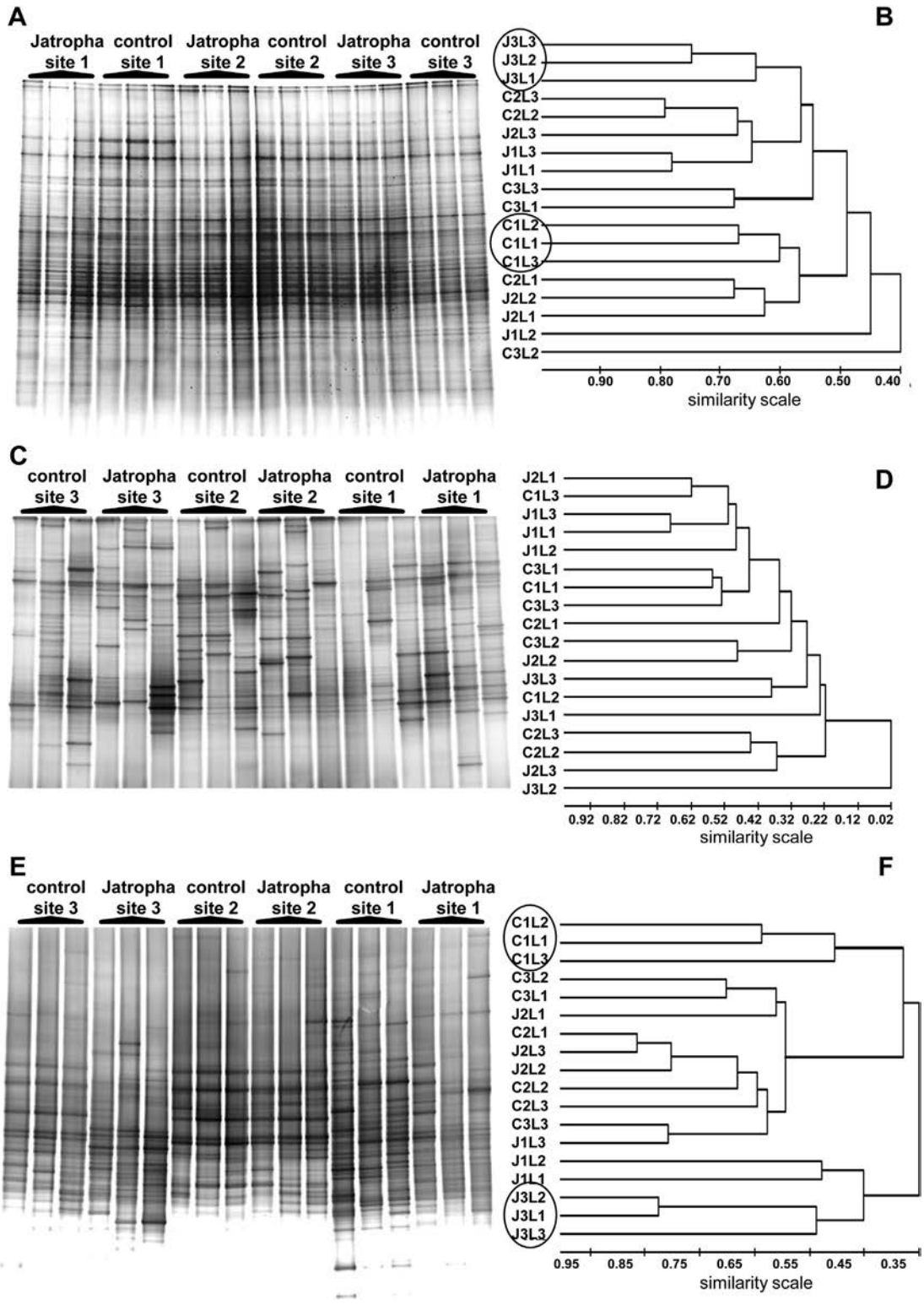


**Fig. 3.** DGGE fingerprints and cluster analyses of the genetic structures of the total bacterial community (A-B), the *Pseudomonas* community (C-D) and the bacterial nitrogen-fixing community (E-F). J1–J3: Jatropha plantation site 1–site 3; C1–C3: control field (fallow plot) site 1–site 3; L1–L3: within field sampling lines. Circled codes point out relevant groupings.

endophytes) that all possess the *nifH* gene used here as a molecular marker. Strong shifts in community composition were only recorded in sites 1 and 3 (Fig. 3F). Only very few studies to date have investigated the effect of exotic plants on non-symbiotic nitrogen-fixing bacteria. A multi-site investigation, targeted to the invasive weed *Ageratina adenophora* in China, revealed that culturable density of nitrogen-fixing bacteria as well as their diversity were significantly favoured in the highly invaded zones (Xu et al., 2012). However, the numerically dominant species that formed the core community were highly similar between invaded and non-invaded

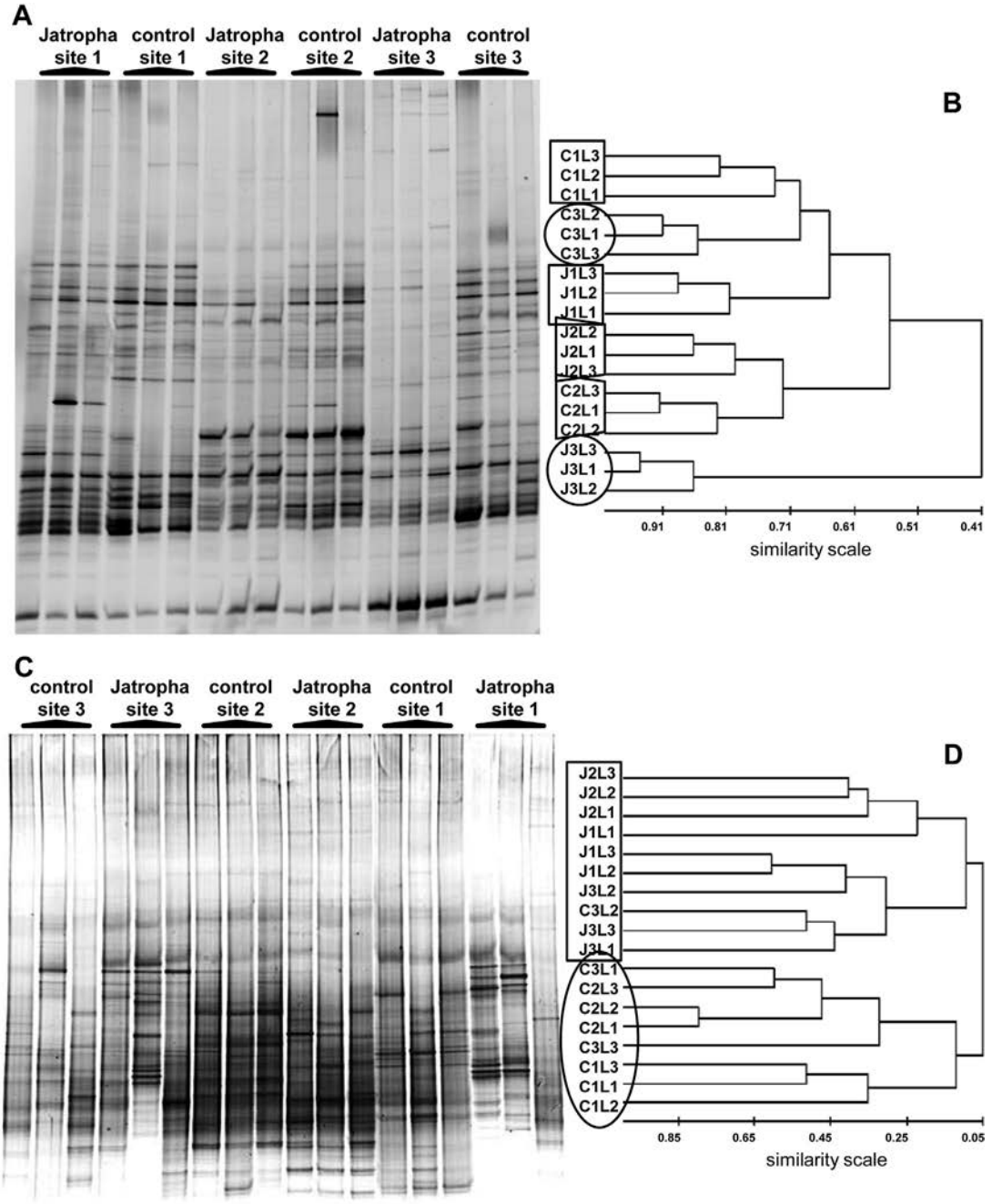


**Fig. 4.** DGGE fingerprints and cluster analyses of the genetic structures of the total fungal community (A-B) and the arbuscular mycorrhizal community (C-D). J1–J3: Jatropha plantation site 1–site 3; C1–C3: control field (fallow plot) site 1–site 3; L1–L3: within field sampling lines. Circled codes point out relevant groupings.

spots. In contrast, only subtle differences were noted in total activity and diversity of nitrogen fixers that inhabited wetland ecosystems facing three types of invasion (Moseman et al., 2009). If soil nitrogen inputs were affected by the recorded shifts in the genetic structure of the nitrogen-fixer community, then the following steps of the nitrogen cycle (ammonification, nitrification, denitrification) could also have been impacted. This assumption is not purely speculative as higher soil N contents were measured in the Jatropha plantations of both sites, especially in site 1 (Table 1). It has already been observed that invasive mechanisms often take advantage of, or favour, higher N availability (Ley and D'Antonio, 1998; Ehrenfeld, 2003; Rout and Callaway, 2009). Thus, this salient result motivates further functional investigations at the scale of the soil N economy (nitrogen fixation rates by diazotrophy, nitrogen-fixer density, Rhizobia density, diversity and symbiotic efficiency).

The *Pseudomonas* genus does not constitute a functional guild *sensu stricto*. But is known to harbour many culturable strains that express beneficial (plant-growth-promoting rhizobacteria, mycorrhiza-helper bacteria) or deleterious effects on plants or possess a genetic potential linked to specific steps of the geochemical cycles (*e.g.* nitrogen-fixing, denitrification genes, phosphorus solubilization, iron sequestration) (Miller et al., 2010). The genetic structure of this community within all control plots was heterogeneous and the situation was unchanged in Jatropha plots (Fig. 3D). Although we cannot explain why the diversity of *Pseudomonas* bacteria was unevenly distributed within fallow plots, this

result could mean that some components of the soil microbiota are insensitive to the changes in their habitat induced by Jatropha growth, in terms of community composition at least. In other words, the ecological impact of Jatropha on soil microbiota is apparently not uniform and depends on the identity of the studied group.

### 4.3. Impact of Jatropha on the total bacterial community

The total bacterial community displayed rather subtle differences in response to Jatropha when compared to fungi or nitrogen-fixing bacteria. Culturable densities were not affected by Jatropha plantations (Table 2). The catabolic signatures associated to the soils influenced by Jatropha were not similar from one site to the next (Fig. 1) and the diversity of these catabolic signatures was not Jatropha-specific, as Jatropha samples did not all gather in a monospecific cluster. In this background, the most clearcut effects of Jatropha were then again recorded in site 3, and especially site 1, without significantly altering the indices of catabolic diversity. Yet, at $P = 0.07$ and only for site 1, the Simpson index of the Jatropha plot was significantly lower than that of the control plot (Table 3), suggesting more homogeneous catabolic potentialities within this Jatropha plot. In contrast to fungi, the most consistent effects of Jatropha on the bacterial catabolic profiles were apparently in line with the significantly higher C–N–P levels registered in the Jatropha plot in site 1. This could suggest that this higher availability in C–N–P elements was involved in the development of distinct catabolic profiles and a higher catabolic evenness. Conversely with DGGE fingerprints, the Simpson index of the Jatropha plot was significantly higher ($P = 0.07$) than that of the control plot (Table 3), suggesting a more heterogeneous genetic structure of the bacterial community within this Jatropha plot. The concomitant and moderate modifications observed at the scales of the genetic structure (Fig. 3B) and of the catabolic signatures (Fig. 1) could indicate that shifts in composition resulted in modified activity patterns. This deduction is supported by the facts that (i) altering organic compound availability in a soil can change microbial catabolic activity patterns while altering community composition (Pennanen et al., 2004) or not (Orwin et al., 2006) and (ii) Jatropha monoculture undoubtably constrained the volume and diversity of organic compounds entering the soil as compared to the herbaceous cover in fallow plots. The absence of a strong effect on this total bacterial community (Figs. 1–3B) contrasts with most studies that assess the same community features (i.e. genetic structure or composition, catabolic diversity) in response to plant invasion. For instance, the invasion of sand dunes by the leguminous tree *Acacia longifolia* significantly impacted the catabolic diversity of the soil microbial community and this impact was related to invasion duration (Marchante et al., 2008). The limited effect of Jatropha on the total bacterial community is also surprising since the mycorrhizal network, and the associated hyphosphere effect, were probably modified in connection with the contrasted plant covers. In this sense, the modified DGGE profiles of AMF-25S rDNA (Fig. 4D) can be viewed as an indirect proof of a modified mycorrhizal network, at least in terms of composition. Bacterial communities are known to be sensitive to the physiological activity of hyphae that release organic compounds in the surrounding soil (Andrade et al., 1997; Johansson et al., 2004; Toljander et al., 2007; Uroz et al., 2007). It was also shown that different mycorrhizas selected for distinct soil bacterial communities (Andrade et al., 1997; Rillig et al., 2006; Marschner et al., 2001). The influence of mycorrhizas on soil bacteria can be determining, as suggested by the work of Kisa et al. (2007). In this study, the negative impact of the exotic *Eucalyptus calmaldulensis* on the native soil microbial catabolic diversity (and mycorrhizal soil infectivity) was counterbalanced by mycorrhization of eucalyptus with a *Glomus intraradices* strain. A negative impact of *Acacia holosericea* on the soil bacterial community was also recorded along with an alteration of the AMF community structure (Remigi et al., 2008). More particularly, the reduced catabolic evenness induced by the leguminous tree was correlated to a weaker resistance of soil bacteria to abiotic stresses (*e.g.* salt, moisture, temperature). Thus, in our case, there seemed to be a poor link between the altered mycorrhizal community and the activity/composition of the soil bacterial community.

Despite the low amplitude of the effects of Jatropha on catabolic potentialities, some substrates appeared as significantly discriminating. The best scores were recorded with α-ketoglutarate, ascorbate, citrate, maleate, malate and trehalose, xylose and galactose but no change was noted concerning amino acids (Fig. 2A–C). Gluconate, sucrose and inositol were secondarily associated to treatment discrimination. Apart from α-ketoglutarate in site 1, several carboxylic acids were preferentially used by the microbial communities of Jatropha soils (site 1: glutamate, gluconate, ascorbate; site 2: glutamate, α-ketoglutarate; site 3: citrate, maleate, malate). An opposite trend was evidenced with carbohydrates only in sites 2 and 3. These substrates could indirectly point out differences in the exudation patterns between Jatropha and fallow plots: the preferential use of these compounds may indicate their higher *in situ* availability. Carboxylic acids are often identified as discriminating substrates in various environmental situations, especially α-ketoglutarate which has a key function in amino acid synthesis in bacteria and fungi (Sanon et al., 2006; Marchante et al., 2008; Remigi et al., 2008).

## 5. Conclusion

Our data indicate that the development of *J. curcas* in arable sub-Sahelian soils can quickly trigger long-lasting compositional shifts in essential microbial communities such as fungi, Glomeromycota and nitrogen-fixing bacteria; this confirms the scarce preliminary results obtained from other agrosystems. However, these shifts operated without altering the diversity levels of these microbial communities, inferred from their molecular fingerprints by calculating the Simpson indices. The consequences of these compositional modifications on soil functioning and plant cover dynamics will have to be studied in further investigations that should focus on the functional diversity of plant/microbe symbiotic associations and N cycle.

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# Impact of the energy crop *Jatropha curcas* L. on the composition of rhizobial populations nodulating cowpea (*Vigna unguiculata* L.) and acacia (*Acacia seyal* L.)

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ABSTRACT

*Jatropha curcas*, a *Euphorbiaceae* species that produces many toxicants, is increasingly planted as an agrofuel plant in Senegal. The purpose of this study was to determine whether soil priming induced by *J. curcas* monoculture could alter the rhizobial populations that nodulate cowpea and acacia, two locally widespread legumes. Soil samples were transferred into a greenhouse from three fields previously cultivated with Jatropha for 1, 2, and 15 years, and the two trap legumes were grown in them. Control soil samples were also taken from adjacent Jatropha-fallow plots. Both legumes tended to develop fewer but larger nodules when grown in Jatropha soils. Nearly all the *nifH* sequences amplified from nodule DNA were affiliated to the *Bradyrhizobium* genus. Only sequences from *Acacia seyal* nodules grown in the most recent Jatropha plantation were related to the *Mesorhizobium* genus, which was much a more conventional finding on *A. seyal* than the unexpected *Bradyrhizobium* genus. Apart from this particular case, only minor differences were found in the respective compositions of Jatropha soil versus control soil rhizobial populations. Lastly, the structure of these rhizobial populations was systematically imbalanced owing to the overwhelming dominance of a very small number of *nifH* genotypes, some of which were identical across soil types or even sites. Despite these weak and sparse effects on rhizobial diversity, future investigations should focus on the characterization of the nitrogen-fixing abilities of the predominant rhizobial strains.



## Introduction

There is worldwide growing interest in renewable energy sources as possible options for lowering fossil fuel dependency. Land use of arable soils to convert plant biomass into fuel, called biofuel or agrofuel (e.g. ethanol and sugarcane), has already been developed [3,29]. In this background, Jatropha (*Jatropha curcas* L.), an oil-producing *Euphorbiaceae* from Central America, has been increasingly introduced into West Africa, Asia, and South America in the last decade owing to its suitability for agrofuel production in tropical and subtropical areas [15]. In Senegal, a national program plans to convert 320,000 hectares to Jatropha culture [8]. However, like many other Jatropha species, this exotic woody shrub contains a cocktail of toxicants (alcaloids, terpenoids, tannins) in almost all of its parts. These molecules have long been used in folk medicine, and different types of plant extracts are known to be toxic to a range of organisms (larvae, cattle, humans) and microorganisms [19,23,27]. The effects of bioactive Jatropha compounds on key soil microbial communities should be assessed since soil microorganism diversity and activity are essential to plant community diversity and productivity [22,39,40].

In this respect, we showed in a previous investigation based on soil DNA from the same soils as those used in this study (see Dieng et al. [9]), that the genetic structures of the fungal, mycorrhizal and bacterial *nifH* nitrogen-fixing communities from Jatropha and control fields were strongly divergent. Shifts in the *nifH* community could point to altered diversity/density of symbiotic nitrogen-fixing bacteria (rhizobia) and in turn altered availability of strains compatible with certain legume species. Additionally, distinct rhizobial strains can exhibit contrasting nitrogen fixation efficiency levels and competitive abilities in the rhizosphere of a given legume

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[17]. Such quantitative and qualitative shifts could further impact grain or woody legume development and performance, as these often rely on specific interactions between compatible legumes and rhizobial partners. These effects should be anticipated since old or unprofitable Jatropha plantations will sooner or later be cultivated again with traditional crops.

Plant-soil microorganism feedback loops were only verified a relatively short time ago, especially in the case of exotic and invasive plants. Batten et al. [2] revealed that invasive plant-induced changes in the soil microbial community could negatively impact native plant performances (e.g. delayed flowering date, lower shoot biomass). More specifically, the soil density of non-symbiotic nitrogen-fixing bacteria, and to a lesser extent their diversity, were significantly altered by the invasive species *Ageratina adenophora* [44]. These changes were associated with increased soil N content, a mechanism of invasiveness that disturbs the competitive coexistence of plants. Regarding rhizobial symbiosis, the introduction of the Australian *Acacia holosericea* into Senegal has drastically affected the genotypic and symbiotic effectiveness of native bradyrhizobia populations associated with *Faidherbia albida*, which has in turn impacted its growth performances [12].

Therefore, the objectives of this study were to assess whether soil chemical and microbial modifications induced by Jatropha growth could impact on growth and the symbiotic rhizobial community of two local legumes, namely the crop *Vigna unguiculata* L. (cowpea) and the agroforestry tree *Acacia seyal*. Both species play important socio-economic roles in Senegal [5,10,14]. Cowpea is a grain legume that is part of the diet of rural populations and is also largely exported [21]. *A. seyal*, like other *Acacia* species (*A. senegal*, *A. laeta*, *A. nilotica*), is valuable in afforestation and agroforestry systems, as well as in regeneration programs of degraded lands [38]. These two trap legumes were grown for several weeks in soils transferred from three Jatropha plantations (1, 2 and 15 years old), as well as adjacent fallow fields considered as control fields. Then, the symbiotic rhizobial communities that nodulate these legumes were described in terms of their nodulation pattern (fresh mass and number of nodules *per* plant) and the molecular diversity of the *nifH* gene.

## Materials and methods

### *Field sites and soil sampling design*

Site location, agricultural management, climatic conditions, soil characteristics (including the genetic fingerprints of several microbial communities) and the sampling design are detailed in Dieng et al. [9]. Briefly, the three Jatropha plantations (aged 1, 2, and 15 years; sites 1, 2, and 3, respectively) were located in West Senegal, in the Fatick region (Alfisol soil type [11]). The three sites were separated from each another by ca. 2 km Jatropha orchards (sites 1 and 2) were planted in fields previously grown with local crops. As such, these arable soils had been submitted to usual agronomic management, but none of the *J. curcas* plots received any mineral or organic fertilizers or pesticides. In each of the three sites, a fallow field adjacent to the Jatropha plantation was selected as a control field that supported spontaneous plant cover composed of grass species. The soil sampling scheme consisted of 3 rows *per* field and one composite soil ($n = 3$) *per* row, resulting in 18 composite soil samples (0–20 cm depth). The soil samples were sieved at 2 mm, homogenized, and transferred to the greenhouse for pot culture.

### *Greenhouse experimental design and nodule sampling*

For each of the 18 composite soils, cowpea and acacia were sown in four replicate pots (500 and 250 $cm^3$, respectively), with one seedling *per* pot. Cowpea and acacia seeds were disinfected as follows: cowpea seeds were treated for 5 min in 33% calcium hypochlorite and rinsed in sterile water, while acacia seeds were treated for 30 min in 96% $H_2SO_4$, rinsed, and immersed in sterile water for 24 h. Then, they were left to germinate on sterile water agar (0.9% w/v) at 37 °C for 2 days (acacia) or 3 days (cowpea). Once rootlets were 1–2 cm long, seedlings were transferred into pots. Cowpea seedlings were grown for 7 weeks and acacia seedlings for 3 months, with daily irrigation. At harvest, fresh shoot and root weights were determined and shoot N–P contents were analyzed from dried sub-samples (70 °C – 3 days) performed by the IRD Laboratory LAMA-US 191 (Dakar, Senegal) certified ISO 9001: 2008 by the Euro Quality System (http://www.lama.ird.sn). Nodules were excised from fresh roots, counted, rinsed, and weighed (total number and fresh weight *per* individual plant). Nodules were then surface-sterilized (5 min in 5% calcium hypochlorite, 5 min in pure ethanol and sterile water rinses) and individually stored at −20 °C in microtiter plate wells filled with sterile 20% glycerol prior to molecular analysis.

### *Nodule DNA extraction and diversity of the nodulating rhizobial populations*

For a given legume species, a maximum of 24 nodules was randomly chosen for each of the 18 composite soil samples, with a maximum of 72 nodules *per* legume species and *per* field. The selected nodules were individually placed in microtiter plate wells, rinsed several times with sterile water and then crushed in 100 μL of 20% glycerol. Bacteroid DNA was extracted from 25 μL aliquots of a 10-fold dilution of the initial tissue homogenate by heat shock (7 cycles of 96 °C for 2 min and 4 °C for 10 s) in an Applied Biosystems Veriti® 96-well thermocycler (Promega, France). Microtiter plates were centrifuged (10 min, 3000 × *g*), and then each supernatant was used as template DNA in a nested PCR amplification of a *nifH* gene fragment used as a rhizobial diversity molecular marker. PCR amplifications were performed with GoTaq® DNA Polymerase (Promega, Lyons, France) on 3 μL of template DNA in a final volume of 25 μL. Undiluted amplicons of the first PCR step (1 μL), obtained with the PolF/PolR primers, were used as target DNA for the second step (PolF/AQER primers) [7]. The reaction mix for these two steps contained 200 μM of each dNTP, 1.5 mM $MgCl_2$, 200 ng $\mu L^{-1}$ of acetylated bovine serum albumin, 0.5 μM of each primer and 1 unit of DNA polymerase. Specificity of the final amplicons (a unique band of ca. 320 bp) was checked by agarose (2%) electrophoresis and positive amplicons were directly sequenced (Sanger technology) by Genoscreen (Lille, France, www.genoscreen.fr).

### *Statistical analyses*

ANOVA analyses were performed with XLSTAT software v2011.1.03 (Addinsoft, Paris, France). Means were classified using Fisher's post-hoc least-significant difference (LSD) test.

Phylogenetic analysis was carried out on the *nifH* sequences obtained in this study and on the closest matches from GenBank. Sequence similarities were determined using Mothur software [33] and *nifH* genotypes were defined on a 100% sequence similarity basis: sequences differing by one nucleotide were considered as belonging to distinct *nifH* genotypes. Sequences were aligned with other published *nifH* sequences using the ClustalX program [37], and the alignments were manually adjusted in GeneDoc [26]. Neighbor-joining (NJ) phylogenetic analyses [32] and pairwise analyses within clusters were carried out using MEGA software version 4 [36].

Sequences related to *nifH* genotypes were deposited in the NCBI GenBank database under accession numbers KJ743845 to KJ743956.

## Results

### *Plant growth performances*

Plants grown in soil transferred from Jatropha plantations were significantly taller and displayed higher shoot biomass than those grown in control soils (fallow fields) but this trend was not significant with site 2 soils (Table 1). Only the root biomass of cowpea seedlings was significantly greater in site 1 Jatropha soil.

Cowpea shoot N content was significantly higher only in site 2 Jatropha soil, while the acacia N content was significantly lower whatever the site (Table 1). A similar pattern was observed with acacia shoot P content, but cowpea P content was significantly higher only in site 1 Jatropha soils.

### *Root nodulation pattern*

Root nodules tended to be less numerous both on cowpea and acacia roots grown in Jatropha soils from all three sites (Table 1). However, this trend was significant only with cowpea grown in site 2 soil. In addition, the average individual nodule weight was higher when legumes were grown in Jatropha soils. This trend was significant with cowpea in soils from sites 1 and 2, and acacia in site 1 soil. On average, nodules formed in Jatropha soils were almost twice as large as those that developed in control soils. No particular relationship was shown between shoot N–P content and nodulation parameters.

### *Rhizobial diversity analysis*

Acacia yielded 292 nodules, while cowpea yielded 432 nodules. The *nifH* sequences were checked and 272 (acacia) and 406 (cowpea) valuable sequences were found for phylogenetic analyses that discriminated 81 genotypes differing by at least one nucleotide.

A total of 45 *nifH* genotypes were identified from cowpea nodules grown in soils from the six fields, with 10 to 14 genotypes *per* field (Fig. 1a , Table S1). Thirty-nine genotypes were affiliated to the *Bradyrhizobium* genus and six others (6 sequences out of 406) to non-symbiotic *Beta-* and *Gammaproteobacteria* (Fig. 1a). Phylogenetic trees describing the distribution of *nifH* genotypes *per* site are available in the supplementary material (Figs. S1a–c).

Within the *Bradyrhizobium* cluster (Fig. 1a), the distribution of *nifH* genotypes into three sub-clusters was unrelated to field type (Jatropha/control) or site origin (Jatropha field age). Among the 39 genotypes, 26 were associated with a single field, and only 47 sequences out of 406. Twelve genotypes (20 sequences) were specific to Jatropha soils and 14 (27 sequences) to control soils. Two genotypes (Ge67, Ge70) were also detected in all six fields. Genotype Ge67 (205 sequences) appeared as the dominant genotype in all fields (between 36 and 73% of total sequences *per* field) except in the site 3 control field (27% of all sequences, ranking second). The second ubiquist genotype Ge70 was consistently a very minor one. Eleven other genotypes (totaling 134 sequences) were detected concomitantly in Jatropha and control fields. For nine of them, the genotype was detected in at least one Jatropha field and its associated adjacent control field. In particular, genotypes Ge35 (4 sequences) and Ge43 (29 sequences) were present in all fields from sites 1 and 2. Overall, the genetic structure of the cowpea rhizobial communities was uneven in each field, since the first two dominant genotypes represented 60.6 to 79.3% of the total sequences (Table S1).

A total of 47 *nifH* genotypes were identified from acacia nodules grown in soils from the six fields, with between 7 and 19 genotypes *per* field (Fig. 1b, Table S2). Thirty-three genotypes were affiliated to the *Bradyrhizobium* genus, three to the *Mesorhizobium* genus and 10 others (11 sequences out of 272) to non-symbiotic *Beta-* and *Gammaproteobacteria* (Fig. 1b). The phylogenetic trees describing the distribution of *nifH* genotypes *per* site are available in supplementary material (Figs. S1d–f).

Within the *Bradyrhizobium* cluster (Fig. 1b), the 33 genotypes were distributed into two sub-clusters. The smallest group harbored 15 genotypes (20 sequences) including eight genotypes (eight sequences) specific to the oldest Jatropha field that yielded 19 genotypes (48 sequences). Contrastingly, the composition of the largest sub-cluster (18 genotypes, 178 sequences) mirrored the diversity of field origins, except for the oldest Jatropha field. In particular, two genotypes (Ge43, Ge67) were detected in all six fields. Genotype Ge43 (80 sequences) was dominant in site 1 control soil, and in site 3 Jatropha and control soils. Genotype Ge67 (54 sequences) appeared to be dominant in site 2 Jatropha and control fields, and was the second largest genotype in site 1 control soil and site 3 Jatropha soil. In addition, 26 out of the 33 *Bradyrhizobium* genotypes were associated with a single field, totaling only 33 sequences out of 272, 14 of which (17 sequences) were specific to Jatropha soils and 12 (16 sequences) to control soils. Within the *Mesorhizobium* cluster (Fig. 1b), two genotypes (Ge78, Ge79) were specifically detected in the soil of the oldest Jatropha plantation (site 1), which accounted for 60 sequences out of 67 retrieved from this field (Table S2). A third and final genotype (Ge77) was associated with this cluster in association with the oldest Jatropha field and it was represented by a single sequence out of 48. Overall, the genetic structure of the acacia rhizobial communities was also uneven in each field, since the first two dominant genotypes represented 52.1 to 89.6% of the total sequences (Table S2).

## Discussion

Very little is currently known about the impact of Jatropha monoculture on the soil nitrogen-fixing bacterial community, especially its symbiotic subset. To test whether Jatropha could alter the symbiotic rhizobial community, the diversity of rhizobia associated with two trap legumes grown in Jatropha-primed soils and in adjacent native soils was surveyed.

Several publications report allelopathic effects of Jatropha residues or tissue extracts but such inhibitory effects depend on Jatropha material amounts and target plant identity [25,43]. However, soil enrichment with foliar residues can also stimulate the growth of some plants, including certain legume species (*Vigna radiata*, *Cyamopsis tetragonoloba*, *Phaseolus aconitifolius*) [34]. Our data did not support the view that soil priming by Jatropha culture induced allelopathic effects on the subsequent growth of acacia and cowpea. On the contrary, their shoot growth, and to some extent their shoot N-P contents, were higher in many cases, especially with soils from sites 1 and 3. In a previous investigation, we had already identified these particular soils as displaying the most clear-cut differences between Jatropha and control fields, especially at the scale of the bacterial *nifH* community [9].

The bacterial genera identified in acacia and cowpea nodules matched those described in the literature [4,10,42,46]. Diouf et al. [10] showed that the composition of *A. seyal* rhizobial communities from 42 different Senegalese soils was dominated by the *Mesorhizobium* (64%) and *Sinorhizobium* (29%) genera. In the current study, *A. seyal* was essentially nodulated by *Bradyrhizobium* strains, except for nodules that formed in site 1 Jatropha soil whose *nifH* sequences – all but 6 – were tightly aligned with *Mesorhizobium plurifarium*. This species was previously detected in *A. seyal* nodules [10], but

**Table 1**
Growth performance and nodulation pattern of trap legumes in soils transferred from Jatropha and control fields.

| | Shoot height (cm) | Shoot biomass (g) | Root biomass (g) | N content (g kg$^{-1}$ dry shoot) | P content (g kg$^{-1}$ dry shoot) | Nodule number (per plant) | Nodule biomass (mg per plant) |
|---|---|---|---|---|---|---|---|
| Cowpea | | | | | | | |
| Jatropha site 1 | 17.25 ± 2.27 a | 0.85 ± 0.05 a | 0.30 ± 0.04 a | 15.26 ± 1.43 a | 1.04 ± 0.17 a | 25.4 ± 4.7 b | 7.08 ± 1.13 a |
| Control site 1 | 11.75 ± 0.40 b | 0.41 ± 0.01 cd | 0.16 ± 0.01 b | 15.21 ± 0.65 a | 0.74 ± 0.07 b | 31.8 ± 8.4 ab | 4.19 ± 0.75 b |
| Jatropha site 2 | 12.62 ± 0.59 b | 0.41 ± 0.03 d | 0.19 ± 0.01 b | 14.61 ± 2.38 a | 1.01 ± 0.30 ab | 25.7 ± 5.1 b | 6.18 ± 1.92 a |
| Control site 2 | 12.37 ± 0.95 b | 0.50 ± 0.03 bc | 0.21 ± 0.02 b | 11.02 ± 1.70 b | 0.73 ± 0.09 b | 41.8 ± 8.6 a | 3.22 ± 0.52 b |
| Jatropha site 3 | 16.72 ± 0.03 a | 0.68 ± 0.08 ab | 0.20 ± 0.03 b | 16.29 ± 0.59 a | 0.96 ± 0.14 ab | 31.5 ± 5.2 ab | 4.05 ± 0.86 b |
| Control site 3 | 12.87 ± 1.45 b | 0.45 ± 0.06 cd | 0.18 ± 0.01 b | 15.62 ± 0.72 a | 0.85 ± 0.05 ab | 39.5 ± 6.5 a | 3.40 ± 0.85 b |
| Acacia | | | | | | | |
| Jatropha site 1 | 21.21 ± 1.33 a | 0.40 ± 0.05 a | 0.30 ± 0.06 a | 16.47 ± 1.38 a | 1.78 ± 0.32 a | 7.2 ± 1.3 ab | 17.97 ± 0.43 a |
| Control site 1 | 13.53 ± 0.23 c | 0.20 ± 0.02 b | 0.28 ± 0.08 a | 13.67 ± 1.47 b | 0.38 ± 0.09 b | 11.4 ± 4.3 a | 8.99 ± 2.30 a |
| Jatropha site 2 | 12.05 ± 0.91 c | 0.20 ± 0.01 b | 0.21 ± 0.02 a | 10.15 ± 0.83 c | 0.41 ± 0.29 b | 1.7 ± 1.4 b | 19.14 ± 17.88 a |
| Control site 2 | 14.12 ± 0.42 c | 0.22 ± 0.02 b | 0.25 ± 0.01 a | 13.00 ± 1.02 b | 0.53 ± 0.21 b | 5.3 ± 5.3 ab | 8.83 ± 3.89 a |
| Jatropha site 3 | 17.66 ± 0.86 b | 0.31 ± 0.03 a | 0.28 ± 0.02 a | 13.88 ± 0.95 b | 1.53 ± 0.63 a | 6.4 ± 4.6 ab | 21.93 ± 12.76 a |
| Control site 3 | 12.66 ± 1.55 c | 0.21 ± 0.03 b | 0.17 ± 0.03 a | 10.66 ± 1.09 c | 0.36 ± 0.25 b | 5.4 ± 6.4 ab | 5.90 ± 1.46 a |

Data are presented as a mean ± standard deviation ($n = 3$). For a given legume species, different letters within a column indicate significant differences ($P < 0.05$).

*A. seyal* is definitely not preferentially associated with *Bradyrhizobium* strains in Senegal. Thus, the overwhelming dominance of this genus associated with either acacia nodules in Jatropha or control soils was surprising. A plausible explanation is that the previous plant covers drastically reduced *Mesorhizobium* spp. populations due to the cropping of legumes with distinct symbiont specificity ranges, such as cowpea [1], groundnut [35], or a forage legume such as the common Sahelian *Zornia glochidiata* [13]. Soil priming by other non-legume crops, such as the wild gramineous species found in all fallow control fields or maize and millet that are classically grown in the area, could also have been unfavorable to *Mesorhizobium* strains. In contrast, rhizobial symbionts associated with *V. unguiculata* were all referred to the *Bradyrhizobium* genus. This is consistent with the literature that depicts cowpea as being preferentially associated with *Bradyrhizobium* symbionts, especially in Senegal [41,42].

A total of 17 *nifH* sequences out of 678 total sequences (2.5%) gathered in non-symbiotic *Beta-* and *Gammaproteobacteria* clusters. They were amplified from both soil culture types and both host plants, but they occurred preferentially from acacia nodules. Such *nifH* sequences probably referred to DNA contamination from diazotroph strains external to the nodules (e.g. cells and DNA adsorption onto the nodule wall) and more likely to dual occupancy of nodules by the symbiotic strain (undetected by PCR) and an endophytic strain. For instance, *Gammaproteobacteria* strains related to the *Pseudomonas*, *Enterobacter* and *Klebsiella* genera were isolated from *Arachis hypogaea* L. (peanut) nodules but did not induce nodule formation in peanut plants [16]. However, such isolations/PCR detections of endophytic strains from nodules are occasionally reported [6,18,24,45].

In this context, most genotypes were affiliated to the *Bradyrhizobium* genus and most of them (26 out of 39 for cowpea, 26 out of 33 for acacia) were associated with a single field. However, these isolated genotypes represented only a small minority within the rhizobial communities. Thus, a few genotypes infected most nodules. The phylogenetic distribution of dominant and minority genotypes showed clusters of composite origins, including site and field types. Some genotypes were even identified in all fields, as in the case of Ge67 that was the largest genotype with cowpea in five fields and with acacia in site 2. Besides these rare but large ubiquist genotypes, numerous occurrences of genotypes shared by adjacent Jatropha and control fields were highlighted for both acacia and cowpea. The only *Bradyrhizobium* cluster that mainly gathered soil-specific genotypes belonged to acacia cultured in site 3 Jatropha soil. However, the number of associated sequences represented one sixth of the sequences from this soil and less than half of the cluster content, so that the ecological significance of this cluster should be tempered.

All these comparative elements led us to conclude that Jatropha culture did not induce a cleavage in rhizobial assemblages. In particular, the absence of a noticeable impact of the Jatropha growing in site 3 could mean that the long-term presence of Jatropha shrubs did not condition greater effects over 15 years. In parallel, the structure of the *Bradyrhizobium* populations nodulating cowpea and acacia, whether grown in Jatropha or control soils, was systematically uneven. Indeed, the cumulated size of the first two largest genotypes accounted for at least 50% of all sequences of a given host plant in a given soil type. In half of the cases, this threshold was even above 70%. Thus, such an imbalanced genetic structure of the nodulating populations was maintained in Jatropha-primed soils whatever the duration of the Jatropha culture.

A marked effect on nodule morphology was shown with cowpea grown in soils of sites 1 and 2, and with acacia in site 1 soil, since nodules were fewer and larger in Jatropha soils. Contrasting effects of the rhizobial genotype on the nodulation pattern have been classically reported. For instance, individual inoculations of soybean with three *Bradyrhizobium japonicum* strains triggered the formation of nodules of variable sizes [30]. Genotype identity of diverse strains within *Rhizobium leguminosarum* bv. *viciae* also proved decisive for the number, size and mass of pea (*Pisum sativum*) nodules including a large nodule phenotype [20]. Since intraspecific diversity among rhizobial strains can induce contrasting nodulation phenotypes, the weak *nifH* sequence variations observed in the current study could also have been sufficient to induce the formation of larger nodules in some Jatropha soils. Additional molecular analyses are needed in order to obtain more details about the genomic and symbiotic divergences among rhizobial populations (e.g. sequencing or RFLP patterns of the ribosomal IGS together with other marker genes, such as *nodD* or *nodA*). In contrast, this point can unambiguously account for the complete shift at the genus level in acacia nodules (site 1): *Mesorhizobium* nodules (Jatropha soil) were on average twice as large as *Bradyrhizobium* nodules (control soil). Additionally, the general pattern with fewer and larger nodules in Jatropha soils (especially for cowpea in site 2) could be explained by the autoregulation of nodulation by plants [28,31]. In particular, legumes can down-regulate the formation of nodules in response to increased soil nitrogen availability. However, the Jatropha and control soil nitrogen contents did not differ in site 2 (0.013 vs. 0.019% N, respectively, see Dieng et al. [9]), although they did differ in site 1 (0.043 vs. 0.013% N). Thus, the autoregulation of nodulation appears as a more likely cause in the case of acacia grown in site 1 soils than for cowpea grown in site 2 soils.

The soil-mediated effects of the establishment of three Jatropha plantations on rhizobial populations did not appear to disrupt legume-rhizobia mutualism. Cowpea and acacia growing in Jatropha-primed soils vs. control soils acquired rhizobial mutualists that were not taxonomically divergent, as depicted by phylogenetic comparisons of partial *nifH* gene sequences. We observed only one exception with acacia grown in soils sampled from the one-year-old Jatropha field. It consisted of a shift from a *Bradyrhizobium* to

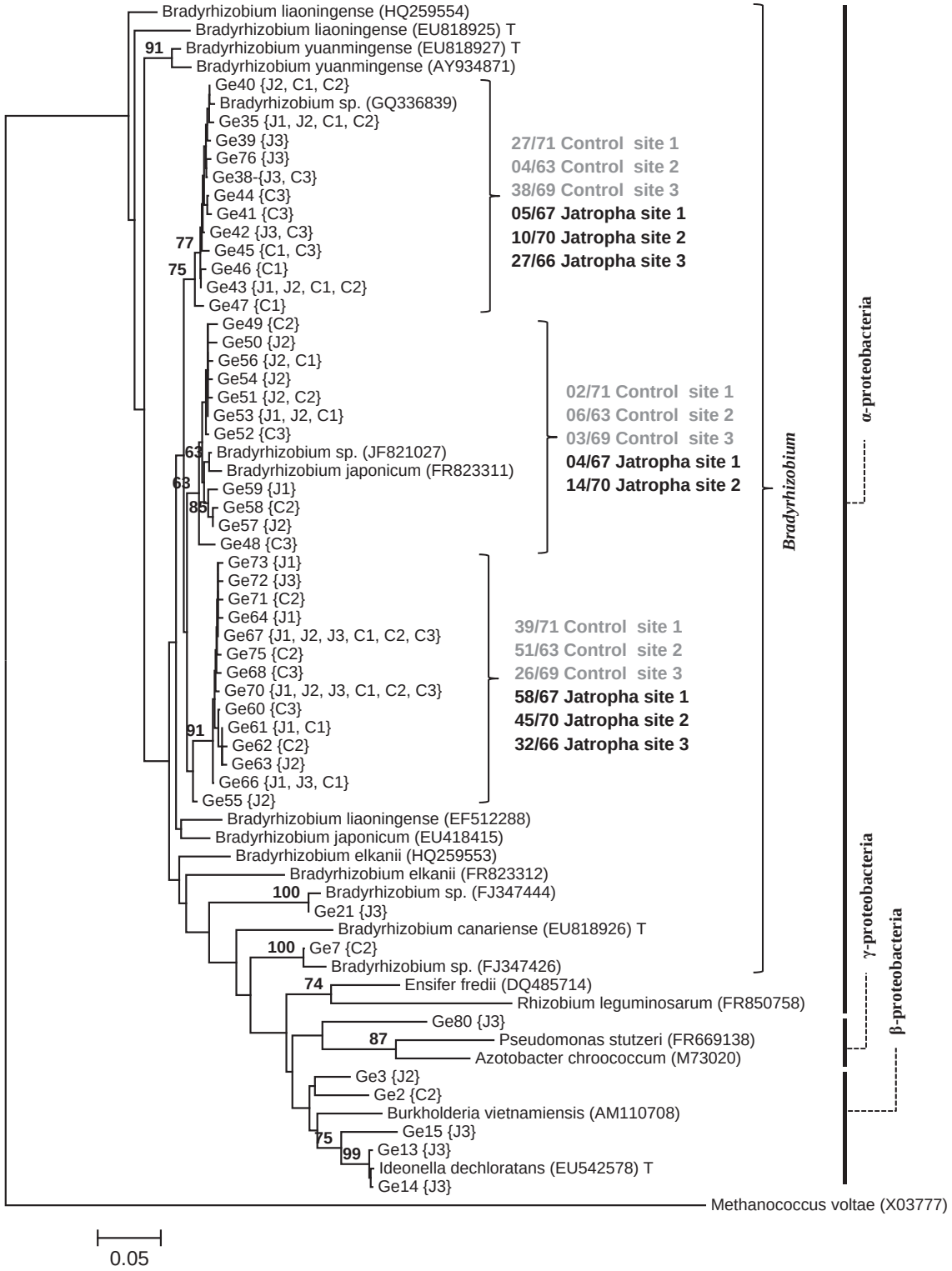


**Fig. 1.** Phylogenetic relationships of partial *nifH* gene sequences amplified from nodule DNA of cowpea (a) and acacia (b) trap cultures grown in Jatropha and control soils from the three sites. The neighbor-joining tree was rooted by a *nifH* sequence of *Methanococcus voltae* as the outgroup. Only bootstrap values ≥60% (1000 replicates) are shown. Accession numbers of GenBank reference sequences are in brackets. A letter T in bold superscript indicates the type strain of the species. The sequences obtained in the present study are labeled from the left (e.g. Ge35 {J1, J2, C1, C2}) according to the genotype identity number (e.g. Ge35) and the list of fields harboring the genotype (e.g. {J1, J2, C1, C2}), with J for Jatropha fields, C for control fields and 1–3 for sites 1 to 3. Brackets delineating major sub-clusters are associated with captions summarizing the distributions of sequences between fields (*per* total number of sequences obtained for the corresponding fields). Large brackets on the right-hand side delineate major clusters. The scale bar represents 5% nucleotide substitutions.

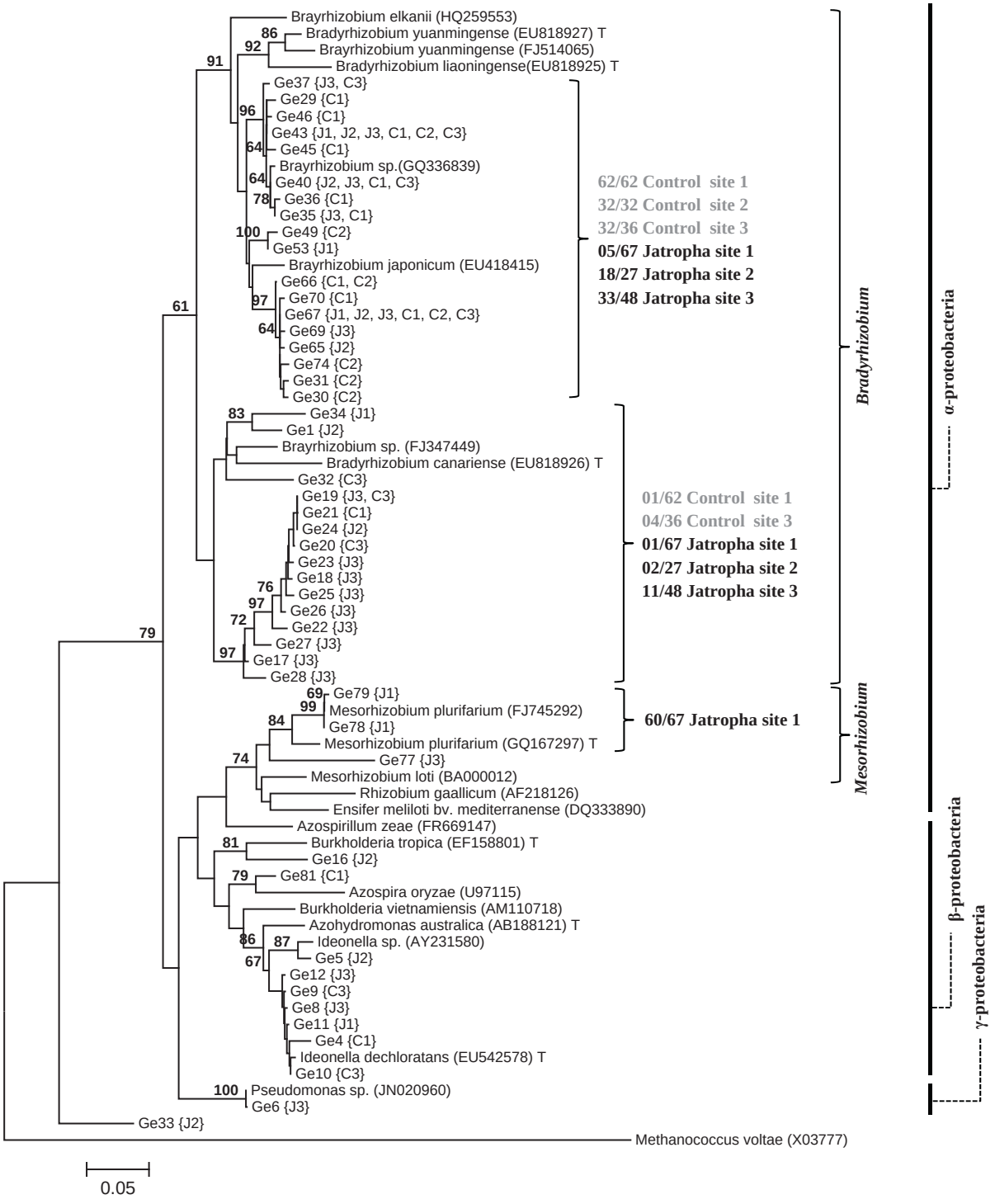


**Fig. 1.** (*Continued*).

a *Mesorhizobium* population. We did not expect to identify only *Bradyrhizobium* species on this acacia species in two fallow fields and their paired Jatropha plantations. Additionally, soil priming by Jatropha did not shift the structure of rhizobial assemblages that were systematically dominated by one or two genotypes accounting for 60 to 79% (cowpea) and 52 to 89% (acacia) of sequences in a given field. Moreover, Jatropha culture did not modify the identity of the largest dominating genotype, which was identical in all six fields in the case of cowpea cultures. In this background of a poor impact on rhizobial assemblages, plant growth and shoot N–F contents were enhanced. Future investigations should focus on the characterization of nitrogen fixation efficiency of the strains that dominate the root nodule bacterial communities. The effects of cross-infections with these dominant strains on legume performances, originally isolated from Jatropha vs. control soils, should also be investigated in order to complete the diagnosis and confirm the absence of noxious effects of Jatropha on rhizobial genetic resources.

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## Appendix A. Supplementary data

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# Cultivation of *Jatropha curcas* L. leads to pronounced mycorrhizal community differences

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ABSTRACT

Plantations of *Jatropha curcas* for biodiesel production in tropical and sub-tropical regions are currently booming. Although *Jatropha* species are well known for synthesizing a range of toxicants, the effects of *J. curcas* monoculture on the symbiotic status of staple crops and trees have hardly been investigated. We transferred to the greenhouse soil samples collected from 3 *J. curcas* plantations established 1, 2 and 15 years ago in Senegal, together with soil samples from the corresponding adjacent fallow plots. We tested whether the modification of the original chemical and microbial soil properties induced by the development of *J. curcas* could alter the growth of common local crops (millet, cowpea) and of a local tree (*Acacia seyal*), all grown in pots. We also investigated effects at the scale of the root mycorrhizal community by determining root colonization patterns (frequency, intensity) and taxonomic diversity (18S rDNA sequencing). Pronounced compositional shifts in root mycorrhizal communities were almost systematically observed between Glomeraceae and Gigasporaceae, or in some cases within Glomeraceae. Biomass and N–P shoot contents were often higher in *J. curcas*-grown soils. In conclusion, the altered composition of the soil mycorrhizal communities in response to the development of *J. curcas* did not have detrimental effects on local staple crops. The functional consequences of such modified mycorrhizal assemblages should now be further investigated under more stressful conditions.



## 1. Introduction

Fast-growing exotic tree species (e.g. pine, eucalyptus, Acacia) introduced as forest plantations in the last decades to accelerate recovery and restoration of degraded tropical lands have had detrimental ecological effects on soil hydrology or wildlife diversity (Dodet and Collet, 2012). More particularly, the introduction of such exotic tree species can result in pronounced changes in the microbial community and ecosystem functioning (Reinhart and Callaway, 2006; van der Putten et al., 2007). For instance, in Burkina Faso the introduction of *Gmelina arborea*, a fast-growing tree native from India, led to a displacement of the herbaceous plant community, concomitant with a decrease of the catabolic potentialities of the soil microbial community (Sanon et al., 2006). Seven years following the plantation of the Australian *Acacia holosericea* in a Senegalese agrosystem, resistance of the soil bacterial community to diverse abiotic stresses was lowered, and so was the diversity of the arbuscular mycorrhizal community (Remigi et al., 2008). The development of *Eucalyptus camaldulensis*, another fast-growing Australian tree, caused a shift in the genetic structure of the bacterial community together with the diversity of the herbaceous plant community in a sahelian soil (Kisa et al., 2007). Besides these intentional introductions of non-native tree species and their side effects that can impact populations of mycorrhizal fungi, some exotic herbaceous species mainly base their ecological strategy on the disruption of plant–fungus mutualism and can prove invasive (Pringle et al., 2009). *Alliaria petiolata*, a non-mycotrophic herbaceous species originating from Europe, is currently invading North American forests. It synthesizes antifungal molecules (isothiocyanate-based compounds) that disrupt associations between native tree seedlings and their arbuscular mycorrhizal fungi (Vaughn and Berhow, 1999; Stinson et al., 2006; Callaway et al., 2008). *Centaurea maculosa* is another well-studied case. Meadows invaded by *C. maculosa* display much lower mycorrhizal diversity than adjacent non-invaded ones, as well as significantly shorter extraradical mycelium (Mummey and Rillig, 2006).

Owing to its suitability for agrofuel production in tropical and subtropical areas, *Jatropha curcas*, an oil-producing Euphorbiaceae native from Central America, is increasingly being introduced in

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West Africa (Burkina Faso, Benin), Asia (India) and South America (Mexico) in the last ten years (Singh et al., 2014). In particular, the former Senegalese government instituted a national program between 2007 and 2012 targeting an annual production of a billion liters of *J. curcas* seed oil (Campbell, 2014). However, this program failed to achieve its goals. Only approximately 2200 ha were planted in 2014, especially at the expense of food crops in rural communities. Moreover, smallholders disappointed with the poor productivity of *J. curcas* (fruit and oil) resolved to alternative crops. Yet, this woody shrub contains a cocktail of toxicants in almost all of its parts, and different kinds of its extracts have proved toxic on a range of organisms and microorganisms (Devappa et al., 2010). The environmental fate of *J. curcas* bioactive compounds, and more precisely their effects on key soil microbiota, needs a thorough evaluation because (i) diversity and activity of the soil microbiota are essential to plant community diversity and productivity (van der Heijden et al., 1998, 2008; Ingleby et al., 2007), and (ii) arable surfaces dedicated to *J. curcas* monoculture are planned to boom (13 million hectares of land scheduled to be under *J. curcas* cultivation worldwide by 2015, Renner et al., 2008). Evidence is lacking to convincingly prove the potential of *J. curcas* for invasiveness (e.g. spontaneous regeneration, seed dispersal, seed-bank longevity) (Negussie et al., 2014). However its potential long-lasting effects on the soil microbiota could be a major issue in terms of food security because they could compromise the development of local staple crops that will sooner or later replace *J. curcas* plantations. Microsymbionts, namely nitrogen-fixing rhizobia and arbuscular mycorrhizal fungi (AMF), are to be given priority owing to their central role in the coexistence and growth of plants in agrosystems constrained by water and nutrient supplies.

In a previous study (Dieng et al., 2014a), we analyzed the genetic structure of the soil mycorrhizal communities in 3 Senegalese *J. curcas* plantations and their adjacent fallow fields. Total DNA was extracted from soil samples representative of each of those 6 fields to generate genetic fingerprints of the Glomeromycota 18S rRNA marker gene. Comparisons revealed a pronounced shift of the genetic structure of the soil mycorrhizal communities in response to *J. curcas* culture. In the present study, we hypothesize that growing common local crops in soils transferred from *J. curcas* versus control fields is appropriate to clarify the impact of this Euphorbiaceae species on the taxonomic diversity of soil infective AMF propagules. We also hypothesize that the expected changes in root AMF diversity can go along with a modification of plant growth performances and shoot N–P contents. Cowpea (*Vigna unguiculata*) and millet (*Pennisetum glaucum*) play important socio-economic roles in Senegal (Diangar et al., 2004; Boys et al., 2007). As a grain legume and a cereal, respectively, they are both part of the diet of rural populations. *Acacia seyal*, like other Acacia species (*A. senegal*, *A. laeta*, *A. nilotica*), is valuable in afforestation and agroforestry systems (acacia gum), as well as in regeneration programs of degraded lands (Traoré et al., 2012). Millet, cowpea and the leguminous tree *A. seyal* were grown in pots filled with soil transferred from *J. curcas* plantations and from their adjacent fallow margins (control soils). Plant height and biomass production were evaluated at harvest after several weeks of culture, along with shoot N–P contents. AMF communities were described in terms of frequency and intensity of root colonization, and molecular diversity (18S rDNA gene sequencing).

## 2. Materials and methods

### 2.1. Field sites and soil sampling design

Sites location, agronomic management, climatic conditions, soil characteristics (Alfisol) and sampling were presented elsewhere (Dieng et al., 2014a). Briefly, smallholders growing *J. curcas* were located in West Senegal. Three neighboring sites were selected, where *J. curcas* had been grown for 1 (site 1) and 2 (site 2) years as a monoculture, and 15 years as a living fence (site 3). None of the *J. curcas* plots received any mineral or organic fertilizers or pesticides. In each site, a fallow zone adjacent to the *J. curcas* plot was selected as a control plot. Control and *J. curcas* soil samples (0–20 cm depth) were taken in April 2009 at the end of the dry season. The sampling scheme consisted of 3 rows per plot (3 *J. curcas* plots and 3 control plots), with one composite soil per row. Along each row (20 m length and 10 m distance between two rows), 3 soil samples were taken around *J. curcas* shrubs, or every 5 m in control plots, and bulked. This sampling procedure resulted in 9 *J. curcas* and 9 control composite soil samples that were sieved at 2 mm, homogenized and transferred to the greenhouse to initiate a pot experiment.

### 2.2. Greenhouse experimental design

For each of the 18 composite soils, 3 local crops (*P. glaucum* – millet, *V. unguiculata* – cowpea, and *A. seyal* – acacia, the last two being leguminous species) were sown in 4 replicate pots containing 400 g of soil for millet and acacia, or 800 g for cowpea, with 1 seedling per pot, i.e. a total of 72 pots per crop type. Several millet seeds were sown per pot, and plantlets were thinned 15 days later. Cowpea and acacia seeds were disinfected as follows: cowpea seeds were treated for 5 min in 33% calcium hypochlorite and rinsed in sterile water, while acacia seeds were treated for 30 min in 96% $H_2SO_4$, rinsed, and immersed in sterile water for 24 h. Then they were left to germinate on sterile water agar (0.9% w/v) at 37 °C for 2 days (acacia) or 3 days (cowpea). Once rootlets were 1–2 cm long, seedlings were transferred into pots. Cowpea, acacia and millet were grown for 7 weeks, 3 months, and 6 months, respectively, with daily irrigation. These cultures were initiated at the beginning of the hot-humid season (May–November) as the most suitable period for plant growth. The first 3 months, natural climatic conditions were on average 75–80% relative humidity, 17–25 °C min–max temperatures and 8.5–9.5 h daily sunshine. The next 3 months, mean daily sunshine declined from 9.5 to 7.5 h, relative humidity was rather stable, and min–max temperatures raised from 20 to 24 °C and from 26 to 30 °C, respectively. At harvest, fresh root weight, fresh/dry (70 °C for 1 week) shoot weight, and shoot height were measured. Dried shoots were used to determine N and P contents (performed by the IRD Laboratory LAMA-US 191, certified ISO 9001:2008 by Euro Quality System, http://www.lama.ird.sn). All fresh root systems were then longitudinally divided into 2 equal parts that were weighed. The first half was used to reveal inner root mycorrhizal structures by trypan blue staining. The second half was dried at 50 °C for 1 week and weighed again. The dry weight of the total root system was inferred from this measurement. Molecular analyses of mycorrhizal diversity were performed on this dried root material.

### 2.3. Description of root mycorrhizal communities

Fresh roots of the 4 plant replicates grown in one of the 18 composite soil samples were cut into 1-cm-long fragments and mixed. Root fragments were cleared in 10% KOH for 1 h at 90 °C, rinsed in water, and stained with trypan blue in lactophenol (0.05%) for 30 min at 90 °C (Phillips and Hayman, 1970). Five batches of 20 root fragments were mounted on slides in 90% glycerol and observed under a stereomicroscope (×40 magnification) to determine mycorrhizal intensity and frequency according to Trouvelot et al. (1986).

The dried root subsamples of the 4 plant replicates were ground and mixed in liquid nitrogen with a mortar and pestle to carry out their homogeneization and their extemporaneous DNA extraction with the DNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France) from 20 mg of crushed roots. DNA extracts were further purified on PVPP and Sepharose 4B columns (Sigma, St.-Quentin Fallavier, France) according to Edel-Hermann et al. (2004). Purified DNA extracts were quantified by fluorescence with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, St. Aubin, France) and the Tecan infinite M200 microtiterplate reader (Tecan, Lyon, France). A composite root DNA extract, representative of a given field, was then elaborated by mixing the 3 DNA extracts obtained from the culture pots containing the 3 composite soils from the field in equimolar amounts. This procedure led to 18 composite DNA extracts (3 plant species, 6 fields). They were used as template DNAs to describe the molecular diversity of mycorrhizal symbionts by PCR amplification of a specific fragment of the glomeromycotean 18S rDNA gene using GoTaq® DNA Polymerase (Promega, Lyon, France). The first PCR step (universal eukaryote primers NS1/NS4) was performed on 4 ng of root DNA according to White et al. (1990). Undiluted amplicons of the first PCR step (2 μL) were then used as templates for the second step using the universal eukaryote primer NS31 and the Glomeromycota-specific primer AM1 (Helgason et al., 1998).

The entire volume of the NS31/AM1 amplicons was resolved in 2% agarose gels and stained with ethidium bromide. Amplicons of the expected size (ca. 550 bp) were purified from gels with the PureLink Quick Gel Extraction Kit (Invitrogen). Purified amplicons were eluted in 30 μL of buffer and quantified at 260 nm with a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Illkirch, France). They were further cloned (28 ng) with the pGEM-T Easy vector (Promega) in XL2-Blue ultracompetent cells (Agilent Technologies, Massy, France) according to the manufacturers' recommendations. For each of the 18 libraries, 96 white clones were randomly selected, and DNA from cell aliquots was extracted in 30 μL of sterile water by 7 heat shocks (96 °C–2 min, 4 °C–10 s). Cell debris was pelleted at 3000 × *g*, and supernatants were used as template DNAs in a PCR amplification using the 17 mer pUC/M13 vector primers (Promega). PCR products of the expected size were sequenced by Genoscreen (Sanger technology, http://www.genoscreen.fr).

### 2.4. Phylogenetic analysis

Sequences including both NS31/AM1 priming sites were compared with sequences found in the GenBank database using the BLASTn algorithm. Sequences not affiliated with the Glomeromycota phylum were discarded. The 18 libraries were then pooled prior to the definition of AMF genotypes whose identification number was unique whatever the plant species or soil origin.

Sequence similarities were determined using Mothur software (Schloss et al., 2009), and AMF genotypes were defined on a 100% sequence similarity basis: sequences differing by one nucleotide were considered as belonging to distinct genotypes. Genotypes were aligned with published Glomeromycota sequences using the ClustalX program (Thompson et al., 1997), and alignments were manually adjusted in GeneDoc (Nicholas and Nicholas, 1997). Neighbor-joining phylogenetic analyses (Kimura 2 parameters, 1000 replications) were performed with the Mega 5.05 program (Tamura et al., 2011). *Endogone pisiformis* and *Mortierella polycephala* were used as outgroups. AMF phylotypes were inferred from the topology of the phylogenetic tree: sequences sharing at least 97% homology and grouped under a minimum bootstrap value of 85% were considered as belonging to a given phylotype. The sampling effort curves (95% confidence intervals) were generated using EstimateS 9.0.0 software (Colwell, 2013).

A total of 533 sequences representative of all the different genotypes were deposited at NCBI GenBank under accession numbers KP226863–KP227041 (acacia), KP227042–KP227196 (cowpea) and KP227197–KP227395 (millet).

### 2.5. Statistics

All statistical analyses were performed using XLSTAT (v2010.5.04) software. Percentage values were arcsin(sqrt)-transformed prior to statistical analysis. Significant differences between dependant variables (plant growth parameters and mycorrhization scores) were determined by analysis of variance using two-way ANOVA (fixed-effects model) with *J. curcas* (presence/absence) and site (sites 1–3) as the two factors, followed by Fisher's LSD test for pairwise multiple comparisons ($\alpha = 0.05$).

## 3. Results

### 3.1. Plant growth performances

As already reported for cowpea and acacia cultures in Dieng et al. (2014b), the 3 plant species grown in soil transferred from *J. curcas* fields were significantly taller and displayed higher shoot biomass than those grown in control soils, but this trend was not significant in site-2 soils (Table 1). The root biomass of millet was significantly greater in site-2 *J. curcas* soils, and the root biomass of cowpea was significantly greater in site-1 *J. curcas* soils. Similar trends were observed with shoot N–P contents: they tended to be higher in plants grown in *J. curcas* soils, especially P contents (Table 1). Yet, no significant gain was noticed with millet. Cowpea shoot N content was significantly higher only in site-2 *J. curcas* soils, while acacia shoot N content was significantly lower in this case but higher in the other cases. Acacia shoot P content was also significantly favored in soils of the oldest (site 3) and the earliest (site 1) *J. curcas* fields, while in cowpea it was significantly favored only in the earliest one.

### 3.2. AMF root colonization

Mycorrhization frequency was high and similar for all plant species (>95%), irrespective of soil origin (Table 1), except with millet grown in site-1 *J. curcas* soils (83%). The intensity of millet and cowpea mycorrhization proved more sensitive to soil origin. It was largely increased in soils originating from *J. curcas* fields as compared to the corresponding control soils. However, a significant decrease was noticed again with millet grown in soils of the earliest *J. curcas* field. Acacia was the least responsive plant: intensity of its mycorrhization increased only in soils transferred from the *J. curcas* living fence.

### 3.3. Diversity of the root AMF community

Among the 1728 clones (96 clones per library), 1623 gave a unique band of the expected size and were subsequently sequenced. Twenty-seven sequences were discarded (poor quality) and BLAST results indicated that 220 sequences were not affiliated to the Glomeromycota phylum, but mainly to ascomycetes and basidiomycetes. The remaining 1376 sequences displayed high similarity (>96%) to Glomeromycota sequences and were split into 533 genotypes differing by at least one nucleotide. However, in the case of cowpea growing in site-2 control soils, all sequences were affiliated to ascomycetes. The entire procedure, including root DNA extraction, was repeated twice, but led to a similar conclusion (data not shown). The sampling effort curves (Fig. S1) displayed plateaus or almost reached their plateau phase, showing that the root AMF communities were described accurately.

**Table 1**
Growth performance and root mycorrhization of the 3 trap cultures grown in soils transferred from *J. curcas* and control fields.

| Crop identity and soil origin | Shoot height (cm) | Shoot dry biomass (g) | Root dry biomass (g) | N content (g $kg^{-1}$ dry shoot) | P content (g $kg^{-1}$ dry shoot) | Mycorrhization intensity (%) | Mycorrhization frequency (%) |
|---|---|---|---|---|---|---|---|
| **Millet** | | | | | | | |
| Site 1 *J. curcas* | 32.92 ± 1.05 a | 0.52 ± 0.03 a | 0.11 ± 0.01 ab | 9.61 ± 2.02 a | 1.70 ± 0.25 a | 6.03 ± 0.37 c | 83.00 ± 2.52 b |
| Site 1 Control | 25.87 ± 0.85 b | 0.30 ± 0.05 b | 0.10 ± 0.03 ab | 10.15 ± 2.20 a | 1.13 ± 0.53 ab | 22.36 ± 3.59 ab | 96.00 ± 1.00 a |
| Site 2 *J. curcas* | 31.03 ± 1.03 a | 0.56 ± 0.08 a | 0.20 ± 0.03 a | 8.80 ± 1.88 a | 1.43 ± 0.05 ab | 28.60 ± 2.80 a | 98.00 ± 0.58 a |
| Site 2 Control | 30.16 ± 1.40 a | 0.46 ± 0.04 ab | 0.08 ± 0.02 b | 8.62 ± 2.44 a | 1.03 ± 0.50 b | 14.07 ± 2.07 bc | 95.33 ± 2.19 a |
| Site 3 *J. curcas* | 33.00 ± 0.09 a | 0.52 ± 0.02 a | 0.09 ± 0.02 b | 7.79 ± 2.36 a | 1.45 ± 0.22 ab | 26.73 ± 2.54 a | 99.33 ± 0.67 a |
| Site 3 Control | 23.84 ± 1.69 b | 0.62 ± 0.14 a | 0.12 ± 0.07 ab | 8.51 ± 0.66 a | 0.96 ± 0.11 b | 15.41 ± 6.96 bc | 97.33 ± 1.76 a |
| **Cowpea** | | | | | | | |
| Site 1 *J. curcas* | 17.25 ± 2.27 a | 0.85 ± 0.05 a | 0.30 ± 0.04 a | 15.26 ± 1.43 a | 1.04 ± 0.17 a | 51.91 ± 1.01 a | 100.00 ± 0.01 a |
| Site 1 Control | 11.75 ± 0.40 b | 0.41 ± 0.01 cd | 0.16 ± 0.01 b | 15.21 ± 0.65 a | 0.74 ± 0.07 b | 23.03 ± 2.69 c | 98.67 ± 0.88 ab |
| Site 2 *J. curcas* | 12.62 ± 0.59 b | 0.41 ± 0.03 d | 0.19 ± 0.01 b | 14.61 ± 2.38 a | 1.01 ± 0.30 ab | 39.75 ± 1.72 b | 99.33 ± 0.33 ab |
| Site 2 Control | 12.37 ± 0.95 b | 0.50 ± 0.03 bc | 0.21 ± 0.02 b | 11.02 ± 1.70 b | 0.73 ± 0.09 b | 29.28 ± 1.14 c | 99.67 ± 0.33 ab |
| Site 3 *J. curcas* | 16.72 ± 0.03 a | 0.68 ± 0.08 ab | 0.20 ± 0.03 b | 16.29 ± 0.59 a | 0.96 ± 0.14 ab | 32.08 ± 4.30 bc | 98.00 ± 1.00 b |
| Site 3 Control | 12.87 ± 1.45 b | 0.45 ± 0.06 cd | 0.18 ± 0.01 b | 15.62 ± 0.72 a | 0.85 ± 0.05 ab | 27.13 ± 4.88 c | 98.33 ± 0.33 ab |
| **Acacia** | | | | | | | |
| Site 1 *J. curcas* | 21.21 ± 1.33 a | 0.40 ± 0.05 a | 0.30 ± 0.06 a | 16.47 ± 1.38 a | 1.78 ± 0.32 a | 30.12 ± 1.16 c | 97.00 ± 1.00 b |
| Site 1 Control | 13.53 ± 0.23 c | 0.20 ± 0.02 b | 0.28 ± 0.08 a | 13.67 ± 1.47 b | 0.38 ± 0.09 b | 33.94 ± 0.80 bc | 99.67 ± 0.33 a |
| Site 2 *J. curcas* | 12.05 ± 0.91 c | 0.20 ± 0.01 b | 0.21 ± 0.02 a | 10.15 ± 0.83 c | 0.41 ± 0.29 b | 42.30 ± 1.50 ab | 98.50 ± 0.76 ab |
| Site 2 Control | 14.12 ± 0.42 c | 0.22 ± 0.02 b | 0.25 ± 0.01 a | 13.00 ± 1.02 b | 0.53 ± 0.21 b | 44.13 ± 2.83 a | 99.33 ± 0.33 a |
| Site 3 *J. curcas* | 17.66 ± 0.86 b | 0.31 ± 0.03 a | 0.28 ± 0.02 a | 13.88 ± 0.95 b | 1.53 ± 0.63 a | 45.07 ± 6.45 a | 99.67 ± 0.33 a |
| Site 3 Control | 12.66 ± 1.55 c | 0.21 ± 0.03 b | 0.17 ± 0.03 a | 10.66 ± 1.09 c | 0.36 ± 0.25 b | 34.05 ± 2.38 bc | 99.67 ± 0.33 a |

For a given crop, different letters within a column indicate significant differences ($P < 0.05$, mean and standard deviation, n = 3).

Acacia roots yielded 179 AMF ribosomal genotypes differing by at least one nucleotide (Fig. 1A), for a total of 505 sequences (Table 2). Their phylogenetic analysis revealed that Glomeraceae (417 sequences) were predominant over Gigasporaceae (80 sequences) and Acaulosporaceae (8 sequences) in this legume tree. The phylogenetic tree (Fig. 1A) further revealed that the AM fungal community assembly of roots grown in site-1 soils was essentially composed of Gigasporaceae in control soil (67 sequences out of 84, Giga1 cluster), but of Glomeraceae in *J. curcas* soil (67 sequences out of 75, Glo1 sub-cluster). Moreover in this case, a few Acaulosporaceae sequences were detected. Contrastingly, almost all AMF sequences retrieved from site-2 control and *J. curcas* soils were interspersed within the same Glomeraceae sub-cluster Glo1. Moreover, 2 genotypes common to both soil types (#2 and #53) gathered a significant number of sequences. For instance, genotype #53 totaled 22 sequences out of 84 in control soil, and 38 out of 86 in *J. curcas* soil. However, acacia roots grown in *J. curcas* soil also harbored a few sequences from Gigasporaceae (13 out of 86). In site 3, the composition of the root AMF community was once again dependent on soil type. All 94 sequences obtained from cultures conducted in control soil were gathered in a localized branching within the Glomeraceae sub-cluster Glo1, with genotype #97 hyperdominant (72 sequences). By contrast, sequences related to the *J. curcas* soil were split among distinct Glo1 branchings and other Glomeraceae sub-clusters, with genotype #113 as the dominant one (26 sequences out of 82, Glo1 sub-cluster).

Millet roots yielded 199 AMF ribosomal genotypes differing by at least one nucleotide (Fig. 1B), for a total of 507 sequences (Table 2). Their phylogenetic analysis revealed that Glomeraceae (387 sequences) were also predominant over Gigasporaceae (120 sequences) in this crop. Genotypes constitutive of AMF root communities systematically belonged to distinct phylogenetic groups, whether millet was grown in control or *J. curcas* soils (Fig. 1B). In site 1, genotypes from *J. curcas* soil almost essentially clustered in the Glomeraceae sub-cluster Glo6 (76 sequences out of 84), while genotypes from control soil were dispatched between the Glomeraceae sub-cluster Glo2 (46 sequences out of 87) and the Gigasporaceae sub-cluster Giga2 (37 sequences). In site-2 control soil, millet was only mycorrhized by Glomeraceae (sub-cluster Glo2, 90 sequences out of 91), while it was associated with Gigasporaceae symbionts in *J. curcas* soil (sub-cluster Giga2, 82 sequences out of 82). In site 3, differences occurred only within Glomeraceae sub-clusters: symbionts isolated from *J. curcas* soil gathered in sub-cluster Glo5 (79 sequences out of 80), while those from control soil were mostly localized in sub-cluster Glo1 (71 sequences out of 83).

Cowpea roots yielded 160 AMF ribosomal genotypes differing by at least one nucleotide (Fig. 1C), for a total of 364 sequences (Table 2). Five of these genotypes were also identified in acacia roots (#29-50-113-154-174). Again, Glomeraceae (272 sequences) were predominant species over Gigasporaceae (92 sequences). Mycorrhization of cowpea roots in the soil of the earlier *J. curcas* field was exclusively due to Glomeraceae, more precisely to an exclusive branching within the Glomeraceae sub-cluster Glo2 (Fig. 1C). By contrast, Gigasporaceae symbionts were essentially involved in the corresponding control soil (sub-cluster Giga1, 72 sequences out of 86). A similar pattern was identified with the living fence. The root AMF communities established from *J. curcas*-primed soil exclusively referred to Glomeraceae (sub-cluster Glo1), while the communities from control soil preferentially harbored Glomeraceae (sub-cluster Glo2, 62 sequences out of 82) besides Gigasporaceae (sub-cluster Giga1, 19 sequences).

## 4. Discussion

The introduction of *J. curcas* as an energy crop in a sub-sahelian agrosystem acted as a potent interference agent of mycorrhizal symbiosis. Soil chemical and microbial modifications inherited from the planting of this Euphorbiaceae induced significant and extended modifications of the mycorrhizal status of 3 socio-economically important crops. The most striking effect was an almost thorough shift of the composition of root mycorrhizal assemblages as compared to control soils. Moreover, these compositional cleavages were often accompanied by enhanced mycorrhization intensity. Far from being detrimental to crops, these changes were associated to improved growth and N–P nutrition.

### *4.1. Mycorrhizal symbiosis was not disrupted despite drastic and systematic shifts in the composition of the root mycorrhizal communities*

Soil changes inherited from *J. curcas* cultivation in the same sites as those of this study triggered drastic shifts in the genetic structure

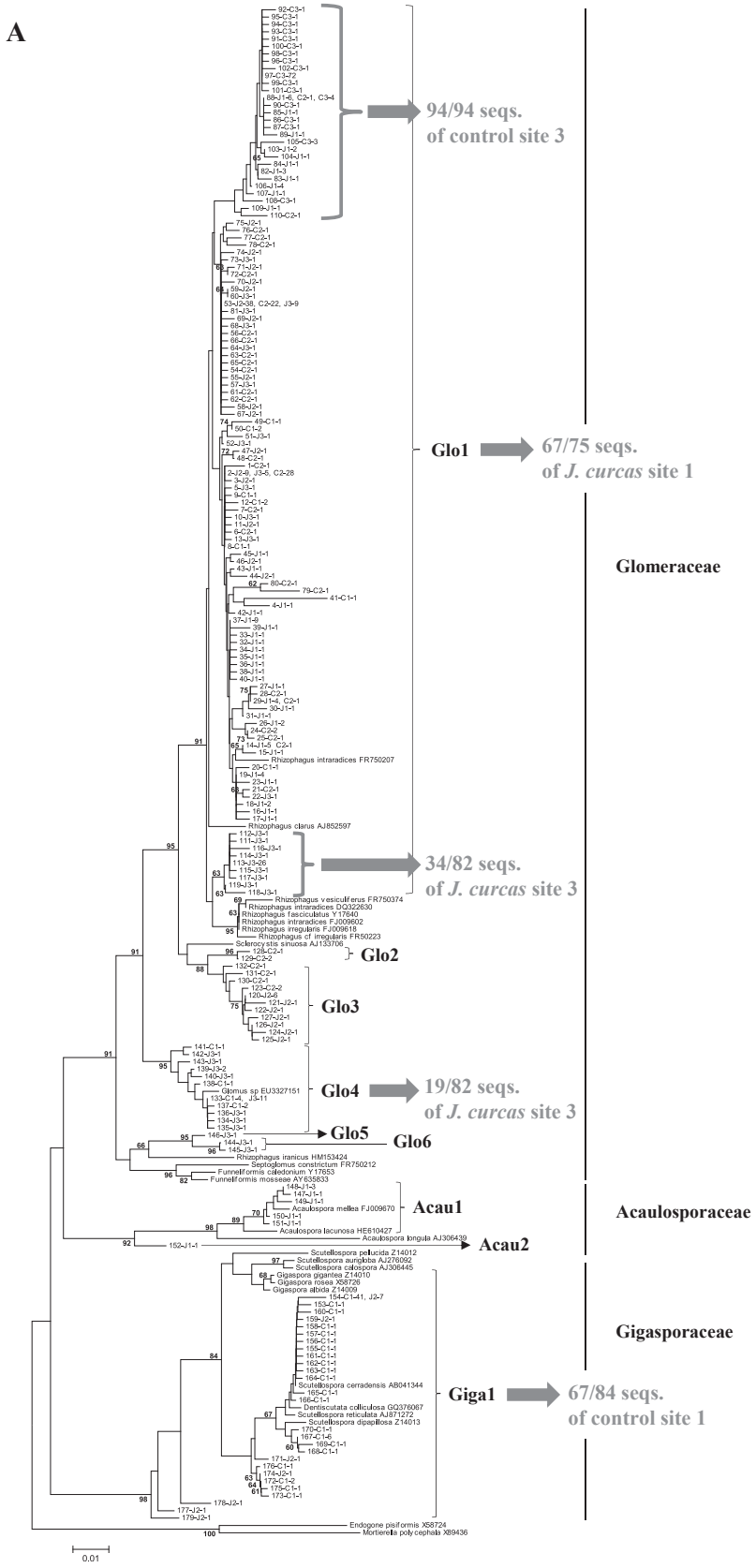


**Fig. 1.** Phylogenetic relationships of partial 18S rDNA gene sequences amplified from root DNA from *A. seyal* (A), millet (B) and cowpea (C) grown in *J. curcas* and control soils from the 3 sites. Sequences are labeled from the left (e.g. 92-C3-1) according to genotype identity number (e.g. 92), field identity (e.g. C3, with J and C for *J. curcas* and control fields, respectively and 1–3 for sites 1–3) and to the number of identical sequences (genotype size, e.g. 1). Neighbor-joining trees were rooted using ribosomal sequences of *Endogone pisiformis* and *Mortierella polycephala* as outgroups. Only bootstrap values ≥60% (1000 replicates) are shown. Internal brackets delineate phylotypes. The scale bar represents 1% nucleotide substitutions. Captions, arrows and brackets in dak grey point out the dominant sequence groupings that support the effects of soil origins on the composition of the root AMF community. "seqs." stands for sequences.

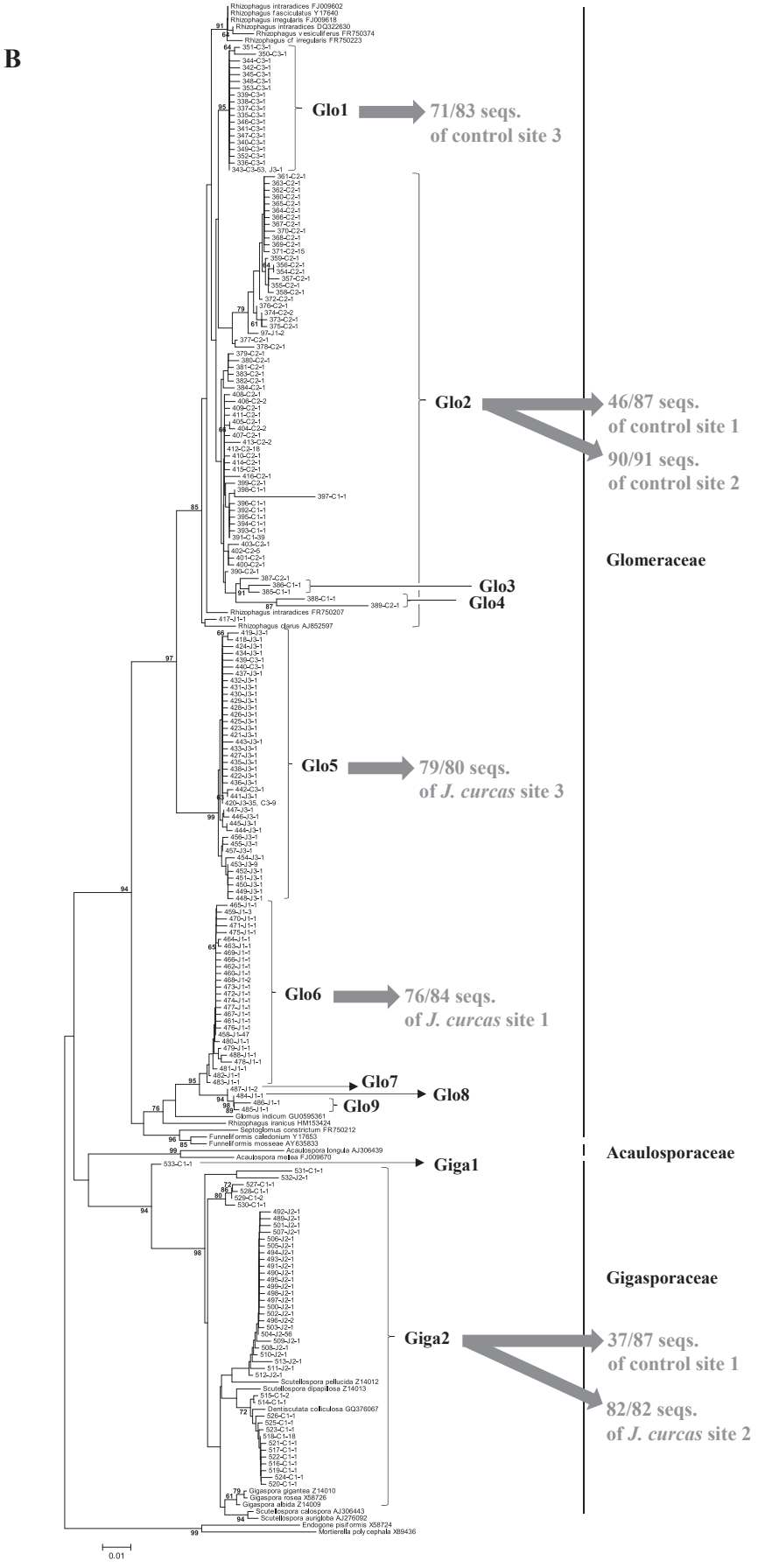


**Fig. 1.** (*continued*).

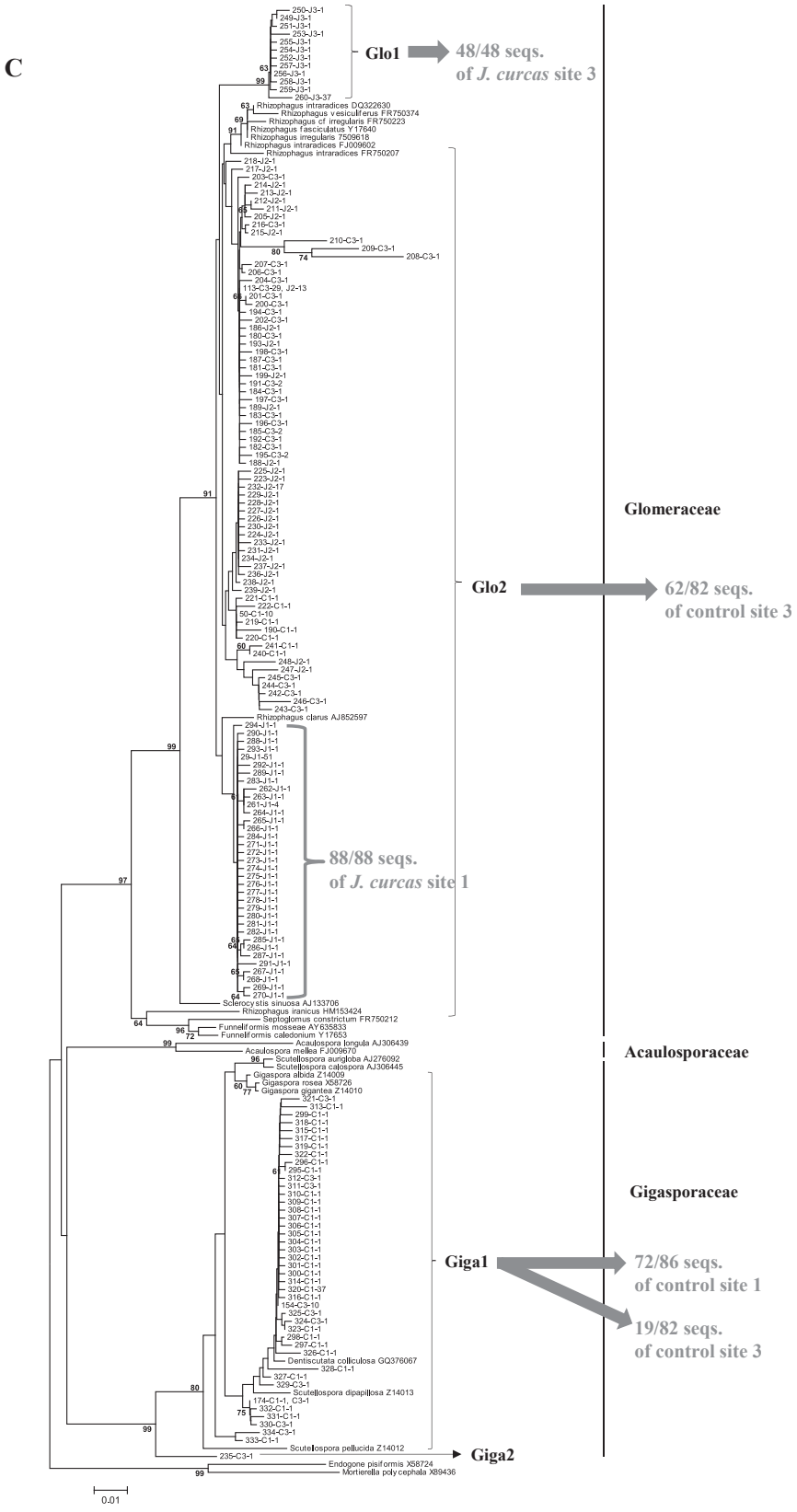


**Fig. 1.** (*continued*).

of the mycorrhizal community, as depicted by soil DNA-based molecular fingerprinting (Dieng et al., 2014a). Our present data unambiguously demonstrate that these qualitative effects are also found at the scale of root mycorrhizal community composition, whatever the trap culture. Being a mycotrophic species (Charoenpakdee et al., 2010; Kamalvanshi et al., 2012), *J. curcas* is unlikely to strictly degrade mycorrhizal mutualism the way invasive species such as *A. petiolata* clearly do (Stinson et al., 2006; Callaway

et al., 2008; Barto et al., 2011). *A. petiolata* excretes glucosinolate substances whose hydrolysis products (allyl isothiocyanate, benzyl isothiocyanate, glucotropaeolin) are directly phytotoxic (Vaughn and Berhow, 1999) and also toxic to Glomeromycota spore germination (Stinson et al., 2006). It progressively erodes the soil infectious potential and allelopathically suppresses local mycotrophic species. Other exotic annual species also consistently reduce mycorrhizal colonization by up to 50%, as exemplified with juvenile *Plantago lanceolata*, *Lotus corniculatus* and *Trifolium pratense* (Tanner and Gange, 2013), or *Acer pseudoplatanus* (Ruckli et al., 2014) in response to the development of the exotic *Impatiens glandulifera*. Similarly to exotic annual plants, some exotic ligneous species also have deleterious effects on mycorrhizal symbiosis in resident trees. This is the case of *Acacia mearnsii*, an Acacia from Australia introduced worldwide to rehabilitate degraded lands, which finally proved highly invasive (Richardson and Rejmanek, 2011). In an invaded Algerian reserve, this Acacia species significantly altered the diversity of the ectomycorrhizal fungal community associated with native *Quercus suber* trees (Boudiaf et al., 2013). In our study, despite the toxicity of *J. curcas* tissues and possibly of its rhizodeposits, mycorrhization intensity was stimulated in most cases, and mycorrhization frequency levels were high. In this respect, it should be emphasized that our mycorrhization values were often very high which could be due to the calculation method chosen to evaluate root mycorrhization. Other procedures quantifying the proportion of root length colonized by AMF, such as the gridline intersect method (Giovannetti and Mosse, 1980) or the magnified intersections method (McGonigle et al., 1990) would probably have given lower scores as the method used here is known to overestimate percentage colonization. Nevertheless, such results rule out the "invasive" scenario, all the more as *J. curcas* monoculture did not impact soil spore density (Dieng et al., 2014a). Although numbers of infective mycorrhizal propagules most probably remained stable, a progressive modification of the phylogenetic composition of soil AMF communities may have been induced during the development of *J. curcas*: preferential associations of some AMF with this Euphorbiaceae species may have formed. Increasing numbers of investigations have shown AMF host specificity patterns over the past decade, ranging from plant species (Vandenkoornhuyse et al., 2003; Davison et al., 2011) to ecological groups (grasses, legumes, non-legume forbs, trees) (Scheublin et al., 2004; Öpik et al., 2009; Yang et al., 2012). Nevertheless, the incidence of these plant–AMF interactions can obviously vary depending on ecosystem type, including plant community structure (e.g. floristic diversity, patchy distribution) and edaphic conditions (Martínez-García and Pugnaire, 2011; Veresoglou and Rillig, 2014). The irregular composition of AMF communities we recorded between sites for a given host plant and also between host plant species for a given soil origin could stem from such preferential association patterns at the plant species level. For all our *J. curcas*-primed soils however, 582 sequences out of 685 (85%) were affiliated to the Glomeraceae family, as against 494 sequences out of 691 (71.5%) in control soils. This could advocate for a preferential association of *J. curcas* with Glomeraceae symbionts. Yet, this global reduction in Glomeraceae representativity in control soils was almost exclusively due to site-1 control soil, where cowpea, acacia, and to a lesser extent millet, were preferentially associated with Gigasporaceae. Preferential association of *J. curcas* with Glomeraceae taxa should further be confirmed by molecular analyses of its root mycorrhizal community.

### 4.2. Shifts in root AMF taxa were often concomitant with higher plant performance

To the best of our knowledge, so far only one study has focused on changes in the molecular diversity of soil AMF following a land use switch to *J. curcas* (Alguacil et al., 2012). The sequencing of 18S rDNA fragments amplified from soil DNA extracts revealed a dominance of Glomeraceae in *J. curcas*-primed soils. Moreover, AMF diversity (Shannon index) was lower than in soil grown with native vegetation. In our case, although we did not evaluate diversity from the Shannon index, such erosion of diversity was not obvious. Overall, phylotype richness was the same in *J. curcas* and control soils, except in site-3 soil grown with acacia (greater richness in *J. curcas* soil) and cowpea (lower richness in *J. curcas* soil). Many studies are dedicated to the negative consequences of altered mycorrhization on native plant community performance in response to allelochemicals secreted by exotic plants (Stinson et al., 2006; Barto et al., 2011; Ruckli et al., 2014). Yet, the patent modifications of mycorrhizal assemblages we evidenced did not impair crops in their growth or in N–P nutrition. This could directly suggest that the functional values of contrasted root mycorrhizal communities were equivalent for a given crop in a given site. In other words, mycorrhizal symbionts adapted to *J. curcas* soil conditions and sustained the same cost/benefit balance as the native mycorrhizal community. Since mycorrhization intensity and plant biometry parameters were often increased in *J. curcas* soils, we could even hypothesize that this modified mycorrhizal diversity in primed soils led to higher efficiency in nutrient acquisition and translocation, and often enhanced plant performance. This beneficial influence could also be linked to side effects of the remodelled AMF diversity, in addition to its intrinsic symbiotic traits. Soil bacterial community composition and activity are known to be impacted by the functioning of AMF mycelial networks, which release fungal exudates in the surrounding soil (Marschner et al., 2001; de Boer et al., 2005; Rillig et al., 2006; Toljander et al., 2007). This phenomenon and its functional consequences are known as the mycorrhizosphere effect (Johansson et al., 2004; Uroz et al., 2007). In the background of the strong AMF diversity shifts evidenced in this paper, it is tempting to assume that the so-called mycorrhizosphere effect associated to *J. curcas* favored some functional bacterial populations directly involved in nutrient availability (free nitrogen fixation, phosphorus solubilization, iron acquisition), phytostimulation (auxin and gibberellin synthesis), or mycorrhization (mycorrhization-helper bacteria, see Frey-Klett et al., 2007; Requena et al., 1997; Artursson et al., 2006).

### 4.3. Beyond the all-or-nothing rule

The invasive leguminous *Acacia dealbata* is also an exotic ligneous species known to release allelopathic compounds that affect resident plants and soil bacterial communities (Lorenzo et al., 2010, 2013a). However, soil leachates used to water cultures of the highly mycorrhizal *P. lanceolata* and collected from several *A. dealabata*-invaded sites did not alter *P. lanceolata* mycorrhization in various control soils (Lorenzo et al., 2013b). Unfortunately, this mechanistic study did not investigate the effects of natural *A. dealabata* leachates on mycorrhizal composition. Similarly, we did not evidence any negative effects of *J. curcas*-primed soils on root colonization of our crops, while we did indeed easily highlight clear-cut effects on the diversity of their mycorrhizal symbionts. The textbook case of *A. petiolata* itself does not only include systematic noxious effects against mycorrhizal symbiosis. In contrast to our results, root colonization of *Acer saccharum* seedlings collected in high natural forest areas invaded by *A. petiolata* was reduced as compared to that of uninvaded areas (Barto et al., 2011). However in half of the cases, significant shifts in the composition of the root mycorrhizal assemblages were evidenced, while overall diversity did not decrease. Monodominant covers of invasive *Centaurea stoebe* and *Euphorbia esula* can even harbor higher abundance and diversity of mycorrhizal fungi than native multi-

**Table 2**
Distribution and number of Glomeromycota 18S rDNA sequences detected from the 3 trap cultures grown in soils transferred from *J. curcas* and control fields, according to the phylotypes inferred from their phylogenetic comparisons (see Fig. 1A–C).

| | Site 1 | | Site 2 | | Site3 | | Total |
|---|---|---|---|---|---|---|---|
| | *J. curcas* soil | Control soil | *J. curcas* soil | Control soil | *J. curcas* soil | Control soil | |
| **Acacia** | | | | | | | |
| Glo1 | ***67*** | 9 | 61 | 76 | ***60***[a] | ***94***[a] | 367 |
| Glo2 | – | – | – | 3 | – | – | 3 |
| Glo3 | – | – | 12 | 5 | – | – | 17 |
| Glo4 | – | 8 | – | – | 19 | – | 27 |
| Glo5 | – | – | – | – | 1 | – | 1 |
| Glo6 | – | – | – | – | 2 | – | 2 |
| Acau1 | 7 | – | – | – | – | – | 7 |
| Acau2 | 1 | – | – | – | – | – | 1 |
| Giga1 | – | ***67*** | 13 | – | – | – | 80 |
| Total | 75 | 84 | 86 | 84 | 82 | 94 | 505 |
| **Millet** | | | | | | | |
| Glo1 | – | – | – | – | 1 | ***71*** | 72 |
| Glo2 | 3 | ***46*** | – | ***90*** | – | – | 139 |
| Glo3 | – | 2 | – | – | – | – | 2 |
| Glo4 | – | 1 | – | 1 | – | – | 2 |
| Glo5 | – | – | – | – | ***79*** | 12 | 91 |
| Glo6 | ***76*** | – | – | – | – | – | 76 |
| Glo7 | 2 | – | – | – | – | – | 2 |
| Glo8 | 1 | – | – | – | – | – | 1 |
| Glo9 | 2 | – | – | – | – | – | 2 |
| Giga1 | – | 1 | – | – | – | – | 1 |
| Giga2 | – | ***37*** | ***82*** | – | – | – | 119 |
| Total | 84 | 87 | 82 | 91 | 80 | 83 | 507 |
| **Cowpea** | | | | | | | |
| Glo1 | – | – | – | ND | ***48*** | – | 48 |
| Glo2 | ***88*** | 14 | 60 | ND | – | ***62*** | 224 |
| Giga1 | – | ***72*** | – | ND | – | 19 | 91 |
| Giga2 | – | – | – | ND | – | 1 | 1 |
| total | 88 | 86 | 60 | ND | 48 | 82 | 364 |

Glo, Acau and Giga stand for Glomeraceae, Acaulosporaceae and Gigasporaceae, respectively. ND: not determined.
For a given trap culture and a given site, numbers in bold and italics highlight the main discriminating phylotypes between *J. curcas* and control soil origins.
[a] These 2 populations of sequences are localized in distinct groupings within the Glomeraceae sub-cluster Glo1 (see Fig. 1A).

species communities of forbs and grasses (Lekberg et al., 2013). The results of these studies suggest that besides severe and systematic allelopathic effects of invasive plants against native mycorrhizal fungi, new bioactive compounds released by exotic plant species in soils can have more subtle effects on fungal symbiosis than a thoroughly depressive impact on both the abundance and diversity of these fungi. The extent of these effects probably depends on the diversity components of the local mycorrhizal community that can harbor more or less tolerant species. Mycorrhizal diversity per se also entails mycorrhizal belowground networks and their diverse associated host plants, that might act jointly to better allow individual mycorrhizal species to cope with novel allelochemicals.

## 5. Conclusion

Mycorrhization of acacia, cowpea and millet crops was significantly impacted in all three *J. curcas*-primed soils as compared to fallow field soils. In particular, deep changes in root mycorrhizal diversity were revealed. Compositional cleavages occurred between Glomeraceae and Gigasporaceae, or within Glomeraceae, depending on the site/crop association. These diversity changes were often accompanied by increased mycorrhization intensity and better growth performance. In light of these results, *J. curcas* monoculture does not seem to degrade the functional outputs of native plants/soil arbuscular mycorrhizal fungi interactions. However, the severe shifts we evidenced in the root mycorrhizal community in response to *J. curcas* plantation were very quick. Therefore such conclusions should be further confirmed by implementing *in situ* trap cultures, so as to include hydric stress that was negligible in the present investigation. Moreover, such an experimental design would advantageously integrate the local mycorrhizal belowground networks that were disrupted by our soil sampling procedure. Such networks are integral parts of the functional value of the taxonomic diversity of root mycorrhizal communities, so their significance should also be tested.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.06.019.

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