

Université Montpellier 2

Habilitation à Diriger des Recherches

Philippe JOURAND

**Rôles des rhizobiums dans l'adaptation des
légumineuses aux contraintes abiotiques des sols : le
modèle *Acacia spirorbis* en Nouvelle-Calédonie :
adaptation à l'ultramafisme.**

Laboratoire de Symbioses Tropicales et Méditerranéennes

LSTM UMR113 (CIRAD, IRD, INRA, Agro-M, UM2)

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Article 1 : Majorel C., Hannibal L., Soupe ME, Carriconde F, Ducouso M, Lebrun M & Jourand P. 2011. <i>Molecular Ecology</i> (soumis le 01/10/2011, N° MEC-11-0835)	
Article 2 : Bhattacharjee R., Jourand P., Chaintreuil C., Dreyfus B., Singh A. & Mukhopadhyay S.N. 2011. <i>Biology and Fertility of Soils</i> (DOI 10.1007/s00374-011-0614-9).	
Article 3 : Renier A., Mania De Faria S., Jourand P., Giraud E., Dreyfus B., Rapior S. & Prin Y. 2011. <i>Journal of Experimental Botany</i> 62:3693-3697.	
Article 4 : Lagrange A., Ducouso M., Jourand P., Majorel C., Amir H. 2011 <i>Canadian Journal of Microbiology</i> 57:21-28.	
Article 5 : Jourand P. et al, 2010, <i>Tree Physiology</i> 30:1311-1319.	
Article 6 : Jourand P., et al 2010 <i>FEMS Microbiology Ecology</i> 72:238-249.	
Article 7 : Renier A., Jourand P., Rapior S., Poinot V., Sy A., Dreyfus B. & Moulin L. 2008. <i>Soil Biology Biochemistry</i> 40:1404-1412.	
Article 8 : Jourand P., et al., 2005 <i>Molecular Plant-Microbe Interactions</i> 18:1061-1068.	
Article 9 : Jourand P., et al. 2004 <i>International Journal of Systematic & Evolutionary Microbiology</i> 54:2269-2273.	
Article 10 : Jourand P., et al, 2004a <i>Nematology</i> 6:79-84.	
Article 11 : Jourand P., et al, 2004b <i>Nematology</i> 6:763-769.	
Article 12 : Giraud E., Hannibal L., Fardoux J., Jaubert M., Jourand P., Dreyfus B., Sturgis J. N. & Verméglio A. 2004 <i>Journal of Biological Chemistry</i> 279:15076-15083	
Article 13. Sy A., Giraud E., Jourand P., Garcia N., Willems A., De Lajudie P., Prin Y., Neyra N., Gillis M., Masson-Boivin C. et Dreyfus B. 2001. <i>Journal of Bacteriology</i> 183:214-220	
Article 14. Gauthier D., Navarro I., Rinaudo G., Jaffré T., Jourand P., Prin Y. 1999. <i>Journal of Soil and Biology</i> 35:199-205	

Curriculum Vitae

Philippe JOURAND

Chargé de Recherche (CR1) - Institut de Recherche pour le Développement (IRD)
Laboratoire des Symbioses Tropicales et Méditerranéennes
LSTM UMR 113 - Equipe Adaptation et Ultramafisme (A&U)

ETAT CIVIL et COORDONNEES

Né le 16/06/1966 à St Denis (93) France, de Nationalité Française, Pacsé.

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TITRES ET DIPLÔMES UNIVERSITAIRES

2003 : Doctorat de l'Université Montpellier 2 - Formation doctorale : Développement et Adaptation des Plantes (DAP) - École Doctorale de Biologie Intégrative - Discipline : Physiologie, Biologie des organismes et Populations. - Mention Très Honorable - Soutenue le 1er décembre 2003 à Montpellier.

Titre : « Les légumineuses du genre *Crotalaria* : symbiose fixatrice d'azote et activités biologiques contre les nématodes phytoparasites »

<u>Jury</u> :	M. LEBRUN, PR, UM2, Président	T. MATEILLE, DR IRD, Examineur
	P. ABAD, DR INRA Antibes, Rapporteur	S. RAPIOR, PR UM1, Co-Directrice de thèse
	B. OLLIVIER, DR IRD Marseille, Rapporteur	B. DREYFUS, DR IRD, Directeur de thèse

2000 : Dérogation au diplôme de DEA Développement et Adaptation des Plantes. École Doctorale de Biologie Intégrative - Université Montpellier 2.

1990 : Ingénieur biochimiste, diplômé d'état, de l'Institut National des Sciences Appliquées de Lyon (INSA), département de Biochimie.

1987 : Brevet de Technicien Supérieur de Biochimie, Ecole Nationale de Physique-Chimie-Biologie (ENCPB, rue Pirandello, Paris 13e).

SITUATION ACTUELLE

Chargé de Recherche (CR1) - Institut de Recherche pour le Développement (IRD), affecté sur le Centre IRD de Nouméa, Nouvelle-Calédonie, représentant du Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM UMR 113) - équipe Adaptation et Ultramaïfisme (A&U).

PARCOURS PROFESSIONNEL

Depuis 2006 : Affectation en Nouvelle-Calédonie, Centre IRD de Nouméa, LSTM UMR113. Chargé du programme de recherche: rôle de la symbiose ectomycorhizienne dans l'adaptation de leurs plantes hôtes aux conditions édaphiques extrêmes (sols ultramaïfiques des maquis miniers de Nouvelle-Calédonie). Application à la re-végétalisation de sites miniers dégradés. Promu Ingénieur de Recherche de 1^{ère} classe (IR1) en 2009 ; détaché dans le corps des Chargés de Recherche de 1^{ère} classe (CR1) depuis 2010.

2005-2006: Affectation en Australie, Adélaïde, South Australia: Université d'Adélaïde, Ecole de la terre et de l'environnement, Laboratoire de physiologie végétale. Poste d'accueil dans le laboratoire du Pr R. Reid : Acquisition des méthodes d'études de la réponse physiologique des végétaux au stress métallique.

1998-2005 : Affectation à Montpellier, France. LSTM UMR113, CIRAD Campus de Baillarguet. Chargé du programme de recherche : Les légumineuses du genre *Crotalaria*: symbiose fixatrice d'azote et activités biologiques contre les nématodes phytoparasites. Utilisations en agriculture biologique. Promu Ingénieur d'Etudes de 1^{ère} classe (IE1) en 2003, puis passage dans le corps des Ingénieurs de Recherche de 2^{ème} classe (IR2) en 2004 par voie de concours interne à l'IRD.

Depuis 1998 : Membre permanent du Laboratoire de Symbioses Tropicales et Méditerranéennes (LSTM UMR113, Unité mixte de recherche CIRAD, IRD, Université Montpellier II, SupAgro, INRA).

1996-1998 : Affectation en Nouvelle-Calédonie, Centre IRD de Nouméa, Laboratoire de Microbiologie (Unité de recherche UR3B, Microbiologie). Poste : Ingénieur d'études participant au programme de recherche sur les symbioses *Frankia* – *Casuarinaceae* et de leur utilisation pour la re-végétalisation des sols dégradés des mines de Nickel

1994-1996 : Affectation au Mexique, Mexico city. Université Autonome de Mexico (UAM-Iztapalapa), Laboratoire de Biotechnologie. Poste : Ingénieur en charge de l'équipement et de l'installation complète d'un laboratoire de microbiologie. Participation au programme de recherche sur l'utilisation des champignons filamenteux dans la valorisation des déchets de l'industrie agro-alimentaire (pulpe de café et bagasse de canne à sucre).

PARCOURS PROFESSIONNEL (SUITE)

1994 : Titularisation dans le corps des Ingénieurs d'Etudes de l'IRD. Intégration de l'Unité de recherche UR3B (Microbiologie), département Milieux et Activités Agricoles (MAA).

1993 : Recruté par l'IRD Ingénieur d'Etudes de 2^{ème} classe (IE2) par voie de concours externe. Affectation à Montpellier, France. Rattaché à l'Unité de Recherche UR3C (Agronomie, Phytopathologie et Parasitologie des végétaux) du Département Milieux et Activités Agricoles de l'IRD (MAA). Poste de recrutement : responsable de la plate forme de biochimie analytique du centre IRD de Brazzaville au Congo. Poste redéployé en raison de la guerre civile survenue au Congo.

1990-1992 : Affectation en Nouvelle-Calédonie : Service National, Volontaire à l'Aide Technique (VAT) : Laboratoire des Bactéries Entéropathogènes, Institut-Pasteur de Nouméa. Poste occupé : ingénieur de recherche participant au programme d'épidémiologie de l'OMS étudiant les bactéries entéropathogènes responsables des diarrhées tropicales infantiles.

COMPETENCES

Discipline scientifiques :

- Compétences acquises au cours de la formation dans les disciplines fondamentales: microbiologie, biochimie et biologie-moléculaire ; dans les disciplines appliquées : techniques de microbiologie (isolement, purification, caractérisation), de biochimie analytique (purification de biomolécules, HPLC, CPG) et techniques de biologie moléculaire (séquençage, génotypage, génie génétique).
- Compétences acquises avec l'expérience professionnelle: écologie, agronomie et biotechnologie végétale (échantillonnage de plantes et de sols, expérimentations des interactions plantes /microorganismes en laboratoire, en serre et in situ).

Informatique : Logiciels de bureautique et statistique (Word, EndNote, Power-Point, Excel, Xlstat), Logiciels de Biologie Moléculaire et Phylogénie (Chromas pro, Sequence Analysis); Logiciels de traitement de l'image (Photoshop, AnalySIS).

Langues : Espagnol (bilingue), Anglais (courant et anglais scientifique).

CENTRES D'INTERET

Passionné de météorologie et d'aviation civile commerciale.

Sports : natation (quotidienne) et randonnée pédestre (montagne).

1) Diffusion de l'information scientifique

1.1. ARTICLES PUBLIES DANS DES REVUES INTERNATIONALES (15)

La liste récapitulative des publications (P) est présentée par ordre chronologique décroissant (de la plus récente à la plus ancienne) avec facteur d'impact (IF année 2010) disponible sur la base de données ISI-Web (5-years impact factor, par rapport à l'année 2010).

- P1.** Majorel C., Hannibal L., Soupe M.E., Carriconde F., Ducousso M., Lebrun M. & **Jourand P.** 2011. Tracking nickel adaptive biomarkers in *Pisolithus albus* from New Caledonia using a transcriptomic approach. *Molecular Ecology* (soumis le 01/10/2011, N° MEC-11-0835) (IF=6,233).
- P2.** Bhattacharjee R., **Jourand P.**, Chaintreuil C., Dreyfus B., Singh A. & Mukhopadhyay S.N. 2011. Indole acétique acid and ACC deaminase producing *Rhizobium leguminosarum* bv. *trifolii* SN10 promote rice growth, and in the process undergo colonisation and chemotaxis. *Biology & Fertility of Soils* DOI 10.1007/s00374-011-0614-9 (IF=2,070).
- P3.** Renier A., Mania De Faria S., **Jourand P.**, Giraud E., Dreyfus B., Rapior S. & Prin Y. 2011. Nodulation of *Crotalaria podocarpa* DC. by *Methylobacterium nodulans* displays very unusual features. *Journal of Experimental Botany* 62:3693-3697. (IF= 4,745).
- P4.** Lagrange A., Ducousso M., **Jourand P.**, Majorel C., Amir H. 2011. New insights into the mycorrhizal status of *Cyperaceae* from ultramafic soils in New Caledonia. *Canadian Journal of Microbiology* 57:21-28 (IF= 1,502).
- P5.** **Jourand P.**, Ducousso M., Reid R., Majorel C., Richert C., Riss J., Lebrun M. 2010. Nickel-tolerant ectomycorrhizal *Pisolithus albus* ultramafic ecotype from nickel mines in New Caledonia strongly enhance growth of the host plant *Eucalyptus globulus* at toxic nickel concentrations. *Tree Physiology* 30:1311-1319 (IF= 2,530).
- P6.** **Jourand P.**, Ducousso M., Majorel C., Hannibal L., Santoni S, Prin Y. & Lebrun M. 2010. Ultramafic soils from New Caledonia structure *Pisolithus albus* in ecotype. *FEMS Microbiology Ecology* 72:238-249 (IF = 3,885).
- P7.** Renier A., **Jourand P.**, Rapior S., Poinot V., Sy A., Dreyfus B. & Moulin L. 2008. Symbiotic properties of *Methylobacterium nodulans* ORS 2060T: a classic process for an atypical symbiont. *Soil Biology Biochemistry* 40:1404-1412. (IF=3,566).
- P8.** **Jourand P.**, Renier A, Rapior S, Mania de Faria S, Prin Y, Galiana A, Giraud E, Dreyfus B. 2005. Role of methylotrophy during symbiosis between *Methylobacterium nodulans* and *Crotalaria podocarpa*. *Molecular Plant-Microbe Interactions* 18:1061-1068. (IF=4,844).

- P9. Jourand P.**, Giraud E., Bena G., Sy A., Willems A., Gillis M., Dreyfus B. and De Lajudie P. **2004.** *Methylobacterium nodulans* sp. nov., for a group of aerobic, facultatively methylotrophic, legume root-nodule-forming and nitrogen-fixing bacteria *International Journal of Systematic & Evolutionary Microbiology* 54:2269-2273. (IF=2,488).
- P10. Jourand P.**, Rapior S., Fargette M. & Mateille T. **2004.** Nematostatic effect of a leaf extract from *Crotalaria virgulata* subsp *grantiana* on *Meloidogyne incognita* and its use to protect tomato roots. *Nematology* 6:79-84. (IF=0,964)
- P11. Jourand P.**, Rapior S. Fargette M. & Mateille T. **2004.** Nematostatic activity of aqueous extracts of West African *Crotalaria* species. *Nematology* 6:763-769. (IF=0,964)
- P12.** Giraud E., Hannibal L., Fardoux J., Jaubert M., **Jourand P.**, Dreyfus B., Sturgis J. N. & Verméglio A. **2004.** Two distinct crt gene clusters for two different functional classes of carotenoid in *Bradyrhizobium*. *Journal of Biological Chemistry* 279:15076-15083. (IF=5,440).
- P13.** Sy A., Giraud E., **Jourand P.**, Garcia N., Willems A., De Lajudie P., Prin Y., Neyra N., Gillis M., Masson-Boivin C. et Dreyfus B. **2001.** A group of *Methylobacterium* nodulates and fixes nitrogen in symbiosis with leguminous plant. *Journal of Bacteriology* 183:214-220. (IF=4,035).
- P14.** Gauthier D., Navarro I., Rinaudo G., Jaffré T., **Jourand P.**, Prin Y. **1999.** Isolation, characterisation (PCR-RFLP) and specificity of *Frankia* from eight *Gymnostoma* species endemic to New Caledonia. *European Journal of Soil and Biology* 35:199-205. (IF=1,445).
- P15.** Begaud E., **Jourand P.**, Morillon M., Mondet D., Germani Y. **1993.** Detection of diarrheogenic *Escherichia coli* in children less than ten years old with and without diarrhea in New-Caledonia using seven acetylaminofluorene labeled DNA probes. *American Journal of Tropical Medicine and Hygiene* 48:26-34. (IF=2,907).

1.2. CHAPITRE D'OUVRAGES SCIENTIFIQUES

Amir H., **Jourand P.**, Cavaloc Y., Ducousso M. **2011.** Role of mycorrhizal fungi on the alleviation of heavy metal toxicity on plant. In : *Mycorrhizal Fungi: Use in Sustainable Agriculture and Forestry*. Z.M. Solaiman, L.K. Abbott and A. Varma (Eds). *Soil Biology series* (sous presse).

Sy A., **Jourand P.**, Giraud E., Ndoye I., De Lajudie P., Boivin-Masson C. Samba P., Neyra M., Willems A., Gillis M. & Dreyfus B. **2000.** Multipurpose legumes of the tropical genus *Crotalaria* are associated with unusual Rhizobia, p537-538. In: Pedrosa F.O. eds, *Nitrogen Fixation: From Molecule to Crop Productivity*. Kluwer Academic Publishers, The Netherlands.

1.3. MEMOIRE DE THESE

Jourand P. 2003. Thèse de Doctorat de l'Université Montpellier 2, Ecole Doctorale (SIBAGHE, ex EDBI) : Titre : Les légumineuses du genre *Crotalaria* : symbioses fixatrices d'azote et activités biologiques contre les nématodes phytoparasites. 183p.

1.4. COMMUNICATIONS A DES CONGRES INTERNATIONAUX (14)

La liste récapitulative des communications orales (CO) et des communications affichées (ou posters) (CA) à des congrès internationaux est présentée par ordre chronologique décroissant (de la plus récente à la plus ancienne).

- C1.** Ducouso M., **Jourand P.**, Majorel C., Hannibal L., Prin Y., Lebrun M. **2011**. Ectomycorrhizal diversity within New Caledonian ultramafic ecosystems and role of *Pisolithus albus* in host plant adaptation toward Nickel toxicity. International workshop: «Mycorrhizae : a biological tool for sustainable development in Africa ». 21-23 February, 2011, Dakar, Senegal (CO).
- C2.** **Jourand P.**, Ducouso M., Reid R., Majorel C., Hannibal L., Prin Y. & Lebrun M. **2010**. *Pisolithus albus* and plant adaptation to nickel toxicity in New Caledonia. 5ème Colloque International « Contamination Métallique : Impact sur l'Environnement, la Santé et la Société » 13-15 Octobre, 2010, Oruro, Bolivie (CO).
- C3.** Ducouso M., **Jourand P.**, Majorel C., Prin Y., Lebrun M. **2010**. Quels enseignements tirer des sols néo-calédoniens naturellement riches en métaux lourds. 1^{er} Congrès international sur les symbioses mycorhiziennes, écosystèmes et environnements en milieu méditerranéen, MYCOMED. 11-13 Octobre 2010, Marrakech, Maroc (CO).
- C4.** Ducouso M., **Jourand P.**, Majorel C., Hannibal L., Prin Y. & Lebrun M. **2010**. Contribution de *Pisolithus albus* à l'adaptation de la plante hôte à la toxicité au nickel en Nouvelle-Calédonie. Secondes Journées Francophones Mycorhizes : Les Mycorhizes et leurs environnements. Bruxelles, Belgique, 15-17 septembre 2010 (CO).
- C5.** Renier A., **Jourand P.**, Rapior S. **2009**. Implication de l'apigénine dans la symbiose *Methylobacterium nodulans*-*Crotalaria podocarpa*. Symposium sur les composés phénoliques « nutraciques ou médicaments ? » 17-18 Décembre 2009, Agadir, Maroc (CA).
- C6.** Ducouso M., **Jourand P.**, Majorel C., Riss J., Prin Y. and Lebrun M. **2009**. Nickel tolerance and population structure of *Pisolithus albus* in New Caledonia. International Conference on Mycorrhiza (ICOM6), 9-14 Août 2009, Belo-Horizonte, Brésil (CO+CA).

- C7.** Ducouso M., **Jourand P.**, Majorel C., Jinakoa A., Riss J., Maurizi L., Goulon C., Prin, Y., Lebrun M. **2009.** Biodiversité des champignons ectomycorhizien des écosystèmes ultramaïques de Nouvelle-Calédonie. 11ème congrès international du pacifique « Pacific Science Inter-Congres », 02-06 Mars 2009, Tahiti, Polynésie-Française (CA).
- C8.** Renier A., Rapior S., Dreyfus B. & **Jourand P.** **2007.** The *Methylobacterium nodulans* / *Crotalaria podocarpa* symbiosis: a classic process for an original model. Congrès international « Rhizosphère 2 », (CIRAD, CNRS, IRD, INRA, SupAgro, UMI), 26-31 Août 2007, Montpellier, France (CA).
- C9.** Pouschat, P., Plouffe G., Keller C., Rose J., Ducouso M., **Jourand P.**, Ambrosi, JP. **2007.** Manganese localization in *Tristanopsis* spp. from soil to plant in New Caledonian ferrallitic soils. Congrès international « Rhizosphère 2 », (CIRAD, CNRS, IRD, INRA, SupAgro, UMI), 26-31 Août 2007, Montpellier, France (CA).
- C10.** Renier A., Rapior S., Prin Y., Galliana A., Giraud E., Dreyfus B. & **Jourand P.** **2005.** Methylo trophy : a great asset in symbiosis process. 14ème Congrès Australien sur la fixation de l'azote, 21-25 Novembre 2005, Katoomba, New-South-Wales, Australie (CA).
- C11.** Dreyfus B., Bena G., **Jourand P.**, Prin Y., Bailly X. & Giraud E. Importance of atypical nitrogen fixing symbioses in natural tropical ecosystems. 6ème Congrès Européen sur la fixation de l'azote, 24-27 Juillet 2004, Toulouse, France (CA+CO).
- C12.** **Jourand P.**, Lambion J., Védie H., Bertrand C., Fargette M., Mateille T., Rapior S., Dreyfus B. Potential of West African *Crotalaria* species in controlling root-knot nematodes parasitizing vegetable crops in organic agriculture. 14ème Congrès International de l'Agriculture Biologique, Août 2002, Victoria, Vancouver, Canada (CO).
- C13.** Sy. A, Giraud E, **Jourand P.**, Garcia N, Willem A., De Lajudie P., Prin Y., Neyra N., Gillis M., Masson-Boivin C., Dreyfus B : A group of *Methylobacterium nodulans* and fixes nitrogen in symbiosis with leguminous plant. Fourth European Nitrogen Fixation Conference, Séville, Espagne (CA).
- C14.** Sy. A., **Jourand P.**, Giraud E., Ndoeye I., De Lajudie P., Boivin C., Samba R., Neyra M., Willems A. Gillis M., Dreyfus B. : "Multipurpose legumes of the tropical genus *Crotalaria* are associated with unusual rhizobia." Congrès international "Nitrogen Fixation", Universidad do Parana, Brésil (CO).

1.5. ARTICLES, RAPPORTS ET COMMUNICATIONS NATIONAUX (6)

Il s'agit d'articles parus dans des revues françaises (A) ou de rapport (R), ou de communication présentées lors de conférences nationales sous forme de poster (P).

- N1. Jourand P., Ducousso. M. & Grenier L. 2010.** Les 24 plus beaux défis de la science : Comment des plantes poussent sur un sol toxique. La Recherche, vol 446, p46. Sophia Publications, Paris, France. (A).
- N2. Jourand P., Ducousso M., Riss J., Polutele S., Lebrun M. & Prin Y. 2007.** Diversité des ectomycorhizes en forêt et en maquis dans le massif du Koniambo ; Importance des ectomycorhizes pour la tolérance au nickel. Rapport ANR Niko. (R).
- N3. Jourand P. 2007.** L'association ectomycorrhizienne *Pisolithus-Myrtaceae* : un modèle d'étude de la symbiose champignon-arbre. Sciences au Sud n°40. Editions IRD, France. (A).
- N4. Renier, S. Rapior, B. Dreyfus & P. Jourand. 2006.** 7èmes Rencontres Plantes-Bactéries (CNRS, IRD, INRA, CEA, CIRAD), 20-24 Mars 2006, Aussois, France. Methylo trophy : from a great assess in symbiosis process to molecular dialog élucidation. (P).
- N5. Lambion J., Védié H., Bertrand C. & Jourand P. 2003.** Etude des moyens de lutte contre les nématodes à galles en maraîchage biologique. Revue Agricole "Alter Agri", 57:2-14. (A).
- N6. Jourand P & Bertrand C. 2001.** Les Crotalaires contre les nématodes. Revue Agricole "Le sillon" 146:34-35. (A).

2) Activités d'Encadrement et de Formation

2.1. ENCADREMENTS D'ETUDIANTS

Les sujets de thèse ou de stage ont été choisis parmi les thématiques de recherche du LSTM UMR113. Les publications ou communications dans des congrès auxquelles les étudiants ont été associés sont développées dans le chapitre « Travaux scientifiques ».

2.1.1. TROISIEME CYCLE UNIVERSITAIRE

Co-encadrement d'étudiants en Doctorat d'Université : 3

- Simon GENSOUS : encadrement de thèse en cours sur la période 2011-2013.

- Etudiant titulaire du Master Biologie Fonctionnelle des Plantes, Université de Montpellier 2, France. Diplôme obtenu en 2010. Cet étudiant est inscrit en doctorat à l'Université de Nouvelle-Calédonie (UNC), Ecole Doctorale du Pacifique (ED 469).
- Directeur de thèse : Pr Hamid Amir (DR, HDR, UNC)
- Sujet de thèse: Les champignons mycorhiziens à arbuscules des maquis miniers de Nouvelle-Calédonie : diversité et rôle dans l'adaptation des plantes à la carence en phosphore et à la toxicité métallique ; relations avec les bactéries à activités PGPR. Ce sujet a obtenu le prix d'encouragement à la recherche de la Province Sud de Nouvelle-Calédonie.

Il s'agit d'un co-encadrement pour l'un des chapitres de la thèse (25% de la thèse) : j'ai contribué à la rédaction du sujet de ce chapitre de thèse, intitulé : étude de la rhizosphère bactérienne des *Costularia*, plantes pionnières des sols ultramafiques en Nouvelle-Calédonie : diversité et rôle dans l'adaptation de la plante (ultramafisme et nutrition phosphatée). Je participe à l'encadrement de l'étudiant sur ce chapitre en collaboration avec le Dr Marc DUCOUSSO (HDR, DR, CIRAD).

- Alexandre LAGRANGE : encadrement de thèse sur la période 2006-2009.

- Thèse de doctorat de l'Université de Nouvelle-Calédonie, Ecole Doctorale du Pacifique (ED 469). La thèse a été soutenue en Septembre 2009, à l'Université de Nouvelle-Calédonie.
- Directeurs de thèses : Dr Marc DUCOUSSO (DR, HDR, CIRAD) et Pr Hamid AMIR (DR, HDR, UNC).
- Sujet de thèse : études écologiques et microbiologique des espèces du genre *Costularia* (*Cyperaceae*) pionnières des sols ultramafiques en Nouvelle-Calédonie : application à la restauration écologique.

- Financement : Il s'agit d'une thèse appuyée par un financement CIFRE obtenu en partenariat avec la société SIRAS Pacifique, PME de Nouvelle-Calédonie (www.siras.nc/), spécialisée dans la Réhabilitation et l'Aménagement de Sites dégradés.

- Jury :

Philippe Hinsinger	CR INRA, UMR 1222 Eco&Sols, Montpellier	Rapporteur
Sergio Miana de Faria	Pr Embrapa, Agrobiologia, Rio de Janeiro, Brazil	Rapporteur
Hamid Amir	Professeur LIVE, Université de Nouvelle-Calédonie	Directeur
Marc Ducouso	Chercheur CIRAD, UMR 113 LSTM, Montpellier	Co-directeur
Fabrice Colin	DR IRD, Centre IRD de Nouméa	Examineur
Laurent L'Huillier	Chercheur IAC, Port Laguerre, Nouvelle-Calédonie	Examineur
Danielle Saintpierre	Chercheur Société Siras Pacifique, Nouméa	Examineur

J'ai participé au co-encadrement de l'étudiant (25% de la thèse) :

- dans la rédaction de l'article (P4) relatif au 3^{ème} chapitre de la thèse, intitulé : « New insights into the mycorrhizal behaviour of *Cyperaceae*. Role of arbuscular mycorrhizas in growth and adaptation of *Costularia comosa* in New Caledonian ultramafic soils ».
- dans l'organisation et la réalisation du 4^{ème} et dernier chapitre de la thèse, intitulé « Caractérisation et sélection de rhizobactéries tolérantes au nickel chez deux espèces de *Costularia* pionnières des sols ultramafiques », avec valorisation des travaux relatifs à ce chapitre sous forme d'une publication internationale en préparation.

Publications internationales associées à la thèse pour lesquelles je suis co-auteur : P4

Devenir de l'étudiant après sa thèse : Alexandre a ouvert un bureau d'étude en environnement intitulé « Bota Environment » basé à Koné, BP1195, Nouvelle-Calédonie, au sein duquel il est gérant et chargé de projets de restauration de milieux dégradés impliquant botanique et environnement.

- **Adeline RENIER: encadrement de thèse sur la période 2004-2007**

- Thèse de doctorat de l'Université Montpellier 2, Ecole Doctorale SIBAGHE, discipline : Biologie Intégrative des Plantes. Thèse soutenue le 18 janvier 2008.
- Directeurs de thèses : Pr Sylvie RAPIOR (PR, HDR, UM1) et Dr Bernard DREYFUS (DR, HDR, IRD).
- Sujet de thèse : Approche pluridisciplinaire de la symbiose *Methylobacterium nodulans* / *Crotalaria podocarpa*.

- Jury :		
Bruno Touraine	Pr UMII, Université Montpellier 2	Président
Philippe Normand	DR CNRS, UMR5557 Lyon	Rapporteur
Stéphane Vuilleumier	Pr Université de Strasbourg	Rapporteur
Charles Rosenberg	DR INRA UMR215, Toulouse	Examineur
Sylvie Rapior	Pr UM1, Montpellier	Directrice
Bernard Dreyfus	DR IRD, LSTM UMR113	Co-directeur

J'ai participé au co-encadrement de la thèse (50% de la thèse) au niveau de:

- i) l'élaboration du sujet et sa validation par l'école doctorale SIBAGUE,
- ii) la recherche des financements : ½ bourse régionale région Languedoc Roussillon et ½ bourse régionale région Guadeloupe,
- iii) l'encadrement scientifique : chapitres I, II et III,
- iv) la participation aux comités de thèse,
- v) l'encadrement de la rédaction du mémoire,
- vi) la participation à la rédaction des publications et des communications.

Publications associées à la thèse : **P3, P7 et P8.**

Communications associées à la thèse : **C5, C8, C10 et N4.**

Devenir de l'étudiant après sa thèse : Adeline a été recrutée en CDI en Mai 2010 au Centre d'expertise en techniques environnementales et végétales (CETEV, 31460 Le Faget, France) où elle est responsable R&D d'outils de diagnostics de maladies des plantes/arbres en zone non agricole.

2.1.2. DEUXIEME CYCLE UNIVERSITAIRE

Co-encadrement d'étudiants en stages de Master (M1 et M2, ex-DEA et ex-DESS) et stages d'Ecole d'Ingénieur (4^{ème} et 5^{ème} année) : 6

2011 : Mathieu GONIN, étudiant en stage de Master 2, spécialité Biologie et Evolution des Plantes, cursus Biotechnologie des Plantes Tropicales, Université Montpellier 2. Stage de 8 mois, de Janvier à Août 2011. Sujet: caractérisation moléculaire et physiologique des bactéries des *Costularia* spp., plantes endémiques et pionnières des maquis miniers de Nouvelle-Calédonie.

Co-encadrement avec Dr Marc DUCOUSSO (DR/HDR CIRAD, LSTM UMR113).

1 Publication en préparation associée au stage.

2011 : Hélène BARAILLE, étudiante en stage de Master 1, spécialité Biologie et Evolution des Plantes, cursus Biotechnologie des Plantes Tropicales, Université Montpellier 2. Stage de 6 mois, de Mars à Août 2011. Sujet: Etude des mécanismes support de l'adaptation au nickel chez l'ectomycorhize *Pisolithus albus* appartenant à l'écotype ultramafique de Nouvelle-Calédonie.

Co-encadrement avec Dr Clarisse MAJOREL (IE2, LSTM UMR113) et Dr Marc DUCOUSSO (DR/HDR CIRAD, LSTM UMR113).

2007 : Jennifer RISS, étudiante en stage de l'Ecole d'Ingénieur ISTOM (Cergy-Pontoise, France). Stage de fin d'études de 9 mois (de janvier 2007 à septembre 2007). Sujet: rôle de l'ectomycorhization dans l'adaptation des Myrtacées exposés à de fortes concentrations en Nickel - Application à la re-végétalisation des maquis miniers.

Co-encadrement avec Dr Marc DUCOUSSO (DR/HDR CIRAD, LSTM UMR113).

1 Publication associée au stage (P5) et une communication (N2).

2004 : Hedi KEBLI, étudiant en stage du DEA « Environnement Tropical et Valorisation de la Biodiversité », Faculté des Sciences exactes et naturelles de l'Université des Antilles et de la Guyane (UAG). Stage de 6 mois réalisé de janvier à juin 2004. Sujet: Expression des gènes symbiotiques (nif) et de méthanol déshydrogénase (mxaf) de *Methylobacterium* nodulant les légumineuses tropicales du genre *Crotalaria*.

Co-encadrement avec Dr Yves PRIN (DR/HDR CIRAD, LSTM UMR113).

2003 : Emilie DROUARD, étudiante en stage du DESS Physiologie Végétale, Université d'Angers. Stage de fin d'études période du 15 juin 2003 - 15 septembre 2003. Sujet: Etude *in vitro* de l'action biologique de l'extrait aqueux racinaire de *Crotalaria grantiana* sur des larves du nématodes phytoparasite *Meloidogyne incognita* au stade infestant j2.

Co-encadrement avec Dr Hélène VEDIE, ingénieur de recherche au Groupement de Recherche en Agriculture Biologique (GRAB, Avignon).

2002 : Céline RONFORT, étudiante en stage de 2ème année, Ecole d'Ingénieur Agricole de Beauvais. Stage de 3 mois (du 28/06/02 au 01/09/02); Sujet: étude de la spécificité de symbiose fixatrice d'azote entre les plantes du genre *Crotalaria* (légumineuses tropicales) et les bactéries des genres *Methylobacterium* et *Bradyrhizobium*.

Co-encadrement avec Dr Bernard DREYFUS (DR IRD, LSTM UMR113).

2.1.3. PREMIER CYCLE UNIVERSITAIRE

Encadrement et co-encadrement d'étudiants en stages de Licence (L2 et L3), et stages de 2ème année d'études d'Ecole d'Ingénieur : 6

2008 : David Maridas, étudiant en Licence de Biologie (L3), Université de Nouvelle-Calédonie (UNC). Stage de 3 mois de Octobre à Décembre 2008. Sujet: isolement, identification et caractérisation moléculaire de la souche endomycorhizienne colonisant *Costularia comosa*. Encadrement de stage dans le cadre de la thèse d'Alexandre Lagrange.

2008 : Loic Bourguine, étudiant en Licence de Biologie (L2), Université de Nouvelle-Calédonie (UNC) : Stage de 3 mois de Octobre à Décembre 2008. Sujet: isolement et caractérisation moléculaire de la rhizosphère bactérienne de 3 espèces de *Costularia*. Encadrement de stage dans le cadre de la thèse d'Alexandre Lagrange.

2007 : Marine Toussirot, étudiante en Licence de Biologie (L2), Université de Nouvelle-Calédonie (UNC). Stage de 3 mois de Octobre à Décembre 2007. Caractérisation moléculaire basée sur le séquençage et l'analyse des ITS du champignon ectomycorhizien *Pisolithus albus* isolé de sols ultramafiques de Nouvelle-Calédonie. Encadrement de stage dans le cadre du projet Ultrabio.

2007 : Nathalie Deveaux, étudiante en Licence de Biologie (L2), Université de Nouvelle-Calédonie (UNC). Stage de 3 mois de Octobre à Décembre 2007. Sujet: isolement et caractérisation physiologique et biochimique de la rhizosphère bactérienne de 3 espèces de *Costularia*. Encadrement de stage dans le cadre de la thèse d'Alexandre Lagrange.

2002 : Frédéric Audebert, étudiant en Licence de Biologie et Physiologie Végétale (L3), Université Montpellier 2. Stage du 26/06/02 au 20/09/02. Sujet: screening d'activité nématocide d'extraits foliaires et racinaires de plantes du genre *Crotalaria* originaires d'Afrique de l'Ouest (Sénégal)».

Co-encadrement avec Dr Thierry MATEILLE (DR IRD) et Dr Mireille FARGETTE (CR IRD), CBGP, Montpellier.

2002 : Guillaume Lafforgue, étudiant en Licence de Biologie et Physiologie Végétale (L3), Université Montpellier 2. Stage du 18/06/02 au 20/09/02. Sujet : Test in vitro de protection d'une culture sensible (tomate) vis à vis du nématode phytoparasite *Meloidogyne incognita*, par des extraits foliaires de *Crotalaria*.

Co-encadrement avec Dr Thierry MATEILLE (DR IRD) et Dr Mireille FARGETTE (CR IRD), CBGP, Montpellier.

2.2. ACTIVITES D'ENSEIGNEMENT UNIVERSITAIRE

2.2.1. Enseignements à l'Université de Nouvelle Calédonie

Années 2009 et 2010 : PCEM1, enseignements de Biochimie : 12h de Travaux Dirigés (TD) et 4h d'examens.

Années 1997 et 1998, Licence de Biologie, Unité de Valeur de Biologie Moléculaire : 24h de Cours Magistraux (CM), 12h de Travaux Dirigés (TD), et 8h d'examens écrits et oraux.

2.2.2. Enseignement à l'Université Montpellier 1

Participation annuelle en 2000, 2001, 2002 au DESS « valorisations des substances naturelles », Université Montpellier 1 sous la direction du Pr Sylvie RAPIOR. Enseignement de 3h de Cours Magistraux (CM) sur le cycle de l'azote et l'utilisation des légumineuses.

2.2.3. Enseignement à l'étranger : Université do Parana, Curitiba, Brésil

Année 2000 : cours de biochimie des protéines, durée 2 semaines : 16h de Cours Magistraux (CM) et 20h de Travaux Pratiques (TP). **Cet enseignement a été dispensé en Espagnol.**

2.3. ENCADREMENT DE PERSONNELS

Il s'agit principalement de l'encadrement de personnels temporaires tels que les volontaires civils à l'aide technique (VCAT, contrat CDD de 1 an renouvelable 1 fois, poste d'ingénieur niveau bac+5, salaires financés par le Ministère de l'Outre Mer). Au cours de la période 2006-2012, j'ai participé à l'encadrement de 3 VCAT, en poste (partiellement ou à temps complet) au LSTM UMR113, IRD de Nouméa, Nouvelle-Calédonie.

2007-2008 : Lucie MAURIZI, VCAT IRD. Poste à temps complet au LSTM UMR113, Centre IRD de Nouméa, Nouvelle-Calédonie. VCAT Recrutée dans le cadre du projet ANR ULTRABIO (voir contrat de recherche, paragraphe 3). Fonctions occupées : chargée d'études en écologie moléculaire sur la diversité des symbioses ectomycorhiziennes des plantes appartenant à la famille des *Fagaceae*, genre *Nothofagus* (espèces végétales dominantes des forêts à galerie des massifs ultramafiques miniers de Nouvelle-Calédonie) et des plantes appartenant à la famille des *Myrtaceae*, genre *Tristania* (espèces végétales dominantes des maquis des massifs ultramafiques miniers de Nouvelle-Calédonie). Encadrement en biologie moléculaire et bioinformatique.

2008-2010 : Marion BEAUVALLET, VCAT CIRAD. Poste à temps complet au LSTM UMR113, Centre IRD de Nouméa, Nouvelle-Calédonie. Recrutée dans le cadre du projet ANR ULTRABIO (voir contrat de recherche, paragraphe 3). Fonctions occupées : chargée d'études en écologie moléculaire sur la diversité des symbioses endomycorhiziennes des plantes appartenant aux genres *Psychotria* (famille des *Rubiaceae*) et *Planchonella* (famille des *Sapotaceae*) présentes sur les massifs ultramafiques de Nouvelle-Calédonie. Encadrement en biologie moléculaire et bioinformatique.

2010-2011 : Marie-Estelle SOUPE, VCAT IRD. Poste à temps complet au LSTM UMR113, Centre IRD de Nouméa, Nouvelle-Calédonie. Recrutée dans le cadre du projet CNRT Ecomine Biotop ((voir contrat de recherche, paragraphe 3). Fonctions occupées : recherche des déterminants moléculaires chez l'ECM *Pisolithus albus* ecotype ultramafique de Nouvelle-Calédonie impliqué dans la tolérance au nickel. Encadrement en microbiologie et biologie cellulaire *in vitro* (CIV). Publication associée aux résultats des travaux effectués: **P1**.

2010-2012 : Léa HATTESCHWEILER, VCAT Université de Nouvelle-Calédonie. Poste à temps partiel au LSTM UMR113, Centre IRD de Nouméa, Nouvelle-Calédonie. Recrutée dans le cadre du projet CNRT Ecomine Biotop (voir contrat de recherche, paragraphe 3). Fonctions occupées : chargées de la coordination et de la réalisation des expérimentations de terrain sur sites miniers prévues dans le cadre du projet Ecomine Biotop. Encadrement dans la gestion et le management des différentes parties du projet.

3) Contrats de recherche et collaborations scientifiques

3.1. CONTRATS DE RECHERCHE

3.1.1. PROJET ANR ECCO NIKO (période 2006-2008)

Intitulé : il s'agit du projet ANR-05-ECCO-005, intitulé « ECCO : Ecosphère Continentale et Risques environnementaux », projet intitulé NIKO « Biogéochimie des éléments métalliques dans le continuum sol-eau-microorganismes-plantes des écosystèmes latéritiques de Nouvelle-Calédonie : activités anthropiques vs cycle naturel ».

Contexte et objectifs du projet : ce projet visait à caractériser le comportement et la dynamique des métaux dans les écosystèmes latéritiques de Nouvelle-Calédonie sur deux bassins versant du massif minier du Koniambo, l'un devant être impacté par les activités minières, l'autre restant naturel et intact d'emprise humaine. Les relations sol - eau - microorganismes et plantes sont au coeur de cette demande. Les métaux ciblés sont Fe, Cr, Ni, Mn et Co que l'on trouve à des teneurs élevées mais d'origine naturelle dans les sols, les écosystèmes ayant par ailleurs un niveau élevé de diversité végétale et d'endémisme avec également la présence de plantes hyperaccumulatrices de Ni. Comment fonctionne ce continuum depuis le minéral vers le biologique ? Quelles sont les stratégies développées par les plantes vis à vis des fortes teneurs en métaux ? Quel est le rôle des communautés bactériennes dans cette dynamique ? Quid des mycorhizes ? Quelles sont les dynamiques de transfert au niveau d'un bassin versant ? Quels sont les impacts de l'activité minière sur les écosystèmes, leurs fonctionnements et les transferts en métaux ? Telles sont les questions auxquelles ce projet souhaitait répondre. Pour cela, nous proposons d'instrumenter et de suivre chacun des écosystèmes dans leurs aspects dynamiques des métaux, diversité microbienne, statut mycorhizien et composition des plantes

Porteur du projet : le coordonnateur du projet a été Jean-Paul AMBROSI, CR1 du CEREGE (UMR CNRS 6635 - UMR IRD 161).

Durée et financement obtenu : 356900 € sur 3 ans dont 19 K€/an pour le LSTM (UMR113), coordonnateur pour le LSTM : Yves PRIN (DR, HDR CIRAD).

Contribution scientifique: communications C7 et C9; N2 et N3.

3.1.2. PROJET ANR BIODIV ULTRABIO (période 2008-2010)

Intitulé: il s'agit d'un projet mené dans le cadre d'un financement ANR Biodiversité BIODIV-2007, intitulé ULTRABIO : Biodiversité et stratégies adaptatives végétales et microbiennes des écosystèmes ultramafiques en Nouvelle-Calédonie.

Contexte et objectif général du projet : Les sols ultramafiques résultent de l'altération de roches ultrabasiques en conditions tropicales. En Nouvelle-Calédonie, ces sols sont caractérisés par des teneurs très élevées en métaux lourds (nickel, chrome, cobalt, manganèse), des teneurs très basses en azote, phosphore et potassium, un excès de magnésium (Ca/Mg faible) et plus de 75 % d'oxyde de fer. Grâce à une approche multidisciplinaire (botanique, écologie, pédologie, géochimie, génétique, biologie moléculaire, microbiologie) ULTRABIO a pour objet d'évaluer le poids des contraintes liées à l'ultramafisme sur l'adaptation des plantes et de leurs microorganismes associés. Pour cela, en relation avec la spéciation des métaux dans le continuum sol-plante, la biodiversité végétale, bactérienne et fongique (mycorrhizienne) et son organisation spatiale seront caractérisées sur des plantes endémiques de Nouvelle-Calédonie adaptées aux sols ultramafiques et croissant également sur des sols volcano-sédimentaires non-métallifères

Porteur du projet : le coordonnateur du projet a été le Pr Michel LEBRUN, directeur de l'UMR113 (LSTM).

Durée et financement obtenu : le financement total accordé par l'ANR a été de 725492€ sur 3 ans (2008-2011). Le financement accordé au LSTM UMR113, implantation secondaire de Nouvelle-Calédonie, a été de 58K€/an.

Contribution scientifique: ma contribution principale à ce projet a porté sur la description moléculaire et la caractérisation fonctionnelle des symbioses ectomycorhiziennes des plantes adaptées aux sols ultramafiques de Nouvelle-Calédonie (work packages 1 et 4 du projet Ultrabio). Les résultats sur l'étude de la diversité génétique des populations de champignons ectomycorhiziens *Pisolithus albus* ont abouti aux publications **P1, P4, P5 et P6** et aux communications **C3, C4, C6, C7, N1 et N3**.

3.1.3. PROJET CNRT Ecomine BIOTOP (période 2010-2012)

Intitulé : CNRT Ecomine BIOTOP : Caractérisation et fonctionnement de sols de surface (topsoils), d'espèces végétales et des champignons associés, des maquis miniers de Nouvelle-Calédonie. Application à la restauration écologique des terrains dégradés.

Porteur du projet : le coordonnateur du projet est le Pr Hamid AMIR, directeur du Laboratoire Insulaire du Vivant et de l'Environnement (LIVE), Université de Nouvelle-Calédonie (UNC).

Objectif général du projet : ce projet propose à la fois une expérimentation sur le terrain concernant le stockage et la gestion du topsoil, des travaux de recherche in situ et des expérimentations en serre et au laboratoire sur les topsoils. La caractérisation et le suivi de la banque de graines, du potentiel mycorrhizogène et de l'activité microbienne permettront d'obtenir les informations nécessaires pour mieux maîtriser sa conservation et son utilisation.

Durée et financement obtenu : 545000€ sur 3 ans dont 39K€/an pour le LSTM (UMR113), implantation secondaire de Nouvelle-Calédonie.

Contribution scientifique: La contribution du LSTM (équipe de Nouméa) à ce projet porte sur 3 actions de recherche :

- 1 : Biodiversité des champignons mycorhiziens et adaptation au milieu ultramafique,
- 2 : Caractérisation moléculaire de champignon ECM : étude du modèle *Pisolithus abus* : déterminants moléculaires impliqués dans la tolérance au nickel.
- 3 : Expérimentation sur terrain : essai d'inoculation contrôlée avec des champignons ECM et MA sur un topsoil remplacé après exploitation minière.

A ce stade de l'avancement du projet, nos travaux ont permis les publications **P1 et P5** les communications **C1, C2, C3 et C4**.

3.1.4. PROJET LEGUMES FOR FUTURE (période 2009-2012)

Intitulé : FP7-KBBE-2009 : Legume-supported cropping systems for Europe

Objectif général du projet : This research project will design new legume-supported cropping systems that reduce the environmental impact of European agricultural systems. The research is designed to support public policy and related commercial objectives: reducing fossil energy inputs into agriculture, nitrogen emissions to the environment, the global environmental impact of European agriculture, increased economic competitiveness of legume crops in Europe, and the development of sustainable European agricultural production systems more generally. The purpose of the research is to inform decision-making across the range of actors that determine change in agricultural systems and supply chains and to enhance and facilitate access to supporting technologies.

Porteur du projet : le coordinateur du projet Dr RM REES, The Scottish Agricultural College, UK.

Implication et contribution du LSTM, partenaire n°10 : Dr P. De Lajudie (DR, IRD) et Dr Yves Prin (DR CIRAD). The « Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), Montpellier, France », was created in 1998 to group together scientists belonging to five French research institutes (IRD, CIRAD, INRA, ENSA-M and the University of Montpellier 2) with a long and internationally recognised experience: in the field of biology, ecology and utilisation of tropical and Mediterranean plant-microbe symbioses, especially rhizobia-legume and mycorrhizas. Specifically the group is reputed for its major contribution to the discovery of the unexpected diversity of rhizobia and description of 6 new genera and 13 new species. The institute has collaborations with numerous developing countries for fundamental research and applications. The laboratory harbours a large collection of wild rhizobial strains (3000 strains) from various tropical and Mediterranean legumes. The group now in LSTM has participated in seven EU-funded international projects related to rhizobium-legume symbioses. Role in the project – Provision of case study (WP1), participate in WP2, 3, 4, 5 & 6.

Financement obtenu : le budget total de ce projet est de 3840 k€ sur 3 ans et le LSTM a obtenu un financement total de 63K€ soit 21K€/an.

Contribution scientifique: ma contribution à ce projet a été de rassembler toutes les données bibliographiques accumulées et les résultats publiés par le LSTM (UMR113) concernant les légumineuses tropicales du genre *Crotalaria*. Cela concerne la valorisation des publications **P11 et P12**, des communications **C12, C14, N5 et N6** et du **mémoire de thèse** (synthèse bibliographique et résultats).

3.2. COLLABORATIONS SCIENTIFIQUES

3.2.1. Collaborations avec des partenaires institutionnels

3.2.1.1. Université Montpellier 1 (UM1) (période 2000-2008)

Collaboration avec le Pr Sylvie RAPIOR, Laboratoire de Botanique, Phytochimie et Mycologie, UMR 5175 Centre Ecologie Fonctionnelle et Evolutive (CEFE), Faculté de Pharmacie, 15 Avenue Charles Flahault, B.P. 14491, 34093 Montpellier cedex 5, France. Nature de la collaboration : encadrement (direction) de ma thèse de doctorat (2000-2003) et co-encadrement de la thèse de doctorat d'Adeline Renier (2004-2007). Cette collaboration a permis les publications **P3, P7, P8, P10 et P11** et les communications **C5, C8, C10, C12 et N4** ainsi que la contribution au mémoire de thèse de A. Renier.

3.2.1.2. Collaborations avec d'autres UMR (période 2000-2011)

UMR 1062 : Centre de Biologie et Génétique des Populations, CBGP, Campus International de Baillarguet, CS 30016, 34988 Montferrier-sur-Lez cedex, France. Collaborations sur la période 2000-2003 avec Dr Thierry MATEILLE (DR, IRD) et Dr Mireille FARGETTE (CR, IRD). Nature de la collaboration : dans le cadre de ma thèse de doctorat, T. Mateille et M. Fargette m'ont encadré sur le sujet : utilisation des légumineuses du genre *Crotalaria* dans la lutte contre les nématodes phytoparasites. Cette collaboration a permis les publications **P10 et P11**, la communication **C12** et la contribution au **mémoire de thèse**.

UMR 232 : Diversité, Adaptation Développement des Plantes (UMR ex DIAPC, DIADE depuis 2011), INRA - Centre de Montpellier, France. Collaboration avec le Dr Sylvain SANTONI, responsable de la plate forme de génotypage, sur la période 2009-2011. Sujet : génotypage des *Pisolithus albus* de Nouvelle-calédonie. Cette collaboration a permis la publication **P6**.

UMR 6635 CNRS et UMR 161 IRD : Centre de recherche et d'enseignement en géosciences de l'environnement (CEREGE), Université d'Aix Marseille III, Aix en Provence. Collaboration avec Dr JP AMBROSI (CR CNRS) sur la période 2005-2008 sur le projet ANR ECCO NIKO. Cette collaboration a permis les communications **C9 et N2**.

3.2.1.3. Université d'Adelaïde, Australie (période 2005-2010)

Collaboration avec le Pr Rob REID, School of Earth and Environmental Sciences, University of Adelaïde, Adelaïde, 5005 South Australia. Collaboration sous forme d'accueil pour la période 2005-2006, puis contribution sur le sujet : base moléculaire des mécanismes de la tolérance au nickel chez le champignon ectomycorhizien *Pisolithus albus* des sols ultramafiques de Nouvelle-Calédonie. Cette collaboration a permis la publication **P5** et la communication **C2**.

3.2.1.4. Université de Nouvelle-Calédonie (UNC) (période 2006 - en cours)

Collaboration avec l'équipe du Pr H. AMIR, Laboratoire Insulaire du Vivant et de l'Environnement, Université de la Nouvelle-Calédonie (UNC), B.P. R4, 98 851 Nouméa, Nouvelle-Calédonie. Nous travaillons en collaboration i) autour des projets porteurs ANR ECCO, ANR ULTRABIO et CNRT Ecomine Biotop et ii) l'encadrement d'étudiants en thèse de doctorats : thèse CIFRE d'Alexandre Lagrange, période 2006-2009 et la thèse de doctorat Province Sud de Simon Gensous, période 2011-2013. Cette collaboration a permis la publication **P4** et le chapitre d'ouvrage « Amir H., Jourand P., Cavaloc Y., Ducouso M. 2011. Role of mycorrhizal fungi on the alleviation of heavy metal toxicity on plant. In : Mycorrhizal Fungi: Use in Sustainable Agriculture and Forestry. Z.M. Solaiman, L.K. Abbott and A. Varma (Eds). *Soil Biology series* ».

3.2.1.5. Institut Agronomique Calédonien (IAC) (période 2006 - en cours)

Collaboration avec le Dr Laurent L'HUILLIER (directeur de l'IAC), le Dr Laurent MAGGIA (DR, CIRAD) et le Dr Marc DUCOUSSO (DR, CIRAD) détachés du CIRAD à l'IAC, Nouméa, Nouvelle-Calédonie. Nous travaillons en collaboration i) autour des projets porteurs ANR ULTRABIO et CNRT Ecomine Biotop et ii) l'encadrement d'étudiants en thèse de doctorats : thèse CIFRE d'Alexandre Lagrange, période 2006-2009 et la thèse de doctorat Province Sud de Simon Gensous, période 2011-2013. Cette collaboration a permis les publications **P1, P4, P5 et P6**, et les communications **C1, C3, C4, C6, C7, C8, C9 et N2**.

3.2.2. Collaborations avec des partenaires privés

3.2.2.1. Groupement de Recherche en Agriculture Biologique GRAB (période 2000-2003)

Collaboration avec Hélène VEDIE et Cyril BERTRAND du GRAB, Avignon, France. Sujet d'étude : l'utilisation des Crotalaires comme moyen de lutte intégrée contre les nématodes phytoparasites en agriculture biologique méditerranéenne. Cette collaboration a permis les communications **C12, N5 et N6**.

3.2.2.2. Société SIRAS Pacifique (période 2006-2009)

La société SIRAS pacifique est une « Société Internationale de Réhabilitation et d'Aménagement des Sites » et a pour vocation première la réhabilitation des sites dégradés par l'activité minière en Nouvelle-Calédonie. Cette société est située 6, rue Edouard Glasser Motor Pool - BP 8173 - Nouméa Cedex Nouvelle-Calédonie (site web : www.siras.nc). Nous avons collaboré avec le Dr Danièle SAINT-PIERRE, responsable du département R&D de cette société jusqu'en 2011. Notre collaboration a consisté à contribuer à l'encadrement de la thèse d'Alexandre Lagrange, dont la société SIRAS a cofinancé la bourse CIFRE. Cette collaboration a permis la publication **P4**.

3.2.2.3. Société Koniambo SA (KNS) (période 2010- en cours)

La société Koniambo SA (KNS, site web : www.koniambonickel.nc) est basée à Koné, Nouvelle-Calédonie. Cette société est chargée i) de la gestion du domaine d'exploitation du nickel sur le Massif du Koniambo ((Koné, Nouvelle-Calédonie) et ii) du montage et de la mise ne route d'une usine pyrométallurgique de traitement du minerai du nickel. Nous collaborons avec le département environnement / service de re-végétalisation dirigé par Antoine Leveau. Nous sommes actuellement engagés en partenariat avec la société KNS dans la demande de financement d'une bourse CIFRE pour une thèse de doctorat de l'Université de Nouvelle-Calédonie, sur le sujet intitulé : Stratégies de re-végétalisation des maquis miniers nickélifères de Nouvelle-Calédonie : étude sur les potentiels biologiques des topsoils et leur gestion en vue de leur utilisation pour la restauration écologique du milieu dégradé.

4) Activités de recherche

4.1. TRAVAUX DE RECHERCHE SUR LES LEGUMINEUSES DU GENRE CROTALARIA

Ces travaux de recherche ont été menés sur la période 1998-2011 :

- i) dans le cadre de mon arrivée et de mon intégration au LSTM (UMR 113) où j'ai été affecté à partir de 1998 ;
- ii) dans le cadre de mon Doctorat de Biologie Intégrative de l'Université Montpellier 2 et réalisé au sein du LSTM (UMR113) pour la période 2000-2003 ;
- iii) dans le cadre de l'encadrement du Doctorat d'Adeline Renier (période 2004-2007), puis de la valorisation des travaux de sa thèse (période 2008-2011)

4.1.1. Introduction générale : Les légumineuses du genre *Crotalaria*

Les crotalaires sont des plantes à fleurs et à fruits (Angiospermes), dicotylédones, qui appartiennent à l'ordre des Fabales, famille des légumineuses (*Fabaceae*), sous-famille des *Papilionoideae*, tribu des *Crotalariae*, genre *Crotalaria* (Polhill & Raven, 1981 ; Lecointre & Leguyader, 2001 ; Spichiger et al., 2002). Le genre *Crotalaria* comprend plus de 600 espèces (Allen & Allen, 1981). Au sein de ce genre, le botaniste Polhill a classé et réparti les espèces de crotalaires en 8 sections botaniques en se basant sur l'analyse morphologique des fleurs (Polhill, 1982). Les crotalaires sont majoritairement des plantes herbacées annuelles comme *Crotalaria atropurpurea*, mais parfois arbustives comme *C. juncea*, et quelquefois pérennes comme *C. cylindrocarpa* (White et al., 1993 ; White, 2002).

La répartition géographique des crotalaires est vaste et couvre principalement les zones sub-tropicales et intertropicales en Afrique, en Amérique, en Asie et en Océanie (Polhill & Raven, 1981). Plus de 500 espèces originaires d'Afrique et de Madagascar ont été répertoriées par Polhill (1982). Mais, si certaines espèces sont rencontrées dans des zones géographiques limitées comme *C. glaucoidea* que l'on trouve en Guinée-Bissau, Mali et Sénégal (Polhill, 1982), d'autres espèces couvrent presque tous les continents. Ainsi, l'espèce *C. pallida* est signalée en Afrique (36 pays), en Asie (17 pays), en Océanie (19 pays), en Amérique Centrale et Amérique du Sud (16 pays). Plus rares, certaines espèces ont été signalées en zone tempérée : ainsi *C. sagittalis* est présente en Amérique du Nord (White et al., 1993; White, 2002).

4.1.2. La symbiose atypique avec des bactéries du genre *Methylobacterium*

4.1.2.1. Introduction

Les crotalaires sont des légumineuses fixatrices d'azote : la méthode de dilution isotopique de l'azote ^{15}N permet de montrer que *C. ochroleuca*, *C. perrotteti* et *C. retusa* sont capables de fixer l'azote atmosphérique (Sy et al., 2000). Comme chez la plupart des légumineuses, le siège de la fixation de l'azote est un organe racinaire appelé nodule. Dans cet organe, se met en place une symbiose entre une bactérie fixatrice d'azote appelée communément rhizobium et la légumineuse (Fischer et al., 1992).

La plupart des souches bactériennes fixatrices d'azote développant une symbiose avec des crotalaires isolées à partir de leurs nodules racinaires appartiennent au genre *Bradyrhizobium* (Trinick & Hadobas, 1989 ; Gao et al., 1994 ; Samba et al., 1999 ; You et al., 2002). Récemment, et de façon inattendue, d'autres souches bactériennes isolées de nodules de crotalaires d'Afrique de l'Ouest, ont été identifiées comme appartenant au genre *Methylobacterium* (Sy et al., 2001a, b).

4.1.2.2. Caractérisation d'une nouvelle espèce bactérienne : *Methylobacterium nodulans*

Cette caractérisation s'est faite en 2 étapes : dans une 1^{ère} étape, la publication de l'article Sy et al., (2001) rapporte i) la description générale de la nouvelle espèce bactérienne et ii) ses caractéristiques métaboliques, nodulantes et fixatrices d'azote. Le résumé de cet article est présenté ci-dessous :

Rhizobia described so far belong to three distinct phylogenetic branches within the α -2 subclass of Proteobacteria. Here we report the discovery of a fourth rhizobial branch involving bacteria of the *Methylobacterium* genus. Rhizobia isolated from *Crotalaria* legumes were assigned to a new species, "*Methylobacterium nodulans*," within the *Methylobacterium* genus on the basis of 16S ribosomal DNA analyses. We demonstrated that these rhizobia facultatively grow on methanol, which is a characteristic of *Methylobacterium* spp. but a unique feature among rhizobia. Genes encoding two key enzymes of methylotrophy and nodulation, the *mxoF* gene, encoding the α subunit of the methanol dehydrogenase, and the *nodA* gene, encoding an acyltransferase involved in Nod factor biosynthesis, were sequenced for the type strain, ORS2060. Plant tests and *nodA* amplification assays showed that "*M. nodulans*" is the only nodulating *Methylobacterium* sp. identified so far. Phylogenetic sequence analysis showed that "*M. nodulans*" NodA is closely related to *Bradyrhizobium* NodA, suggesting that this gene was acquired by horizontal gene transfer.

Dans une 2^{ème} étape, la publication Jourand et al. (2004), rapporte la description complète de la nouvelle espèce bactérienne avec la récapitulation des propriétés et caractéristiques et propose le nom *Methylobacterium nodulans*. Le résumé de cet article est présenté ci-dessous :

Data on 72 non-pigmented bacterial strains that specifically induce nitrogen-fixing root nodules on the legume species *Crotalaria glaucoidea*, *C. perrottetii* and *C. podocarpa* are reviewed. By SDS-PAGE analysis of total protein patterns and by 16S rRNA PCR-RFLP, these strains form a homogeneous group that is separate from other legume root-nodule-forming bacteria. The 16S rRNA gene-based phylogeny indicates that these bacteria belong to the genus *Methylobacterium*. They can grow on C1 compounds such as methanol, formate and formaldehyde but not methylamine as sole carbon source, and carry an *mxoF* gene, encoding methanol dehydrogenase, which supports their methylotrophic metabolism. Presence of a *nodA* nodulation gene, and ability to nodulate plants of *Crotalaria* species and to fix nitrogen are features that separate the strains currently included in this group from other members of the genus *Methylobacterium*.

The present study includes additional genotypic and phenotypic characterization of this novel *Methylobacterium* species, i.e. nifH gene sequence, morphology, physiology, enzymic and carbon source assimilation tests and antibiotic resistance. The name *Methylobacterium nodulans* sp. nov. (type strain, ORS 2060^T=CNCM I 2342^T=LMG 21967^T) is proposed for this group of root-nodule-forming bacteria.

4.1.2.3. Expression de la méthylotrophie dans la symbiose fixatrice d'azote

La méthylotrophie est une fonction facultative chez les *Methylobacterium* : il s'agit de la capacité pour ces bactéries à dégrader des composés mono-carbonés (excepté le méthane et le CO₂), comme source unique de carbone ou d'énergie (Whittenbury, et al., 1970 ; Wittenbury & Dalton, 1981 ; Anthony, 1982 ; 1991 ; Lidstrom, 1992).

L'étude de la fonction méthylotrophe a débuté par la caractérisation des enzymes impliquées dans les processus biochimiques d'oxydation de ces composés C1 et de leur transformation en formaldéhyde, composé central du métabolisme méthylotrophique (Anthony, 1982). La dégradation du méthanol en formaldéhyde s'effectue grâce à une enzyme périplasmique, la méthanol-déshydrogénase (MDH) (Anthony, 1992). C'est une protéine tétramérique de configuration $\alpha_2\beta_2$ dont chaque sous-unité α et β fait respectivement 66 et 9 Kda (Nunn & Anthony, 1988 ; Nunn et al., 1989). La sous-unité α est associée à un groupement prosthétique, la pyrrolo-quinoline-quinone (PQQ), et interagit avec un ion Ca²⁺. Ces deux cofacteurs sont essentiels au bon fonctionnement de la MDH (Richardson & Anthony, 1992 ; Anthony et al., 1994). De plus, cette enzyme nécessite un transporteur d'électron spécifique, le cytochrome CL (Anthony et al., 1994).

Nous avons étudié l'expression de la méthylotrophie dans la symbiose fixatrice d'azote entre *Methylobacterium nodulans* et sa plante hôte *Crotalaria podocarpa*. Les résultats de ces travaux sont rapportés dans la publication Jourand et al., (2005) dont le résumé est présenté ci-dessous :

Some rare leguminous plants of the genus *Crotalaria* are specifically nodulated by the methylotrophic bacterium *Methylobacterium nodulans*. In this study, the expression and rôle of bacterial methylotrophy were investigated during symbiosis between *M. nodulans*, strain ORS 2060T, and its host legume, *Crotalaria podocarpa*. Using lacZ fusion to the mxaF gene, we showed that the methylotroph genes are expressed in the root nodules, suggesting methylotrophic activity during symbiosis. In addition, loss of the bacterial methylotrophic function significantly affected plant development. Indeed, inoculation of *M. nodulans* non-methylotroph mutants in *C. podocarpa* decreased the total root nodule number per plant up to 60%, decreased the whole-plant nitrogen fixation capacity up to 42%, and reduced the total dry plant biomass up to 46% compared with the wild-type strain. In contrast, inoculation of the legume *C. podocarpa* with nonmethylotrophic mutants complemented with functional mxa genes restored the symbiotic wild phenotype. These results demonstrate the key role of methylotrophy during symbiosis between *M. nodulans* and *C. podocarpa*.

4.1.2.4. Structure et fonctionnement du nodule fixateur d'azote

Suite à la mise en évidence de l'expression de la méthylotrophie de *Methylobacterium nodulans* dans le nodule fixateur d'azote chez la plante hôte *Crotalaria podocarpa*, nous avons complété les travaux sur le plan histologique pour comprendre l'organisation et le fonctionnement du nodule et essayer d'établir un lien entre l'expression de la fonction méthylotrophique exprimée par la bactérie et le métabolisme énergétique général dans le nodule. Les résultats de ces travaux sont rapportés dans la publication Renier et al., (2011) dont le résumé est présenté ci-dessous

Crotalaria are plants of the Fabaceae family whose nodulation characteristics have been little explored despite the recent discovery of their unexpected ability to be efficiently nodulated in symbiosis with bacteria of the genus *Methylobacterium*. It has been shown that methylophony plays a key role in this unusual symbiotic system, as it is expressed within the nodule and as non-methylophony mutants had a depleting effect on plant growth response. Within the nodule, *Methylobacterium* is thus able to obtain carbon both from host plant photosynthesis and from methylophony. In this context, the aim of the present study was to show the histological and cytological impacts of both symbiotic and methylophony metabolism within *Crotalaria podocarpa* nodules. It was established that if *Crotalaria* nodules are multilobed, each lobe has the morphology of indeterminate nodules but with a different anatomy; that is, without root hair infection or infection threads. In the fixation zone, bacteroids display a spherical shape and there is no uninfected cell. *Crotalaria* nodulation by *Methylobacterium* displayed some very unusual characteristics such as starch storage within bacteroid-filled cells of the fixation zone and also the complete lysis of apical nodular tissues (where bacteria have a free-living shape and express methylophony). This lysis could possibly reflect the bacterial degradation of plant wall pectins through bacterial pectin methyl esterases, thus producing methanol as a substrate, allowing bacterial multiplication before release from the nodule.

4.1.2.5. Support moléculaire classique de cette symbiose atypique

Enfin pour compléter cette étude sur cette symbiose atypique, nous avons voulu savoir quel était le support du dialogue moléculaire entre la bactérie et sa plante hôte, en comparaison avec le dialogue moléculaire classique connu entre les légumineuses et les rhizobiums nodulants et fixateurs d'azote. Les résultats de ces travaux sont rapportés dans la publication Renier et al., (2008) dont le résumé est présenté ci-dessous :

Some legume species of the *Crotalaria* genus are specifically nodulated by methylophony bacteria belonging to the *Methylobacterium nodulans* species. The feature of this symbiotic bacterium is its ability to oxidize methanol, a property based on the presence of a methanol dehydrogenase enzyme. Despite a good knowledge of this property and its implication in symbiosis, the molecular dialogue between *M. nodulans* and *Crotalaria podocarpa* leading to symbiosis is largely unknown, except the presence of a *nodA* nodulation gene in the genome of *M. nodulans* ORS 2060.

To investigate if *M. nodulans* ORS 2060 produces Nod factors, molecules considered as the major bacteria-to-plant signals essential for the establishment of rhizobia-legume symbiosis, we identified and sequenced a nodDABCUIJHQ cluster from a genomic library of ORS 2060. Phylogenetic analyses of nod genes revealed that *M. nodulans* ORS 2060 form a branch together with *Burkholderia tuberum* STM678 and a strain of *Methylobacterium* sp. (4-46) isolated from Lotononis, and distinct from all the other rhizobia. To analyse the regulation of ORS 2060 nod genes, we constructed a nodA-LacZ promoter fusion to monitor the nod gene expression with various flavonoids. The flavone apigenin was found to be the strongest inducer of nod gene expression in *M. nodulans* ORS 2060. This latter flavonoid was used to induce ORS 2060, and Nod factors were purified by high-performance liquid chromatography (HPLC) and further characterized by mass spectrometry. One major Nod factor structure was identified as a pentamer of chitin substituted by C18:1 or C16:0 acyl chains on the non-reducing end and 6-O-sulphated on the other end, suggesting a classic symbiotic dialogue between *M. nodulans* and *C. podocarpa*.

4.1.2.6. Conclusions

Nous avons caractérisé la symbiose fixatrice d'azote originale entre des bactéries du genre *Methylobacterium* avec trois espèces de *Crotalaria*, i.e. *C. glaucoïdes*, *C. perrottetii* et *C. podocarpa*. Ces *Crotalaria* sont nodulées par l'espèce bactérienne, *Methylobacterium nodulans*, qui présente non seulement la fonction métabolique méthylotrophique mais aussi les capacités à noduler et à fixer l'azote. Cette symbiose *Methylobacterium nodulans* / *Crotalaria podocarpa* est une symbiose originale. En effet, la méthylotrophie, propriété remarquable de la bactérie, s'exprime à l'apex du nodule et modifie localement le métabolisme: une dégradation apicale marquée des tissus est observée suite à une digestion des parois végétales, libérant du méthanol directement utilisable par la bactérie. Grâce à ce métabolisme, le bactéroïde -de taille et de forme inhabituellement grandes- fournirait l'énergie nécessaire à l'activité de la nitrogénase, et permettrait à la plante d'accumuler des réserves carbonées sous forme d'amyloplastes dans les cellules infectées. Cette propriété méthylotrophique bactérienne apporte également à la plante-hôte un gain de biomasse (>40 % par rapport aux mutants non méthylotrophes). En revanche, à l'échelle de l'espèce, la Crotalaire n'est pas avantagée, en termes de fixation d'azote, à s'associer avec *M. nodulans* ou avec *Bradyrhizobium* sp., autre genre bactérien décrit en symbiose avec ces plantes. L'originalité de cette association symbiotique se situe également au niveau du dialogue moléculaire : les gènes nod de *M. nodulans* sont insensibles aux isoflavones, flavonoïdes inducteurs des *Bradyrhizobium*, mais sont induits par les flavanones et les flavones. L'analyse phylogénétique des gènes nod révèle que *M. nodulans* forme une branche particulière avec *Methylobacterium* sp. 4-46 et *Burkholderia tuberum* STM678, isolées de nodules de légumineuses appartenant à la tribu des Crotalariae. Au niveau du signal bactérien, une structure unique de facteur Nod a été caractérisée chez *M. nodulans* ORS2060^T : MnV(C18:1, S). Enfin, si la symbiose fonctionnelle *M. nodulans*/ *Crotalaria* a été identifiée seulement chez trois espèces de plante, la capacité du partenaire bactérien à former des pseudonodules avec d'autres *Crotalaria* laisse entrevoir un rôle clé d'autres composés pendant le processus d'infection suggérant d'autres mécanismes moléculaires impliqués dans la spécificité d'hôte

4.1.3. Utilisation des crotalaires pour le contrôle des populations de nématodes phytoparasites

Dans le cadre de ma thèse (2000-2003), j'ai souhaité mener des travaux en accord avec les fondements de la politique scientifique de mon employeur, l'IRD, c'est à dire la recherche développement pour les pays du Sud. C'est pourquoi un ensemble de travaux de recherche ont été menés sur les utilisations potentielles des légumineuses du genre *Crotalaria* en agriculture dans la lutte contre les nématodes phytoparasites, application principale de ces plantes en agriculture des pays du Sud très largement mentionnée dans la littérature (au 01/07/2011, il existe 181 références bibliographiques à ce sujet disponibles dans la base de données CAB abstract couvrant la période de 1973-2011).

4.1.3.1. Introduction

Les utilisations agronomiques des crotalaires sont multiples : les moins toxiques d'entre-elles ont été testées avec succès comme fourrage pour le bétail (Machado et al., 1994). L'espèce arbustive, *C. juncea*, est cultivée en Inde pour la production de fibres et la fabrication de cordages (130.000 tonnes par an) (Vietmeyer, 1979). Grâce à leur potentiel fixateur d'azote, de nombreuses espèces ont été utilisées avec succès comme fertilisant azoté (Dumont & Nguyen 1938 ; Sandanam et al., 1976 ; Mwambazi et al., 1998 ; Becker & Johnson, 1999 ; Fischler et al., 1999). Enfin, plusieurs espèces de crotalaires sont résistantes aux nématodes phytoparasites des plantes cultivées (Silva et al., 1989a, b ; Desaegeer & Rao, 1999). La culture de ces espèces résistantes comme plantes d'assolement permet de réduire sensiblement certaines populations de nématodes dans les sols cultivés (Brodie & Murphy, 1975 ; Moura, 1991 ; Shepherd & Barker, 1993 ; Desaegeer & Rao, 2000).

4.1.3.2. Utilisation d'un extrait de *Crotalaria* en protection de culture

Dans un 1^{er} temps nous avons voulu vérifier le potentiel phytoprotecteur d'un extrait de *Crotalaria* sur une culture maraîchère vis à vis d'un nématode phytoparasite appartenant au genre *Meloidogyne*. Pour cela nous avons testé un extrait de *Crotalaria grantiana* sur des plants de tomates infestés par *Meloidogyne incognita*. Les résultats de ces travaux ont fait l'objet d'une publication (P11) dont le résumé est présenté ci-dessous :

An aqueous extract from leaves of *Crotalaria virgulata* subsp. *grantiana* was assayed for its effects on second-stage juveniles of *Meloidogyne incognita*. The biological activity was nematostatic; nematodes were not killed but were completely paralysed in a 1 mg/ml (w/v) extract: the LD₅₀ equivalent was estimated to be 0.5 mg/ml. The effect was reversible: juveniles previously paralysed by *C. grantiana* extract recovered complete mobility in water and were able to infest a susceptible tomato plant. Freeze-dried aqueous extract from *C. grantiana* leaves added to a sterile sandy substrate at 1 mg/ml protected susceptible tomato plants from *M. incognita* infestation.

This suggests a promising use of *C. grantiana* as both a green manure and natural alternative to synthetic chemicals in nematode population control, especially in integrated pest management for vegetable crops in organic agriculture of tropical and temperate areas.

4.1.3.3. Activité nématostatique des extraits de *Crotalaria*

Dans un 2^{ème} temps, nous avons choisi d'étudier sur 15 espèces de crotalaires originaires d'Afrique de l'Ouest la sensibilité de ces légumineuses à certaines espèces de *Meloidogyne*. Ensuite, nous avons criblé l'activité des extraits de feuilles et de racines de ces espèces 15 crotalaires sur des juvéniles de *Meloidogyne*. L'objectif de cette étude était de montrer que les crotalaires d'Afrique de l'Ouest nodulées par les *Methylobacterium* comme celles nodulées par les *Bradyrhizobium* présentent des propriétés similaires de résistance et de toxicité vis à vis de nématodes du genre *Meloidogyne* et donc un même potentiel agronomique dans l'utilisation en agriculture comme moyen de lutte contre les nématodes phytoparasites. Les résultats de ces travaux ont fait l'objet d'une publication (P11) dont le résumé est présenté ci-dessous :

The *in vitro* paralysis of second-stage juveniles of *Meloidogyne incognita*, *M. javanica* and *M. mayaguensis* by aqueous extracts of 15 West African *Crotalaria* species was analysed. A multivariate analysis distinguished four groups, based on their nematostatic activity: i) *C. glaucoidea* extracts were not active; ii) the root extracts from *C. goreensis*, *C. lathyroides* and *C. perrottetii* were more active than the shoot extracts; iii) the shoot extracts from *C. comosa* and *C. cylindrocarpa* were more active than the root extracts; iv) both shoot and root extracts of *C. atropurpurea*, *C. barkae*, *C. grantiana*, *C. hyssopifolia*, *C. pallida*, *C. podocarpa*, *C. retusa*, *C. senegalensis* and *C. sphaerocarpa* have nematostatic effects depending on either the *Crotalaria* species or plant tissue from which the extracts were derived. However, considering the greater biomass contribution of the leaves and stems compared to the roots when the plants are used as green manure, *C. barkae*, *C. grantiana*, *C. pallida* and *C. podocarpa* are the most efficient *Crotalaria* species, whatever the *Meloidogyne* species targeted.

4.1.3.4. Essais expérimentaux de contrôles de population de nématodes au champ

Des essais expérimentaux au champ d'utilisation de *Crotalaria* pour le contrôle de population de nématodes phytoparasites ont été réalisés simultanément en agriculture biologique méditerranéenne en France et tropicale au Sénégal.

Les résultats des travaux menés au champ en agriculture biologique méditerranéenne en France ont fait l'objet de la communication orale (C12) au 14^{ème} congrès international de l'agriculture biologique en 2002 qui s'est tenu à Vancouver au Canada (Jourand et al., 2002), dont les résultats majeurs sont résumés ci-dessous :

First, among the 22 *Crotalaria* species investigated, only *C. grantiana*, *C. juncea*, *C. podocarpa*, *C. retusa*, *C. spectabilis* and *C. sphaerocarpa* were able to grow correctly in Mediterranean conditions. After 3 months, their fresh vegetative biomass reached 1.8 kg/m² for *C. spectabilis*, 1.6 kg/m² for *C. juncea* and *C. retusa*, 1.2 kg/m² for *C. grantiana*, 1 kg/m² for *C. sphaerocarpa* and 0.4 kg/m² for *C. podocarpa*. Total nitrates measured before and after green manuring increased from 100 ppm to 250 ppm for *C. grantiana*, *C. juncea*, *C. retusa*, *C. spectabilis*, *C. sphaerocarpa* and confirmed a good nitrogen fixing ability. Then, as the resistance of *C. juncea* and *C. spectabilis* to nematodes *Meloidogyne incognita* and *M. javanica* as well as the nematostatic activity of their aqueous leave and root extracts were previously confirmed, we demonstrated also the resistance of the other 20 West African *Crotalaria* species to these both nematodes species. In addition, leave and root aqueous extracts of *C. goreensis*, *C. grantiana*, *C. hyssopifolia*, *C. podocarpa*, *C. retusa*, and *C. sphaerocarpa* showed the highest in vitro nematostatic activity on juveniles of both *Meloidogyne* species. Finally, in the field experiment, a previous crop of *C. grantiana* highly protected the cucumber plants from *M. incognita* as the root gall index was significantly lower than on cucumber cropped in untreated areas (5.3 and 17.5 respectively).

Les résultats des travaux menés au champ en agriculture biologique tropicale au Sénégal sont publiés dans mon mémoire de thèse (Jourand, 2003) dont les résultats majeurs sont résumés ci-dessous :

Sur les parcelles d'un sol infesté par des nématodes phytoparasites de l'IRD de Dakar (Sénégal), on a cultivé successivement du gombo puis sur certaines parcelles des crotalaires (*C. comosa* et *C. senegalensis*) tandis que d'autres sont restées nues. Enfin, après les crotalaires, on a cultivé sur toutes les parcelles, des tomates puis du gombo (*Hibiscus esculentum*). Les parcelles ayant eu précédent cultural avec l'une ou l'autre espèce de crotalaire (*C. comosa* ou *C. senegalensis*) donnent des résultats comparables : les pieds de tomate présentent un même développement végétatif, un indice de galles identiques et des rendements similaires. De plus, la végétation aérienne de ces pieds de tomates est nettement plus vigoureuse que celle des pieds de tomates ayant poussé sur les surfaces témoins. Les racines sont majoritairement saines, avec peu de galles ; elles présentent un système racinaire abondant et très développé comparativement aux racines des plants témoins qui ont un système réduit avec de nombreuses galles. L'indice de galles des surfaces témoins et des surfaces assolées avec *C. comosa* et *C. senegalensis* était respectivement de 2,9 ; de 0,9 et de 1,2. Corrélativement, en fin de cycle, les rendements des parcelles témoins étaient significativement moins élevés que les autres parcelles soit : 1,15 kg/pied (témoin) versus 1,86 kg/pied (après *C. comosa*) et 1,94 kg /pied (après *C. senegalensis*).

Sur les surfaces assolées avec l'une ou l'autre espèce de crotalaires on n'observe pas de différence notable de végétation, d'indice de galles et de rendement. Les plantes des parcelles témoins sont chétives, rabougries et affichent une chlorose diffuse du feuillage alors que les plantes des parcelles après culture de crotalaire ont un développement et un faciès normal. Le système racinaire est nécrotique et porte peu de racines secondaires. De plus il est, dans les cas extrêmes, réduit au pivot entouré d'un manchon de grosses galles.

L'indice de galles moyen des surfaces témoins (3,5) est proche du maximum de l'échelle de cotation ; il est plus élevé que celui des autres parcelles (2,3 et 2.1), lui-même proche de celui relevé avant la mise en place du dispositif expérimental (2,5). Les rendements des parcelles assolées avec *C. comosa* et *C. senegalensis* sont respectivement, en fin de récolte, de 2,57 kg/10 pieds et de 2,65 kg/10 pieds et ils sont significativement plus élevés que les témoins 1,57 kg /10 pieds.

4.1.3.5. Conclusions

Nous avons testé l'activité biologique des trois *Crotalaria* nodulées par *Methylobacterium nodulans* et de douze autres espèces de *Crotalaria* nodulées par *Bradyrhizobium* sp. vis à vis de nématodes phytoparasites majeurs des plantes cultivées. Elles sont toutes résistantes aux principaux phytoparasites du genre *Meloidogyne*. Les extraits aqueux de feuilles et de racines de la majorité d'entre-elles paralysent réversiblement les nématodes juvéniles au stade infestant, suggérant un fort potentiel nématostatique de leur engrais vert. Les essais agronomiques en conditions méditerranéennes ou tropicales montrent que la culture de la plupart de ces *Crotalaires*, comme leur engrais vert, permettent de réduire fortement la population dominante de nématodes protégeant ainsi efficacement les cultures maraîchères sensibles. Les résultats obtenus laissent entrevoir une utilisation prometteuse de ces légumineuses en agriculture comme alternative naturelle aux engrais azotés et aux nématicides chimiques.

4.2. TRAVAUX DE RECHERCHE SUR LA SYMBIOSE ECTOMYCORHIZIENNE

4.2.1. D'une symbiose atypique mais classique à une symbiose en milieu extrême

Après avoir consacré plus de 10 ans de travaux de recherche sur la symbiose atypique entre *Methylobacterium nodulans* et *Crotalaria podocarpa*, je travaille depuis 2005 sur la symbiose ectomycorhizienne entre le champignon *Pisolithus albus* et ses plantes hôtes, majoritairement appartenant à la famille *Myrtaceae* et des *Fabaceae*, symbiose atypique car se développant dans conditions édaphiques extrêmes : les sols ultramafiques des maquis miniers de Nouvelle-Calédonie.

En effet, l'histoire géologique unique de la Nouvelle-Calédonie, archipel tropical du sud-est du Pacifique a conduit à la présence de sols ultramafiques riches en nickel sur un tiers de sa surface. Ces sols extrêmes sont caractérisés par l'omniprésence du fer (jusqu'à 85%), des concentrations élevées en métaux lourds (Ni, Cr, Co, Mn), un déséquilibre important du rapport Ca/Mg par un excès de Mg et des concentrations très faibles en N, P et K, éléments essentiels de la nutrition des plantes (Brooks, 1987). Ces sols, sous un climat tropical à sub-tropical constant depuis plusieurs millions d'années ont engendré le développement d'écosystèmes endémiques uniques au monde avec un taux d'endémisme proche de 80%, faisant de cet archipel un « hot spot » de la biodiversité (Jaffré et al, 1980 ; Myers, 2000).

Parmi ces écosystèmes, certains sont dominés par des plantes ectomycorhiziennes, *Acacia spirorbis*, *Tristaniopsis* spp., *Melaleuca* spp., *Sannantha* spp., souvent associées avec le champignon ectomycorhizien dominant *Pisolithus albus* (Perrier et al, 2006 ; Jourand et al, 2010a).

4.2.2. Diversité des populations de *Pisolithus albus* de Nouvelle-Calédonie

Notre approche a consisté dans un 1^{er} temps à caractériser les populations de *Pisolithus albus* de Nouvelle-Calédonie. L'ensemble des travaux est rapporté dans la publication Jourand et al. (2010a) et dont le résumé des travaux est présenté ci-dessous :

Isolates of ectomycorrhizal *Pisolithus albus* were sampled from both ultramafic and volcano-sedimentary soils in New Caledonia, a tropical hotspot of biodiversity, to investigate the relationships between genetic diversity and edaphic constraint through tolerance to nickel (Ni). Carpophore description, spore morphology and phylogenetic analysis based on internal transcribed spacer (ITS) rDNA sequences confirmed that all isolates belong to *P. albus* and are closely related to other Australasian specimens. Using molecular tools, ITS-restriction fragment length polymorphism and amplified fragment length polymorphism markers, we showed the existence of two distinct genetic clusters within *P. albus*: ultramafic and volcano-sedimentary. Mycelia response to Ni toxicity supports such a population structure. *Pisolithus albus* from ultramafic soils included isolates with a high diversity of in vitro Ni tolerance, with both Ni-tolerant isolates (average Ni EC₅₀ at 575 mM) and Ni-sensitive isolates (average Ni EC₅₀ at 37 mM). In contrast, all isolates from volcano-sedimentary soils were found to be Ni sensitive (average Ni EC₅₀ at 32 mM). We highlight that (1) *P. albus* population from ultramafic soils of New Caledonia are genetically structured in ecotype, and that (2) Ni tolérance among ultramafic isolates suggests an adaptive physiological response to Ni toxicity.

4.2.3. Rôle de la symbiose ectomycorhizienne dans l'adaptation de la plante hôte

Dans un 2^{ème} temps, nous avons cherché à mettre en évidence le rôle du champignon ectomycorhizien symbiotique dans l'adaptation de sa plante hôte à la contrainte édaphique ultramafique. Nous avons commencé notre approche par une étude de l'impact du champignon ectomycorhizien *Pisolithus albus* sur la tolérance au nickel (métal lourd le plus toxique rencontré dans ces sols extrêmes) lors de la symbiose avec une plante hôte modèle de la famille des Myrtaceae, i.e. *Eucalyptus globulus*. L'ensemble des travaux est rapporté dans la publication Jourand et al. (2010b) et dont le résumé des travaux est présenté ci-dessous :

Ectomycorrhizal (ECM) *Pisolithus albus* (Cooke & Massee), belonging to the ultramafic ecotype isolated in nickel-rich serpentine soils from New Caledonia (a tropical hotspot of biodiversity) and showing in vitro adaptive nickel tolerance, were inoculated to *Eucalyptus globulus* Labill used as a Myrtaceae plant-host model to study ectomycorrhizal symbiosis.

Plants were then exposed to a nickel (Ni) dose-response experiment with increased Ni treatments up to 60 mg kg⁻¹ soil as extractable Ni content in serpentine soils. Results showed that plants inoculated with ultramafic ECM *P. albus* were able to tolerate high and toxic concentrations of Ni (up to 60 µg g⁻¹) while uninoculated controls were not. At the highest Ni concentration tested, root growth was more than 20-fold higher and shoot growth more than 30-fold higher in ECM plants compared with control plants. The improved growth in ECM plants was associated with a 2.4-fold reduction in root Ni concentration but a massive 60-fold reduction in transfer of Ni from root to shoots. *In vitro*, *P. albus* strains could withstand high Ni concentrations but accumulated very little Ni in its tissue. The lower Ni uptake by mycorrhizal plants could not be explained by increased release of metal-complexing chelates since these were 5- to 12-fold lower in mycorrhizal plants at high Ni concentrations. It is proposed that the fungal sheath covering the plant roots acts as an effective barrier to limit transfer of Ni from soil into the root tissue. The degree of tolerance conferred by the ultramafic *P. albus* isolates to growth of the host tree species is considerably greater than previously reported for other ECM. The primary mechanisms underlying this improved growth were identified as reduced Ni uptake into the roots and markedly reduced transfer from root to shoot in mycorrhizal plants. The fact that these positive responses were observed at Ni concentrations commonly observed in serpentine soils suggests that ultramafic ecotypes of *P. albus* could play an important role in the adaptation of tree species to soils containing high concentrations of heavy metals and aid in strategies for ecological restoration.

4.2.4. Déterminants moléculaires de la tolérance au nickel

Suite à la caractérisation de la population de champignon ECM *Pisolithus albus* (Jourand et al., 2010a) et suite à l'étude de son rôle dans l'adaptation de sa plante hôte dans la tolérance au nickel (Jourand et al., 2010b), nous avons menés des travaux pour rechercher et identifier des déterminants génétiques impliqués dans l'adaptation au « syndrome serpentinique », avec utilisation du nickel comme marqueur de l'ultramafisme, par analyse transcriptomique massive chez *Pisolithus albus*. A partir des souches de *P. albus* sensibles et hypertolérantes au nickel isolées ultramafiques et caractérisées au LSTM (Jourand et al., 2010a,b), une étude transcriptomique par séquençage à haut débit a été développée. L'ensemble des travaux est rapporté dans la publication Majorel et al. (2011) soumise pour publication dans la revue *Molecular Ecology* et dont le résumé des travaux est présenté ci-dessous :

Ectomycorrhizal fungus *Pisolithus albus*, tolerant to nickel, isolated from ultramafic soils in New Caledonia, a hotspot of biodiversity in the South Pacific, was screened to track specific nickel-responsive genes. We used pyrosequencing and quantitative polymerase chain reaction approaches to explore and compare the transcriptomes of a Ni-tolerant isolate of *P. albus* that had been grown with nickel at a concentration found in ultramafic soils. A total of 1,071,375 sequencing reads were assembled into 19,518 putative genes. Comparison of levels of expression revealed that 30% of the identified genes were modulated by nickel stress. The genes whose expression was induced most markedly by nickel encoded products that are involved in many putative biological functions, such as the modification of cellular components (53%), and the regulation of biological processes (27%) or molecular functions (20%).

Furthermore, genes up regulated by nickel were expressed exclusively in nickel tolerant isolates, thereby identifying themselves as specific biomarkers of the tolerance of the fungus to nickel. The result that *P. albus* nickel tolerant isolates express nickel specific genes, argue in favour an adaptive response system to nickel-rich environments. On the contrary, the no detection of these genes in nickel sensitive isolates, suggests that these isolates could prosper in ecological niches where nickel might be at very low level. Furthermore, the analysis of specific biomarkers for nickel tolerance in *P. albus* that can distinguish between the two nickel phenotypes within the ultramafic ecotype should help to elucidate how fungal species can adapt to extreme environments.

4.2.5. Conclusions et perspectives

Nous avons montré que les populations de *Pisolithus albus* de Nouvelle-Calédonie i) sont structurées en écotype en fonction de la contrainte édaphique ultramafique et en particuliers en fonction de leur tolérance sensibilité le nickel, métal lourd le plus toxique rencontré dans ces sols, ii) présentent une variabilité dans leur tolérance au nickel, liée à la présence de déterminants moléculaires spécifiques de l'adaptation à la présence de ce métal, et iii) ces *P. albus* tolérant au nickel sont capables de protéger leur plante hôte contre ce métal. Tous ces résultats suggèrent le fort potentiel de ces champignons pour leur utilisation en symbiose avec leurs plantes hôtes dans les stratégies de restauration écologique des sites miniers après exploitation. Nous poursuivons nos travaux i) en élargissant l'étude du support moléculaire de l'adaptation de *P. albus* (écotype ultramafique) à plusieurs paramètres clés de l'ultramafisme (autres métaux tels que Co, Cr et Mn, ainsi que le fort déséquilibre du ratio Ca/Mg), et ii) en procédant à des essais d'inoculation de plantes hôte (*Myrtaceae* endémique) sur terrains miniers à restaurer.

5) Projet de recherche

5. PROJET DE RECHERCHE

5.1. Intitulé « Rôles des rhizobiums dans l'adaptation des légumineuses aux contraintes abiotiques des sols : le modèle *Acacia spirorbis* en Nouvelle-Calédonie : adaptation à l'ultramafisme ».

5.2. Contexte

La Nouvelle-Calédonie est une île du pacifique sud qui, suite au phénomène géologique rare de l'obduction, a été recouverte d'un manteau de roches ophiolitiques issu de la croûte océanique. L'altération supergène de ce manteau depuis plus de 30 millions d'années a conduit à sa disparition sur les deux tiers de ce territoire et à l'existence sur environ un tiers de roches dites « ultramafiques » altérées qui se caractérisent par un pourcentage en silice extrêmement faible (moins de 45 % en poids) et contiennent plus de 90 % de minéraux riches en fer et magnésium (caractère ma-fique : Mg et Fe). Ces roches sont également connues pour leur richesse en métaux lourds notamment le cobalt, le chrome, le manganèse et le nickel (Brooks, 1987). Ces particularités édaphiques sont probablement à l'origine de la diversité des écosystèmes terrestres de la Nouvelle-Calédonie considérée comme un « Hot-spot » de biodiversité (Myers et al., 2000) avec une flore de plus de 3000 espèces de végétaux supérieurs dont 80 % sont endémiques (Jaffré, 1974).

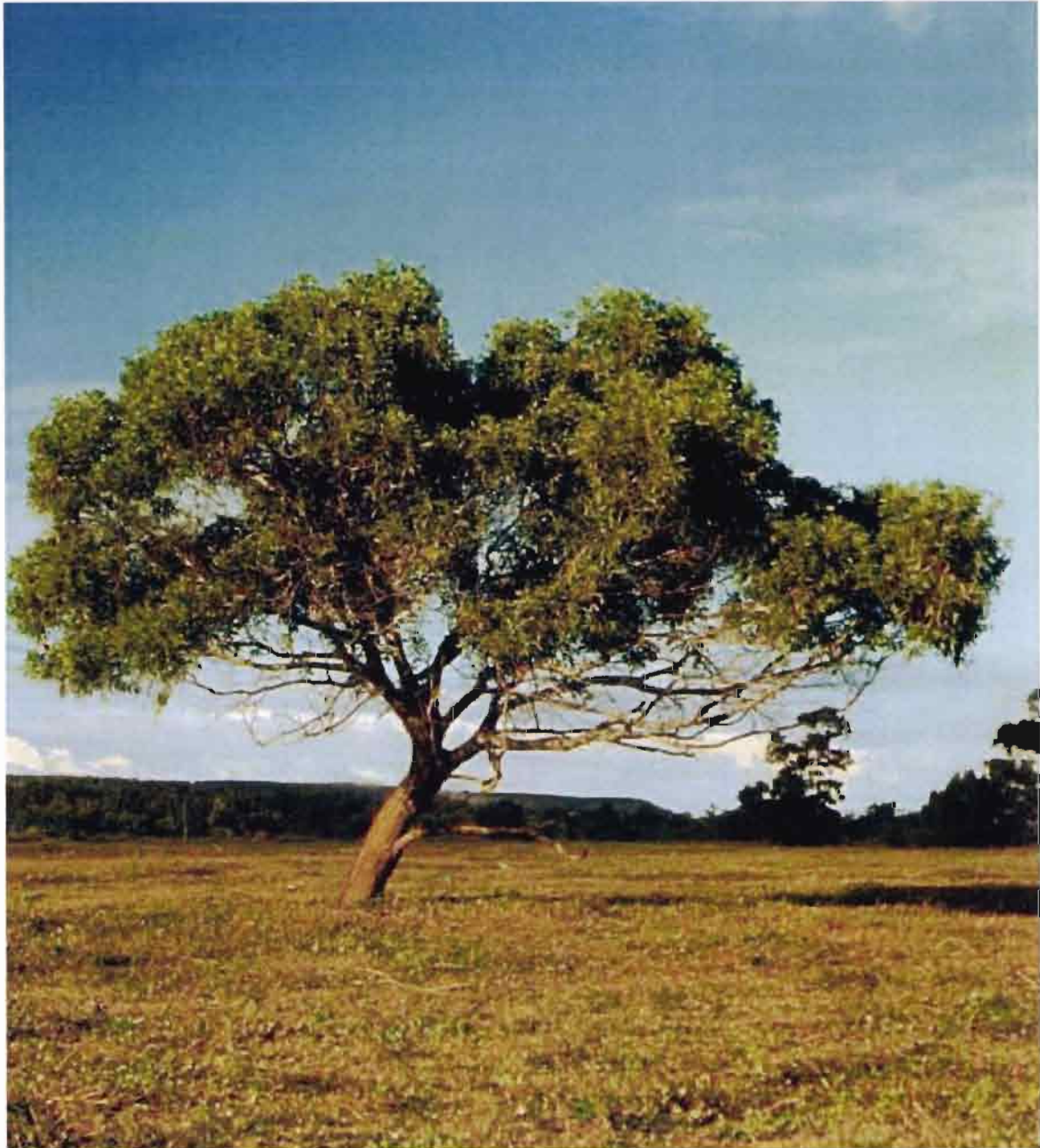
Parmi cette flore, la famille des légumineuses (3^{ème} plus grande famille de plantes à fleurs, comportant plus de 700 genres et 19000 espèces) est aussi présente en Nouvelle-Calédonie. Cependant, les observations floristiques de Jaffré et al. (1992) indiquent un faible taux d'endémisme même si les trois sous familles *Caesalpinioideae*, *Mimosoideae* et *Papilionoideae* sont présentes. Parmi, les légumineuses rencontrées, on trouve l'espèce dominante *Acacia spirorbis* décrite en 1825 par Labillardière en Nouvelle-Calédonie. Cette espèce est considérée comme une des légumineuses potentiellement endémiques des archipels mélanésien de Nouvelle-Calédonie et du Vanuatu. Elle est considérée comme voisine de l'espèce australienne *Acacia solandri* au point que Pedley (1990) propose de regrouper les deux espèces et de créer deux sous-espèces : *A. spirorbis* subsp. *solandri* en Australie et *A. spirorbis* subsp. *spirorbis* en Nouvelle-Calédonie et au Vanuatu.

En Nouvelle-Calédonie, *Acacia spirorbis* est capable de croître, de se développer et de montrer un caractère envahissant sur pratiquement tous les types de sol. On le retrouve sur les calcaires coralliens soulevés en bord de mer ou à l'intérieur des terres, dans les milieux serpentiniques à la base des massifs ultrabasiques, sur les colluvions saprolitiques et latéritiques des massifs ultrabasiques jusqu'à 400 m d'altitude, sur les schertz (silice concrétionnée) et les sables blancs, dans des schistes, des grès, des argiles ou dans des ponces volcaniques riches en aluminium (îles Loyautés). *A. spirorbis* pousse donc sur des substrats ayant une grande diversité de milieux à pH et nature variés (fer sur les milieux ultramafiques, silice sur les schertz, aluminium sur ponces volcaniques, calcium sur les calcaires coralliens) et semble adaptée aux toxicités polymétalliques, aux déséquilibres du rapport Ca/Mg et aux carences diverses.

Seule l'altitude semble représenter une limite à son ubiquité puisque l'espèce ne dépasse pas spontanément 400 m d'altitude. Cependant des plantations ont été réalisées et réussies dans les massifs miniers au delà de 900 m.

Voici quelques illustrations d'*Acacia spirorbis* de Nouvelle-Calédonie

1-1) Arbre entier sur sol volcano-sédimentaire



1-2) Arbre entier buissonnant sur sol ultramafique



2-1) Feuillage



2-2) Feuille



3) Fleurs



4) Fruits



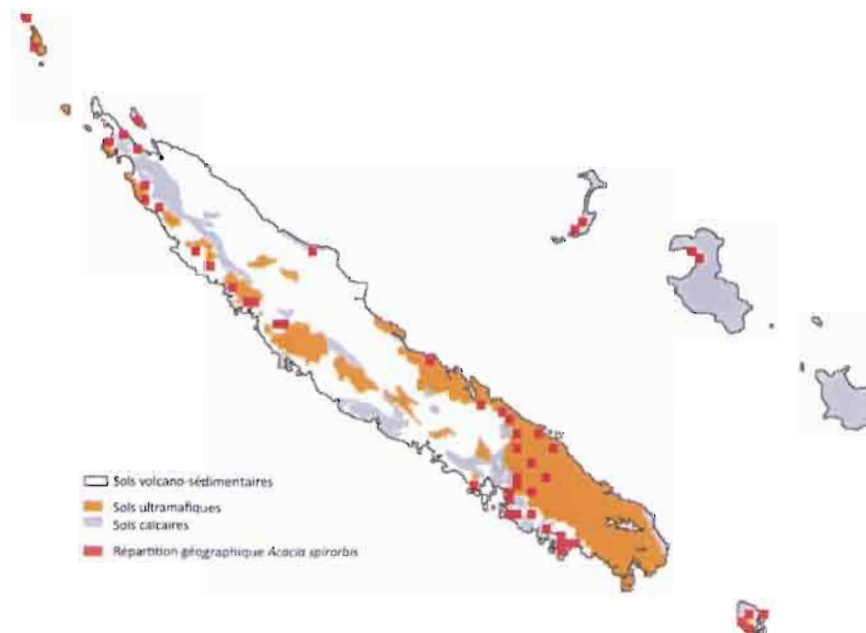
5) Graines



5) Nodules fixateurs d'azote sur racines d'*Acacia spirorbis*



6) Répartition géographique d'*Acacia spirorbis* sur l'archipel néo-calédonien.



Comme la majorité des légumineuses, et la plupart des *Acacia* (De Faria et al., 1989), *A. spirorbis* développe des nodules fixateurs d'azote en présence de rhizobiums symbiotiques (Ducousso, communication personnelle). Cette symbiose avec les rhizobiums permet à la légumineuse de s'implanter même en absence de composés azotés dans le sol.

A ce jour, aucune publication n'existe sur les bactéries symbiotiques d'*Acacia spirorbis*. Cependant, on peut supposer que, comme chez la plupart des légumineuses, le mécanisme de cette symbiose est classique. Probablement, l'initiation de la symbiose est contrôlée par des molécules signales sécrétées par les deux partenaires : *A. spirorbis* doit exsuder des flavonoïdes dans la rhizosphère, flavonoïdes qui sont reconnus par le rhizobium compatible.

Cette reconnaissance doit déclencher l'étape d'infection bactérienne de la plante. La bactérie synthétise probablement des facteurs Nod (FNs) signal de reconnaissance pour la plante hôte comme dans le processus symbiotique classique décrit entre les légumineuses et les rhizobiums (Perret et al., 2000). Les FN ont tous la même structure chimique de base (polysaccharides) dont la synthèse est assurée par les gènes bactériens nod communs nodA, nodB et nodC (Perret et al, 2000). Une fois la symbiose établit et l'organe appelé nodule formé, les gènes bactériens nifH, nifD et nifK gènes de structure du complexe nitrogénase organisés généralement en opéron (nifHDK), sont activés engendrant la synthèse de la nitrogénase, enzyme permettant le processus de transformation de l'azote atmosphérique (N_2) en azote assimilable par la plante (NH_4^+) (Postgate, 1998).

Acacia spirorbis représentant une espèce endémique de légumineuse adaptée aux sols ultramafiques, mais également capable de coloniser d'autres substrats, les études sur le couple symbiotique *A. spirorbis* / rhizobiums sur en fonction des écosystèmes sont potentiellement très intéressantes. Ces études pourraient en effet contribuer à identifier i) la diversité des rhizobiums symbiotiques, ii) la nature de flavonoïdes produits par la plante et, iii) la structure des facteurs nod synthétisés.

De plus, comme il a été récemment démontré lors de l'étude d'une autre symbiose classique légumineuse / rhizobium, développée par une légumineuse endémique de Nouvelle-Calédonie (*Serianthes calycina*), la flore symbiotique est adaptée au substrat ultramafique (Chaintreuil et al., 2007). On peut raisonnablement penser que la flore bactérienne symbiotique d' *A. spirorbis* présente aussi les mêmes capacités d'adaptation aux contraintes édaphiques quand elles existent. Cela se pourrait se traduire par l'existence de déterminants génétiques moléculaires (gènes) impliqués dans les mécanismes de tolérance aux contraintes édaphiques. La recherche et la caractérisation de tels déterminants et l'étude de leurs flux en fonction i) des genres et espèces bactérienne symbiotiques rencontrés, et ii) des écosystèmes variés sur les quels se développe *A. spirorbis*, pourrait aboutir à argumenter en faveur de l'hypothèse générale des flux de gènes entre microorganismes support de leur adaptation à la contrainte édaphique ultramafique et de comprendre en quoi ces microorganismes adaptées contribuent à leur tour à l'adaptation de leur plante hôte (Kazakou et al, 2008).

5.3. Etude de la diversité des rhizobiums associés à *Acacia spirorbis* en fonction des sols de prélèvement et des écosystèmes

La question posée est de savoir si on observe une diversité des populations de rhizobiums symbiotiques selon le type de sol sur lequel *Acacia spirorbis* se développe, i.e. sols ultramafiques, sols calcaires enrichis en aluminium et sols volcano-sédimentaires.

5.3.1. Caractéristiques physico-chimiques de sols

Il s'agira de collecter des échantillons de sols rhizosphériques au pied de plusieurs individus d'*Acacia spirorbis*, sur plusieurs sites et sur les 3 types de sols mentionnés. L'étude des caractéristiques physiques et les analyses chimiques des sols rhizosphériques seront conduites comme précédemment décrit par Perrier et al. (2006). Les résultats seront intégrés aux résultats menés sur la diversité des rhizobiums symbiotiques et du fonctionnement des symbioses chez *Acacia spirorbis*.

5.3.2. Diversité des bactéries symbiotiques fixatrices d'azote du groupe des rhizobiums

Principe : il s'agira de collecter des nodules sur plusieurs individus d'*Acacia spirorbis*, sur plusieurs sites et sur les 3 types de sols mentionnés afin d'étudier i) la structure des populations des rhizobiums symbiotiques et/ou la présence de bactéries nodulantes et fixatrices d'azote appartenant à d'autres genres que les rhizobiums, ii) la capacité de ces bactéries à noduler et, iii) l'efficacité de la fixation de l'azote.

Méthodologie : l'isolement des rhizobiums à partir de nodules se fera selon la méthode décrite par Chaintreuil et al, (2000). L'extraction de l'ADN génomique des souches de rhizobium isolées se fera selon le protocole décrit par Pitcher et al, (1989). Les marqueurs phylogénétiques de l'ADN génomique bactérien tel que les gènes 16SrRNA et recA seront amplifiés par PCR. Les ADN amplifiés seront séquencés puis analysés par bioinformatique afin de mener les analyses phylogénétiques (Gaunt et al 2001). Les gènes de nodulations (nod) et de la fixation de l'azote (nif) seront également recherchés et caractérisés selon la méthode décrite dans Jourand et al, (2004). Des tests de nodulation seront effectués afin de confirmer que les isolats sont bien capables de noduler *Acacia spirorbis*. Ils seront réalisés *in vitro* selon la méthode décrite par Jourand et al, (2005) sur des plants cultivés à partir de graines récoltés en Nouvelle-Calédonie. Les plants présentant des nodules seront testés pour leur capacité à fixer l'azote sera mesurée par la capacité à réduire l'acétylène mesurée par chromatographie en phase gazeuse selon la méthode décrite dans Jourand et al. (2005).

Résultats attendus : ces travaux permettront de connaître la structure de la (ou des) population(s) de bactéries symbiotiques nodulantes et fixatrices d'azote (genres, espèces) en symbiose avec *Acacia spirorbis*. Ils devraient aussi permettre d'effectuer des comparaisons entre ces communautés en fonction des sols et des écosystèmes spécifiques sur lesquels les prélèvements auront été effectués et renforcer (ou non) l'hypothèse que sur les sols ultramafiques ces communautés bactériennes sont riches et diversifiées (Herrera et al., 2007 ; Rjakumar et al., 2009).

Contribution à la formation : les travaux d'isolement de souches bactériennes, d'identification, de caractérisation moléculaire, et d'études physiologiques pourront faire l'objet de stages niveau master 2 ou d'école d'ingénieur, réalisés dans le cadre des formations universitaires ou des écoles d'ingénieur menées dans le domaine de l'écologie microbienne ou plus largement dans le domaine de l'environnement.

Collaborations envisagées : ces travaux se feront en étroite collaboration avec l'équipe 2 du LSTM (UMR113) dirigée par le Dr Lionel Moulin (HDR), spécialiste de la diversité des symbioses microbiennes nodulantes et fixatrices d'azote avec les légumineuses.

5.4. Adaptation des microorganismes symbiotiques à la contrainte édaphique extrême

La question posée est de savoir quelles sont les bases moléculaires de l'adaptation de ces microorganismes symbiotiques aux conditions extrêmes de la contrainte édaphique, en particuliers la présence des métaux lourds, le déséquilibre du ratio Ca/Mg en faveur de l'élément Mg et le stress hydrique. Dans ce projet de recherche, on se restreindra à la question de l'étude de la tolérance des microorganismes symbiotiques aux métaux lourds.

Principe : chez les souches bactériennes nodulantes et fixatrices d'azote, isolées de nodules d'*Acacia spirorbis* lors des travaux précédents, il s'agira de rechercher la présence de gènes de tolérance aux métaux lourds présents sur les sols néocalédoniens : aluminium sur sols calcaires et multi métaux sur sols ultramafiques (Co, Cr, Mn et Ni).

Cette étude peut-être abordée :

- soit par une approche "sans à priori" : en sélectionnant les souches de microorganismes tolérantes à un métal donné et en analysant les différences de leur transcriptome (RNA) en présence ou en absence du métal, par des méthodes générales de séquençage massif du transcriptome tel que le pyroséquençage (Ahmadian et al., 2006)

- soit en se basant sur la bibliographie existante référençant les différents gènes et déterminants moléculaires de la tolérance au métal déjà identifiés chez les bactéries et chez d'autres microorganismes : ce sont les gènes impliqués dans i) les processus d'immobilisation et d'exclusion du métal dans le proche environnement extérieur à la cellule (sécrétions de molécules fixant le métal : acides organiques, protéines, polysaccharides, acides gras), ii) les processus de transport membranaires (influx ou efflux du métal), iii) des processus de stockage intracellulaire, (fixation à des protéines, ou à des enzymes et à leurs substrats), iv) des processus de détoxification par des mécanismes métaboliques complexes.

Ainsi, en choisissant la deuxième approche, l'étude de la tolérance aux métaux Ni et Co se basera sur i) des mesures des niveaux de tolérance in vitro (recherche et détermination des IC_{50}), et ii) recherche, identification, séquençage, mutation et complémentation des gènes de tolérance au Ni et Co: le gène NreB impliqué dans la résistance au nickel ; le gène NccA impliqué dans la tolérance au nickel, cobalt et cadmium ; le gène CnrA impliqué dans la résistance au nickel et au cobalt ; et enfin le gène CzcA impliqué dans la tolérance au cobalt, zinc, et cadmium.

L'étude de la tolérance au Cr sous sa forme Cr(VI) (forme majoritaire de l'élément Cr biodisponible dans les sols ultramafiques selon Becquer et al., 2003) se basera aussi sur i) des mesures des niveaux de tolérance *in vitro* (recherche et détermination des IC₅₀), et ii) recherche, identification, séquençage, mutation et complémentation des gènes de tolérance codant pour des transporteurs membranaires tel que le gène ChrA, ou des enzymes détoxifiantes telles que les chromates réductases ou encore des enzymes impliquées dans la réponse au stress oxydatif telle que les super oxyde dismutase (SOD) (Ramirez-Diaz et al., 2008).

L'étude de la tolérance au Mn se basera aussi sur i) des mesures des niveaux de tolérance *in vitro* (recherche et détermination des IC₅₀), et ii) recherche, identification, séquençage, mutation et complémentation des gènes de tolérance codant principalement pour des transporteurs membranaires protéiques de type Nramp (natural resistance-associated macrophage protein) tels que les gènes de la famille mntH et mntS (Kehres et al, 2000 ; Groot et al, 2005).

En revanche, la recherche de gènes de tolérance à l'aluminium chez les bactéries semble moins évidente : selon Beris et al. (2011), je cite « the molecular studies on aluminium tolerance in bacteria are limited. However, there are numerous studies on aluminium resistance in several plants (Sugimoto et al., 2004). Generally, these studies were conducted at the molecular level, for example, cloning the genes induced by Al. There are just a few studies on Al tolerance mechanisms in Al-tolerant microorganisms. The acidophilic bacterium, *Arthrobacter viscosus* ALRJ6 has a gene responsible for the aluminium resistance (Jo et al., 1997). The cloned fragment of this gene has been designated as *alu1-p* but the full sequence of the gene is still unknown. In addition, six Al-tolerant acidophilic microorganisms that possess a high level of resistance to Al (up to 300 mM) were characterized (Kawai et al., 2000). The relationship between ATPase and Al tolerance has not been studied in bacteria. Thus far, a relationship between ATPase and Al tolerance has only been seen in plants and yeast, e.g. the ALR1 (aluminium resistance) gene encodes the major Mg²⁺ uptake system in yeast (Lee and Gardner, 2006), also the potential role of vacuolar H⁺-ATPase and the F-ATPase in Al and NaCl tolerance in wheat (*Triticum aestivum* L.). Here we have characterized a new ATPase belonging to the PP-loop ATPase superfamily from *Anoxybacillus gonensis* G2 (Belduz et al., 2003) ». Aussi, la possibilité d'isoler des bactéries nodulantes et fixatrices d'azote à partir de nodules d'*Acacia spirorbis* laisse-t-elle entrevoir des études novatrices dans ce domaine et prometteuses quand à la recherche de déterminants moléculaires support de la tolérance à l'aluminium et aux mécanismes biochimiques qui en découleraient.

Méthodologie : l'ensemble des ces études sera mené comme rapportés dans l'article de Jourand et al., (2005) et Chaintreuil et al. (2007) : 1) recherche, identification et séquençage des gènes connus marqueurs de la tolérance au métal, 2) amplification et clonage pour obtention de mutants ayant perdue leur tolérance au métal par recombinaison homologue, 3) vérification de la mutation par restauration de la tolérance par complémentation, et 4) tests sur la plante hôte pour mesurer l'impact de la perte de la tolérance au métal sur la symbiose en termes de nodulation, de fixation d'azote et de croissance de la plante.

Résultats attendus : il s'agira i) de mettre en évidence la nature des déterminants moléculaires support de la tolérance à tel ou tel métal chez les espèces de rhizobiums rencontrés dans les nodules fixateurs d'azote d' *Acacia spirorbis*, et ii) de montrer d'éventuels flux de gènes entre les souches bactériennes d'une espèce à une autre traduisant une réponse adaptative à la contrainte édaphique.

Contribution à la formation : l'ensemble de ces travaux de génie génétique et d'études physiologiques sur la plante pourront faire l'objet d'une thèse de doctorat réalisée dans le cadre des formations universitaires dans le domaine de l'écologie microbienne ou plus largement dans le domaine de l'environnement.

Collaborations partenariats : cette étude se fera en collaboration avec l'équipe 1 du LSTM dirigée par Eric Giraud qui maîtrise l'ensemble des approches et des outils de la génétique moléculaire bactérienne.

5.5. Rôle de ces symbioses dans l'adaptation de la plante

La question posée est de savoir si les rhizobiums symbiotiques adaptés à la contrainte édaphique extrême du sol ultramafique, en particuliers la présence des métaux lourds, i) ont développé des mécanismes symbiotiques avec leur plante hôte différents de ceux connus et décrits dans la littérature, et ii) si l'adaptation de ces microorganismes contribue, au travers de la symbiose, à l'adaptation de leur plante hôte à ces conditions extrêmes.

5.5.1. Nature du dialogue moléculaire plante-microorganisme

Principe : nous proposons de déterminer i) la nature moléculaire biochimique des flavonoïdes inducteurs de la symbiose nodulante fixatrice d'azote, sécrétés par la plante hôte (*Acacia spirorbis*) et de vérifier s'il existe ou non une spécificité du langage moléculaire de la plante en fonction de sol sur lequel elle se développe, i.e. sol ultramafique, sol volcano-sédimentaire ou sol calcaire riche en aluminium.

De la même façon, on étudiera le langage moléculaire bactérien en réponse à l'induction de la plante, en déterminant la molécule biochimique des facteurs de nodulation (Nod factors) produits par les bactéries afin d'étudier une éventuelle spécificité du langage moléculaire bactérien en fonction de sol de prélèvement.

Méthodologie : pour ces études on s'appuiera sur les méthodes d'isolement, d'extraction, de purification et d'analyses biochimiques de molécules (HPLC et spectrométrie de masse) largement décrites dans la thèse d'Adeline Renier (2008) et dans Renier et al. (2008).

Résultats attendus : la détermination de la nature moléculaire biochimique des flavonoïdes inducteurs de la plante hôte et des facteurs de nodulation bactériens permettra de mettre en évidence (ou non) un langage moléculaire adapté (ou non) à la contrainte édaphique.

Collaborations partenariat : ces études se feront en collaboration avec l'équipe du Pr Sylvie Rapior (UMR 5275 CEFE, Université Montpellier 1) spécialiste de l'isolement et de l'analyse moléculaire structurale des biomolécules produites par les plantes comme les polyphénols (flavonoïdes).

5.5.2. Essais *in vitro* et en conditions semi-contrôlées (serre)

Principe : il s'agira de montrer i) l'expression de ces déterminants moléculaires bactérien impliqués dans la tolérance aux métaux lourds lors de l'établissement de la symbiose nodulante et fixatrice d'azote de jeunes plantes d'*Acacia spirorbis*, et ii) de montrer les conséquences de l'expression de ces déterminants sur la physiologie de la plante hôte.

Méthodologie : ces tests seront menés *in vitro* et en conditions contrôlées (serre) par inoculation de la plante hôte soumis à différentes conditions de milieu en rapport avec les conditions édaphiques. Les mesures et analyses se feront i) au niveau des nodules fixateurs d'azote pour la mise en évidence de l'expression à l'aide de gène rapporteur comme décrit dans Jourand et al, (2005); et ii) au niveau de la plante : croissance, biomasse, fixation de l'azote et nutrition minérale (études flux des métaux lourds et autres nutriments en fonction des conditions de milieux selon les contraintes édaphiques) selon les méthodes décrites par Jourand et al, (2005 et 2010b).

5.5.3. Contribution à la formation

L'ensemble de ces travaux biochimie de tests physiologiques sur la plante pourra faire l'objet faire de stages niveau master 2 ou d'école d'ingénieur, réalisés dans le cadre des formations universitaires ou des écoles d'ingénieur, ou bien de façon plus intégrée sous forme d'une thèse de doctorat réalisée dans le cadre des formations universitaires dans le domaine de l'écologie microbienne ou plus largement dans le domaine de l'environnement.

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7) Fiche de synthèse

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2005-2006 : Affectation en Australie, Adélaïde, South Australia: Université d'Adélaïde, Ecole de la terre et de l'environnement, Laboratoire de physiologie végétale. Poste d'accueil dans le laboratoire du Pr Reid : Acquisition des méthodes d'études de la réponse physiologique des végétaux au stress métallique.

1998-2005 : Affectation à Montpellier, France. LSTM UMR113, CIRAD Campus de Baillarguet. Chargé du programme de recherche : Les légumineuses du genre *Crotalaria*: symbiose fixatrice d'azote et activités biologiques contre les nématodes phytoparasites. Utilisations en agriculture biologique. **Promu Ingénieur d'Etudes de 1^{ère} classe (IE1) en 2003, puis passage dans le corps des Ingénieurs de Recherche de 2^{ème} classe (IR2) en 2004 par voie de concours interne à l'IRD.**

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1996-1998 : Affectation en Nouvelle-Calédonie, Centre IRD de Nouméa, Laboratoire de Microbiologie (Unité de recherche UR3B, Microbiologie). Poste : Ingénieur d'études participant au programme de recherche sur les symbioses Frankia – Casuarinaceae et leur utilisation pour la re-végétalisation des sols dégradés des mines de Nickel.

1994-1996 : Affectation au Mexique, Mexico city. Université Autonome de Mexico (UAM-Iztapalapa), Laboratoire de Biotechnologie. Poste : Ingénieur en charge de l'équipement et de l'installation complète d'un laboratoire de microbiologie. Participation au programme de recherche sur l'utilisation des champignons filamenteux dans la valorisation des déchets de l'industrie agro-alimentaire (pulpe de café et bagasse de canne à sucre).

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ENCADREMENTS D'ETUDIANTS

- Participation à l'encadrement de **3 étudiants** en **Doctorat d'Université** : Simon Gensous de l'Université Nouvelle-Calédonie (UNC, thèse en cours), Alexandre Lagrange (UNC, bourse CIFRE, 2009) et Adeline Renier (Université Montpellier 2, 2008).
- Participation à l'encadrement de **6 étudiants** en stage de **Master** (M1 et M2, ex-DEA et ex-DESS) et stages **d'Ecole d'Ingénieur** (4ème et 5ème année). Encadrement et co-encadrement de **6 étudiants** en stages de **Licence** (L2 et L3), et stages de 3ème année d'études **d'Ecole d'Ingénieur**.

ACTIVITES DE RECHERCHE :

Thématiques de recherche : de 1998 à 2005 : **thématique** de mon **Doctorat d'Université** : les légumineuses du genre *Crotalaria* : symbiose fixatrice d'azote et activités biologiques contre les nématodes phytoparasites. Depuis 2005 : étude de la **symbiose ectmycorrhizienne** *Pisolithus albus* / **Myrtaceae** en milieu tropicale extrême : sols ultramafiques des maquis des mines de nickel en Nouvelle-Calédonie.

Contrats de recherche : Participation à 4 projets de recherche : 2 ANR : ECCO (NIKO 356 K€ sur 2005-2008) et BIODIV (ULTRABIO, 725K€ sur 2007-2010); 1 projet européen : FP7-KBBE-2009 (Legume-supported cropping systems for Europe); 1 projet Nouvelle-Calédonie CNRT Ecomine Biotop (545 K€ sur 2010-2012).

Collaborations scientifiques : équipes de recherche institutionnelles **françaises** : CEFE UM1, UMR 1062 CBGP, UMR 232 DIADE, UMR 6635 CEREGE, Université Nouvelle-Calédonie (UNC) et Institut Agronomique Calédonien (IAC), **et internationales** : Université d'Adélaïde, Australie. **Collaborations avec partenaires privés** : GRAB, Avignon; SIRAS Nouméa et KNS Koniambo, Koné, Nouvelle-Calédonie.

Projet de recherche: Rôles des rhizobiums dans l'adaptation des légumineuses aux contraintes abiotiques des sols : le modèle *Acacia spirorbis* en Nouvelle-Calédonie : adaptation à l'ultramafisme. Ce projet porte à la fois i) sur l'étude de la diversité et de l'adaptation des symbioses avec des rhizobiums fixateurs d'azote, ii) sur l'étude du support moléculaire de l'adaptation des bactéries aux conditions édaphiques, et iii) sur les conséquences de la symbiose sur l'adaptation de la plante hôte à la contrainte édaphique.

Publications internationales retenues HDR: 14 (2 avant la thèse ; 3 en thèse, et 9 après la thèse)

P1. Majorel C., Hannibal L., Soupe ME, Carriconde F, Ducouso M, Lebrun M & Jourand P. 2011. *Molecular Ecology* (soumis le 01/10/2011, N° MEC-11-0835) (IF = 6,633)

P2. Bhattacharjee R., Jourand P., Chaintreuil C., Dreyfus B., Singh A. & Mukhopadhyay S.N. 2011. *Biology & Fertility of Soils* (sous presse). (IF=2,070).

P3. Renier A., Mania De Faria S., Jourand P., Giraud E., Dreyfus B., Rapior S. & Prin Y. 2011. *Journal of Experimental Botany* 62:3693-3697 (IF=4,745)

P4. Lagrange A., Ducouso M., Jourand P., Majorel C., Amir H. 2011 *Canadian Journal of Microbiology* 57:21-28 (IF= 1,502)

P5. Jourand P. et al, 2010, *Tree Physiology* 30:1311-1319 (IF= 2,530)

P6. Jourand P., et al 2010 *FEMS Microbiology Ecology* 72:238-249 (3,885)

P7. Renier A., Jourand P., Rapior S., Poinsot V., Sy A., Dreyfus B. & Moulin L. 2008. *Soil Biology Biochemistry* 40:1404-1412 (IF= 3,566)

P8. Jourand P., et al., 2005 *Molecular Plant-Microbe Interactions* 18:1061-1068 (IF=4,844)

P9. Jourand P., et al. 2004 *International Journal of Systematic & Evolutionary Microbiology* 54:2269-2273 (IF=2,488)

P10. Jourand P., et al, 2004 *Nematology* 6:79-84 (IF=0,964)

P11. Jourand P., et al, 2004 *Nematology* 6:763-769 (If=0,964)

P12. Giraud E., Hannibal L., Fardoux J., Jaubert M., Jourand P., Dreyfus B., Sturgis J. N. & Verméglio A. 2004 *Journal of Biological Chemistry* 279:15076-15083 (IF=5,440)

P13. Sy A., Giraud E., Jourand P., Garcia N., Willems A., De Lajudie P., Prin Y., Neyra N., Gillis M., Masson-Boivin C. et Dreyfus B. 2001. *Journal of Bacteriology* 183:214-220 (IF=4,035)

P14. Gauthier D., Navarro I., Rinaudo G., Jaffré T., Jourand P., Prin Y. 1999. *Journal of Soil and Biology* 35:199-205 (IF=1,445)

Communications: 14 internationales (dont 9 communications orales et 9 posters) **et 6 nationales.**

Enseignement Universitaire (2001-2011): 43h de CM, 24h de TD, 12h d'examens et 20h de TP.

**Tirés à part des 14 publications
retenues pour l'HDR**

Tracking nickel-adaptive biomarkers in *Pisolithus albus* from New Caledonia using a transcriptomic approach

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Abstract

The fungus *Pisolithus albus* forms ectomycorrhizal (ECM) associations with plants growing on extreme ultramafic soils, which are naturally rich in heavy metals such as nickel. Both nickel-tolerant and nickel-sensitive isolates of *P. albus* are found in ultramafic soils in New Caledonia, a biodiversity hotspot in the Southwest Pacific. The aim of this work was to monitor the expression of genes involved in the specific molecular response to nickel in a nickel-tolerant *P. albus* isolate. We used pyrosequencing and quantitative polymerase chain reaction (qPCR) approaches to investigate and compare the transcriptomes of the nickel-tolerant isolate MD06-337 in the presence and absence of nickel. A total of 1 071 375 sequencing reads were assembled to infer expression patterns of 19 518 putative genes. Comparison of expression levels revealed that 30% of the identified genes were modulated by nickel treatment. The genes, for which expression was induced most markedly by nickel, encoded products that were putatively involved in a variety of biological functions, such as the modification of cellular components (53%), regulation of biological processes (27%) and molecular functions (20%). The 10 genes that pyrosequencing analysis indicated were induced the most by nickel were characterized further by qPCR analysis of both nickel-tolerant and nickel-sensitive *P. albus* isolates. Five of these genes were expressed exclusively in nickel-tolerant isolates as well as in ECM samples *in situ*, which identified them as potential biomarkers for nickel tolerance in this species. These results clearly suggest a positive transcriptomic response of the fungus to nickel-rich environments. The presence of both nickel-tolerant and nickel-sensitive fungal phenotypes in ultramafic soils might reflect environment-dependent phenotypic responses to variations in the effective concentrations of nickel in heterogeneous ultramafic habitats.

Keywords: ectomycorrhiza, New Caledonia, nickel, *Pisolithus albus*, pyrosequencing

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Introduction

The presence of high levels of heavy metals in terrestrial environments can result from natural or human-related factors, such as pollution. It may influence the ecology and evolution of plants, fungi, animals and

other organisms (Branco & Ree 2010; Colpaert *et al.* 2011). In the presence of high levels of heavy metals, ectomycorrhizal (ECM) fungal populations are differentiated into those that are highly tolerant of metal toxicity and those that are sensitive to such metals (Hartley *et al.* 1997).

In recent years, several studies have characterized and reviewed the various molecular mechanisms and cellular responses to stress that enable ECM fungi to

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tolerate heavy metals (Meharg 2003; Bellion *et al.* 2006). These mechanisms involve the uptake and subcellular sequestration of the metal (Blaudez *et al.* 2000a), intracellular binding of the metal (Jacob *et al.* 2004), responses to oxidative stress by the production of molecules that are involved in detoxification processes (Lanfranco 2007; Ramesh *et al.* 2009) and the synthesis of proteins involved in the transport and accumulation of metals. Additional mechanisms include antioxidative responses (Muller *et al.* 2007a; Ruytinx *et al.* 2011), extracellular chelation and binding of the metal to the cell wall (Bellion *et al.* 2006). The involvement of all of the above-mentioned mechanisms was confirmed recently using the complete genome and transcriptome data of the ECM fungal species *Tuber melanosporum* (Bolchi *et al.* 2011), which enabled the detection of genes related to metal homeostasis in this plant-symbiotic ascomycete. The genes that were identified were subdivided into three major functional classes, namely those involved in: (i) transport of metals, (ii) defence against the effects of oxidative stress and (iii) detoxification of metals (Bolchi *et al.* 2011). Such molecular processes could play a key role in enabling plants, fungi or microorganisms to persist in extreme habitats, such as serpentine soils, which are naturally rich in heavy metals (Branco 2009; Gonçalves *et al.* 2009; Rajkumar *et al.* 2009; Wright & Stanton 2011).

Serpentine soils are derived from ultramafic bedrock and are characterized by high concentrations of magnesium, iron and various heavy metals, such as chromium, cobalt, nickel and manganese. They are also extremely deficient in elements that are essential for plant nutrition, including nitrogen, phosphorus and potassium (Brooks 1987). Previous studies have shown that serpentine soils are characterized by a high biological diversity of plants and microorganisms that use various mechanisms to cope with the edaphic conditions (in particular, adaptation to toxic heavy metals, such as nickel) (Kazakou *et al.* 2008; Branco & Ree 2010). For example, it was shown recently that samples of the ECM fungus *Cenococcum geophilum* that were isolated from serpentine soils were highly tolerant of nickel, which suggests that the organism had adapted to its presence (Gonçalves *et al.* 2009). Recently, similar findings of fungal tolerance to nickel were observed in *Pisolithus albus* (Cooke & Masee) (Jourand *et al.* 2010a).

Pisolithus albus belongs to the genus *Pisolithus* Alb. & Schwein., a major ECM genus in the *Boletales* order, members of which are distributed globally and form ECM associations with a broad range of angiosperm and gymnosperm trees and shrubs (Marx 1977; Martin *et al.* 2002). *Pisolithus albus* grows on serpentine soils in New Caledonia, a tropical archipelago in the Southwest Pacific that is considered to be a hotspot of biodiversity

(Myers *et al.* 2000; Jourand *et al.* 2010a). Serpentine soils cover a third of the surface area of the main island (Perrier *et al.* 2006). In New Caledonia, isolates of *P. albus* from these soils cluster genetically into a single group that comprises both nickel-tolerant and nickel-sensitive isolates (Jourand *et al.* 2010a). It was suggested that the observed phenotypic variations in nickel tolerance might arise from variations in the concentrations of bioavailable nickel in these soils. In addition, the nickel-tolerant isolates of *P. albus* were found to improve the growth and tolerance of nickel in plant hosts that were exposed to toxic concentrations of the metal (Jourand *et al.* 2010b).

Given these findings, it is important to clarify the molecular mechanisms of ECM adaptation to serpentine soils and tolerance of nickel in particular. In the study reported here, we examined changes in the transcriptome upon exposure of a single nickel-tolerant *P. albus* isolate from the ultramafic soils of New Caledonia to nickel to identify biomarkers that are involved in the molecular responses to the metal. Data on the complete transcriptome were obtained using pyrosequencing. We identified genes for which expression was changed by the presence of nickel and verified their differential expression in nickel-tolerant and nickel-sensitive isolates by using real-time quantitative PCR (RT-qPCR). The use of these procedures enabled us to generate the first database of expressed sequence tags (ESTs) from a nickel-tolerant fungus and to identify five genes expressed exclusively in nickel-tolerant isolates of *P. albus*. The latter suggests that this species shows a molecular adaptive response to nickel-rich environments.

Materials and methods

Fungal material (in vitro and in situ)

The ECM fungal isolates used in the study were isolated from sporocarps collected from ultramafic soils in New Caledonia at collection sites 1, 2, 3, 4 and 5 described in Table 1 and Fig. S1 (Supporting information). Anatomical and morphological characterizations led to their identification as isolates of the fungal species *Pisolithus albus*. The Ni-tolerant isolate MD06-337, which was described previously (Jourand *et al.* 2010b), was also used in the study. The other isolates of *P. albus* that were included in the study were new samples and are described in Table 1. Whereas MD09-045 and MD09-001 are nickel-tolerant isolates, MD09-063 and MD09-078 are nickel-sensitive isolates. To confirm their identities at the genus and species levels, these new isolates were analysed phylogenetically on the basis of their internal transcribed spacer (ITS) rDNA sequences as described previously (Jourand *et al.* 2010a). The analysed ITS sequences were deposited in the EMBL Nucleotide

Table 1 Isolates of *Pisolithus albus* used in this study. In addition to description of the sites where isolates were sampled (map location and GPS coordinates), details regarding the accession number of ITS sequences and *in vitro* tolerance of nickel are provided for each isolate

Site description: ultramafic soils		<i>Pisolithus albus</i> isolates					
Site on map	Map location, GPS coordinates	Isolate code	DNA-ITS EMBL accession code	Nickel phenotype	*Ni EC ₅₀ μ M MMN medium	*Ni EC ₅₀ μ M UF-like medium	References
1	Trazy-Guerioum, Koniombo Massif (21°00'28" S; 164°49'50" E)	MD06-337	AM947121	Tolerant	569 $\pm$ 3	1800 $\pm$ 5	Jourand <i>et al.</i> 2010a
2	Pindjen Waterfall Road Koné (21°02'19" S; 164°46'26" E)	MD09-078	FR852890	Sensitive	42 $\pm$ 4	65 $\pm$ 1	This study
3	Pindai (21°19'49" S; 164°58'22" E)	MD09-063	FR852893	Sensitive	76 $\pm$ 2	29 $\pm$ 2	This study
4	Mont-Dore, Plum Road (22°15'17" S; 166°36'46" E)	MD09-045	FR852892	Tolerant	>1000	2600 $\pm$ 5	This study
5	Bois du Sud (22°10'18" S; 166°44'49" E)	MD09-001	FR852891	Tolerant	1194 $\pm$ 3	1500 $\pm$ 5	This study

EC₅₀, the effective concentration that inhibits growth by 50% (Blaudez *et al.* 2000b); ITS, internal transcribed spacer.

*Ni EC₅₀ was determined in two media: Melin-Norkrans medium as reference medium (Blaudez *et al.* 2000b) and UF-like medium.

Sequence Database. To evaluate the expression of the nickel biomarkers in fungal material *in situ* further, samples from ECM root tips were collected at the study site where the nickel-tolerant isolate MD06-337 and the nickel-sensitive isolate MD09-078 were found. These fungal materials were stored in liquid nitrogen.

Growth conditions

To generate stock cultures from each sporocarp, a fresh piece of pileus trama was transferred aseptically to solid modified Melin-Norkrans (MMN) medium (Blaudez *et al.* 2000b). The MMN medium had the following components: KH₂PO₄ (0.5 g/L; K and P = 3.7 mM), (NH₄)₂HPO₄ (0.25 g/L; N = 3.78 mM and P = 3.67 mM), CaCl₂ (0.05 g/L; Ca = 0.34 mM), NaCl (0.025 g/L; NaCl = 0.43 mM), MgSO₄·7H₂O (0.15 g/L; Mg = 0.61 mM), thiamine hydrochloride (100 μ g/L), FeCl₃·6H₂O (0.03 g/L), glucose (10 g/L), malt extract (3 g/L) and agar (14 g/L). The pH was adjusted to 5.6 using 1 M HCl to match the pH measured in ultramafic soils as reported by Perrier *et al.* (2006). The medium was autoclaved for 20 min at 120 °C. All fungal isolates were maintained as subcultures at 30 °C in this medium.

For further experiments, MMN medium was altered to mimic the extreme conditions of ultramafic soils. In the Koniombo Massif, where the *P. albus* isolate MD06-337 was sampled, the concentrations of chemical elements in the soil according to Jourand *et al.* (2010a) were as follows: N (3.6 g/kg, N = 257 mM), P

(0.07 g/kg, P = 2.25 mM), K (0.09 g/kg, K = 2.3 mM), Ca (0.26 g/kg, Ca = 6.5 mM) and Mg (4.1 g/kg, K = 170 mM), with an unbalanced molar ratio of Ca/Mg (1/26) and an excess of extractable Ni (Ni-diethylene triamine pentaacetic acid) (19 mg/kg, Ni = 327 μ M). To mimic these conditions, the MMN medium was altered to generate what is henceforth referred to as UF-like medium, which contained the following components: KH₂PO₄ (0.25 g/L; P = 1.83 mM and K = 1.83 mM), (NH₄)₂HPO₄ (0.125 g/L; N = 1.8 mM and P = 0.9 mM), NH₄NO₃ (10 g/L; N = 250 mM, CaCl₂ (0.05 g/L; Ca = 0.34 mM, KCl (0.033 g/L; K = 0.44 mM, NaCl (0.025 g/L), MgSO₄·7H₂O (1.95 g/L; Mg = 7.9 mM, thiamine hydrochloride (100 μ g/L), FeCl₃·6H₂O (0.03 g/L), glucose (1 g/L) and malt extract (0.3 g/L). The pH was adjusted to 5.6 using 1 M HCl to match the pH measured in ultramafic soils as reported by Perrier *et al.* (2006). The medium was autoclaved for 20 min at 120 °C. When needed, nickel was added after autoclaving under sterile conditions from a stock solution of NiCl₂ (1 M) to the required final concentrations. To check the nickel tolerance of all the isolates used in the study, the EC₅₀ of Ni was measured in both the reference MMN medium and the UF-like medium as described previously (Jourand *et al.* 2010a).

Extraction of total RNA

Total RNA was extracted from mycelium that had been cultivated *in vitro* as well as from frozen fungal

material that was collected *in situ*. A cetyltrimethyl ammonium bromide (CTAB)-based protocol was used as described by Salzman *et al.* (1999). Briefly, 1–5 g of frozen mycelium or ECM tissue was ground in liquid nitrogen using a mortar and pestle. Then, 0.6–1 mL of extraction buffer (2% CTAB, 2% polyvinylpyrrolidone PVP-K-40, 100 mM Tris-HCl, 25 mM EDTA and 2 M NaCl) was added to each tube, and the samples were incubated at 60 °C for 15 min. An equal volume of a mixture of chloroform:isoamyl alcohol (24:1) was added and mixed immediately for 2 min using a vortex mixer. The samples were then centrifuged at 10 000 g for 10 min at 4 °C. Supernatants solutions were transferred to a new tube, and a 1/3 volume of 8 M LiCl was added to each tube. The samples were mixed by inversion and stored at 4 °C overnight. Total RNA was purified using the silica membrane from an RNeasy Plant Mini kit (Qiagen, Courtaboeuf, France), in accordance with the manufacturer's instructions. The purified RNA was quantified using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) at wavelengths of 230, 260 and 280 nm. The integrity of the RNA was verified by separating sample components in 1.2% agarose gels, as described by Salzman *et al.* (1999).

Processing of 454 pyrosequencing data and assembly of cDNA sequences from the nickel-tolerant Pisolithus albus isolate (MD06-337)

To compare the gene expression in the nickel-tolerant *P. albus* isolate (MD06-337) in the presence and absence of nickel, two sets of total RNA were isolated using the CTAB-based protocol from 15-day-old liquid cultures of the isolate cultivated in UF-like medium with or without nickel (250 µM NiCl₂). The two sets of extracted RNA were sent to GATC Biotech (Constance, Germany), where the cDNA was pyrosequenced in accordance with the established procedures used by the company. The primer sequences were removed from the data. Sequence reads were then assembled into contigs using the Genomics Workbench software (CLC Bio, Aarhus, Denmark). Expression analysis was performed using the RPKM (reads per kilobase of exon model per million mapped reads) values (Mortazavi *et al.* 2008). The RPKM values, which are used to standardize sequence counts as a function of contig length and absolute size of the data set (i.e. mapped reads: assembled reads), were calculated as follows: (number of reads per contig) × (1000/length of contig) × (1 × 10⁶/total number of mapped reads in the assembly) (Mortazavi *et al.* 2008). On the basis of the ratio of the RPKM values obtained in the presence and absence of nickel (which represents the fold induction of gene expression in response to

nickel), we selected the genes for which expression was increased more than twofold above the background level for the pyrosequencing, as commonly performed in previous studies using comparative transcriptomic approaches (Chelaifa *et al.* 2010). This cut-off level for the induction of gene expression was used previously in another analysis of the transcriptomic response to a heavy metal in another ECM fungus (Jacob *et al.* 2004). The genes that presented both the highest RPKM values in the presence of nickel (RPKM ≥ 10) and the lowest RPKM values in the absence of nickel were selected as candidate biomarkers of nickel tolerance in *P. albus*. In addition, to identify genes, the contig sequences that were obtained by pyrosequencing were compared with sequences stored in the GenBank nonredundant database (nr), *Laccaria bicolor* database (<http://mycor.nancy.inra.fr/imgc/laccariagenome/>) and *Pisolithus microcarpus* database (free access was provided generously by the F. Martin (INRA laboratory, Nancy, France) using BLASTX with an E-value cut-off of 10⁻⁵, as described previously for other pyrosequencing transcriptome analyses (Schmid *et al.* 2010). All assembled contigs and their respective RPKM values sequences of the genes selected from the pyrosequencing data analysis were deposited in the EMBL Nucleotide Sequence Database and referenced under the accession numbers shown in Table 2.

Gene annotation using Gene Ontology (GO) terms

To identify the global functional categories of the unique contigs (with respect to the standard Gene Ontology (GO) categories of biological process, molecular function and cellular component), the two transcriptomic sequence banks and the pyrosequencing contig data obtained from *P. albus* MD06-337 in the presence or absence of nickel were analysed using the GO database. This analysis was based on the similarities of the sequences to those of known proteins in the GenBank nr protein database (E-value cut-off of 10⁻⁵), which had been annotated with GO terms using the software BLAST2GO V2.4.8 (Götz *et al.* 2008).

Quantitative PCR analysis

To confirm the nickel-responsive differential expression revealed by pyrosequencing, we used RT-qPCR to verify the fold induction of the genes that had been selected as biomarkers of nickel tolerance. This technique is the most sensitive and reproducible method to quantify gene expression, as described recently in a study using the pyrosequencing approach (Chelaifa *et al.* 2010). For the RT-qPCR experiments, *P. albus* isolates were grown in UF-like medium that contained

Table 2 Candidate biomarker genes, shown by pyrosequencing analysis to be up-regulated by nickel, which show differential expression in nickel-tolerant *Pisolithus albus* isolate MD06-337. EMBL accession codes are provided for all DNA sequences, as are the closest homologs identified by BlastN analysis

Pyrosequencing analysis					Gene sequence and BlastN identification			
No. Contig	No. gene	Size (bp)	RPKM 0 μ M Ni	RPKM 250 μ M Ni	EMBL accession number	Species	E-value	% Identity
C10864	1	517	0	64	FR852894	<i>Pisolithus microcarpus</i>	2E ⁻³³	100%
C6406	2	411	0	65	FR852895	<i>P. microcarpus</i>	1E ⁻¹⁰⁵	96%
C9200	3	443	0	10	FR852896	<i>P. microcarpus</i>	3E ⁻⁹⁴	95%
C7318	4	411	0	90	FR852897	<i>P. microcarpus</i>	2E ⁻⁷⁹	95%
C10795	5	504	0	90	FR852898	<i>P. microcarpus</i>	1E ⁻¹³⁰	99%
C15327	6	235	0	17	FR852899	<i>P. microcarpus</i>	1E ⁻¹¹²	99%
C17653	7	1199	1	27	FR852900	<i>P. microcarpus</i>	9E ⁻⁴⁴	98%
C18133	8	1125	1	35	FR852901	<i>P. microcarpus</i>	1E ⁻¹⁵¹	99%
C10491	9	665	6	30	FR852902	<i>P. microcarpus</i>	9E ⁻⁹²	97%
C4552	10	1303	3	42	FR852903	<i>P. microcarpus</i>	1E ⁻¹⁵⁵	99%

bp, base pair; RPKM, reads per kilobase of exon model per million mapped reads (Mortazavi *et al.* 2008).

either 50 or 250 μ M nickel provided as NiCl₂. Flasks that contained 250 mL of UF-like medium were each inoculated with five plugs of mycelium from the MMN stock culture. The cultures were allowed to grow in the dark for 15 days, with continuous shaking at 130 rpm at 30 °C. The RNA was extracted from three biological replicates of the fungal isolate MD06-337, which had been cultivated *in vitro* as described above, and analysed by RT-qPCR. At the same time, we tested RNA samples extracted from ECM of the MD06-337 and MD09-078 isolates that had been isolated *in situ*. Furthermore, we analysed RNA samples extracted from other *P. albus* isolates (already described in Table 1). Aliquots of total RNA (1 μ g) were treated with TurboDNase I (Applied Biosystems, Carlsbad, CA, USA) and used for cDNA synthesis, which was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in accordance with the manufacturer's protocol. Real-time quantitative PCR was performed in duplicate in a volume of 20 μ L, which contained 5 μ L of diluted cDNA (1 ng of total RNA equivalent), 1 $\times$ SYBR Green PCR Master Mix (Applied Biosystems) and 0.3 μ M of each primer. Primers were designed from the *P. albus* contig data, which were obtained from the results of the pyrosequencing using the Primer Express software (Applied Biosystems). The primers amplified a gene fragment of 150 bp in length at an annealing temperature of 60 °C, as shown in Table S1 (Supporting information). PCR was applied to test the specificity of the primers and the efficiency of amplification (Table S1, Supporting information). The amplification profile consisted of an initial denaturation step at 95 °C for 15 min, then 40 cycles that each comprised a 95 °C step for 10 s, 59 °C for 30 s and 72 °C for

32 s, followed by a dissociation stage, in accordance with the manufacturer's instructions. Real-time PCR was performed using an ABI 7300 Sequence Detection System (Applied Biosystems) in the presence of SYBR Green. A melting curve analysis was performed at the end of each RT-qPCR by gradually increasing the temperature from 60 to 95 °C, while recording the change in fluorescence. A single peak at the melting temperature of the PCR product confirmed primer specificity, indicating that RT-qPCR had been performed with no contamination or formation of primer dimers.

Data and statistical analysis of qPCR

Data were analysed using two methods. The REST 2009 method, which is available at <http://www.genequantification.de/rest-2009.html> (Pfaffl *et al.* 2002), was used to determine the ratios of transcripts, whereas the 2^{- $\Delta\Delta C_t$} method was used to analyse mRNA accumulation (Livak & Schmittgen 2001). Each analysis was normalized with two reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and elongation factor 4 α (EF4 α). For each treatment, we performed three technical replicates for each of the three biological replicates per isolate and per nickel treatment, to evaluate the relative expression. In the REST method, the RT-qPCR results were analysed using the hypothesis test [*P*(*H*1)], which represents the probability that the alternate hypothesis differentiates between the sample and control groups only owing to chance. In the 2^{- $\Delta\Delta C_t$} method, one-way analysis of variance (ANOVA) was performed and the results were compared by Student's *t*-test (*P* < 0.05) using the XLSTAT software (Addinsoft 2005).

Results

Phylogenetic analysis of the fungal ITS sequence

To confirm that the fungal samples used in the study belong to the species *Pisolithus albus*, we analysed the ITS sequences (ITS1/5.8S rRNA gene/ITS2) of each isolate. The ITS sequence is a commonly used phylogenetic marker for Dikarya (Ascomycetes and Basidiomycetes) and some lower fungi. The sequences were deposited in the EMBL Database and referenced under the accession numbers reported in Table 1. The phylogenetic analysis was performed as described previously (Jourand *et al.* 2010a). The results, which are presented as supplementary data in Fig. S2 (Supporting information) confirmed that the isolates belong to *P. albus*.

DNA pyrosequencing, assembly and sequence analysis of the nickel-tolerant *Pisolithus albus* isolate MD06-337

We obtained two non-normalized cDNA libraries from the nickel-tolerant *P. albus* isolate MD06-337 grown in the presence and absence of nickel. Pyrosequencing of these libraries yielded 1 071 375 single reads, which comprised 542 680 trimmed reads from *P. albus* grown in medium that contained nickel and 528 695 trimmed reads from *P. albus* grown in the absence of nickel. The average read lengths were 238 and 236 bp, respectively (Table S2, Supporting information). We used a two-step assembly procedure to process these reads and generated two groups of contigs by excluding low-quality sequences (sequences shorter than the length threshold, i.e. <200 bp, were discarded). The two groups corresponded to 18 800 contigs from *P. albus* exposed to nickel and 16 636 contigs from *P. albus* that had not been exposed to nickel. These two groups of contigs were pooled and reassembled to highlight the genes that were expressed differently in response to nickel. A final group of 19 518 contiguous sequences (from 813 651 total read counts) from *P. albus* MD06-337 was obtained, with an average length of 698 bp. There were also 254 424 singleton reads (24%; Table S2, Supporting information). Hereafter, the contiguous sequences are referred to as genes. Of the 19 518 genes that were obtained through the *de novo* assembly of the sequence reads, the expression of 30% of the genes was modulated by nickel. Further analysis identified 4211 genes (21%) that were up-regulated by nickel and 1763 genes (9%) that were down-regulated by nickel. The global statistical distribution of these 19 518 genes is presented on a scatter plot in Fig. 1. To identify the greatest possible number of descriptive annotations for each sequence, global gene prediction was carried out on the basis of a

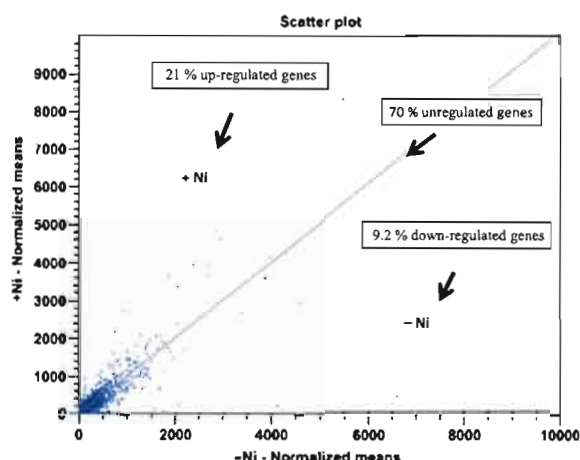


Fig. 1 Analysis of 454 pyrosequencing data. The scatter plot represents gene expression levels in a free-living mycelium of the nickel-tolerant *P. albus* isolate (MD06-337) grown without nickel or with nickel at 250 μ M. The expression levels of genes were normalized using a scale of 0–10 000. Each circle in the plot represents the level of expression of one gene.

set of BLASTX searches (Table S2, Supporting information). Among the 19 518 unique contigs, the global BLASTX analysis indicated that 11 489 contigs (59%) had significant matches in the GenBank database, whereas 10 926 contigs (56%) had matches in the *Laccaria bicolor* database and 15 150 contigs (77.6%) in the *Pisolithus microcarpus* database (Table S2b, Supporting information).

GO annotation of genes expressed differentially in *Pisolithus albus*

To determine the global variation in gene expression in the MD06-337 isolate of *P. albus* in the presence and absence of nickel, we assigned GO terms to the sequences identified in the two pyrosequencing transcriptomic databanks. The result showed that 23% and 25% of the sequences from the databanks for *P. albus* grown in the presence and absence of nickel, respectively, could be assigned a GO term (Fig. 2). The distribution of sequences among the following primary GO categories was analysed: cellular component, molecular function and biological process. This showed differences in distribution between the two conditions within the categories of biological process and cellular component (Fig. 2). To focus on the factors that underlie nickel tolerance in *P. albus*, we also performed a GO analysis of the 4211 contigs (21%) that corresponded to genes that were up-regulated by nickel (Fig. S3, Supporting information). The analysis indicated that only 12.6% of the up-regulated genes could be assigned to the three GO categories, among which 53% were assigned to cellular components, 27% were assigned to

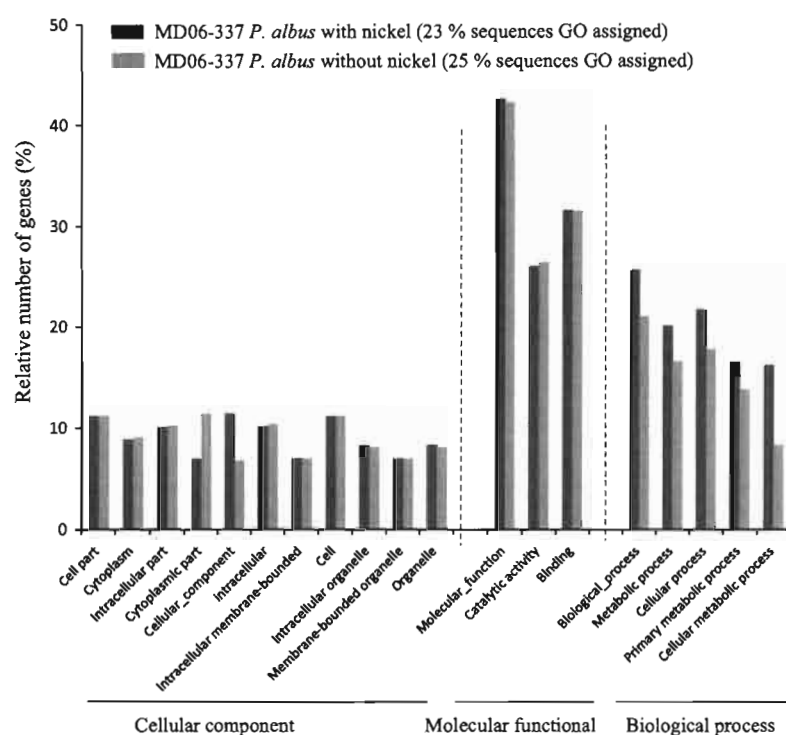


Fig. 2 Assignment of functional Gene Ontology terms and distribution of total sequences for two transcriptomes of the nickel-tolerant isolate MD06-337 of *P. albus* with (in the presence of 250 μ M NiCl_2) and without nickel, in terms of the categories of biological process, molecular function and cellular component.

biological processes and 20% were assigned to molecular function (Fig. S3, Supporting information).

Tracking of nickel biomarkers in nickel-tolerant MD06-337 *Pisolithus albus*

Among the genes that were expressed differentially in response to nickel (4211 genes), we selected the 10 genes that showed the greatest variation on the basis of the RPKM values obtained in the pyrosequencing analysis, as shown in Table 2. The names of the sequences correspond to the contig numbers (No. contigs in Table 2). All nucleotide sequences have been deposited in the EMBL Database, and their accession number and lengths (in base pairs) are summarized in Table 2. These DNA sequences were analysed using BLASTN, and the sequences were found to be identical in approximately 95–100% of cases to sequences in the *Pisolithus microcarpus* database (currently not publicly available, personal communication from Martin F., INRA Nancy, France) (Table 2). In addition, the function of the genes was determined using BLASTX sequence alignments (with a threshold of $<1\text{E}^{-03}$). The results showed homologies to putative proteins from the following species: *Laccaria bicolor* (seven genes, No. 2, 3, 4, 6, 8, 9 and 10), *Coprinopsis cinerea* (two genes, No. 5 and 7) and *Saccharomyces cerevisiae* (gene No. 1). Full data from the BLASTX search are presented in Table 3. The analysis of gene

function showed that genes No. 1, 3, 5, 6, 7, 8, 9 and 10 encoded proteins with predicted functions, whereas genes No. 2 and 4 encoded proteins with no known function (Table 3). The genes with a predicted function encoded for (i) cell-wall components (genes No. 1 and 7), (ii) components involved in metabolic processes and amino acid transport (genes No. 3, 6, 8 and 9), (iii) components involved in RNA processing (gene No. 10) and (iv) components involved in metallopeptidase processes (gene No. 5).

Validation of pyrosequencing results by RT-qPCR in nickel-tolerant MD06-337 *Pisolithus albus*

The 10 selected genes were analysed by RT-qPCR to confirm the differential expression that was indicated by the pyrosequencing data. For this purpose, specific primer sequences were designed from the DNA sequences obtained by pyrosequencing (Table S1, Supporting information). These primers were used in RT-qPCR to quantify gene expression in *P. albus* MD06-337 using two sets of cDNA. The first set corresponded to the cDNAs that were used for the pyrosequencing, whereas the second set corresponded to newly synthesized independent cDNAs from three independent mycelia. Both sets contained cDNAs that were obtained from *P. albus* grown in the presence of nickel and those of *P. albus* grown in the absence of nickel. The

Table 3 Functional description and assignment of GO terms for 10 candidate biomarker genes (No. 1–10), up-regulated following exposure to nickel, as selected from pyrosequencing analyses that revealed differential expression in nickel-tolerant *Pisolithus albus* isolate MD06-337

No. contig	No. gene	BLASTX functional identification					
		Best predicted function	Species	E-value	Blast hit	Functional classification	GO terms
C10864	1	GPI-anchored cell surface glycoprotein-like (AGA1)	<i>Saccharomyces cerevisiae</i>	5E ⁻⁰³	YNR044W	Cell-wall anchorage	No hits found
C6406	2	Unknown function	<i>Laccaria bicolor</i>	3E ⁻⁰⁸	XM_001881526.1	Unknown function	No hits found
C9200	3	Alpha-aminoadipate reductase Lys1p (LbLYS1)	<i>L. bicolor</i>	8E ⁻⁶⁴	XM_001879583.1	Secondary metabolite biosynthesis	GO:0003824
C7318	4	Unknown function	<i>L. bicolor</i>	3E ⁻⁰⁷	XP_001878436.1	Unknown function	No hits found
C10795	5	Vacuolar protein sorting-associated protein 70	<i>Coprinopsis cinerea</i>	4E ⁻²⁰	XP_001837057.2	Metalloprotease activity	GO:0004180
C15327	6	S-adenosylmethionine-dependent methyltransferase	<i>L. bicolor</i>	2E ⁻²¹	XP_001874602.1	Methyl transferase	GO:0016740
C17653	7	Class III chitinase	<i>C. cinerea</i>	5E ⁻⁰⁸	XM_001828384.2	Chitin catabolic process	No hits found
C18133	8	Cytochrome P450 (CYP)	<i>L. bicolor</i>	4E ⁻⁴⁰	XP_001878510.1	Monoxygenase activity	GO:0009055
C10491	9	APC amino acid permease	<i>L. bicolor</i>	4E ⁻³⁹	XP_001875612.1	Amino acid transport	GO:0006560
C4552	10	bZip transcription factor	<i>L. bicolor</i>	9E ⁻¹⁰	XP_569310.1	RNA processing	GO:0009267

GO, Gene Ontology.

RT-qPCR results for the selected genes are presented in Table 4 and are given as the ratio of mRNA expression in the presence of nickel to mRNA expression in the absence of nickel (ratio +Ni/-Ni). The relative expression levels that were obtained from the cDNAs used in

the pyrosequencing analysis and from the new cDNA samples (means $\pm$ SD of the three replicates) are compared in Table 4. The data show that there were no significant differences between gene expression profiles in relation to the cDNA template used (pyrosequenced

Table 4 Validation of the pyrosequencing data by RT-qPCR. Relative induction of expression by nickel of the 10 selected genes (up-regulated by nickel: No. 1–10) was measured in the MD06-337 nickel-tolerant isolate, using GAPDH and EF4 α as reference genes

No. contigs/genes	C10864	C6406	C9200	C7318	C10795	C15327	C17653	C18133	C10491	C4552
Relative expression (cDNA 454)*	10***	10.4***	6.3	6.5	4.2***	8***	3.5	39***	17.3***	7.7***
(1) P(H1)	0.0001	0.0001	0.49	0.49	0.0001	0.0001	0.49	0.0001	0.0001	0.0001
†Relative expression (3n)	5.1**	4.1*	2.3	8***	5*	6.5**	6***	8.5	39.5**	5.2***
(2) P(H1)	0.004	0.05	0.175	0.0001	0.022	0.007	0.0001	0.066	0.005	0.0001

Relative expression values were determined by normalizing expression levels against those of the housekeeping genes that encode glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and elongation factor 4 α (EF4 α).

*Values in line (1) represent the relative expression levels of the 10 genes as determined using the cDNA used for the pyrosequencing analysis of MD06-337 isolate.

†Values in line (2) represent the means of three biological independent relative expressions qPCR on the MD06-337 isolate.

All statistical computation and analyses of qPCR data were carried out using the REST 2009 software (Pfaffl *et al.* 2002).

We used the hypothesis tests P(H1).

*P < 0.05; **P < 0.01; ***P < 0.001.

Values indicate the mean $\pm$ SD (n = 3).

DNA or new cDNA; $P > 0.05$). The ratios of gene expression that were obtained using RT-qPCR confirmed the transcriptomic profile of nickel-tolerant *P. albus* MD06-337 that was obtained by pyrosequencing. We also used the REST method to compare the relative expression patterns of the 10 selected genes in the nickel-tolerant isolate MD06-337 at 50 μM , with expression patterns in the presence of 250 μM of nickel. The results are presented in Fig. S4 (Supporting information) and show similar profiles for the two concentrations of nickel tested. In addition, RT-qPCR was performed to verify the levels of mRNA expression of the 10 genes selected in nickel tolerant MD06-337 ECM root tips sampled in situ. The results (Table 5) showed that the selected genes were also expressed in symbiotic

ECM root tips sampled in situ, except for gene No.7, which was not detected in ECM root tips.

Comparison of expression patterns in nickel-tolerant (MD06-337) and nickel-sensitive (MD09-078) isolates of *Pisolithus albus*

We compared the expression profiles of the 10 selected genes that were up-regulated by nickel between the nickel-tolerant isolate (MD06-337) and a nickel-sensitive isolate (MD09-078) from the ultramafic soils. As shown in Fig. 3, the levels of expression of the genes analysed differed significantly between the nickel-sensitive isolate MD09-078 and the nickel-tolerant isolate MD09-337 ($P < 0.05$). Genes No. 1, 2, 5, 6 and 7 were expressed

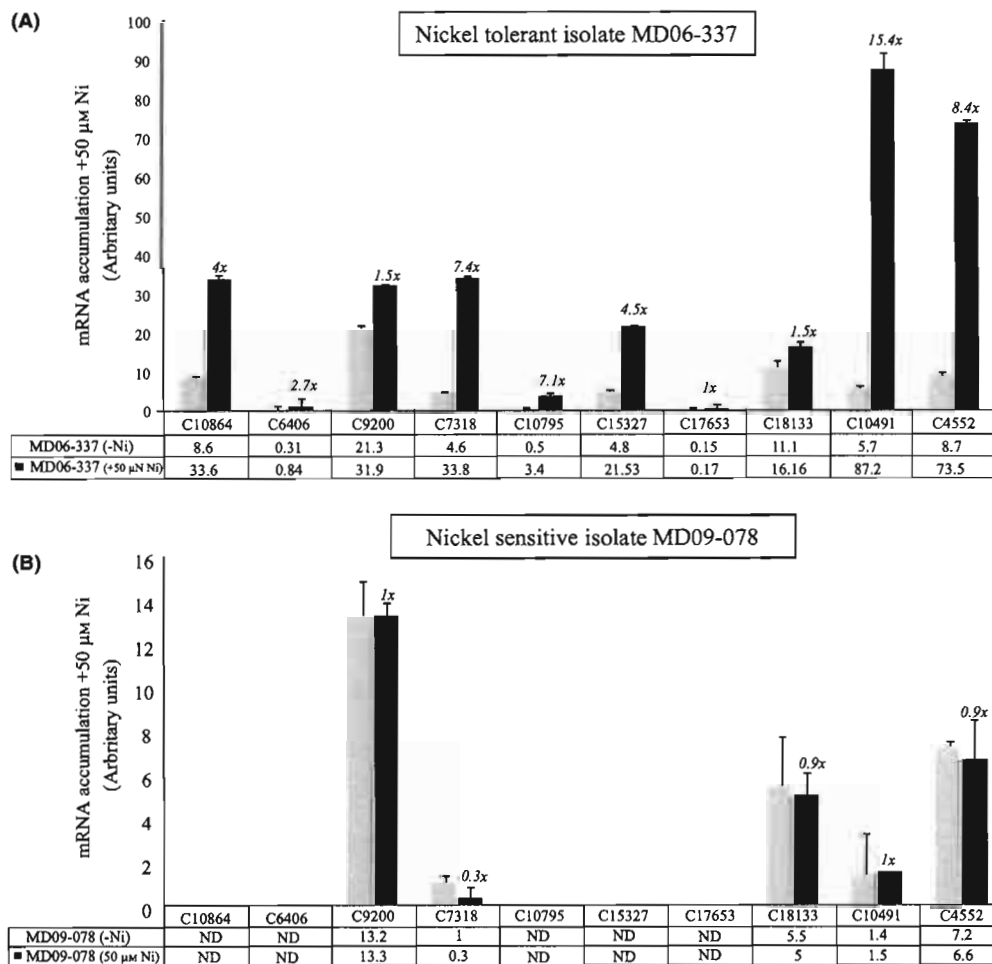


Fig. 3 Comparison of patterns of mRNA accumulation in *P. albus* isolates grown without nickel or with nickel (provided as 50 μM NiCl_2). Expression of the 10 genes up-regulated most by nickel, as determined by pyrosequencing, in nickel-tolerant (MD06-337) and nickel-sensitive (MD09-078) isolates. Transcript accumulation was quantified by RT-qPCR, using the $2^{-\Delta\Delta\text{Ct}}$ method with glyceraldehyde-3-phosphate dehydrogenase and EF4 α as reference genes for normalization, and expressed as arbitrary units. The data indicate mean values $\pm$ SD, calculated from three technical replicates with triplicate biological samples. The fold induction by nickel is presented above the columns in italics. ND: mRNA nondetected (Ct values >37).

Table 5 *In situ* validation of the pyrosequencing data by RT-qPCR. Relative induction of expression by nickel of the 10 selected genes (up-regulated by nickel: No. 1–10) in ectomycorrhizal *Pisolithus albus* root tips (nickel-tolerant MD06-337 isolate and nickel-sensitive MD09-078 isolate) *in situ* was measured by comparison with the nickel-tolerant isolate MD06-337 and nickel-sensitive isolate MD09-078, *in vitro*. Transcript accumulation was quantified by RT-qPCR using the $2^{-\Delta\Delta Ct}$ method with normalization using two reference genes, GAPDH and EF4 α

No. gene	No. 1 C10864	No. 2 C6406	No. 3 C9200	No. 4 C7318	No. 5 C10795	No. 6 C15327	No. 7 C17653	No. 8 C18133	No. 9 C10491	No. 10 C4552
mRNA accumulation										
ECM (MD06-337) <i>in situ</i>	11	0.4	8.4	5.1	1.2	1.3	nd	0.8	3	7
Nickel-tolerant isolate										
Control (<i>in vitro</i>)	10	10.4	6.3	6.5	4.2	8	3.5	39	17.3	7.7
MD06-337										
(UF-like + 250 μ M nickel)										
ECM (MD09-078) <i>in situ</i>	0.25	0.74	48.2	35.7	0.24	0.41	0.24	18.5	0.34	26.6
Nickel-sensitive isolate										
Control (<i>in vitro</i>)	nd	nd	22	3	0.30	0.24	0.21	8	1.5	3
MD09-078										
(UF-like + 50 μ M nickel)										

nd, not detected (CT > 37); ECM, ectomycorrhizal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

very weakly or were not detectable (ND) in the MD09-078 isolate, whereas they were expressed strongly in MD06-337 isolate. As shown in Fig. 3, the normalized expression levels varied from 0.15 to 87 arbitrary units. The remaining genes, No. 3, 4, 8, 9 and 10, were expressed at substantial levels in both isolates. However, whereas in the nickel-sensitive isolate, the expression of these genes (No. 3, 4, 8, 9 and 10) was not affected by nickel (there was no significant induction), in the nickel-tolerant isolate, nickel induced an increase in expression of these genes of between 1.5- and 15-fold in the nickel-tolerant isolate. From this comparison of gene expression levels between nickel-sensitive and nickel-tolerant isolates, we identified genes No. 1, 2, 5, 6 and 7 as showing the most significant differences in terms of levels of expression. We concluded that they show the greatest promise as molecular markers (biomarkers) of tolerance to nickel in *P. albus* from the ultramafic soils of New Caledonia. We also included gene No. 9 in this set of promising biomarkers because it was induced strongly by nickel in the nickel-tolerant isolate. These six selected genes were used as biomarkers for the final analysis of nickel tolerance. In addition, RT-qPCR was performed to verify the levels of mRNA expression for the 10 selected genes in ECM samples from nickel-sensitive *P. albus* isolate MD09-078 sampled *in situ* (Table 5). The RT-qPCR results showed that the six genes that were selected as biomarkers were either not expressed, or only weakly expressed, in nickel-sensitive isolate from ECM root tips sampled *in situ*. These results are in agreement with those obtained by *in vitro* experiments conducted using the nickel-sensitive *P. albus* isolate MD09-078.

Comparison of biomarker expression between nickel-tolerant and nickel-sensitive *Pisolithus albus* isolates

We tested the expression levels of the six biomarkers in five isolates of *P. albus* from the ultramafic soils of New Caledonia. These included three nickel-tolerant isolates (MD06-337, MD09-045 and MD09-001) and two nickel-sensitive isolates (MD09-078 and MD09-063). The six biomarkers were expressed in all three nickel-tolerant isolates (MD06-337, MD09-045 and MD09-001), but they were not expressed in any of the nickel-sensitive isolates, as shown in Fig. 4. In the nickel-tolerant isolates, the presence of 50 μ M nickel increased the expression of the six genes by between one- and ninefold (Fig. 4).

Discussion

We have used a transcriptomic approach to characterize the expression of potential biomarkers of nickel tolerance in the ECM fungus *Pisolithus albus* from the ultramafic soils of New Caledonia. The demonstrations that 21% of all genes in *P. albus* are up-regulated in the presence of nickel and that six genes are expressed exclusively in nickel-tolerant isolates of *P. albus* together suggest the presence of a molecular transcriptomic adaptive response to nickel-rich environments in this species.

Transcriptomic database of nickel-tolerant *Pisolithus albus*

In previous studies, genes that are regulated by heavy metals in ECM fungi have been identified by

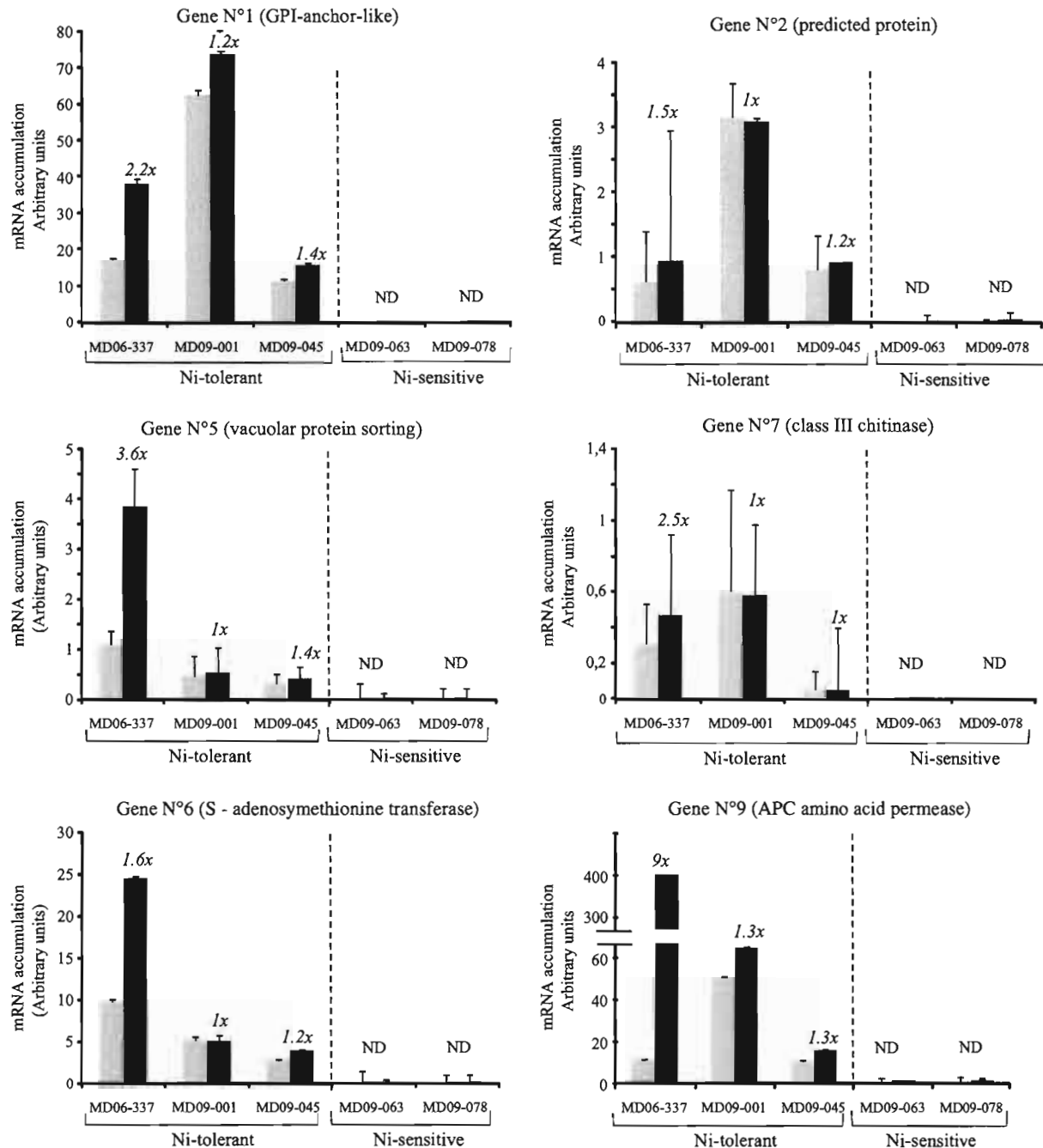


Fig. 4 Comparison of mRNA accumulation profiles for the six selected up-regulated genes in five *P. albus* isolates from ultramafic soil in the presence of nickel 50 µM (black columns) and in the absence of nickel (grey columns). Three nickel-tolerant isolates (MD06-337, MD09-045 and MD07-001) and two nickel-sensitive isolates (MD09-078 and MD09-063) were compared. Transcript accumulation was quantified by qPCR using $2^{-\Delta\Delta CT}$ method with normalization to two reference genes, glyceraldehyde-3-phosphate dehydrogenase and EF4 α , and is expressed as arbitrary units. The data indicate mean values $\pm$ SD values, calculated from three technical replicates with triplicate biological samples. The fold induction by nickel is presented above the black columns in italics. ND: mRNA nondetected (Ct values >37).

transcriptomic approaches, such as DNA microarray or cDNA-AFLP (amplified fragment length polymorphism) analysis (Jacob *et al.* 2004; Muller *et al.* 2007a). These studies were conducted mostly on ECM samples isolated from soils polluted with heavy metals as a result of human activities (Jacob *et al.* 2004; Muller *et al.* 2007b; Ruytinx *et al.* 2011). Here, we report a transcriptome database, generated using pyrosequencing, for an isolate (MD06-337) of the ECM fungus *P. albus*, which was isolated from extreme soils that naturally contain high concentrations of heavy metals, particularly nickel, and is tolerant of nickel. To the best of our knowledge, this is the first repository of its kind.

We also characterized molecular biomarkers that were associated with tolerance to nickel in *P. albus*. The reference database that we generated for *P. albus* comprised 19 518 contigs, of which 56% were also found in the *Laccaria bicolor* database (Martin *et al.* 2008) and 77.6% in the *Pisolithus microcarpus* database (F. Martin, *P. microcarpus* database version 1.0; INRA laboratory, Nancy, France). Moreover, the differential transcriptomic analyses that we conducted showed that, after growth for 15 days in the presence of nickel, 30% of all genes in the *P. albus* genome varied in their levels of expression relative to the expression levels in the absence of nickel. Analysis of the changes in the transcriptome of *P. albus* between these two conditions showed that of the differentially expressed genes, 21% were up-regulated following exposure to nickel and 9% were down-regulated following exposure to nickel. In a previous study, it was demonstrated that environmental changes could markedly affect gene expression profiles (Gibson 2008). We also identified the genes for which expression was affected the most by exposure to nickel. Our results suggest that, at a minimum, all of the 5855 genes that showed differential expression in the nickel-tolerant *P. albus* isolate in response to nickel contribute to tolerance of the fungus to the extreme ultramafic environment in New Caledonia. The experiments that were based on GO analysis and functional genetic tools suggest also the role of these genes as (i) biomarkers of tolerance to nickel and (ii) putative adaptive mechanisms of nickel tolerance in *P. albus*. Among the sequences obtained from the pyrosequencing analysis of *P. albus* grown in the presence and absence of nickel, only 25% and 23%, respectively, were assigned to one or more GO categories. Comparison of the results showed that the global distributions of GO terms for the two nickel conditions were similar for the category of 'molecular function', but not for the categories of 'biological process' and 'cellular component'. Moreover, a large number of orphan genes were present in the two databanks, which suggests that the accuracy of the functional annotation was limited. This limited accuracy

can probably be explained by the fact there is no reference transcriptome available for *P. albus*, and there is no transcriptome available for related species under similar conditions. Indeed, our database is the first to be based on the full transcriptome from an organism isolated from an environment that naturally contains high concentrations of a heavy metal.

Most previous studies that involved comparative transcriptomic approaches have focussed initially on genes up-regulated by environmental stimuli or developmental cues (Chelaifa *et al.* 2010). In our study, we focussed on the GO distribution of the genes for which expression was up-regulated by nickel. The majority of genes up-regulated by nickel belonged to the GO category 'cellular component'. Information on the annotations of these genes is valuable for the further investigation of gene functions, cellular structures and biological processes that might be involved in the tolerance of fungi to nickel via extracellular and intracellular mechanisms, as suggested by Bellion *et al.* (2006).

Predicted function of the specific biomarkers

In previous studies involving fungi, many genes involved in the response to stress induced by heavy metals were found to encode proteins that function as metal transporters or metal-binding proteins (Jacob *et al.* 2004; Bellion *et al.* 2006; Bolchi *et al.* 2011). However, in the present study, most of the nickel biomarkers did not belong to classes of proteins that are typically involved in response mechanisms to stress induced by heavy metals. This suggests that the mechanisms that underlie the tolerance to nickel in *P. albus* from ultramafic soils differ from those of other fungi.

Two of the biomarkers identified, namely levels of mRNA transcripts that encode chitinase-like and glycosylphosphatidylinositol (GPI) cell-wall structural proteins, are involved in extracellular processes and encode putative cell-wall proteins. Recently, it was suggested that modifications of structural elements of the cell wall, such as the rearrangement of chitin and biosynthesis of glucan- or galactosamine-containing polymers, might play a key role in modulating the integrity of the cell wall and its capacity to immobilize heavy metals. In this way, such modifications could confer tolerance to metals and affect the ability of fungi to survive in stressful environments (Meharg 2003; Bellion *et al.* 2006; Fuchs & Mylonakis 2009). Our findings regarding the expression of genes that are involved in the rearrangement of the cell wall in the presence of nickel suggest that these genes play a substantial role in modifying the chemical composition and hence the integrity of the cell wall in *P. albus*. This provides an effective barrier that can chelate the heavy metal.

The other biomarkers that were up-regulated in the presence of nickel were predicted to function in intracellular mechanisms such as resistance to oxidative stress, transcriptional regulation and subcellular compartmentalization. One biomarker encodes a putative S-adenosylmethionine-methyl (SAM) transferase. In the fungus *Podospora anserina*, overexpression of SAM transferase increases resistance to metal-induced oxidative stress by counteracting the pro-oxidant effect of phenolic compounds in the presence of heavy metal. This overexpression of SAM transferase leads to a decrease in the formation of reactive oxygen species (ROS) (Kunstmann & Osiewacz 2009), which are known to cause irreversible and lethal oxidative damage to cells (Gill & Tuteja 2010). Our results in *P. albus* suggest that SAM transferase is also overexpressed in the presence of nickel and thus might increase fungal resistance to metal-dependent oxidative stress by methylating phenolic compounds, which are known to accumulate in *P. albus*.

Another biomarker encodes a basic leucine zipper (bZIP) transcription factor. Previous studies on *Saccharomyces cerevisiae* have characterized AP-1 as a master regulator that orchestrates responses to metal/metalloid stress (Wysocki & Tamas 2010). Another study showed that a transcription factor related to AP-1-like (a member of the bZIP family) is up-regulated in the fungus *Paxillus involutus* upon exposure to cadmium. This suggests that Zip1-like proteins could play a critical role in the transcription of genes that are essential for protection against cadmium stress (Jacob *et al.* 2004). The overexpression of a bZip transcription factor in *P. albus* in the presence of nickel suggests that members of the bZIP family might act as regulators of genes involved in tolerance of metals.

Another of the selected biomarkers encodes a putative vacuole targeting-related protein. A previous study on vacuolar-defective mutants of *S. cerevisiae* confirmed the essential role that the vacuole plays in the detoxification of metals as such as nickel, zinc, cobalt and manganese. It was hypothesized that compartmentalization of these toxic metals in the vacuole prevents them from harming the cell (Ramsay & Gadd 1997). In the present study, the overexpression of a vacuole targeting-related protein in the presence of nickel suggests that the vacuole may play a key role in metal detoxification in *P. albus*.

Regarding the other biomarkers, no known function related to nickel tolerance could be identified. Taken together, the results for predicted protein function agree with the results of the GO analyses, in which the genes that were up-regulated by nickel were classified into the established GO categories 'cellular components', 'molecular function' and 'biological processes'.

Characterization of biomarker expression in *Pisolithus albus*

Comparison of the levels of expression of the 10 genes that were up-regulated most strongly in response to nickel (Fig. 3) revealed marked differences between the nickel-tolerant and nickel-sensitive phenotypes of different *P. albus* isolates from ultramafic soils. It also enabled us to identify five genes that were expressed exclusively in nickel-tolerant isolates. The remaining five genes were not selected as candidate biomarkers because they were expressed in both phenotypes. However, these genes might also be of value in predicting nickel tolerance because their level of expression was higher in the nickel-tolerant isolate than in the nickel-sensitive one, and their induction by nickel was observed only in the nickel-tolerant isolate. To strengthen the robustness of the predictive value of these final five biomarkers, levels of the appropriate transcripts were analysed in additional nickel-tolerant and nickel-sensitive isolates of *P. albus*. The data (Fig. 4) confirmed that these five final selected biomarkers of *P. albus* are suitable for identifying nickel-tolerant fungal isolates.

Given that ECM symbioses are known to play a major role in the fitness of plants in the presence of heavy metals (Jentschke & Godbold 2000), these biomarkers should also be analysed in the context of symbiotic interactions between ECM fungi and plants. Preliminary results from *in vitro* assays of nickel-tolerant *P. albus* (MD06-337) and nickel-sensitive *P. albus* (MD09-078) in symbioses with two members of the *Myrtaceae*, the model species *Eucalyptus globulus* and *Tristanopsis* sp., which is endemic to New Caledonia, show that these biomarkers are expressed exclusively in nickel-tolerant ECM symbioses (data not shown). This confirms their suitability for monitoring the adaptation of ECM symbioses to nickel both *in vitro* and *in situ*.

Such candidate gene approaches, which involve the identification of individual genes linked to nickel tolerance, provide a starting point for investigating the molecular basis of adaptation to heavy metal content in soils and contribute to understanding tolerance for ultramafic soils in natural fungal populations (Brady *et al.* 2005). Interestingly, for each gene, the level of expression that was induced by nickel varied among the isolates tested. This intraspecific variation in transcript profiles was also observed in cadmium-tolerant *Suillus luteus* isolates, which suggests that such variation helps fungal species to persist in novel environments (Ruytinx *et al.* 2011). Interestingly, for each gene, the level of expression that was induced by nickel varied among the isolates tested, for both nickel-tolerant and nickel-sensitive isolates of *P. albus*.

The presence of both nickel-tolerant and nickel-sensitive fungal phenotypes in the same ultramafic habitat could be explained by variation in the effective concentration of nickel in such soils. Recent studies have shown that the ecotoxicological risk of nickel to organisms in ultramafic soils depends on its availability and that nickel shows local concentration gradients and variation in metal mobility (Becquer *et al.* 2006; Echevarria *et al.* 2006; Wright & Stanton 2011). Edaphic variation could have induced a variety of responses to cope with nickel toxicity in ultramafic soils (Branco 2009). Alternatively, elevated levels of magnesium in the soils could explain the presence of the nickel-sensitive isolates. Indeed, high concentrations of magnesium were found exclusively in the soils at the sites where the two sensitive isolates MD09-078 and MD09-063 were sampled (Table S3, Supporting information). Previous studies have shown that in yeast, the magnesium transporter allows low-affinity uptake of other divalent cations such as nickel, manganese, zinc and cobalt and that the *GAI* mutant of the yeast *Schizosaccharomyces pombe*, which is defective in the transport of magnesium, is resistant to nickel and cobalt (Sarikaya *et al.* 2006). In addition, magnesium is known to be an antagonist of nickel (Sarikaya *et al.* 2006). These observations could indicate that magnesium prevents the nickel uptake, thereby alleviating nickel toxicity in ultramafic soils and allowing the growth of fungi with a nickel-sensitive phenotype. These fungal responses to nickel could result from environment-dependent phenotypic expression or from genetic adaptation (or specialization) to local biotic and abiotic conditions (Branco 2009; Wolfe *et al.* 2009). Selection in ultramafic environments is associated with multiple constraints in addition to nickel concentration. These include pH, metal abundance, shortage of macronutrients, cation exchange capacity and water deficit. These constraints might have favoured genetic differentiation of *P. albus* between ultramafic and non-ultramafic sites as previously described (Jourand *et al.* 2010a,b). Further experiments should be carried out to assess the existence of local adaptation or phenotypic plasticity of *P. albus* to nickel in ultramafic soils. These experiments should include comparison of fitness parameters for isolates of *P. albus* that have been collected from their native soils and cultivated under different nickel conditions, and analysis of the physiological and molecular responses to nickel in the presence of the other metals that are found in ultramafic soils.

Conclusion

We have described transcriptomic differences in the molecular response to nickel between nickel-tolerant and nickel-sensitive isolates of *P. albus* from ultramafic

soils. As a result, we have been able to identify specific biomarkers of nickel tolerance, which are expressed in nickel-tolerant isolates *in vitro* as well as *in situ* in ECM isolates. We suggest (i) that these results reflect a positive transcriptomic response of the fungus to nickel-rich environments and (ii) the presence of both nickel-tolerant and nickel-sensitive fungal phenotypes in ultramafic soils might reflect environment-dependent phenotypic responses to variations in the effective concentrations of nickel in heterogeneous ultramafic habitats. In addition, the results of our study suggest new directions for genetic research in terms of the selection of novel isolates that are tolerant to nickel or various other ultramafic conditions such as elevated levels of chromium, cobalt, or manganese, a low calcium:magnesium ratio, or low levels of essential macro-/micronutrients and drought stress.

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The main research interest of the authors focuses on the role of ectomycorrhizal symbiosis in plant adaptation to extreme edaphic conditions as found in ultramafic soils (deficiency in major plant nutrients, unbalanced ratio Ca/Mg and high contents in heavy metals such as Co, Cr, Mn and Ni).

Data accessibility

Internal transcribed spacer DNA sequences: EMBL accessions FR852890, FR852891, FR852892, FR852893. The trimmed pyrosequencing contig sequences (Table 2) are available in the EMBL Database under the following accession numbers: contig C10864: FR852894; contig C6406: FR852895; contig C9200: FR852896; contig C7318: FR852897; contig C10795: FR852898; contig C15327: FR852899; contig C17653: FR852900; contig C18133: FR852901; contig C10491: FR852902; contig C4552: FR852903. mRNA accumulation data: The data used for Fig. 3 are included in the figure. The data used for Fig. 4 are available in Table S4 (Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 primer sequences (Primer express software), amplification size and efficiency of the selected ESTs and two reference genes (GAPDH and EF4) used for qPCR analysis.

Table S2 Pyrosequencing 454 data from *P. albus* MD06-337 isolate.

Table S3 Geochemical characteristics of rhizospheric soils site: major element content.

Table S4 Comparison of mRNA accumulation profiles for the six selected up-regulated genes in five *P. albus* isolates from ultramafic soil.

Fig. S1 General geographical map describing the New Caledonian archipelago with location of ultramafic massifs (in grey) and sites where *P. albus* isolates were collected (1, 2, 3, 4 & 5).

Fig. S2 Phylogenetic synthetic relationships among representative *Pisolithus* sp., in particular *P. albus* isolates from New Caledonia included in this study, i.e. MD06-337, MD09-078, MD09-045, MD09-063 and MD09-001 and worldwide reference isolates.

Fig. S3 Functional GO terms assignment and distribution of sequences GO terms of 4211 genes nickel up regulated from the differential transcriptome of MD06-337 nickel tolerant isolate from *P. albus* with +250 µM of nickel and without nickel, among Gene Ontology (GO) biological process, molecular function and cellular component.

Fig. S4 Relative expression patterns of 10 nickel selected genes from nickel tolerant *P. albus* MD06-337 isolate with two reference genes GAPDH and EIF4a. Panel A and B show respectively, relative expression patterns of 10 nickel with 250 or 50 µM of nickel, after 15 days of growth.

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Indole acetic acid and ACC deaminase-producing *Rhizobium leguminosarum* bv. *trifolii* SN10 promote rice growth, and in the process undergo colonization and chemotaxis

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Abstract This study focuses on the chemotaxis, colonization and rice growth promoting ability of indole acetic acid (IAA) and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase-producing rhizobacteria *Rhizobium leguminosarum* bv. *trifolii* SN10, previously isolated from root nodules of *Trifolium alexandrinum* L. We show here that *R. leguminosarum* bv. *trifolii* SN10 promote the growth of four different rice varieties grown in India in terms of biomass, root branching and N content. In addition, using scanning electron microscopy and viable cell counts, we provide evidence that the bacteria successfully colonize the root surface of the rice variety which showed maximum growth promotion upon inoculation. Not only this, *R. leguminosarum* bv. *trifolii* SN10

exhibit a strong chemotaxis response towards the rice seed and root exudates despite the presence of a bacteriostatic phenolic compound, 7-hydroxycoumarin (umbelliferone). Further, *R. leguminosarum* bv. *trifolii* SN10 secretion of phytohormones such as IAA and ACC deaminase suggest the potential of the plant growth promoting rhizobacteria to be used as biofertilizer to enhance rice crop production in the subcontinent.

Keywords Chemotaxis · Flavonoids · Plant growth promoting rhizobacteria (PGPR) · *Rhizobium* · Rice

Introduction

Nitrogen-fixing bacteria, especially *Rhizobia* and *Bradyrhizobia*, have been known for many years as specific beneficial symbionts for legumes. However, studies mainly in the last decade have revealed that roots of gramineous plant-like rice (Chaintreuil et al. 2000; Yanni et al. 1997), wheat (Hilali et al. 2001), sugarcane and maize (reviewed in Bhattacharjee et al. 2008) can also host as a third niche for the nitrogen-fixing bacteria. Such plant–bacteria association was found to have great potential in improving production of non-leguminous crops, manifested in terms of increased seedling vigor, yield, growth and nutrient uptake, photosynthetic activity, stomatal conductance and N content (reviewed in Bhattacharjee et al. 2008).

The benefits conferred by rhizobial association with non-legumes have been believed to be primarily the result of plant growth promoting rhizobacterial (PGPR) activities (Hilali et al. 2001; Yanni et al. 2001). PGPR exert their growth promoting effect by a wide variety of mechanisms. These include production of growth promoting compounds, such

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as, phytohormones (indole acetic acid (IAA), gibberellins and cytokinins) and siderophores or solubilization of mineral phosphates (Glick 1995; Nelson 2004). In addition, PGPR exert antagonism towards plant pathogens by releasing metabolites like antibiotics, cell wall lytic enzymes or by inducing defense systemic responses (Nelson 2004). PGPR interaction with host plant is complex and not yet fully understood. Plant root colonization by the bacteria is considered as a primary step towards the successful initiation of the plant–microbe interaction (Nelson 2004). Colonization in turn depends on bacterial motility and chemotaxis (specific migration towards chemicals) towards the plant root release or exudates (Bashan et al. 2004). Root exudates are a rich source of nutrients for rhizospheric bacteria that undergo chemotaxis and colonize the plant root surface (Bais et al. 2006). PGPR that make their way to the plant interior are termed endophytes. In either case, the PGPR exert their beneficiary effect on the plant in terms of growth and development.

One such PGPR isolated from rice (*Oryza sativa* L.), cultivated in the field of interior Egypt, was *Rhizobium leguminosarum* bv. *trifolii* (Yanni et al. 1997). This bacterium harbored the potential to improve growth and development of various rice and wheat varieties grown in Egypt. In India, rice forms the staple food crop for more than 60% of the population. With increasing demand, the need to increase production is leading to an excessive application of chemical fertilizers. In the light of growing environmental concerns associated with chemical fertilizer, rhizobial association with cereal is gaining importance as it promises to provide a cleaner path towards maintaining crop production targets. Considering the benefits that would arise from the interaction of rice and PGPR, in this study we have assessed the growth-promoting activities of a phytohormone-producing strain of *R. leguminosarum* bv. *trifolii* (isolated from Berseem clover) on rice. Furthermore, to understand this rice–*Rhizobium* interaction, we have investigated the involvement of rhizobial chemotaxis and plant root colonization.

Materials and methods

Plant seeds and bacterial strains

The strain SN10 of *R. leguminosarum* bv. *trifolii* used in this study was obtained from CCS Haryana Agricultural University, Hisar, India (Gaur et al. 2002). *Escherichia coli* DH5 α strain was provided from Bethesda Research Laboratories (Gaithersburg, MD). The bacterial cultures were maintained on yeast extract mannitol (YEM) medium (Vincent 1970). Rice seeds were obtained from IARI, Delhi India. Seeds of *Trifolium alexandrinum* L. were obtained

from LSTM collection centre, Montpellier, France and IARI, New Delhi, India.

Gnotobiotic rice growth promotion studies

De-hulled seeds of rice were surface-sterilized by soaking in 96% ethanol for 15 min followed by 0.2% HgCl₂ for 5 min. The seeds were then rinsed several times and allowed to imbibe overnight in the dark in sterile water. To allow the germination of the seeds and to check contamination, the surface sterilized seeds were transferred to plates containing Cambel's media (Chaintreuil et al. 2000). The plates were incubated in the dark at 28°C till the plantlets developed 1- to 2-cm long roots. Rice plantlets showing no contamination were soaked for 5 min in an axenic mid-log phase (OD₆₀₀, ~0.6 to 0.8) rhizobial culture in YEM (Vincent 1970). As control, plants were inoculated with suspension of *E. coli* or heat-killed rhizobia in YEM. All the plantlets (inoculated and controls) were then transferred to 25- $\times$ 200-mm tubes (one seedling per tube) containing 20 ml of either N-free Jensen's medium (Vincent 1970) or Jensen's medium containing KNO₃ (10 mM), supplemented with 0.8% agar. The plants were grown for 6 weeks in a growth chamber at 28°C, with a 12-h night and day cycle, adding nutrient solution (N-free Jensen's medium and Jensen's media with 10 mM KNO₃+0.2% agar) every week, under aseptic conditions. Twenty plants were used in each experiment, and three independent experiments were performed. For the dry weight, both shoots and roots were oven-dried at 60°C until the weight became constant (~5 days). The N content of the shoot was estimated by the Kjeldahl method (AOAC 1990).

Bacterial colonization counting and scanning electron microscopy

Rice roots from gnotobiotic seedlings (six plants) after 10 days of growth were carefully removed from each tube, excised, washed with sterile water, blotted dry, divided into two parts. One portion (three roots per plant) was used for viable cell count after taking the fresh weight. The number of bacteria present in the external rooting medium and on the root surface was determined by viable count method as described previously (Yanni et al. 2001). The CFU obtained from three roots in each of the three tubes were then averaged with the SD. The other portion was used for scanning electron microscopy (SEM). Roots for microscopic analysis were carefully rinsed with distilled deionised water and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7) overnight. Tissues were then washed in 0.1 M phosphate buffer, four times for 15 min each. This was followed by distilled H₂O wash 3 $\times$ 5 min. Tissues were dehydrated in graded ethanol series. Samples were then

lyophilized for 3–4 h for critical drying. The moisture-free samples were coated with silver using the Sputter Coater E5100 (Polaron, England) by plasma coating. The thickness of the film was about 100–150 Å. The coated samples were then observed under SEM (Cambridge Setero Scan 360, UK).

Rice seed and root exudates collection

A hundred surface-sterilized seeds of *O. sativa* L. ssp. *japonica* cv. Kdulam, were soaked in 20 ml of sterile water overnight in the dark at 28°C. The solution thus obtained was termed as seed exudates for this study. For root exudates, the germinated seeds were grown on a paper support in 20 tubes containing liquid Jensen media (five seedlings per tube) for 10 days at 28°C with an 18-h light and 6-h dark period in gnotobiotic conditions. The strength of the media was reduced to 1/10 to minimize the interference of nutrient. The nutrient solution was filtered through a 0.2-µm Millipore filter. A batch of rice cultivar Kdulam was inoculated with *R. leguminosarum* bv. *trifolii* SN10. In order to remove the bacteria, root exudates obtained from this batch were centrifuged at 1,200×g for 20 min at 4°C prior to filtration. The exudates were lyophilized and were kept at –20°C for later use.

Chemotaxis assay

The chemotactic responses of the bacterial species towards rice exudates were evaluated using: (1) swarm plate assay (Adler 1973) and (2) Pfeffer's capillary tube assay (Adler et al. 1973). The exudates used for this assay were prepared by dissolving 10 mg of the lyophilized exudates in 0.5 ml of sterile distilled water. In these tests, *T. alexandrium* L. exudates were used as positive control while Phosphate Buffer Saline (PBS, pH 7.0) was used as negative control. Similarly, other chemotaxis assays were conducted by testing chemicals at a final concentration of 10 mM prepared in PBS (pH 7.0)

Detection and quantification of glucose, proline and flavonoid

Detection and quantification of D-glucose was performed using the glucose oxidase peroxidase method. This was done using the GOD–POD Merckotest^R Glucose (Merck) as described in the user's manual. For estimation of L-proline, acid ninhydrin was used as described before (Bates et al. 1973). For the detection of flavonoid compounds, lyophilized seed exudates and the root exudates were extracted with 80% methanol (~160 mg.ml⁻¹) overnight at 4°C, with shaking. The flavonoids were purified by using a Sep-Pak C18 column (Waters) as described in the user's

manual. The high-performance liquid chromatography (HPLC) equipment used for the separation and characterization of the exudates consisted of a Hewlett-Packard 1090 M liquid chromatography system equipped with a diode array detector (200–400 nm). Chromatographic runs were performed on a Waters Symmetry C18 (250×4.6 mm) column. The solvent system and elution profiles used were as described previously (Mathesius et al. 1998). For mass spectrometry (JASCO-1585, Great Dunmow, UK Ltd.) electrospray spectra were obtained using the following: LC/MS system 2790 alliance equipped with a 996 photodiode array detector connected to a Q-TOF I (Micromass UK Ltd., Manchester) electrospray mass spectrometer operated in the positive ionization mode, electrospray; capillary voltage, 3 kV; cone voltage, 40 V; source block temperature, 150°C; desolvation temperature, 250°C. The mass range scanned was 50–1,500 Da, at a rate of 3 s per scan. The column and the conditions used to analyze the samples were the same as used for HPLC/DAD analysis. The synthetic flavonoid used in the identification 7-hydroxycoumarin was obtained from Indofine Chemical Company, Inc., USA. The standards were prepared as indicated by the provider and stored in dark bottles at –20°C.

Detection of 1-aminocyclopropane-1-carboxylic acid deaminase, indole acetic acid

The ACC deaminase activity test was performed as already described (Belimov et al. 2001). Estimation of IAA was done using Salkowski's reagent as described previously (Patten and Glick 2002).

Umbelliferone tolerance test

Bacterial culture dilutions from 10⁻¹ to 10⁻⁹ of either *R. leguminosarum* bv. *trifolii* SN10 or *E. coli* DH5α were prepared from a fresh culture at OD_{600nm}=1, in liquid YEM and plated on YEM containing plates. Sterile filter paper discs similar to those used for antibiograms were treated with 50 µl of umbelliferone concentrations ranging from 1 nM to 10 mM in solution in diluted NaOH (0.01 M), and were placed on the bacteria-plated YEM plates. Diluted NaOH without umbelliferone was used as control. Plates were incubated for 48 h at 30°C. Bacterial tolerance or sensitivity was measured by the radial inhibition of growth reported in millimeters at the lowest concentration tested.

Statistical analysis

Data were analyzed by ANOVA, and the means were compared following Fisher's test of least significant difference to assess the effects of inoculation on plant growth promotion.

Results

IAA and ACC deaminase production by *R. leguminosarum* bv. *trifolii* SN10

In a culture filtrate, *R. leguminosarum* bv. *trifolii* SN10 previously isolated from roots of the leguminous plant *T. alexandrinum* (Berseem clover) synthesize IAA to a final concentration of $2 \pm 0.1 \mu\text{g.ml}^{-1}$. The IAA production by the bacteria increased in the presence of L-tryptophan in the media. In the presence of this amino acid at $100 \mu\text{g.ml}^{-1}$ in the media, the IAA production increased from $2 \pm 0.1 \mu\text{g.ml}^{-1}$ to $25 \pm 1 \mu\text{g.ml}^{-1}$. *R. leguminosarum* bv. *trifolii* SN10 were able to grow on medium containing ACC as the sole N source, confirming the ability to produce ACC deaminase. The ACC deaminase activity of *R. leguminosarum* bv. *trifolii* SN10 was measured to be $15 \pm 2 (\mu\text{mol NH}_4^+.\text{mg}^{-1} \text{ of protein.h}^{-1})$.

Effect of *R. leguminosarum* bv. *trifolii* SN10 inoculation on rice growth

R. leguminosarum bv. *trifolii* SN10 were inoculated to four varieties of rice in the absence of basal N. The performance of the bacteria was scored after 30 days of growth in terms

of plant biomass, root branching and increase in N content. Plants inoculated with heat-killed rhizobia in YEM were used as control. In N-free media, rhizobial inoculation led to an increase in total plant biomass (shoot and root) ranging from 19–61% when compared to heat-killed rhizobia inoculated plants used as the control (Fig. 1a). A similar pattern of increase was observed in the root branching of the four rice varieties upon rhizobial inoculation (Fig. 1b, d). There was minimal change in the N content of the inoculated plants in the absence of basal N in the media (Fig. 1c). Among the four varieties tested, performance of *R. leguminosarum* bv. *trifolii* SN10 was significantly high when inoculated to local variety *Oryza sativa japonica* cv. Kdulam where the increase of plant biomass was almost 60%. To investigate the effect of the presence of a N source on the performance of *R. leguminosarum* bv. *trifolii* SN10, we added 10 mM of KNO_3 to the media. As *O. sativa japonica* cv. Kdulam showed maximum response to *R. leguminosarum* bv. *trifolii* SN10 inoculation, for the rest of the studies, we used this plant microbe combination. Without rhizobial inoculation, plant biomass was increased by 25% in plants grown in the presence of KNO_3 compared to the control plants grown without a N source. However, the biomass increased two times upon the addition of *R. leguminosarum* bv. *trifolii* SN10 to KNO_3 -

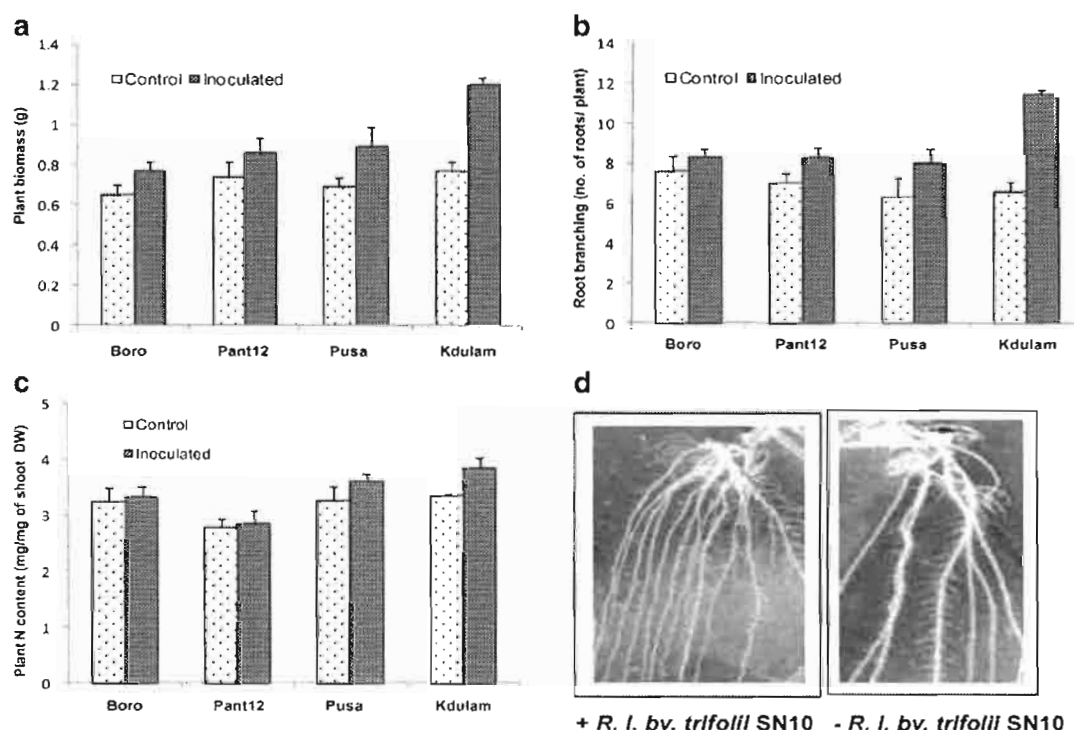


Fig. 1 Growth promotion of different rice varieties after 30 days of inoculation with *R. leguminosarum* bv. *trifolii* SN10 **a** total plant biomass; **b** root branching; **c** plant N content of the four rice varieties

with and without rhizobial inoculation, *DW* dry weight; **d** root branching observed in *O. sativa* L. subsp. *japonica* cv. Kdulam after inoculation with *R. leguminosarum* bv. *trifolii* SN10

containing media when compared to the control (+KNO₃; *R. leguminosarum* bv. *trifolii* SN10) (Table 1). Rhizobial inoculation also led to a change in root morphology in terms of root branching. When compared to the control, root branching increased to 64% in N-free medium, and to 82% in medium supplemented with N (Table 1). Of significant importance is the increase in N content in N-supplemented *R. leguminosarum* bv. *trifolii* SN10-inoculated plants. There was an increase of the shoot N content to 28% when compared to heat-killed rhizobial inoculation (Table 1).

External colonization of rice roots by *R. leguminosarum* bv. *trifolii* SN10

PGPR are known to colonize plant roots while exerting their growth promoting effect (Nelson 2004). We therefore investigated the ability of *R. leguminosarum* bv. *trifolii* SN10 to colonize rice roots by dilution plating and SEM. Roots from inoculated rice were scored for adhering bacteria for 10 days. Uninoculated and *E. coli*-inoculated plants were used as control. Loosely attached bacteria were removed by washing rice roots thoroughly with water. Washing the roots reduced the number of colonies observed by dilution plating in *E. coli*-inoculated plants. In contrast, there was only a 10% reduction in the *R. leguminosarum* bv. *trifolii* SN10-inoculated plants. Overall, the colonization of *R. leguminosarum* bv. *trifolii* SN10 on rice roots were 95–100% more than the *E. coli* and uninoculated plants (Table 2). Colonization was further confirmed by SEM. The roots of 10-day-old rice-inoculated plants were densely populated with *R. leguminosarum* bv. *trifolii* SN10 (Fig. 2).

Chemotaxis towards rice root exudates

Colonization of rice roots by *R. leguminosarum* bv. *trifolii* SN10 prompted us to investigate the in vitro chemotactic motility of the bacteria towards rice root release (exudates) *T. alexandrium* L. is the natural leguminous host plant for

R. leguminosarum bv. *trifolii*, so the root/seed exudates of the plant were used as positive control for this experiment. The average distance moved by *R. leguminosarum* bv. *trifolii* SN10 in rice (Kdulam) seed exudates containing plate was equivalent to the distance traveled in the presence of *T. alexandrium* L. seed exudates and root exudates. When exudates and free compounds were replaced by PBS in the assay, there was no swarm by *R. leguminosarum* bv. *trifolii* (Table 3). *E. coli* did not show any significant movement in the presence of the exudates. Further corroboration of these results was obtained by Pfeffer's capillary tube (Table 3). Pfeffer's capillary tube result showed that seed exudates attracted almost twofold higher bacteria than rice root exudates.

In addition, sugars and amino acids earlier reported to be present in rice seed and root exudates in the first week of germination were individually tested (Bacilio-Jimenez et al. 2003). Among the free sugars and amino acids tested (listed in Table 3), *R. leguminosarum* bv. *trifolii* SN10 showed a significant chemotactic response towards D-glucose (10 mM) and L-proline (10 mM) in both soft and capillary tube. *R. leguminosarum* bv. *trifolii* SN10 exhibited a positive response even at lower concentrations (2 and 5 mM) of D-glucose and L-proline. The bacterial strain exhibited a significantly lower chemotactic response to other amino acids and sugars in soft agar and capillary tube. However, *E. coli* exhibited a positive chemotaxis towards glucose (data not shown).

Detection of D-glucose and L-proline in rice root and seed exudates

A chemotaxis-promoting compound glucose was found to be an important constituent of *O. sativa* subsp. *japonica* cv. Kdulam seed exudates ($1.16 \pm 0.1 \mu\text{g} \cdot \text{mg}^{-1}$ of seed exudates) and root exudates ($0.25 \pm 0.05 \mu\text{g} \cdot \text{mg}^{-1}$ of root exudates). In addition, proline was also detected but at a lower concentration than glucose in both seed exudates

Table 1 Plant growth and physiological parameters after inoculation of *O. sativa* L. subsp. *japonica* cv. Kdulam with *R. leguminosarum* bv. *trifolii* SN10

Inoculation	Medium	Shoot DW (g)	Root DW (g)	Root Branching (number of roots per plant)	N content (in mg/g of shoot DW)
<i>R. leguminosarum</i> bv. <i>trifolii</i> SN10	N-free	$0.84 \pm 0.05\text{b}$	$0.40 \pm 0.06\text{a}$	$11.3 \pm 0.5\text{a}$	$3.85 \pm 0.05\text{a}$
	KNO ₃ (10 mM)	$1.00 \pm 0.05\text{a}$	$0.42 \pm 0.06\text{a}$	$12.0 \pm 0.5\text{a}$	$5.25 \pm 0.05\text{b}$
Heat-killed rhizobia in YEM	N-free	$0.52 \pm 0.04\text{b}$	$0.27 \pm 0.05\text{b}$	$6.9 \pm 0.1\text{b}$	$3.35 \pm 0.05\text{c}$
	KNO ₃ (10 mM)	$0.76 \pm 0.05\text{b}$	$0.25 \pm 0.05\text{b}$	$6.6 \pm 0.1\text{b}$	4.10 ± 0.05

Plants inoculated with heat-killed inoculum of *R. leguminosarum* bv. *trifolii* SN10 in YEM medium were the control. Results are means $\pm$ standard deviation of three independent experiments with each treatment measured on 20 plants. All data with the same letters are not significantly different ($P \leq 0.05$)

DW dry weight

Table 2 Bacterial colonization of *O. sativa* sp. *japonica* cv. Kdulam after inoculation with either *R. leguminosarum* bv. *trifolii* SN10, *E. coli* DH5 α , killed inocula or sterile YEM (controls)

	Rhizosphere (growth media) in CFU/ml	Rice root in CFU/gfw of tissue
<i>R. leguminosarum</i> bv. <i>trifolii</i> SN10	$6.3 \times 10^7 (\pm 0.4 \times 10^7)$ a	$6.7 \times 10^5 (\pm 0.7 \times 10^5)$ b
<i>E. coli</i> DH5 α	$1.2 \times 10^3 (\pm 0.5 \times 10^3)$ c	ND
Controls	ND	ND

Results are means $\pm$ standard deviation of three independent experiments with each treatment measured on three repetitions. All data with the same letters are not significantly different ($P \leq 0.05$)

CFU colony forming unit, gfw gram fresh weight, ND not detected

($0.66 \pm 0.05 \mu\text{g} \cdot \text{mg}^{-1}$) and root exudates ($0.25 \pm 0.05 \mu\text{g} \cdot \text{mg}^{-1}$). These concentrations corresponded from 13.2 to 23.2 mM of D-glucose and 5 mM of L-proline in root and seed exudates respectively, which are in the range of concentrations of free D-glucose and L-proline tested for positive chemotaxis of *R. leguminosarum* bv. *trifolii* SN10.

Rice root releases a phenolic compound that inhibit *E. coli* growth

Phenolic compounds released by plants can act as biocontrol for microbial growth (Siqueira et al. 1991). We therefore investigated the rice root exudates for the presence of phenolic compounds that inhibited *E. coli* chemotaxis in seed and root exudates despite of the presence of a high concentration of glucose in the latter. HPLC-MS analysis indicated that a phenolic compound 7-hydroxycoumarin (retention time of 15.40 min and absorption maxima of 322.85 nm) is present at a detectable concentration in uninfected exudates (Fig. 3a). The mass spectrometry analysis

of both molecular ions $(M+H)^+$ and $(M+AcN)^+$ of this compound gives m/z 163.06 and 204.08 which is in agreement with the structure of umbelliferone (data not shown). However, its concentration steeply increases from $15 \pm 1.5 \mu\text{g} \cdot \text{g}^{-1}$ (without rhizobia) to $58 \pm 2 \mu\text{g} \cdot \text{g}^{-1}$ in *E. coli* and *R. leguminosarum* bv. *trifolii* SN10-infected exudates (Fig. 3b). We therefore checked the sensitivity of *R. leguminosarum* bv. *trifolii* SN10 towards different concentrations of 7-hydroxycoumarin in vitro. At a high concentration of 10 mM, 7-hydroxycoumarin does not have any adverse effect on rhizobial survival. However, in the case of *E. coli*, hollow zones indicating bacterial inhibitions were observed at all concentrations tested.

Discussion

Over the years, the growing demand of rice has led to the indiscriminate use of chemical fertilizers around the world. With arising concerns about the effects of fertilizer use on the

Fig. 2 Colonization of roots of *O. sativa* L. subsp. *japonica* cv. Kdulam, *R. leguminosarum* bv. *trifolii* SN10, 10 days after inoculation. SEM photograph showing general colonization of the surface of secondary roots a uninoculated rice plant, b–d rice plant inoculated with *R. leguminosarum* bv. *trifolii* SN10

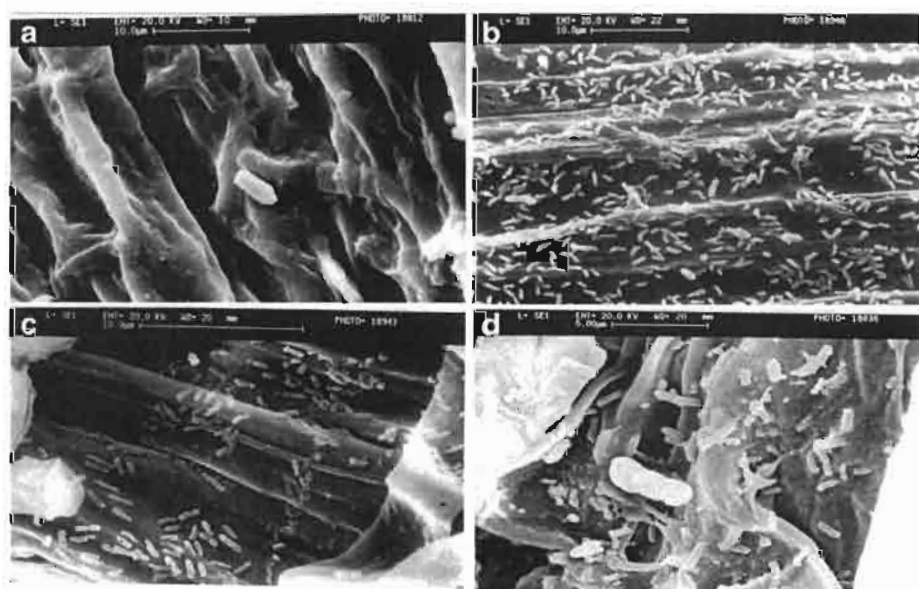


Table 3 Induction of chemotaxis of *Rhizobium leguminosarum* bv. *trifolii* SN10

Chemo attractant	Swarm diameter ^a	Number of bacteria ($\times 10^3$) in Pfeffer capillary tube (Adler 1973)
Plant exudates		
<i>Oryza sativa</i> L. ssp. <i>japonica</i> cv. Kdulam		
Seed exudates	++	25 $\pm$ 1
Root exudates	++	17 $\pm$ 1.5
<i>Trifolium alexandrinum</i>		
Seed exudates (20 mg/ml)	++	30 $\pm$ 2.75
Root exudates (20 mg/ml)	++	27 $\pm$ 2.5
Chemicals		
Amino acids (10 mM)		
L-Proline	+	12 $\pm$ 2
L-Histidine	–	0.05 $\pm$ 0.1
L-Valine	–	0.25 $\pm$ 0.05
L-Alanine	–	0.20 $\pm$ 0.07
L-Glycine	–	0.07 $\pm$ 0.02
Carbohydrates (10 mM)		
D-Glucose	++	21 $\pm$ 1.5
D-Mannose	–	1.5 $\pm$ 0.5
D-Galactose, D-arabinose, D-xylose	–	2.7 $\pm$ 0.25
D-Glucuronic acid	–	0.04 $\pm$ 0.01
Control		
Phosphate buffer (pH 7, 10 mM)	–	0.02 $\pm$ 0.05

++ swarm diameter between 6 and 8 mm, + swarm diameter between 3 and 6 mm, – swarm diameter between 1 and 2 mm

^aRefers to the movement of the swarm diameter on the 2nd day after inoculation

environment, the quest for non-consequential alternatives is being promoted. India is one of the largest rice producers in the world. In this context, we found that a clover symbiont synthesizing ACC deaminase and IAA, *R. leguminosarum* bv. *trifolii* SN10, could enhance growth of various varieties of rice grown in the subcontinent. In this association, *R. leguminosarum* bv. *trifolii* SN10 acts as a PGPR, undergoes chemotaxis and successfully colonizes the plant resisting the inhibitory effect of an antimicrobial compound released by the plant.

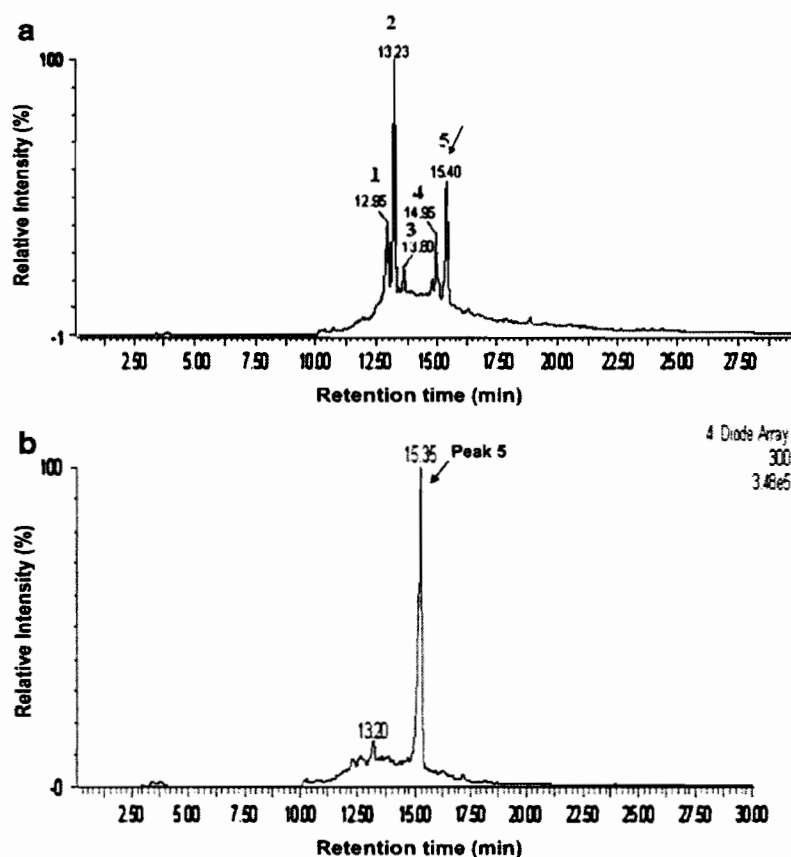
As a general feature, we observed that *R. leguminosarum* bv. *trifolii* SN10 isolated from the legume *T. alexandrinum*, has stimulatory effects on different rice varieties evaluated in terms of shoot and root biomass and root branching. However, the effect of the bacteria is dependent on the variety of rice. Emphasis on the selection of the combination of bacteria and plant for obtaining optimum response has been made previously by other workers (Biswas et al. 2000a; Pedraza et al. 2010; Yanni et al. 2001). *R. leguminosarum* bv. *trifolii* are known to fix N in vitro as well as in association with legume (O'gara and Shanmugam 1978; Kishinevsky et al. 1992). However, it has been observed that *R. leguminosarum* bv. *trifolii* does not fix N in association with rice (Biswas et al. 2000; Yanni et al. 1997, 2001). Thus, the performance of the bacteria improved further when used in supplementation to N in the media. We can therefore

speculate that IAA and ACC deaminase production by *R. leguminosarum* bv. *trifolii* SN10 may account for the observed increase in plant biomass in biotised plants (Glick 1995; Khalid et al. 2005; Shaharoona et al. 2006; Zahir et al. 2011). Root branching would provide an increased surface area for adsorption of nutrients (Yanni et al. 1997) in soil explaining the additional gain in rice plant biomass and N content upon *R. leguminosarum* bv. *trifolii* SN10 inoculation.

In addition to phytohormone production by the bacteria, plant growth promotion also depends on the establishment of the inoculated strain in the rhizosphere. Thus colonization of the host plant by PGPR is a prerequisite for exerting a growth-promoting effect on the host plant (Larcher et al. 2003). A huge colonization on the surface of *O. sativa japonica* roots by *R. leguminosarum* bv. *trifolii* SN10 as against the *E. coli*-inoculated batch, indicates the specific rice root colonizing capacity of the bacteria. *R. leguminosarum* bv. *trifolii* SN10 adherence to the root was significant as loosely attached bacteria could be removed by washing. Our observation is thus in accordance with other studies which have shown that bacteria that promote plant growth also colonize the host plant (Larcher et al. 2003; Pedraza et al. 2010; Vande Broek et al. 1998).

It has been suggested that a prerequisite for the effective colonization of roots is positive chemotaxis towards root exudates (Vande Broek et al. 1998). A positive chemotaxis

Fig. 3 Flavonoid analysis of rice root exudates. HPLC profile of rice root exudates **a** without rhizobial inoculation, **b** with rhizobial inoculation



of *R. leguminosarum* bv. *trifolii* SN10 towards *O. sativa japonica* cv. Kdulam exudates therefore suggests that rice seed and root exudates release compounds that attract bacteria towards the plant leading to colonization. We also observed that *R. leguminosarum* bv. *trifolii* SN10 showed a significant chemotaxis towards glucose and proline which constituted considerable percentages of the seed and root exudates. The higher chemotaxis of *R. leguminosarum* bv. *trifolii* SN10 towards seed exudates than root exudates similar to the observation of Kato and Yasuhiro (2006) may be due to the higher concentration of glucose and proline in seed exudates. Most microorganisms use glucose, and in the case of proline as a source of C, thus *E. coli* showed positive chemotaxis towards glucose in the plate assay (Adler et al. 1973; Vilchez et al. 2000). Chemotaxis was also observed by some endophytic bacteria towards rice root exudates (Bacilio-Jimenez et al. 2003), *Azospirillum* towards strawberry (Pedraza et al. 2010) and *Nostoc* towards non-host *Trifolium repens* root extract (Nilsson et al. 2006). Our results suggest that in the case of this rice–*Rhizobium* interaction, biomolecules like glucose and proline function as major chemoattractants for the chemotaxis of *R. leguminosarum* bv. *trifolii* SN10.

The accumulation of high concentrations of 7-hydroxycoumarin upon inoculation with *R. leguminosarum* bv. *trifolii* SN10 in rice (*O. sativa japonica* cv. Kdulam) root exudates is a stress response towards microbial invasion. The coumarins are known to inhibit the growth and sporulation of fungal plant pathogens and to provide as well a defense tool against hostile microorganisms (Matern et al. 1999; Weinmann 1997). This high concentration of 7-hydroxycoumarin in these rice exudates produced upon microbial invasion might be the inhibitory compound that restricts *E. coli* chemotaxis (Duncan et al. 1998) as *E. coli* is sensitive to umbelliferone at any concentration.

The present results clearly indicate that *R. leguminosarum* bv. *trifolii* SN10, a symbiont of legumes can colonize rice root surfaces and confer beneficiary effects by enhancing root branching, increasing the biomass and N content of the plant, under laboratory conditions. Their colonization is subject to chemotaxis towards biomolecules released by rice root exudates. *R. leguminosarum* bv. *trifolii* SN10 confers the growth promoting activity to *O. sativa japonica* cv. Kdulam as a PGPR and resists the antimicrobial activity of the root release. Thus, *R. leguminosarum* bv. *trifolii* SN10 stands as a potential candidate for further field

studies which would be helpful in extending their use as a PGPR for rice in India.

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RESEARCH PAPER

Nodulation of *Crotalaria podocarpa* DC. by *Methylobacterium nodulans* displays very unusual features

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Abstract

Crotalaria are plants of the Fabaceae family whose nodulation characteristics have been little explored despite the recent discovery of their unexpected ability to be efficiently nodulated in symbiosis with bacteria of the genus *Methylobacterium*. It has been shown that methylotrophy plays a key role in this unusual symbiotic system, as it is expressed within the nodule and as non-methylotroph mutants had a depleting effect on plant growth response. Within the nodule, *Methylobacterium* is thus able to obtain carbon both from host plant photosynthesis and from methylotrophy. In this context, the aim of the present study was to show the histological and cytological impacts of both symbiotic and methylotrophic metabolism within *Crotalaria podocarpa* nodules. It was established that if *Crotalaria* nodules are multilobed, each lobe has the morphology of indeterminate nodules but with a different anatomy; that is, without root hair infection or infection threads. In the fixation zone, bacteroids display a spherical shape and there is no uninfected cell. *Crotalaria* nodulation by *Methylobacterium* displayed some very unusual characteristics such as starch storage within bacteroid-filled cells of the fixation zone and also the complete lysis of apical nodular tissues (where bacteria have a free-living shape and express methylotrophy). This lysis could possibly reflect the bacterial degradation of plant wall pectins through bacterial pectin methyl esterases, thus producing methanol as a substrate, allowing bacterial multiplication before release from the nodule.

Key words: *Crotalaria*, Fabaceae, *Methylobacterium*, methylotrophy, nitrogen fixation, nodulation, symbiosis.

Introduction

Among the *Crotalariae* tribe of the Papilionoideae sub-family, two genera were recently described as forming efficient nodulation with α -Proteobacteriu belonging to the genus *Methylobacterium*: *Crotalaria* (Sy *et al.*, 2001a) and *Lotononis* (Jaftha *et al.*, 2002). Depending on their symbionts, two groups can be distinguished among *Crotalaria* species. The first group contains species which are efficiently nodulated by *Methylobacterium* strains, whereas the second group contains species which are efficiently nodulated by *Bradyrhizobium* strains (Sy *et al.*, 2001b).

Methylobacterium strains are characterized by the ability to utilize, as a sole source of carbon and energy, methanol and other C1 compounds as well as a variety of multicarbon substrates (Lidstrom, 2006). *Methylobacterium nodulans* was originally isolated from *Crotalaria podocarpa* (Sy *et al.*, 2001b). *Methylobacterium nodulans* was proved to possess a *mx* genes cluster, coding for methanol dehydrogenase (MDH) (Sy *et al.*, 2001a). Inactivation of methylotrophy (through insertional mutagenesis in the *mx* gene) was found to drastically affect nodule number and plant growth

response (Jourand *et al.*, 2005). This is a major point of difference between *Crotalaria* and *Lotononis* as, in this last genus, *Methylobacterium* strains are unable to utilize methanol (Ardley *et al.*, 2009). The ability to use methanol, which is a plant by-product essentially resulting from pectin methyl esterase activity, was believed to give a competitive advantage to *Methylobacterium* for nodulation. Here the histological and cytological characteristics of the *C. podocarpa* nodulation by the *M. nodulans* ORS 2060^T strain are described.

Materials and methods

Bacterial strains and growth conditions

Methylobacterium nodulans strain ORS 2060^T (Samba *et al.*, 1999; Sy *et al.*, 2001a; Jourand *et al.*, 2004) and the methylo-trophic mutant A5:ORS 2060 *mTn5-GGm-mxaF* (Jourand *et al.*, 2005) were grown aerobically at 37 °C in YM medium (Vincent, 1970). To evaluate their growth capacity with pectins as the sole carbon and energy sources, both strains were cultivated on liquid minimum M72 medium (Green 1992) supplemented with 1% (w/v) of either 0% or 60% methylated polygalacturonic acid, and their OD_{600 nm} was measured after 170 h.

Plant tests

Crotalaria podocarpa seeds were obtained from IRD (Bel-Air, Dakar, Senegal). Germination and cultivation were conducted in Gibson tubes in plant culture rooms at 28 °C under a 16 h photoperiod and a light intensity of 60 microEinstein (μE) m⁻² s⁻¹ light as described by Le Roux *et al.* (2009). On day 15 after germination, plant roots were inoculated with 5 ml of bacterial culture, reaching an optical density at 600 nm of 1. Sterile distilled water was used for control.

Histological and cytological studies

Freshly harvested nodules were either directly sectioned to a thickness of 30 μm using a Vibratome (Leica France) or fixed for 2 h in 0.1 M sodium cacodylate-buffered 2.5% glutaraldehyde, pH 7.4 and washed three times in the same buffer. They were post-fixed for 45 min in 0.1 M sodium cacodylate-buffered 1% osmium tetroxide, pH 7.4, and washed three times for 20 min in the same buffer before dehydration in a graded series of ethanol. After impregnation in LR White resin (Newman *et al.*, 1982) and inclusion, polymerization was carried out at 60 °C for 24 h. Semi- and ultrathin sections were made with a Diatome (Leica, France) diamond knife. For transmission electronic microscopy, ultrathin sections were routinely treated with uranyl acetate and lead citrate and observed on a Jeol 1200EX transmission electron microscope. For light microscopy, semi-thin sections were stained according to standard procedures (Clarke, 1981) with 0.1% (w/v) toluidine blue in a 1% borax solution, 0.005% (w/v) ruthenium red, 0.01% (w/v) calcofluor white M2R, 0.1% (w/v) acridine orange, or standard periodic acid–Schiff (PAS), and observed on an Olympus Provis microscope.

Results

Mature (i.e. 2–4 weeks old) *C. podocarpa* nodules are multilobed (Fig. 1A), anchored to the root at a single point from which vascularization ramifies to all the lobes (Fig. 1B). Within one mature lobe (Fig. 1B) the morphology is apparently that of an indeterminate nodule as in alfalfa (Vasse *et al.*, 1990), with an apical meristematic zone (I), an

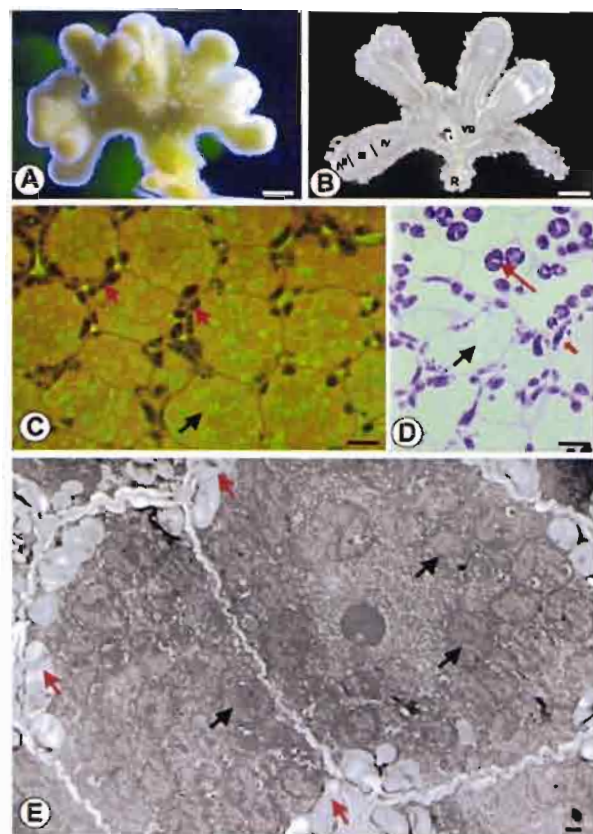


Fig. 1. Histological analysis of a *Crotalaria podocarpa*–*Methylobacterium* strain ORS 2060^T root nodule. (A) General view of a nodule with its typical crotalarioid shape, i.e. lobed and flattened. Bar=1 mm. (B) Longitudinal section through a multilobular nodule showing the anchoring of the vascular bundles (VB) on the root system (R), and the different nodule zones (numbered I–IV according to Vasse *et al.*, 1990). Bar=1 mm. (C and D) Histological staining of resin-embedded sections through fixation zone III. (C) Acridine orange staining observed under fluorescence microscopy showing the general occurrence of both bacteroids (black arrow) and amyloplasts (red arrows) within infected cells. Bar=8 μm. (D) Periodic acid–Schiff stain, observed under a light microscope, showing the different shape of amyloplasts (red arrows) between cortical uninfected cells (round shape, large arrow) and central infected cells (flattened shape, in the peripheral position, small arrow). Black arrow: bacteroids. Bar=8 μm. (E) Transmission electron microscope images of sections through infected cells in fixation zone III of a nodule, illustrating amyloplasts (red arrows) and spherical bacteroids (black arrows) co-existing within active cells of fixation zone III. Bar=2 μm.

invasion zone (II), a fixation zone (III), and a senescence zone (IV).

Within zone II, bacteria are present as classical rods both in the intercellular spaces and intracellularly (not illustrated). No infection threads delivering bacteria to young plant cells were observed in this zone. Within fixation zone III, bacteroids are globally spherical to ovoid (Fig. 1C–E), with a mean diameter of ~4 μm. All the plant cells are

infected, lacking uninfected interstitial cells. Amyloplasts are abundant in the peripheral (cortical) uninfected cells of the nodules, clearly stained by PAS (Fig. 1D). More surprisingly, they also are frequently observed with a more densely packed and flattened shape, within the bacteroid-filled cells of fixation zone III (Fig. 1C–E). In nodules induced by the methylotrophy-defective mutant A5 (Supplementary Fig. S1A available at *JXB* online), spherical bacteroids also co-exist with amyloplasts in fixation zone III.

In older nodules (i.e. 5–6 weeks old), the invasion zone II and the whole apex has an unusual shape, with a progressive loss of plant cell wall integrity, clearly revealed by cell wall stains such as Calcofluor White (Fig. 2A, B), toluidine blue (Fig. 2C), and ruthenium red (Supplementary Fig. S1B at *JXB* online). The whole zone is totally disorganized, with no more plant cell wall, but only longitudinally oriented cell remnants that seem, from histological stainings, to be of polysaccharidic nature (Fig. 2A, B, Supplementary Fig. S1B). In this zone, bacteria are often surrounded by a white halo (Fig. 2C, D), suggesting a lytic activity. In older, 6-week-old nodules, this zone sometimes is completely empty, with images looking like dental decay (Fig. 2E). Interestingly, nodules induced by mutant A5 did not present these apical degradations (Fig. 2F).

Bacterial growth of strains ORS 2060^T and A5 with pectins as the sole carbon and energy sources is presented in Supplementary Fig. S2 at *JXB* online. On polygalacturonic acid, neither of the two strains was able to grow. On 60% methylated pectin, only ORS 2060^T was able to grow.

Discussion

Crotalaria are tropical legumes with poorly explored nodulation properties. The early steps of the nodulation process of *C. podocarpa* by *M. nodulans* have not been described in detail. Root hairs are present, sometimes being deformed following inoculation, but infection threads were never observed. Together with the lack of interstitial cells, this would justify more studies to depict the penetration and internalization of bacteria clearly, and the induction of the nodule primordium.

Corby (1988), in his extensive morphotyping of legume nodules, attributed to *Crotalaria* nodules a particular type: crotalarioid. In *Lotononis*, also a member of the Crotalariae and nodulated by *Methylobacterium*, nodules are quite different, being lupinoid or coralloid and often completely enveloping or girdling the root (Yates *et al.*, 2007). With *C. podocarpa*, it was observed that each lobe has the morphology of an indeterminate nodule with a zonation as described by Vasse *et al.* (1990), but not with all the features described in *Medicago*, such as the presence of infection threads. These nodules have a limited growth, relayed by lateral branching and emergence of new lobes.

Within mature nodules, the dual shape of bacteria (i.e. rods and spherical bacteroids) is observed, in zone II and III, respectively. In *Lotononis*, symbiosomes appear spherical in

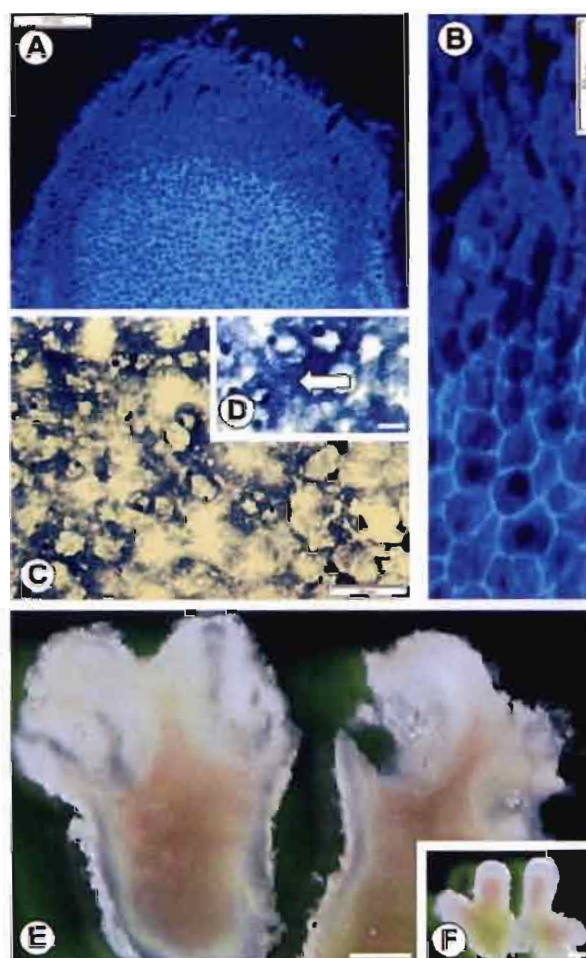


Fig. 2. Histological analysis of *Crotalaria podocarpa* root nodules after inoculation with either *Methylobacterium* strain ORS 2060^T or the methylotrophy-defective mutant A5. (A) Longitudinal Vibratome section through a mature nodule stained with Calcofluor White, under fluorescence microscopy, showing the progressive disorganization of the plant cell wall between fixation zone II and the nodule apex. Bar=200 µm. (B) Magnification of a section through the apical zone of a mature nodule stained with Calcofluor White showing the loss of plant cell wall integrity in this zone. Bar=50 µm. (C and D) Longitudinal Vibratome section through the apical part of a mature nodule stained with toluidine blue illustrating the total disorganization of the nodule tissue, and the presence of numerous unstained rod-shaped bacteria (white arrow) in the densely stained polysaccharidic deposits. Bar=20 µm. (E) Thick hand-cut longitudinal sections of 6-week-old nodules with their emptied apical zones, opened to the outside. Bar=1 mm. (F) Thick hand-cut longitudinal sections of 6-week-old nodules obtained in the same conditions as in E but with the non-methylotrophic mutant strain A5. No apical decay is observed. Bar=1 mm.

sections but they are 'normally oblong in wet mounts' (Yates *et al.*, 2007). Large spherical bacteroids were described in *Arachis* spp. (Sutton, 1983), among the Dalbergioids, a tribe phylogenetically distant from the Crotalarioids.

The occurrence of amyloplasts in nitrogen fixation zone III, co-existing with the spherical, fully differentiated bacteroids is a striking characteristic of *Methylobacterium* nodules. This association was proved to be symbiotically effective, as it was shown to induce higher plant growth responses than the association with the methylotrophy-defective mutant A5 (Jourand *et al.*, 2005). In *Medicago sativa*, it is assumed that, in central zone III, the metabolism of bacteroids is largely devoted to symbiotic functions such as nitrogen fixation and nutrient exchanges with the plant. Using *nif* mutants, Vasse *et al.* (1990) showed that the abundance of amyloplasts within zone III can be indicative of a non-efficient association. Amyloplasts were reported as particularly abundant in interzone II–III—a transition zone limited to 1–3 cell layers—and absent from fixation zone III in nitrogen-fixing alfalfa nodules (Vasse *et al.*, 1990). However, in efficient alfalfa nodules, amyloplasts seem relatively rare in the nodule cortex (Vasse *et al.*, 1990). Noticeably, amyloplasts also co-exist with bacteroids within nodules of the *mxoF* mutant strain A5, suggesting that starch storage has no direct link to methylotrophy. In *C. podocarpa*, Jourand *et al.* (2005), using *mxoF::LacZ* expression mutants, showed that methylotrophy would be restricted to free-living bacteria in zone II at the nodule apex, suggesting that after their differentiation bacteroids are no longer able to carry out methylotrophy.

Degeneration of the apical nodule tissue is another striking feature of *Methylobacterium*–*C. podocarpa* symbioses. In this zone, where the bacterial cells had their free-living shape, an intense lytic activity affects zones I and II in 6-week-old nodules. Despite efforts in the present study, it was not possible to fix and preserve this degenerated zone: it systematically emptied during passage through the different fixation, dehydration, and substitution baths required for inclusion in paraffin or plastic resins. This zone is likely to be a kind of soft ‘jelly’ whose aspect after cutting with a Vibratome (i.e. fresh slicing at 30 μ m thickness, without any fixation treatment) is apparently always out of focus as if this putative ‘jelly’ was limiting the resolution of microscopic observation and photography (e.g. Fig. 2C, D, Supplementary Fig. S1B at *JXB* online). The spontaneous emptying of these zones was observed in hand-cut sections of older, 6-week-old nodules (Fig. 2A). In this apical tissue, methylotrophy was intense at younger developmental stages, suggesting that methanol is present inside the nodule and subsequently used by the bacteria (Jourand *et al.*, 2005). These authors showed that methanol was detected at a substantially higher rate in the nodule than in the root and other plant tissues. In plants, methanol can be produced from different sources, among which is cell wall pectin demethylation (Downie *et al.*, 2004). During symbiosis between *M. nodulans* and *Crotalaria* spp., it is hypothesized that methanol could be produced from pectin cell wall demethylation. It was shown (Supplementary Fig. S2 at *JXB* online) that strain 2060^T was able to use methylated pectins—but not polygalacturonic acid—in pure culture, thus potentially being able to degrade plant cell walls progressively, to produce and use methanol. The

non-methylotrophic mutant strain A5 (Supplementary Fig. S2) was not able to use methylated pectins. Nodules formed by this mutant A5 do not present apical degradation (Fig. 2B), indicating a link between methylotrophy and this unusual characteristic. Strain ORS 2060^T would thus degrade plant cell walls in apical zones—where it is in a free-living shape—of the nodule, leading to the production of methanol from pectin methyl esterase activity, and allowing methylotrophy. The findings of Jourand *et al.* (2005) that methylotrophy and nitrogen fixation exist in two distinct and contiguous zones of the mature nodule, the apical zone II and the fixation zone III, respectively, were confirmed here. Since a loss of methylotrophy significantly affected nitrogen fixation efficiency (Jourand *et al.*, 2005), functional relationships should exist between these two zones, which remain to be uncovered.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. (A) Section through a nodule fixation zone of *Methylobacterium nodulans* non-methylotrophic mutant strain A5 after periodic acid–Schiff stain. There is no visible impact of the loss of methylotrophic ability in this zone. Bar=20 μ m. (B) Longitudinal section through a 6-week-old *M. nodulans* strain ORS 2060^T nodule, after staining with ruthenium red. Plant cell walls are totally disorganized in the nodule apex. Bar=200 μ m.

Figure S2. Growth response (optical density at 600 nm) of *Methylobacterium nodulans* strain ORS 2060^T (open lozenges) and its mutant A5 (open triangles) on minimum M72 medium (Green 1992) supplemented with 1% (w/v) of either 0% or 60% methylated polygalacturonic acid.

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New insights into the mycorrhizal status of Cyperaceae from ultramafic soils in New Caledonia

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Abstract: In New Caledonia, a hot spot of biodiversity, plants from the Cyperaceae family are mostly endemic and considered pioneers of the nickel-rich natural serpentine ecosystem. The aim of the study was to highlight the mycorrhizal status of these Cyperaceae and to bring new insights into the role of this symbiosis in plant tolerance to ultramafic soils. Nine Cyperaceae species were studied and presented evidence of root colonization by arbuscular mycorrhizas (AMs), with frequencies ranging from 8% to 57%. The highest level of AM colonization was observed in plants from the endemic dominant genus *Costularia*. Molecular evidence demonstrated the presence of *Glomus* sp. inside the roots. In a controlled greenhouse assay, AM inoculation of *Costularia comosa* grown under ultramafic conditions significantly enhanced plant growth, with an increase in biomass by up to 2.4-fold for shoots and 1.2-fold for roots, and also reduced nickel content in roots by 2.5-fold, as compared with the controls. All these data support our hypotheses (i) that a relationship exists between the mycorrhizal status of Cyperaceae and their habitat, and (ii) that AM have a positive role in plant tolerance to ultramafic soils (mineral nutrition and metal tolerance), suggesting the use of these pioneer plants with AM management as potential tools for nickel mine site rehabilitation in New Caledonia.

Key words: arbuscular mycorrhiza, Cyperaceae, ultramafic soil, New Caledonia.

Résumé : En Nouvelle-Calédonie, un point chaud de la biodiversité, les plantes appartenant à la famille des Cypéracées sont principalement endémiques et sont considérées comme pionnières de l'écosystème naturel de la serpentine riche en nickel. Cette étude visait à éclaircir le statut mycorrhizien de ces Cypéracées et d'apporter un nouvel aperçu de rôle de cette symbiose dans la tolérance des végétaux aux sols ultrabasiques. Neuf espèces de Cypéracées ont été étudiées et la colonisation des racines par des mycorhizes arbusculaires (MA) a été mise en évidence avec des fréquences variant de 8 % à 57 %. Le niveau de MA le plus élevé a été noté chez les plantes du genre endémique dominant *Costularia*. Les données moléculaires ont démontré la présence de *Glomus* sp. à l'intérieur des racines. Lors d'un essai en serre contrôlée, l'inoculation de MA chez *Costularia comosa* cultivée en conditions ultrabasiques a significativement accru la croissance des plants, avec une augmentation de la biomasse des pousses de 2,4 fois et des racines de 1,2 fois, réduisant aussi le contenu en nickel des racines de 2,5 fois, tout cela par rapport au contrôle. Toutes ces données plaident en faveur de l'hypothèse (i) d'une relation entre le statut en mycorhize des Cypéracées et leur habitat, et (ii) du rôle positif des MA dans la tolérance aux sols ultrabasiques (nutrition minérale et tolérance aux métaux), suggérant que ces plantes pionnières parallèlement à la gestion des MA puissent constituer des outils potentiels de réhabilitation des sites miniers de nickel en Nouvelle-Calédonie.

Mots-clés : mycorhizes arbusculaires, Cypéracées, sol ultrabasique, Nouvelle-Calédonie.

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Introduction

New Caledonia is a tropical archipelago in the South Pacific Ocean. One third of the main island is covered by ultramafic soils, a weathered product of serpentine bedrocks, characterized by low contents of the major nutrients (N, P, and K), an unbalanced Ca/Mg ratio, and the presence of iron oxides with high concentrations of heavy metals (Ni, Cr, Mn, and Co) (Latham 1985; Brooks 1987; Perrier et al. 2006a). As a result, specific biological endemic ecosystems have evolved, making the main island a biodiversity hot spot (Myers et al. 2000). Among noteworthy endemic plants colonizing this archipelago are sedges belonging to the Cyperaceae family, which are very abundant and considered the main pioneer plants of ultramafic maquis, where they constitute almost exclusively the herbaceous stratum (Jaffré 1992). According to Bruhl (1995), the Cyperaceae is a cosmopolitan family of monocotyledonous plants with about 5000 species. Ecologically, the Cyperaceae are also very important, as they are able to colonize a wide range of habitats at almost all latitudes (Bruhl 1995). However, the mycorrhizal status of this plant family is still unclear. In general, Cyperaceae are considered nonmycorrhizal (NM) or only weakly mycorrhizal (Meney et al. 1993; Miller et al. 1999; Wang and Qiu 2006), while several recent studies have reported arbuscular mycorrhizal (AM) associations within Cyperaceae (Amir et al. 1997; Muthukumar et al. 2004; Gai et al. 2006; Perrier et al. 2006b). In a recent review, Brundrett (2009) suggested that there is a continuum extending from fully NM to AM species in the Cyperaceae (a number of 4500 species were considered) resulting from continuing adaptation to variable habitat conditions. Brundrett suggested that the balance of information collected about the mycorrhizal status of Cyperaceae from various habitats (from arctic, alpine habitats to dry, warm arides or to wetlands and disturbed habitats) indicates that this plant family is predominantly NM, with only sporadic glomeromycotan fungus colonization in roots. Previous data about the mycorrhizal status of such sedges from New Caledonia reported that AM structures were observed at low intensity in 3 Cyperaceae species, suggesting they might contribute to plant adaptation to the extreme ultramafic soil conditions (Perrier et al. 2006b).

The aims of the study were (i) to evaluate the AM symbiotic status of Cyperaceae in New Caledonian ultramafic soils by extending the study to most Cyperaceae species dominating such an extreme ecosystem, and (ii) to test the role of AM symbioses on plant growth and survival in these soils. For this purpose, we investigated the occurrence of mycorrhizal colonization of 9 Cyperaceae species from New Caledonian ultramafic maquis ecosystems by microscopic observation to quantify the presence of AM structures within roots. We also performed a molecular analysis to identify the fungi associated with sedges. The relationships between the extent of AM colonization and plant growth, survival, and nutrient status was assessed in greenhouse experiments using *Costularia comosa* (C.B. Clarke) Kük, one of the most frequent cyperaceous species found in New Caledonia ultramafic soils.

Materials and methods

Study sites

Four sites located in ultramafic ligno-herbaceous maquis ecosystems were chosen for this study (see Fig. S1²). Site 1 is located in Plum area (22°16'59"S, 166°39'12"E) on colluvial lateritic soil. Site 2, also composed of colluvial lateritic soil, is located on the Koniambo Massif (21°00'39"S, 164°49'08"E). Site 3 is located in the Plaine des Lacs region (22°13'06"S, 166°52'49"E) on alluvial lateritic soil. Site 4 is located in the Prony area (22°19'28"S, 166°49'25"E) on nodular ferrallitic soil. Jaffré (1992) and Perrier et al. (2006a, 2006b) have already reported a full description of the areas where the sites are located, including climate, geology, geomorphology, and vegetation structure of these ultramafic sites. Soils present in these 4 sites are highly weathered oxisols (Latham 1985; Perrier et al. 2006a) with an iron (Fe) concentration range from 290 to 535 g·kg⁻¹ with diethylene triamine pentaacetic acid extractable concentrations ranging from 81 to 238 mg·kg⁻¹. The 3 other main characteristics of these slightly acidic soils (pH 5.2–6.1) are a strong deficiency in phosphorus and potassium (P_{total}, 64–86 mg·kg⁻¹; P_{Olsen}, 1–5 mg·kg⁻¹; K, 20–84 mg·kg⁻¹), an unbalanced calcium to magnesium ratio (Ca, 0.1–1.5 g·kg⁻¹; Mg, 3.1–88.4 g·kg⁻¹), and a high content of putatively toxic heavy metals (Ni, 3.2–6.5 g·kg⁻¹; Cr, 12.7–22.8 g·kg⁻¹; Mn, 5.2–11.0 g·kg⁻¹; Co, 0.4–1.1 g·kg⁻¹) with diethylene triamine pentaacetic acid extractable concentrations as follows: Ni, 0.2–250 mg·kg⁻¹; Co, 0.3–120 mg·kg⁻¹; Cr, 0–0.8 mg·kg⁻¹; Mn, 0.9–1200 mg·kg⁻¹ (Perrier et al. 2006b). These soils are well drained and are characterized by successions of short periods of optimal moisture and variable periods of drought (Perrier et al. 2006a). Sedge species present in each site have been identified according to morphological features using the Noumea Herbarium, New Caledonia.

Plant root sampling for microscopic observation and molecular analysis

One to 5 g (fresh mass) of thin roots per plant were collected and then fixed in a solution of 33% ethanol, 33% glycerol, and 34% water. Plants were sampled during the rainy season from January to April. Root identity was ascertained by tracing from the clump to the root tips. For each sedge species, 8 independent plants were randomly sampled. The presence of lateral swollen dauciform roots was directly observed by visual examination in the field and later confirmed by stereomicroscope observation in the laboratory.

Staining, observation, and characterization of root fungal colonization

Plant root fungal colonization was investigated to assess both the level and frequency of mycorrhizal colonization. Roots were stained in Trypan blue (Koske and Gemma 1989) to assess the presence of AM colonization. For each individual sample, at least 25 root segments of 1 cm length were mounted in 10% glycerol on glass slides for examination with a light microscope. A root segment was considered to be mycorrhizal when arbuscules, vesicles, or hyphal coils were clearly visible. The frequency of AM coloniza-

² Supplementary data for this article are available on the Journal website at <http://cjnm.nrc.ca>.

tion (F (%)) was calculated for each root sampled as follows: (the ratio of the number of mycorrhizal root fragments/the total number of root fragments) $\times 100$. The intensity of AM colonization in the root system (M (%)), which reflects the abundance or the density of AM structures in the roots. Both F and M were determined according to Trouvelot et al. (1986).

Molecular analyses

Molecular analyses were carried out to identify the mycorrhizal fungi colonizing the plant root tissues and to assess their diversity. We analysed thin root fragments of the 3 most frequent sedge species, *C. comosa*, *Costularia nervosa*, and *Costularia pubescens*. A total of 142 DNA samples were extracted for amplification: 30 DNA samples were extracted from 3 plants of *C. nervosa*, 60 DNA samples from 3 plants of *C. pubescens*, and 52 DNA samples from 2 plants of *C. comosa*. Total genomic fungal DNA was extracted from root fragments using the DNAeasy Plant Qia-gen kit (Qiagen, Courtaboeuf, France). An approximately 550-bp partial fragment of the DNA region encoding for 18S small subunit rDNA (SSU) was amplified with primers AM1 (5'-GTTTCCCGTAAGGCGCCGAA-3') (Helgason et al. 1999) and NS31 (5'-TTGGAGGGCAAGTCTGGTGCC-3') (Simon et al. 1992). PCR reactions were performed as previously described by Helgason et al. (1999). PCR products were analysed by electrophoresis in 1% (m/v) agarose gel containing ethidium bromide. PCR products of 550 bp were purified using the Qiagen Gel Extraction kit (Qiagen) and pooled according to plant species and then cloned in the pGEMt vector in *Escherichia coli* by using the Promega pGEMt kit (Promega, Charbonnières, France). Then, 50 *E. coli* clones corresponding to each amplified DNA fragment pooled per plant were analysed as follows: after reamplification of the 550 bp DNA fragment by PCR, PCR products were purified and then sequenced using Big Dye Terminator chemistry and a ABI3730 capillary electrophoresis-based genetic analyser (Macrogen, Seoul, Korea). DNA sequence analyses were performed by using Chromas Pro 5.2 software (Technelysium Pty Ltd., Shannon, Ireland). A DNA consensus sequence was then deposited in the EMBL nucleotide database for assignment of accession numbers that are further reported.

Greenhouse experiment

A greenhouse experiment was carried out to assess the effect of mycorrhizal inoculation (with AM) of Cyperaceae when growing in ultramafic soil conditions. *Costularia comosa* was chosen in the present study because we showed this species with a high mycorrhizal index. In addition, seeds of *C. comosa* were easily available with a germination rate above 50%. Seeds of *Costularia comosa* were sown in sterilized sand and grown for 1 month before the transfer of young plants into plastic pots (0.6 L) containing a sterilized (autoclave 30 min; 120 °C) mixture of 5 volumes of sieved (2 mm) lateritic topsoil from site 1, with 1 volume of commercial compost (N, 1.7 mg·g⁻¹; P_{total}, 150 mg·kg⁻¹; P _{Olsen}, 7 mg·kg⁻¹; K, 139 mg·kg⁻¹; terreau universel, Agrofino, France). Plants were cultivated for 12 months under greenhouse conditions (15–25 °C, daily watering to field capacity, with partial shading, 50% net). The addition of commercial

compost was necessary because the young *C. comosa* plants are very fragile and many of them do not survive when transferred on a pure lateritic fine soil.

Two different AM inocula were prepared as follows. (i) Strain SFONL (*Glomus etunicatum*), originally isolated from the rhizosphere of *Alphitonia neocaledonica* (Rhamnaceae) in a lateritic soil located at Ouenarou, New Caledonia (22°10'26"S, 166°44'24"E) (Amir and Lagrange 2008). This SFONL strain is known for its capacity to promote plant growth (of *Alphitonia neocaledonica*, *Xanthostemon multiflorus*, and *Cloezia artensis*) under greenhouse conditions (Amir and Lagrange 2008). The SFONL strain was maintained via trap culture on sorghum. A partial 18S rDNA sequence of this strain is available under accession No. FM997956. *Costularia comosa* inoculation with SFONL strain was realized by coating 1-month-old roots with 5 mL of a suspension containing ca. 170 spores, during transfer into the plastic pots. (ii) Spores from the rhizosphere of *C. comosa* from site 1 were isolated (wet-sieving and decanting method) together with young roots to prepare a "natural inoculum". This inoculum consisted of a 5 mL suspension of crushed roots (ca. 250 mg) containing ca. 115 spores and was used as previously described to inoculate *C. comosa*. Under these conditions, it was possible to compare the effects on plant growth of the natural inoculum with those obtained with SFONL strain.

Thirty *C. comosa* plants were inoculated with the SFONL strain, the natural inoculum, or distilled water as negative control and were grown in individual pots randomly distributed in the greenhouse. To ascertain the effect of these 2 mycorrhizal treatments, 30 sorghum plants (a highly mycotrophic species use as positive control) were treated in the same conditions and grown for 12 weeks.

Costularia comosa survival rates were measured after 2, 8, and 12 months of growth. After 12 months, the number and length of leaves and the dry masses of both leaves and roots were measured to assess the effects of AM inoculation on plant growth. In addition, the tissue content of the major plant mineral nutrients N, P, and K was measured as an indicator of AM effects on plant growth. Similarly, Ni content was quantified in plant leaves and roots: Ni content is used as one major ultramafic indicator of AM effect on plant adaptation to ultramafism. The Ni quantification was carried out the chemistry laboratory of IRD (LAMA IRD Noumea) as described in Perrier et al. (2006b). F and M were determined according to Trouvelot et al. (1986).

All data were subjected to a one-way analysis of variance (standard ANOVA). In addition, the results were also analysed with the multiple comparison test of Tukey–Kramer ($p \leq 0.05$). XLSTAT computer software was used to carry out both ANOVA and correlation tests (Addinsoft 2005).

Results

Field mycorrhizal survey

The frequency of AM colonization (F) and the intensity of AM colonization (M) of the 9 studied species of Cyperaceae species are presented in Table 1. Among the 5 genera studied, *Costularia*, present on these 4 study sites, showed the greatest AM colonization ($F = 32.4\%$ and $M = 5.8\%$) and did not show important changes in mycorrhizal fre-

Table 1. Frequency (*F*) and intensity (*M*) of arbuscular mycorrhizal colonization within 9 sedge species collected in the field from 4 different ligno-herbaceous maquis sites.

Species		<i>F</i> (%)	<i>M</i> (%)
<i>Beaumea deplanchei</i> Boeck.	Site 1	7.9±5.1	2.6±2.1
	Site 4	0	0
<i>Costularia arundinacea</i> Kuek	Site 3	43.2±16.1	6.7±4.2
<i>Costularia comosa</i> (C.B. Clarke) Kük	Site 1	29.3±10.4	6.2±3.1
	Site 2	21.5±12.2	1.6±1.1
<i>Costularia nervosa</i> Raynal	Site 1	32.6±13.4	7.3±3.9
	Site 3	57.1±23.7	11.8±5.8
	Site 4	22.2±11.8	3.8±2.7
<i>Costularia pubescens</i> Raynal	Site 1	28.3±9.8	5.2±3.1
	Site 4	25.0±16.8	3.8±2.0
<i>Gahnia aspera</i> (R.Br.) Spreng	Site 4	18.1±8.9	3.1±1.8
<i>Lepidosperma perteres</i> C.B. Clarke	Site 3	24.2±14.8	3.8±2.5
	Site 4	16.7±7.2	2.1±1.7
<i>Schoenus juvenis</i> C.B. Clarke	Site 1	10.0±7.8	6.1±4.7
	Site 4	19.7±13.9	2.7±1.8
<i>Schoenus neocaledonicus</i> C.B. Clarke	Site 1	8.7±6.1	0.7±0.6
	Site 4	8.1±5.9	2.1±1.8

Note: *F* = (number of mycorrhizal root fragments/total number of root fragments) × 100; *M* = (95n₅ + 70n₄ + 30n₃ + 5n₂ + n₁)/(total number of root fragments), where n₅ is the number of root fragments with more than 90% colonization, n₄ is the number of root fragments with 89%–50% colonization, n₃ is the number of root fragments with 49%–10% colonization, n₂ is the number of root fragments with 9%–1% colonization, and n₁ is the number of root fragments with <1% colonization). Values are the means ± standard deviation.

quency and intensity between the 4 sites. In contrast, the genera *Beaumea* and *Schoenus* presented weak AM colonization (*F* = 6.1% and *M* = 1.4%). The most frequently observed mycorrhizal structures were long, straight hyphae, which followed the length of the root cortex. We also observed, although with a lower frequency, *Glomus*-type vesicles with a subspherical shape eventually compressed against the cell wall (Fig. 1B). Extraradical spores and intracellular arbuscules have also been observed (Figs. 1A and 1D). All 136 examined specimens developed dauciform roots, with some showing evidence of AM colonization (Fig. 1C).

Molecular identification of a *Glomus* species associated with AM in Cyperaceae

The DNA sequencing of the PCR 550-bp cloned products revealed one unique consensus DNA sequence showing strong homologies (blast homology 92%) to *Glomus* group B closely related to *Glomus etunicatum*, *G. claroideum* and to the SFONL strain used in this study. The DNA consensus sequence has been deposited in EMBL nucleotide database under accession No. FM997957.

Effects of AM inoculation on *Costularia comosa* root colonization; survival; growth; and N, P, K, and Ni contents

AM treatments were tested on sorghum used as positive controls. The analysis of AM colonization of sorghum revealed that all analysed root fragments presented mycorrhizal structures when treated with either natural or SFONL inoculum, with mycorrhizal intensity values (*M*) of 37% and 60%, respectively. In contrast, uninoculated sorghum

plants never presented any mycorrhizal structures. This result indicated that our inoculum protocol and growth conditions allowed an efficient plant mycorrhizal colonization.

Table 2 shows *Costularia comosa* dry mass variations and major element contents (N, P, K, and Ni) of shoots and roots of plants either inoculated with AM fungi (natural inoculum or strain SFONL) or uninoculated. *Costularia comosa* inoculated with SFONL strain showed a fungal root colonization frequency value (*F*) of 62% and an intensity value (*M*) of 5%. In contrast, the uninoculated control *C. comosa* showed *F* and *M* values of 12.7% and 1%, respectively, which were significantly different from those presented by roots inoculated with SFONL strain. Natural inoculum led to an intermediate mycorrhizal colonization with *F* and *M* values of 31% and 1.7%, respectively. As observed in situ, AM colonization was weak and structures mainly corresponded to extra- and intra-cortical AM hyphae and *Glomus*-type vesicles. The occurrence of vesicles was higher with the SFONL strain. Intracellular arbuscules were observed with the 2 inoculation treatments but at lower frequency.

The survival rates were different depending on inoculation treatments, with 42%, 58%, and 70%, respectively, for control, natural inoculum and strain SFONL treatments. We also observed that survival rates reached stable values 6 months after the beginning of the assay. We then analysed the effect of mycorrhizal colonization on plant growth parameters. SFONL inoculation increased root and shoot dry mass by 124% and 246%, respectively, when compared with controls (uninoculated plants). Similarly, the mean values of root and shoot biomass, in natural inoculum treatment, were enhanced by 48% and 92%, respectively, but the differences with the control were not statistically significant.

Fig. 1. Microscopic observation of arbuscular mycorrhizas in *Costularia comosa* after staining with Trypan blue (Koske and Gemma 1989) and mounting in 10% glycerol. (A) Arbuscules observed in a plant inoculated with strain SFONL. (B) *Glomus*-type vesicles in the cortex of plant root, (C) infection by arbuscular mycorrhizas in a dauciform root, and (D) a spore of *Glomus* sp. on the surface of root. The bars represent 50 μm .

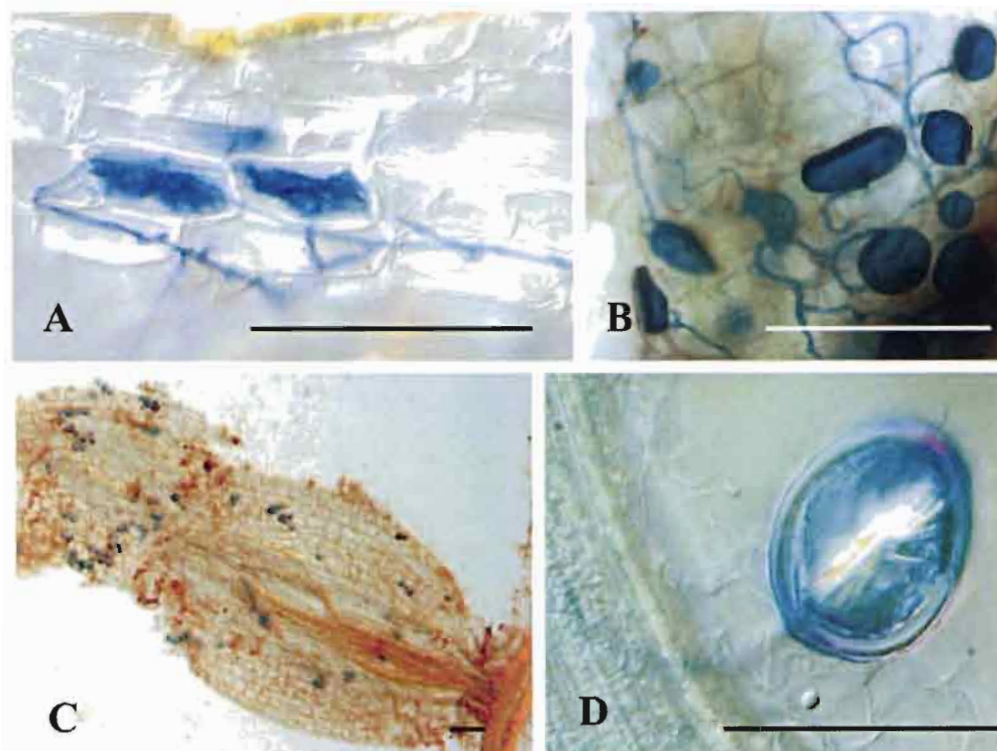


Table 2. Dry mass (g) and N, P, K, and Ni contents ($\mu\text{g}\cdot\text{g}^{-1}$) of shoots and roots of *Costularia comosa* inoculated with arbuscular mycorrhizal fungi (natural inoculum or strain SFONL) or uninoculated.

	Control (uninoculated)	Natural AM inoculum	SFONL strain (<i>Glomus</i>)
<i>F</i> (%)	12.7 $\pm$ 11.0a	31.0 $\pm$ 13.0b	62.0 $\pm$ 17.0c
<i>M</i> (%)	1.0 $\pm$ 1.6a	1.7 $\pm$ 0.9a	5.0 $\pm$ 1.5b
Dry mass			
Shoots	0.089 $\pm$ 0.062a	0.171 $\pm$ 0.057a	0.308 $\pm$ 0.081b
Roots	0.106 $\pm$ 0.071a'	0.157 $\pm$ 0.045a'	0.238 $\pm$ 0.046b'
N			
Shoots	8600 $\pm$ 960a	8800 $\pm$ 790a	8300 $\pm$ 950a
Roots	4200 $\pm$ 620a'	4130 $\pm$ 545a'	3730 $\pm$ 586a'
P			
Shoots	433 $\pm$ 71a	386 $\pm$ 55a	415 $\pm$ 59a
Roots	253 $\pm$ 21a'	236 $\pm$ 32a'	232 $\pm$ 29a'
K			
Shoots	12 350 $\pm$ 1 088a	11 233 $\pm$ 2 055a	12 430 $\pm$ 1 683a
Roots	3450 $\pm$ 3940b'	3400 $\pm$ 350b'	5160 $\pm$ 476a'
Ni			
Shoots	21 $\pm$ 9a	32 $\pm$ 12a	21 $\pm$ 7a
Roots	1789 $\pm$ 198a'	1239 $\pm$ 127b'	718 $\pm$ 132c'

Note: Numbers followed by the same letter for each arbuscular mycorrhizal (AM) colonization parameter, each element, and plant shoot and root dry mass (shoot or root) are not significantly different at $p \leq 0.05$.

cant. Then, to appreciate the overall link between plant growth and mycorrhizal colonization, we assessed a total correlation test. Highly significant total positive correlations were obtained: $r = 0.511$ between shoot dry mass and F ($p \leq 0.001$); $r = 0.417$ between root dry mass and F ($p \leq 0.01$); $r = 0.486$ between shoot dry mass and M ($p \leq 0.001$); $r = 0.405$ between root dry mass and M ($p \leq 0.01$).

Variations in plant element content revealed that only K concentration increased significantly in roots of plants inoculated with SFONL, while K, N, and P concentrations in shoot were not affected by SFONL or natural inoculation. In contrast, the Ni content in roots was reduced significantly 2.5 times in SFONL-inoculated plants and 1.4 times in plants treated with natural inoculum, all when compared with controls.

Discussion

New Caledonian Cyperaceae species are generally mycorrhizal

Species belonging to the Cyperaceae family are generally considered not or weakly mycorrhizal (Meney et al. 1993; Miller et al. 1999; Wang and Qiu 2006). Our results showed the presence of AM in the roots of 9 Cyperaceae species examined from New Caledonia ultramafic ligno-herbaceous maquis. These observations are in accordance with previous studies showing a weak colonization of sedge roots by AM fungi forming more frequently intracortical hyphae and vesicles (Muthukumar et al. 2004; Gai et al. 2006; Perrier et al. 2006b). Indeed, AM colonization frequency and intensity varied greatly between genera and species. For example plants from the genus *Costularia*, the most abundant sedge genus in the ligno-herbaceous ultramafic maquis, presented the most developed AM colonization among the studied species. In contrast, plants from the genus *Baumea* presented the lowest level of AM mycorrhization. However, the level of mycorrhizal colonization of Cyperaceae from New Caledonia, even in the genus *Costularia*, was relatively low and intracellular arbuscules were observed but not frequent. The unique 18S rDNA consensus sequence we isolated is the first molecular proof, to our knowledge, of the presence of *Glomus* into the root of a Cyperaceae. In addition, it shows an absence of fungal diversity of mycorrhizal colonization, which might be related to the extreme conditions of the habitat (ultramafic soils). This result is also in agreement with previous data reviewed by Brundrett (2009) who reported that in the Cyperaceae, the only AM colonization noticed is mostly due to the sparse glomeromycotan fungi colonization.

Role of arbuscular mycorrhizas in *Costularia comosa*

AM fungi are relatively abundant in New Caledonian ultramafic soils and seem to play a role in the adaptation of different plant species to ultramafic conditions (Amir et al. 1997; Perrier et al. 2006b; Amir and Lagrange 2008). The presence of AM at a low level of colonization in our observations on Cyperaceae raised the question of the functional effect of AM colonization on sedge survival and growth in ultramafic soils (low NPK nutrient contents, unbalanced Ca/Mg ratio, and presence of high heavy metals concentrations). Our results, obtained using plants grown in green-

house, showed that even a weak AM colonization with a selected AM strain significantly improved *C. comosa* survival and growth. The effects of AM colonization on *C. comosa* are also supported by a highly significant positive correlation between plant growth and AM colonization parameters. The presence of low levels of mycorrhization in the controls is probably due to cross-contaminations (only 6 plants were found mycorrhizal in a set of 30 plants), as currently occurs in greenhouse conditions when an experiment duration is over a few months. However, the results presented are means with high standard deviations and do not affect the statistical significance of the whole results. These highly significant correlations indicated that plants with the better growth were those presenting the higher mycorrhizal colonization, and this is true for all the treatments, even the natural inoculum, which did not show a significant difference with the control, because of the high variance of the values. If we consider the low concentrations of mineral elements needed by these plants (Lagrange 2009), a low level of root colonization is probably sufficient to increase the plant absorption, and consequently the plant growth, as observed in greenhouse experiment. These results clearly indicate that the AM symbiosis is functional in *C. comosa*. For species belonging to plant families generally considered NM or weakly mycorrhizal, Brundrett (2009) suggested that the presence of intraradical mycelium and vesicles is not sufficient to attest that the species is mycorrhizal, because their presence could be due to a saprophytic growth inside the plant without any functionality. In the case of *C. comosa*, the presence of arbuscules and the positive effect of AM inoculum on plant growth and survival in greenhouse conditions truly suggest that (i) the plant belongs to the AM group and (ii) the mycorrhizal colonization is functional. Lagrange (2009) performed a field experiment with *C. comosa* in ultramafic soil amended with P, N, and K. He reported that mycorrhizal colonization, including arbuscule frequency, was greatly enhanced by a moderate P addition. He concluded that the low concentrations of P, in the majority of ultramafic soils, reduce the efficiency of the mycorrhizal symbiosis in natural conditions for this plant, as suggested by Lambers et al. (2009) for Australian species. Thus, our data on Cyperaceae from New Caledonian ultramafic soils argue in favour of Brundrett's hypothesis that among plants from Cyperaceae family, there is a continuum between NM to AM varying to both genus and species levels and to habitats and environmental conditions (Brundrett 2009).

Mycorrhizal inoculation with AM is known to (i) improve plant growth by leading to an increase of total P and N accumulated in each plant, and by contributing to plant K uptake, and (ii) improve host plant heavy metal tolerance (Marschner 1995). In our experiment, concentrations of P and N were not clearly enhanced, but as SFONL-inoculated plants had a higher dry mass, they also contained more N, P, and K than uninoculated control plants, suggesting that AM colonization participate in nutrient assimilation. A significant increase of K concentration was observed in roots of *C. comosa* inoculated by SFONL. This suggests that the mycorrhizal strain might improve the plant K uptake, as previously reported by George et al. (1992) showing that hyphal structures contribute to about 10% of the total K in mycor-

rhizal *Agropyron repens*. In addition, inoculation by the SFONL strain significantly reduced Ni concentration in roots, suggesting a role of AM in Ni avoidance. While the underlying mechanism is still not known, the protective effect of AM against Ni is probably one of the factors favouring the growth of mycorrhizal plants on ultramafic soil (Amir and Ducouso 2010).

Multiple strategies of New Caledonian pioneer sedge to colonize an adverse environment

Our study showed a beneficial effect of AM colonization on *Costularia* development. We observed also that all samples of the 9 sedge species studied under field conditions and all samples of greenhouse experience developed dauciform roots. This root organ, which belongs to the so-called cluster roots, is characterized by lateral swellings that develop remarkably dense hairy root clusters (Shane et al. 2006). Dauciform roots are mostly found in particular genera of the Cyperaceae family (Lambers et al. 2006) and only appear when plants grow on a low-P soil (Miller 2005; Shane et al. 2005). These structures are considered a plant adaptation to survive stressful soil conditions, notably P deficiency (Lambers et al. 2008).

Therefore, New Caledonian sedge species developing on highly weathered ultramafic soil cumulate at least 2 adaptive structures favouring nutrient acquisition: arbuscular mycorrhizas and dauciform roots. Lambers et al. (2008) noticed that plants of extremely infertile soils are frequently nonmycorrhizal but sometimes compensate for this lack of symbiosis by developing cluster roots. However, Boulet and Lambers (2005) have reported the case of a species endemic to Australian ultramafic soils, *Hakea verrucosa* (Proteaceae), which cumulates the presence of AM and cluster roots. The additional effects of these 2 adaptation strategies could be responsible for the Cyperaceae pioneer behaviour allowing plants to survive and to colonize bare soils. These results designate endemic Cyperaceae and AM management as potential tools for the improvement of mine site rehabilitation in New Caledonia.

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Nickel-tolerant ectomycorrhizal *Pisolithus albus* ultramafic ecotype isolated from nickel mines in New Caledonia strongly enhance growth of the host plant *Eucalyptus globulus* at toxic nickel concentrations

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Summary Ectomycorrhizal (ECM) *Pisolithus albus* (Cooke & Masee), belonging to the ultramafic ecotype isolated in nickel-rich serpentine soils from New Caledonia (a tropical hotspot of biodiversity) and showing in vitro adaptive nickel tolerance, were inoculated to *Eucalyptus globulus* Labill used as a Myrtaceae plant-host model to study ectomycorrhizal symbiosis. Plants were then exposed to a nickel (Ni) dose-response experiment with increased Ni treatments up to 60 mg kg⁻¹ soil as extractable Ni content in serpentine soils. Results showed that plants inoculated with ultramafic ECM *P. albus* were able to tolerate high and toxic concentrations of Ni (up to 60 µg g⁻¹) while uninoculated controls were not. At the highest Ni concentration tested, root growth was more than 20-fold higher and shoot growth more than 30-fold higher in ECM plants compared with control plants. The improved growth in ECM plants was associated with a 2.4-fold reduction in root Ni concentration but a massive 60-fold reduction in transfer of Ni from root to shoots. In vitro, *P. albus* strains could withstand high Ni concentrations but accumulated very little Ni in its tissue. The lower Ni uptake by mycorrhizal plants could not be explained by increased release of metal-complexing chelates since these were 5- to 12-fold lower in mycorrhizal plants at high Ni concentrations. It is proposed that the fungal sheath covering the plant roots acts as an effective barrier to limit transfer of Ni from soil into the root tissue. The degree of tolerance conferred by the ultramafic *P. albus* isolates to growth of the host tree species is considerably greater than previously reported for other ECM. The primary mechanisms underlying this improved growth were identified as reduced Ni uptake into the roots and markedly reduced transfer from root to shoot

in mycorrhizal plants. The fact that these positive responses were observed at Ni concentrations commonly observed in serpentine soils suggests that ultramafic ecotypes of *P. albus* could play an important role in the adaptation of tree species to soils containing high concentrations of heavy metals and aid in strategies for ecological restoration.

Keywords: ectomycorrhiza, *Eucalyptus*, New Caledonia, nickel, *Pisolithus*, serpentine.

Introduction

Nickel-rich ultramafic soils (typically known as 'serpentes' in the botanical and ecological literature) are a weathered product of serpentine bedrocks and are characterized by low available concentrations of major plant nutrients (N, P, K), unbalanced Ca/Mg ratio and high levels of phytotoxic heavy metals, in particular Ni (Brooks 1987, Chiarucci and Baker 2007). As a consequence of these extreme edaphic conditions, the ultramafic soils are considered as highly disadvantageous for most plants and produce-specialized habitats, which frequently host unique plant communities and 'islands' of biodiversity with many restricted and endemic taxa (Chiarucci and Baker 2007, Kazakou et al. 2008). Among the ultramafic extreme soil properties, heavy metal tolerance, especially to Ni, is regarded as a crucial factor for plant survival on serpentine soils (Kazakou et al. 2008). In ultramafic soils, total Ni, which may reach up to 10 g kg⁻¹ compared with the average 50 mg kg⁻¹ in cultivated soils, appears to be also an

element both bioavailable and phytotoxic (L'Huillier and Edinghoff 1996, Echevarria et al. 2006). To grow on such high concentrations of Ni as found in serpentine environments (often coinciding with high concentrations of other heavy metals), plants have developed two major physiological behaviours encompassing (i) exclusion of the absorption of the toxic metal by the roots, or (ii) metal hyperaccumulation with internal complexation and compartmentation (Kazakou et al. 2008). In addition, plants of serpentine soils are in interactions with various microorganisms' communities such as ectomycorrhizal fungi (ECM) (Moser et al. 2005, Gonçalves et al. 2007, 2009, Jourand et al. 2010), arbuscular mycorrhizal fungi (AM) (Schechter and Bruns 2008) and bacteria (Héry et al. 2003, Chaintreuil et al. 2007). All these microorganism communities might play an important role in plant adaptation to the ultramafic constraint. Thus, the significant role in plant growth in serpentine soils played by the rhizospheric bacteria has recently been reviewed (Rajkumar et al. 2009). Such a role is also suggested for ECM and AM communities (Gonçalves et al. 2007, 2009, Schechter and Bruns 2008). Moreover, in most of the studies, microorganisms (ECM, AM and bacteria) were found to be adapted to high Ni content as found in ultramafic soils (Amir et al. 2007, Chaintreuil et al. 2007, Jourand et al. 2010).

In New Caledonia, a tropical archipelago in the South Pacific ocean, Ni-rich ultramafic soils cover one-third of the main island. As a result, specific biological endemic ecosystems have developed, making the main island a biodiversity hot spot (Myers et al. 2000). In New Caledonia, many plants growing on these metal-rich lateritic soils have been reported to be mycorrhizal, which might be consistent with the putative role of these mycorrhizal symbioses in endemic plant adaptation to ultramafic soils containing high levels of bioavailable heavy metals (in particular, diethylene triamine pentaacetic acid (DTPA)-Ni may reach 60 mg kg^{-1}) (Perrier et al. 2006). Recently, a large collection of ECM from the genus *Pisolithus* Alb. and Schwein was collected (Jourand et al. 2010). This fungal genus is one of the major ectomycorrhizal Boletale distributed on a worldwide scale and forms ectomycorrhizal associations with a broad range of angiosperm and gymnosperm tree species (Marx 1977, Martin et al. 2002). Many previous studies have reported associations of *Pisolithus* with Myrtaceae, of which *Eucalyptus* sp. have been often used as a model for studying plant-fungal symbiotic interactions from molecular biology, physiology and biochemistry, to forestry (Burgess et al. 1996, Brundrett et al. 2005, Duplessis et al. 2005). By contrast, while the role of ECM such as *Laccaria*, *Paxillus* and *Suillus* in plant adaptation to heavy metals (Cd, Cu or Zn) have been widely studied and reported (Hartley et al. 1997, Adriaensen et al. 2003, 2005, 2006), only a few studies have reported interactions between *Eucalyptus* sp. and *Pisolithus* sp. with various metal tolerances to improve the capacity of plants to adapt to metal-contaminated soils (Aggangan et al. 1998, Khosla and Reddy 2008). In New Caledonian soils, *Pisolithus* sp. is very abundant and develops ectomycorrhizal symbioses with

many plants from various genera of the *Myrtaceae* family, e.g., *Melaleuca*, *Tristaniaopsis* and *Sannantha* (previously *Ba-bingtonia* according to Wilson et al. 2007) (Perrier 2005). In addition, by using molecular tools, it was recently demonstrated that the population of isolates from New Caledonian ultramafic soils clustered into one ecotype (Jourand et al. 2010).

The aim of the current study was to test the role of ectomycorrhizal Ni-tolerant *Pisolithus albus* (Cooke & Masee), belonging to the ultramafic ecotype isolated in New Caledonia, on the growth of *Eucalyptus globulus* used as a model host plant when exposed to high concentrations of Ni.

Materials and methods

Fungal ultramafic ecotype isolates and site collection descriptions

A map location of New Caledonia archipelago including studied sites is presented in ESM Figure 1 (available as Supplementary Data at *Tree Physiology* Online). The ultramafic sites were the Koniambo Massif (sites 1 and 2, ESM Figure 1) located in the Northern Province of New Caledonia ($20^{\circ}59' \text{ S}$, $164^{\circ}49' \text{ E}$) and Mont Dore (site 3, ESM Figure 1) located in the south of the main island ($22^{\circ}15'21'' \text{ S}$; $164^{\circ}36'42'' \text{ E}$). A full description of the ultramafic sites including climate, geology, geomorphology, vegetation structure, site geographical location, soil sampling and mineral analyses has been previously given in Jourand et al. (2010). Location, description and Ni contents in soil (total Ni and extractable DTPA-Ni contents) are summarized in Table 1a. The two Ni-tolerant fungal isolates, i.e., MD07-168 and MD07-217, have been already collected, identified and described as *P. albus* ultramafic ecotype Ni-tolerant (Jourand et al. 2010). An additional Ni-tolerant isolate, MD06-337, which was included in this study, was characterized by using (i) morphological description and (ii) molecular methods based upon internal transcribed spacer (ITS) rDNA sequencing and ITS-restriction fragment length polymorphism (RFLP) profile as reported in Jourand et al. (2010). The MD06-337 isolate belongs to *P. albus* ultramafic ecotype (for the ITS-RFLP profile of the isolate MD06-337 and comparison with representative ITS-RFLP profile of New Caledonian *P. albus* ecotype, see ESM Figure 2, available as Supplementary Data). *P. albus* ultramafic ecotype description of isolates, site location, DNA ITS sequence accession number in GenBank and in vitro Ni tolerance (Ni EC_{50}) are summarized in Table 1b. Stock cultures from each sporocarp were obtained by transferring aseptically a piece of the pileus trama to solid modified Melin-Norkrans medium (Marx 1969) containing: KH_2PO_4 (0.5 g l^{-1}), $(\text{NH}_4)_2\text{HPO}_4$ (0.25 g l^{-1}), CaCl_2 (0.05 g l^{-1}), NaCl (0.025 g l^{-1}), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.15 g l^{-1}), thiamine hydrochloride ($100 \mu\text{g l}^{-1}$), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.03 g l^{-1}), glucose (10 g l^{-1}), malt extract (3 g l^{-1}) and agar agar (14 g l^{-1}). The pH was adjusted to 5.6 with 1 M HCl and the medium autoclaved for 20 min at 120°C . All fungal strains were maintained as sub-

Table 1a. Site location, soil description and nickel content.

Site number on map	Location and GPS coordinates	Soil characteristic	Total Ni (g kg ⁻¹)	DTPA-Ni (mg kg ⁻¹)	Reference
1	Trazy-Guerioum, Koniambo massif, Kone (21°00'28" S; 164°49'50" E)	Ultramafic	3.3 ± 0.4	12 ± 3	This study
2	Pindjen Water Fall Road, Kone (20°02'30" S; 164°46'09" E)	Ultramafic	3.5 ± 0.7	19 ± 3	Jourand et al. (2010)
3	Mont-Dore, Plum Road (22°15'18" S; 166°36'42" E)	Ultramafic	2.8 ± 0.5	25 ± 2	Jourand et al. (2010)

Table 1b. *Pisolithus albus* isolate ultramafic ecotype description, site location and in vitro Ni tolerance.

<i>P. albus</i> isolate code	Site location	DNA ITS GenBank accession number	In vitro Ni tolerance (Ni EC ₅₀ in mg kg ⁻¹)	In vitro mycelial Ni content when exposed at Ni EC ₅₀ (μmol g ⁻¹ DW tissue)	Reference
MD06-337	1	AM947121	35.7 ± 3.5	0.59 ± 0.05	This study
MD07-168	2	AM947110	41.3 ± 2.3	0.71 ± 0.04	Jourand et al. (2010)
MD07-217	3	AM947114	32.5 ± 2.3	0.56 ± 0.05	Jourand et al. (2010)

DW, dry weight.

cultures at 24 °C on the same medium. To test mycelial Ni content, in vitro cultures of mycelium were grown for 2 weeks in liquid modified Melin–Norkrans medium with Ni added at final concentrations of 0, 0.6, 3, 6 and 30–60 mg kg⁻¹ of solid agar medium.

Plant growth and treatments

E. globulus Labill. subsp. *bicostata* (Maiden et al.) J.B. Kirkp. seed lot N°16731 was obtained from the Australian Tree Seed Centre (Kingston, ACT, Australia). A set of experiments was carried out in a glasshouse as described in Brundrett et al. (1996). Briefly, *Eucalyptus* seeds were pre-treated for 2 min in 70% ethanol supplemented with 0.1% Tween 20, then surface sterilized for 5 min in H₂O₂ (30%) and washed three times in sterile water. Germination occurred after 1 week at 28 °C in the dark. Seedlings were then placed for a week in a growth chamber with an 18/6-h day/night cycle at a light intensity of 200 μmol m⁻² s⁻¹ photosynthetic active radiation and at temperature 25/18 °C. Two-week-old seedlings were transplanted into plastic containers of 1 l capacity filled with sterilized neutral growth substrate composed of 50% acid-washed sand and 50% vermiculite. Plants were inoculated with 10 ml of 2-week-old mycelial slurry in solution at 10 mg ml⁻¹ prepared from mycelium cultivated as described by Brundrett et al. (1996). Controls were uninoculated plants. All plants received nutrient by watering the containers to field capacity with a nutrient solution in which the final concentrations of mineral elements expressed in mg kg⁻¹ of substrate (w/w) were: NH₄NO₃, 36; K₂SO₄, 111.6; Ca(H₂PO₄)₂·H₂O, 40; CaSO₄·2H₂O, 51.5; MgSO₄·7H₂O, 33.7; FeCl₃, 11; MnSO₄·4H₂O, 16.9; CuSO₄·5H₂O, 8.2; ZnSO₄·7H₂O, 9.2; Na₂B₄O₇·10H₂O 1.1; (NH₄)₆Mo₇O₂₄·4H₂O, 0.46; CoCl₂·6H₂O, 0.34. The pH of the nutritive solution was

adjusted to 5.6 with 1 M HCl. Plants were allowed to grow for 1 month before exposure to Ni. Then Ni was added from NiCl₂ solution (1 M) so that the final Ni concentration in containers ranged from 0, 0.6, 3, 6 and 30–60 mg kg⁻¹ of substrate. Plants were grown again for 3 months with average maximum and minimum temperatures of 25 ± 2 °C and 20 ± 2 °C, respec

tively. Watering was applied by spraying 2 min/h, eight times per day. At weeks 4 and 8, a top dressing of NH₄NO₃ was applied at 28.8 mg kg⁻¹ of substrate. All experiments were carried out on three independent assays, each treatment containing 15 plant replicates.

Analyses

Plants were harvested 12 weeks after the beginning of Ni treatment. Root exudates were collected, filtered and lyophilized as described by Silva et al. (2004). Lyophilized root exudates were re-suspended with ultrapure water (20 ml). Then organic acids were determined by high-performance liquid chromatography (HPLC) (Laboratoire LIVE, Nouméa, Université de Nouvelle-Calédonie) with the following conditions: HPLC Waters Alliance system®, with PDA detector at 210 nm (Waters, Milford, MA, USA); octadecyl silane (ODS) C18 column 100 V 4.6 mm diameter and 250 mm length (Tosoh Bioscience, Lyon, France); mobile phase was used at a flow of 0.7 ml min⁻¹ and consisted of a gradient composed of 98% H₃PO₄ at 0.1% in H₂O HPLC grade (v/v) and 2% CH₃CN HPLC grade. Calibration was made with standard organic acids from Sigma-Aldrich kit no.47264 (Sigma-Aldrich, L'isle d'Abeau, France). Total free thiols were also assayed in lyophilized root exudates using Ellman's test and reagent (5,5'-dithio-bis-2-nitrobenzoic acid) as described in Bulaj et al. (1998). Plant root surface was estimated

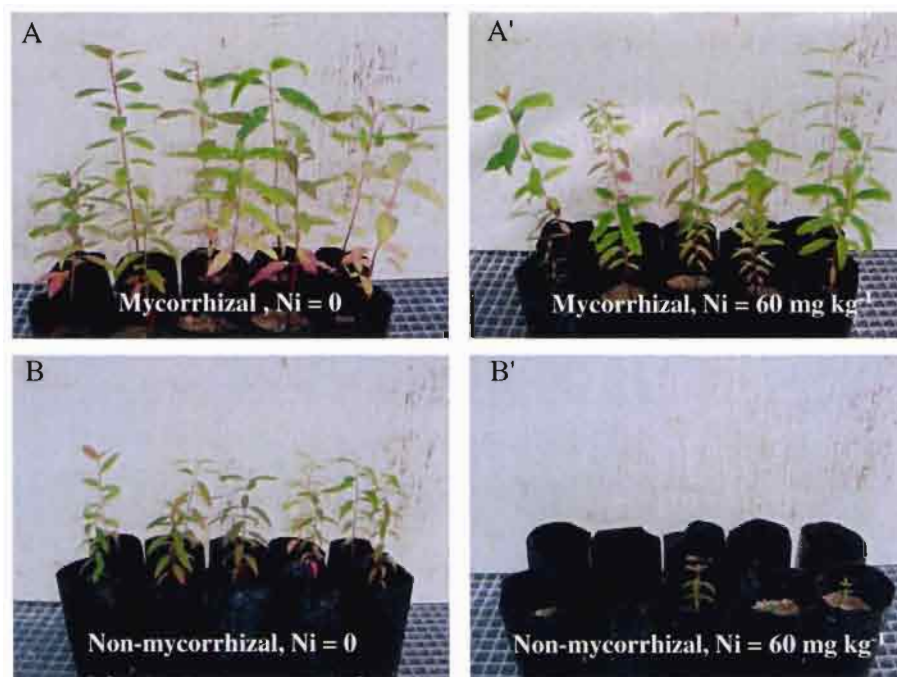


Figure 1. *Eucalyptus globulus* seedlings after 12 weeks' growth. A and A': mycorrhizal; B and B': non-mycorrhizal (controls). A and B: no nickel added; A' and B' seedlings treated with Ni. This figure appears in color in the online version of *Tree Physiology*.

by measuring root surface area of each plant as previously described by Benjamin and Nielsen (2005). Shoot and root dry biomass were measured after drying for 72 h at 60 °C. Samples of dried plant tissues (shoot or root) were collected for mineral analyses and also nitrogen, hydrogen, carbon and sulphur (CHNS) analyses, which were performed, respectively, by inductively coupled plasma-optical emission spectrometry and by elementary analysis with a Thermoquest AS2100, at Laboratoire de Chimie (IRD, Nouméa, New Caledonia) as reported in Perrier et al. (2006). Similarly, mycelial Ni content was measured in *P. albus* tissue after in vitro culture in liquid medium.

Ergosterol is the prominent sterol in most fungi and is either absent or a minor component of higher plants and so was used to estimate fungal biomass (Martin et al. 1990). Ergosterol analysis was carried out in dried roots and in pure mycelium after methanolic extraction as reported in Martin et al. (1990), followed by HPLC analysis with the following conditions: HPLC Waters Alliance system®, with PDA detector at 280 nm, column Agilent Extend C18 2.1 mm diameter and 150 mm length (Agilent, Santa Clara, CA, USA); mobile phase consisted of MeOH HPLC grade 100% at a flow of 0.2 ml min⁻¹. A standard ergosterol was used for calibration (Sigma-Aldrich, L'isle d'Abeau, France). On the basis of the ergosterol determination, relative fungal biomass in plant roots was determined by using as conversion factor the mean of ergosterol per gram dry weight of mycelium, which was calculated after measuring ergosterol extracted from pure culture dried mycelium as previously described in Martin et al. (1990).

Statistics and data presentation

All data were subjected to a two-way analysis of variance (ANOVA), and means were compared using the honestly significant difference (HSD) of Tukey test at $P < 0.05$ using XLSTAT computer software (Addinsoft 2005). As no significant differences were noticed between sets of data from the three independent treatments of plants inoculated with ECM *P. albus* ultramafic ecotype, the three sets of data were considered as one homogeneous set of data named 'ultramafic' to simplify data presentation in both graphics and tables. Nevertheless, full data of each independent treatment are presented in Supplementary Data.

Results

Plant growth

Plant growth summarized data are presented in Figures 1 and 2. Full data are accessible as ESM Table 1 (available as Supplementary Data). Without Ni, ectomycorrhizal treatment with ultramafic *P. albus* induced enhancement of plant growth (Figure 1A) when compared with non-inoculated plants (controls) (Figure 1B). High concentrations of Ni severely affected growth of non-inoculated plants (Figure 1B'), but ECM plants were much less affected (Figure 1A'). The response of plant growth to a range of Ni concentrations is detailed in Figure 2A and B. Concentrations of Ni up to 6 mg kg⁻¹ had little or no effect on plant growth, but higher concentrations (30 and 60 mg kg⁻¹) significantly reduced growth

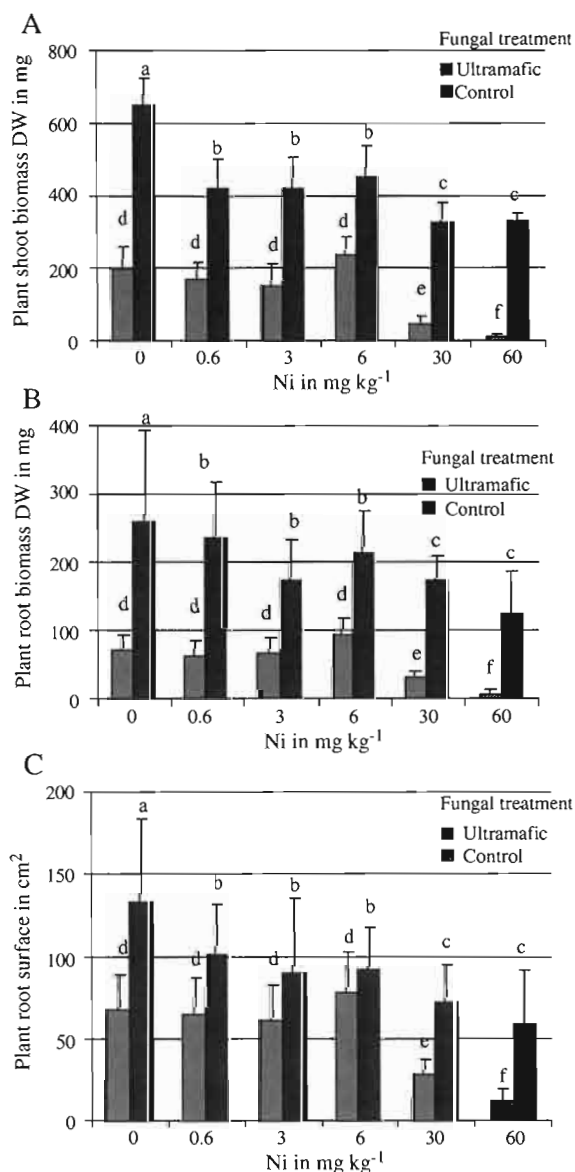


Figure 2. Plant growth parameters. A: shoot biomass; B: root biomass; C: root surface. Bars represent means, and error bars represent standard deviation of means ($n = 15$) of three independent experiments. Different letters above columns indicate significant differences as determined by the Tukey test ($P < 0.05$).

(Figure 2). However, ECM plants were much less sensitive to Ni than non-mycorrhizal plants, maintaining approximately 50% of maximum growth of both roots and shoots at 6 mg kg⁻¹ Ni. In comparison, root growth of the non-mycorrhizal control plants was only 8% of maximum and shoot growth only 5%.

Fungal growth in plant symbiosis

Plant root surface is one indicator of ECM symbiosis between *Pisolithus* and *Eucalyptus*. Full data are accessible as ESM

Table 1; summarized data are reported in Figure 2C. When no Ni was added, ECM inoculation significantly enhanced root surface area by 49%; at low Ni treatments (0.6–6 mg kg⁻¹) root surface increased by 15–36%, and at high and toxic concentrations of Ni (30 and 60 mg kg⁻¹), the root surface area of ECM plants was enhanced by 61 and 79%, all when compared with controls.

Ergosterol was also measured in roots as it is a major sterol in fungi but occurs at very low levels in plants and consequently is a good indicator of the level of mycorrhizal colonization of roots. In pure cultures of fungal isolates, the ergosterol concentration varied from 7.8 ± 0.3 to 9.8 ± 0.2 mg kg⁻¹ of mycelium dry weight (Table 2, ESM). Data for ergosterol determinations in plant roots and conversion into relative fungal biomass are summarized in Table 2 (full data are reported in ESM Table 2, available as Supplementary Data). Without Ni, roots had a mean level of 19.7% ectomycorrhization. At low Ni concentrations (0.6–6 mg kg⁻¹), the level of root ECM colonization varied from 15.6 to 27.8%. At high Ni concentrations (30 and 60 mg kg⁻¹), the level of root colonization was significantly reduced to around 9%. No ergosterol was detected in roots of non-inoculated plants.

Nickel accumulation in plants and ECM

The Ni content of roots and shoots of ECM and non-inoculated plants exposed to a range of Ni concentrations is summarized in Table 3 (full data are reported in ESM Table 3, available as Supplementary Data). In roots, Ni accumulation was linearly related to the external Ni concentration, but in ECM roots it was less than half that of the uninoculated controls. In shoots, Ni concentrations of ECM and control plants were both low up to 6 mg kg⁻¹ Ni, but at higher concentrations, shoot Ni concentrations increased dramatically in control plants but not in ECM plants so that at 30 mg kg⁻¹ Ni, shoots of control plants contained 22-fold more Ni and at 60 mg kg⁻¹ 60-fold more. For all other major elements analysed, i.e., N, P, K, Ca and Mg, no significant differences in concentration were noted in either shoots or roots in response to Ni treatments or fungal treatment (ESM Table 3).

When the Ni-tolerant *P. albus* ecotypes were grown in vitro, a linear relationship existed between Ni concentrations in the medium and in the mycelium. Data are summarized in Table 1b (full data are presented in ESM Table 4, available as Supplementary Data). The mean slope of linear regression line from full data (ESM Table 4) was $53 \text{ ng } \mu\text{M}^{-1} \text{ Ni}$ ($R^2 = 0.999$). Using this slope, the mean Ni tissue concentrations at EC₅₀ (as reported in Table 1b) in mycelium for tolerant ecotypes would be $0.62 \text{ } \mu\text{mol g}^{-1} \text{ DW}$.

Free thiols and oxalic acid contents in plant root exudates

To determine whether Ni tolerance was related to the release of metal binding compounds, exudates from roots were analysed. The two principal chemical components of the exudate solution were non-protein thiols and oxalate. In Figure 3, the amounts of these compounds are expressed as the amount of

Table 2. Ergosterol determination and conversion into mycelial biomass in plant root tissue.

	Fungal treatment	Ni treatment (mg kg ⁻¹)					
		Ni = 0	Ni = 0.6	Ni = 3	Ni = 6	Ni = 30	Ni = 60
Ergosterol assay (μmol g ⁻¹ of root DW biomass)	Control	Not detected					
	Ultramafic isolates	4.4 ± 0.5 c	4.7 ± 0.5 c	3.5 ± 0.5 b	6.3 ± 0.7 d	2.0 ± 0.2 a	2.0 ± 0.2 a
Relative mycorrhizal biomass (% of total root DW biomass)	Control	Not detected					
	Ultramafic isolates	19.7 ± 2.2 c	20.8 ± 2.2 c	15.6 ± 2.2 b	27.8 ± 3.4 d	9.2 ± 1.1 a	9.1 ± 1.1 a

Means (± standard deviation) were calculated from three independent experiments with 15 replicates for each experiment. Means followed by the same letter are not significantly different ($P < 0.05$). DW, dry weight.

Table 3. Plant tissue nickel content (expressed in μmol of Ni per g of DW plant tissue).

Plant tissue	Fungal treatment	Ni treatment (in mg kg ⁻¹)					
		Ni = 0	Ni = 0.6	Ni = 3	Ni = 6	Ni = 30	Ni = 60
Shoot	Control	0.20 ± 0.03 a	0.29 ± 0.02 a	0.54 ± 0.07 b	0.54 ± 0.03 b	13.7 ± 0.9 d	105.3 ± 9.5 e
	Ultramafic isolates	0.41 ± 0.03 ab	0.22 ± 0.03 a	0.29 ± 0.07 a	0.41 ± 0.02 ab	0.63 ± 0.07 b	1.77 ± 0.2 c
Root	Control	0.61 ± 0.12 a'	2.86 ± 0.47 c'	8.07 ± 1.87 f'	14.7 ± 2.1 g'	86.8 ± 7.3 j'	144.8 ± 13.2 k'
	Ultramafic isolates	0.37 ± 0.08 a'	1.36 ± 0.17 b'	4.14 ± 0.54 d'	6.06 ± 0.75 e'	32.3 ± 2.5 h'	61.0 ± 8.4 i'

Means (± standard deviation) were calculated from three independent experiments with 15 replicates for each experiment. Means followed by the same letter are not significantly different ($P < 0.05$). DW, dry weight.

exudate per gram dry weight of root (full data are reported in ESM Table 5, available as Supplementary Data). Control plants excreted significantly more thiols and oxalate, with the increase being more evident at higher Ni concentrations. Control plants released 5-fold more thiols at 30 and 60 mg kg⁻¹ Ni (Figure 3A), and 12- and 8-fold more oxalate at 30 and 60 mg kg⁻¹ Ni, respectively (Figure 3B).

Discussion

Ectomycorrhizal ultramafic P. albus improves plant-host growth and tolerance to nickel

The promotion of plant growth by ECM symbiosis has been widely reviewed: ECM associations are known to affect plant root growth, improving general plant mineral nutrition (Marschner 1995). The contribution of ECM symbiosis to lowering metal cation toxicity for the host plant has also been widely described (Marschner 1995, Hartley et al. 1997, Jenstchke and Godbold 2000). These studies and recent others (e.g., Adriaensen et al. 2003, 2005, 2006) were conducted with fungi isolated from metal-polluted sites and contribute to tree adaptation to adverse soil conditions. In a recent study, we described ecotypes of *P. albus* isolated from natural ultramafic soils that had extreme tolerance to Ni. In the current study, we have examined their capacity to improve the metal tolerance of *E. globulus*, using Myrtaceae plant-host model as ECM. The results clearly indicate that the Ni tolerance of the ECM has a substantial beneficial effect on the plant host. Ultramafic ECM isolates produced significant increases in growth in both the absence and low concentrations of Ni

(from 0.6 to 6 mg kg⁻¹). Plant root surface was greatly increased, and the high level of mycorrhizal colonization is consistent with previous data on the interaction between *Pisolithus* and *Eucalyptus* (Martin et al. 1990, Brundrett et al. 1996). At low Ni concentrations, the increase in both shoot and root biomass noticed in ECM plants compared with non-inoculated plants is probably a consequence of better mineral nutrition (Marschner 1995, Finlay 2004). However, at toxic levels of Ni (30 and 60 mg kg⁻¹), the contribution of the ECM symbiosis with ultramafic *P. albus* to host Ni tolerance was more substantial. While controls (uninoculated plants) showed a strong decrease in biomass (shoot and root) and root surface, ECM plants were able to tolerate highly toxic Ni concentrations (up to 60 mg kg⁻¹).

The basis of Ni tolerance in E. globulus associated with P. albus

The results provide some interesting insights into the possible mechanism by which Ni-tolerant *P. albus* increases the apparent tolerance of its eucalypt host. Tolerance to Ni is associated with a broad spectrum of physiological responses between exclusion and hyperaccumulation strategies. In the latter, internal Ni is detoxified by complexation with organic acids, thiols, nicotianamine or histidine and stored in vacuoles (Gabbriellini et al. 1990, Pollard et al. 2002, Kim et al. 2005, Pianelli et al. 2005). The lower total accumulation of Ni in ectomycorrhizal roots in this study argues against hyperaccumulation and internal detoxification as the primary mechanism.

The lower Ni accumulation in ECM plants might also be attributed to immobilization of Ni in the rhizosphere by complexing agents released from the roots or by either reduced

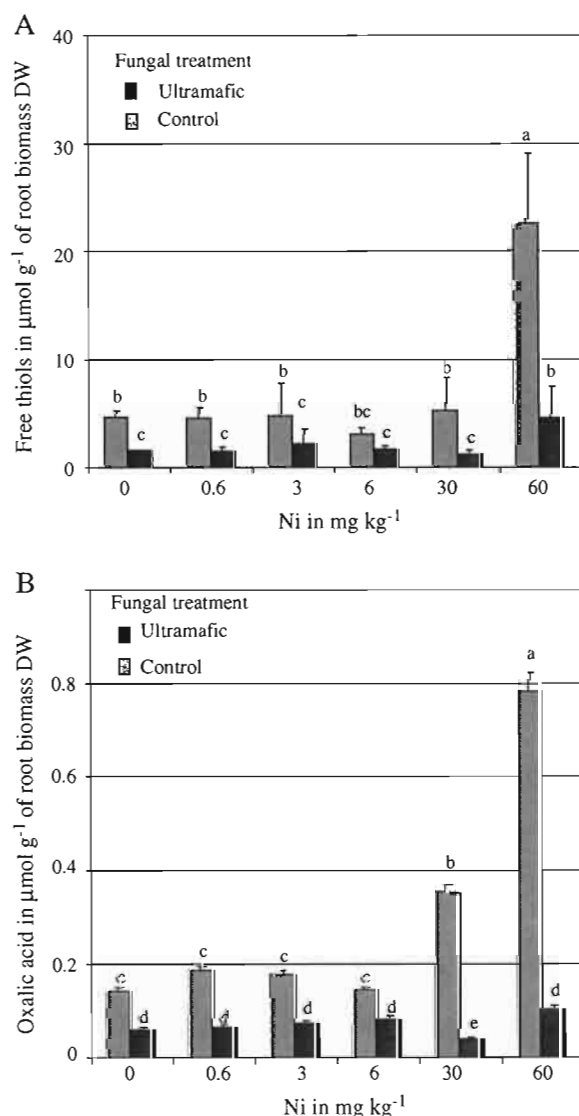


Figure 3. Total free thiols (A) and oxalic acid (B) in plant root exudates. Bars represent means, and error bars represent standard deviation of means ($n = 15$) of three independent experiments. Different letters above columns indicate significant differences as determined by the Tukey test ($P < 0.05$).

uptake or increased efflux by the fungus. The first possibility was directly tested by measuring the release from roots of organic acids and thiols, which are known to have high affinities for Ni. Excretion of oxalate and non-protein thiols was relatively unresponsive to Ni addition, with significantly higher exudation only being observed at Ni concentrations that caused strong inhibition of growth. This contrasts with the tolerance to Al toxicity in *E. globulus* where exposure to low concentrations of Al induced a large increase in the excretion of organic acids such as malate and citrate (Silva et al. 2004). In our study, control plants had higher rates of excretion of both thiols and oxalate compared with mycorrhizal plants. This may be a response to higher Ni concentra-

tions in the roots of control plants or alternatively to a constitutively higher release of these compounds from root tissue; in mycorrhizal plants, part of the 'root' mass is contributed by fungal hyphae. However, the important conclusion is that mycorrhizal roots do not excrete more metal-complexing exudates than control roots.

The alternative is that the ECM is able to restrict Ni uptake by acting as a filter between the soil and the plant root. In pure culture, *P. albus* accumulated very low concentrations of Ni compared with those measured in plant roots, which may allow it to act as an effective barrier to transfer of Ni from the soil to the root. Perhaps the most striking observation about Ni uptake in these experiments was the remarkably small amount of Ni that was transferred to shoots in the ECM plants. The shoot concentrations in ECM plants were 20-fold and 60-fold lower than in the control plants at 30 mg kg^{-1} and 60 mg kg^{-1} Ni, respectively. Yet, the Ni concentrations in roots were only 2.7- and 2.4-fold lower than the controls at these same external Ni concentrations. Intuitively, it seems hard to envisage how a fungus that wraps around the outside of a root could so profoundly influence the transfer from root to shoot, especially when the fungal biomass represents less than 10% of the root (at high Ni concentrations). However, it has long been known that translocation of Ca to the shoot is greatest from apical root sections, which is where the endodermal barrier is leaky (Clarkson 1984). Pineros et al. (1998) showed that Cd was also preferentially absorbed at the root tips of barley and *Thlaspi*, while Berkelaar and Hale (2000) found that Cd uptake in wheat was correlated with the number of root tips. It is reasonable to propose that Ni uptake and translocation may be similar to that of Ca and Cd and that, since root colonization by ectomycorrhizal fungi is generally concentrated around root tips, this pathway could be restricted in ECM plants. At the same time, the protective effects of the ECM around the root apex would enhance root growth.

The identification of ultramafic ecotype *P. albus* as previously reported in Jourand et al. (2010) and its role in plant-host adaptation to toxic Ni concentrations in ultramafic soils (this present study) constitute an important step in evaluating the potential of ECM symbioses for plant adaptation to ultramafic soils containing high concentrations of heavy metals, which is a prerequisite for their use in strategies for ecological restoration of mine sites as suggested by Reddell et al. (1999), Perrier et al. (2006) and, more recently, Khosla and Reddy (2008).

Supplementary data

Supplementary data mentioned in the text are available to subscribers at *Tree Physiology* online.

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RESEARCH ARTICLE

Ultramafic soils from New Caledonia structure *Pisolithus albus* in ecotype

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Abstract

Isolates of ectomycorrhizal *Pisolithus albus* were sampled from both ultramafic and volcano-sedimentary soils in New Caledonia, a tropical hotspot of biodiversity, to investigate the relationships between genetic diversity and edaphic constraint through tolerance to nickel (Ni). Carpophore description, spore morphology and phylogenetic analysis based on internal transcribed spacer (ITS) rDNA sequences confirmed that all isolates belong to *P. albus* and are closely related to other Australasian specimens. Using molecular tools, ITS-restriction fragment length polymorphism and amplified fragment length polymorphism markers, we showed the existence of two distinct genetic clusters within *P. albus*: ultramafic and volcano-sedimentary. Mycelia response to Ni toxicity supports such a population structure. *Pisolithus albus* from ultramafic soils included isolates with a high diversity of *in vitro* Ni tolerance, with both Ni-tolerant isolates (average Ni EC₅₀ at 575 µM) and Ni-sensitive isolates (average Ni EC₅₀ at 37 µM). In contrast, all isolates from volcano-sedimentary soils were found to be Ni sensitive (average Ni EC₅₀ at 32 µM). We highlight that (1) *P. albus* population from ultramafic soils of New Caledonia are genetically structured in ecotype, and that (2) Ni tolerance among ultramafic isolates suggests an adaptive physiological response to Ni toxicity.

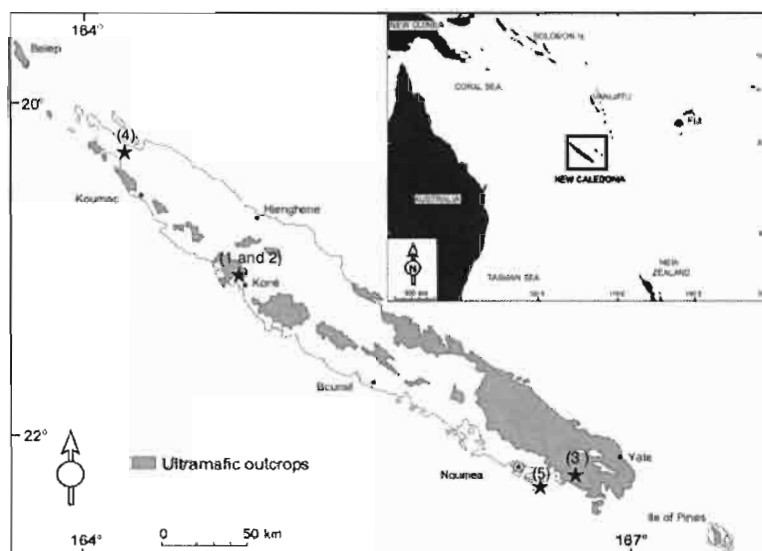
Introduction

Ultramafic soil structures are a weathered product of ultramafic bedrocks, and are characterized by low available concentrations of major plant nutrients (N, P, K), unbalanced Ca/Mg quotient and high levels of phytotoxic heavy metals, in particular nickel (Ni), chromium, manganese and cobalt (Brooks, 1987). Ni that may reach up to 10 g kg⁻¹ in ultramafic soils as compared with the average 50 mg kg⁻¹ in cultivated soils appears to be also the most bioavailable and phytotoxic element (Wenzel & Jockwer, 1999; Echevarria *et al.*, 2006). Further, other stress factors such as low content in organic matter and poor water retention are often associated with these environments (Brady *et al.*, 2005). Altogether, the ultramafic soil conditions strongly restrict plant and microbial growth and are known to select adapted

populations to metal tolerance. Specific ecotypes were noticed either for plants (Proctor & Nagy, 1992; Proctor, 2003; Chiarucci & Baker, 2007), ectomycorrhizal fungi (Panaccione *et al.*, 2001; Moser *et al.*, 2005; Gonçalves *et al.*, 2009), arbuscular mycorrhizal fungi (Schechter & Bruns, 2008), or for microorganisms (Stoppel & Schlegel, 1995).

New Caledonia, a tropical archipelago located in the South Pacific Ocean, is a biodiversity hotspot where specific biological endemic ecosystems have evolved (Myers *et al.*, 2000). It is generally thought that the major reason for this high level of diversity is the presence of natural Ni-rich ultramafic soils that cover one-third of the main island (Latham, 1981). In these soils, a specific flora (Jaffré, 1992) as well as adapted fungi and microorganism populations have been reported (Amir & Pineau 1998a, b; Navarro *et al.*, 1999; Héry *et al.*, 2003; Chaintreuil *et al.*, 2007). Association

Fig. 1. General geographical map describing New Caledonian archipelago, location of ultramafic massifs (in gray) and sites where *Pisolithus* sp. isolates were collected (1, 2 and 3: ultramafic sites; 4 and 5: volcano-sedimentary sites).



of toxic metal-tolerant ectomycorrhizal fungi with plants is important for the establishment of sensitive plants in metal-polluted environment (Krzmaric *et al.*, 2009). In this study, we focused on the fungi from the genus *Pisolithus* Alb. & Schwein. *Pisolithus* is a major ectomycorrhizal genus in the *Boletales*, distributed on a worldwide scale and forming ectomycorrhizal associations with a broad range of angiosperm and gymnosperm tree and shrub species (Marx, 1977; Martin *et al.*, 2002). *Pisolithus* is also regarded as an early colonizer that persists on sites subject to edaphic stresses (Anderson *et al.*, 1998). In New Caledonia, *Pisolithus* is very abundant and develops ectomycorrhizal symbiosis with many plants from various genera of the *Myrtaceae*, for example *Babingtonia*, *Melaleuca*, *Tristanopsis* and one *Mimosaceae*, *Acacia spirorbis* (Perrier, 2005).

In this work, our objectives were: (1) to characterize at the molecular level *Pisolithus albus* from both ultramafic and volcano-sedimentary soils in New Caledonia by internal transcribed spacer (ITS) sequencing, (2) to assess *in vitro* the Ni-tolerance phenotype of isolates' mycelia from either soil type and (3) to determine by amplified fragment length polymorphism (AFLP) the genetic structure of those strains that have been phenotyped. Our ultimate goal was to investigate whether the *in vitro* Ni-tolerance phenotype relates to a specific genetic structure of the ultramafic population of *P. albus*.

Materials and methods

Site description and soil characterization

A map location of New Caledonia archipelago including studied sites is presented in Fig. 1. The ultramafic sites

(ferralsol) were the Koniambo Massif (sites 1 and 2, Fig. 1) located in the Northern Province of New Caledonia (20°59'S, 164°49'E) and Mont Dore (site 3, Fig. 1) located in the Southern province (22°15'21"S; 164°36'42"E). Jaffré (1974) and Perrier *et al.* (2006a, b) have already reported a full description, including climate, geology, geomorphology and vegetation structure of these ultramafic sites. Nonserpentine sites (sites 4 and 5, Fig. 1) were also selected for isolation of *Pisolithus*. The volcano-sedimentary sites are situated in the extreme north of New Caledonia, near Poum (site 4, Fig. 1; 20°12'58–59"S, 164°04'40–43"E) and Ouen Toro Hill (site 5, Fig. 1; 22°18'29–33"S and 166°27'09–15"E), both ferric Acrisol soils (Latham, 1981; Gillespie & Jaffré, 2003) and vegetation consisting of savannah with trees or sclerophyll forest (Morat *et al.*, 1981). Soil sampling and mineral analyses were performed at Laboratoire de Chimie (IRD, Noumea, New Caledonia) as described by Perrier *et al.* (2006a, b).

Fungal isolates and culture conditions

Sporocarps of ectomycorrhizal fungi identified as *P. albus* were collected. The collection number, geographical localization and putative plant host are reported in Table 1a. Sporocarps mineral Ni concentration analysis was performed on an aseptic piece of the *pileus trama* dry tissue at Laboratoire de Chimie (IRD) as described in Perrier *et al.* (2006b). Stock cultures from each sporocarp were obtained by transferring aseptically a piece of the *pileus trama* to a solid modified Melin–Norkrans (MMN) medium (Marx, 1969) containing: KH_2PO_4 (0.5 g L⁻¹), $(\text{NH}_4)_2\text{HPO}_4$ (0.25 g L⁻¹), CaCl_2 (0.05 g L⁻¹), NaCl (0.025 g L⁻¹), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.15 g L⁻¹), thiamine hydrochloride (100 µg L⁻¹), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.03 g L⁻¹), glucose (10 g L⁻¹), malt extract (3 g L⁻¹) and agar (14 g L⁻¹). The pH

was adjusted to 5.6 with 1 M HCl and the medium was autoclaved for 20 min at 120 °C. All fungal strains were maintained as subcultures at 24 °C on the same medium.

ITS sequencing and phylogenetic analysis

Total genomic fungal DNA was extracted and purified from mycelial cultures using the DNAeasy Plant Qiagen Kit (Qiagen, Courtabœuf, France). An approximately 650-base pair (bp) fragment of nuclear ribosomal ITS rDNA containing the 5.8S region was amplified with specific primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). PCR reactions were prepared in a total volume of 25 µL containing aliquots of 1 µL of genomic DNA at 50 ng µL⁻¹, 1 µM of each primer, 1.5 U of *Taq* DNA polymerase (Promega, Charbonnières, France), 1 × Promega *Taq* polymerase buffer, 2 mM MgCl₂ and 200 µM dNTP. Amplification was performed with a DNA thermocycler Mastercycler® Eppendorf system (Eppendorf, Hamburg, Germany) programmed as follows: one cycle for 5 min at 95 °C followed by 35 cycles at 95 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for

7 min. Mock-DNA controls were included to test for the presence of DNA contamination in the reagents. PCR products were separated by electrophoresis in 1% w/v agarose gel in 1 × TAE with ethidium bromide at 10 µg mL⁻¹ in the running buffer as described previously by Moyersoen *et al.*, 2003. Amplified ITS products were purified using the Qiagen Gel extraction Kit (Qiagen). DNA sequencing was performed by MacroGen, using ABI chemistry and an ABI3730 capillary electrophoresis-based genetic analyzer (MacroGen, Seoul, Korea). All ITS sequences were then deposited in the EMBL nucleotide database for assignment of accession numbers (Table 1a). ITS sequences (including ITS1, 5.8S rRNA gene and ITS2) from this study were then aligned together with *Pisolithus* reference ITS sequences (Table 1b), and *Suillus luteus* ITS sequence was included as an outgroup. The alignment was produced using MUSCLE 3.6 (Edgar, 2004) and corrected manually using GENEDEC (Nicholas *et al.*, 1997). Molecular phylogeny of ITS sequences was built under the maximum parsimony (MP) criterion (to treat gaps as informative) using PAUP4 (Swofford, 2000). MP parameters were as follows: initial tree by random addition (1000 replicates), gaps treated as fifth base, character state optimization by accelerated transformation,

Table 1a. *Pisolithus* isolates included in the study along with details of their collection number, putative host plant, site collection reference, geographical origin (locality, GPS coordinates, map localization) and EMBL accession code for ITS sequences

Isolate	Putative host plants	Site number	Map location, locality and GPS coordinates	ITS accession code	Reference
MD07-112	<i>As</i>	1	Main road, Koniambo massif, Kone (21°00'10"S; 164°47'00"E)	AM947078	This study
MD07-113	<i>As</i>	1	Main road, Koniambo massif, Kone (21°00'07"S; 164°46'51"E)	AM947079	This study
MD07-114	<i>As</i>	1	Main road, Koniambo massif, Kone (21°00'06"S; 164°46'46"E)	AM947080	This study
MD07-116	<i>As</i>	1	Main road, Koniambo massif, Kone (21°00'09"S; 164°46'28"E)	AM947081	This study
MD07-117	<i>As</i>	1	Main road, Koniambo massif, Kone (21°00'22"S; 164°46'08"E)	AM947082	This study
MD07-118	<i>As</i>	1	Main road, Koniambo massif, Kone (21°00'28"S; 164°45'56"E)	AM947083	This study
MD07-165	<i>As</i> , <i>B</i> , <i>Mq</i>	2	Pindjen waterfall road, Kone (20°02'30"S; 164°46'09"E)	AM947107	This study
MD07-166	<i>As</i> , <i>B</i> , <i>Mq</i>	2	Pindjen waterfall road, Kone (20°02'30"S; 164°46'09"E)	AM947108	This study
MD07-167	<i>As</i> , <i>B</i> , <i>Mq</i>	2	Pindjen waterfall road, Kone (20°02'30"S; 164°46'09"E)	AM947109	This study
MD07-168	<i>As</i> , <i>B</i> , <i>Mq</i>	2	Pindjen waterfall road, Kone (20°02'30"S; 164°46'09"E)	AM947110	This study
MD07-169	<i>As</i> , <i>B</i> , <i>Mq</i>	2	Pindjen waterfall road, Kone (20°02'30"S; 164°46'09"E)	AM947111	This study
MD07-214	<i>As</i> , <i>B</i>	3	Mont-Dore, Plum road (22°15'21"S; 166°36'16"E)	AM947112	This study
MD07-215	<i>As</i> , <i>B</i>	3	Mont-Dore, Plum road (22°15'21"S; 166°36'16"E)	AM947113	This study
MD07-217	<i>As</i> , <i>B</i>	3	Mont-Dore, Plum road (22°15'18"S; 166°36'42"E)	AM947114	This study
MD07-218	<i>As</i> , <i>B</i>	3	Mont-Dore, Plum road (22°15'18"S; 166°36'42"E)	AM947115	This study
MD07-219	<i>As</i> , <i>B</i>	3	Mont-Dore, Plum road (22°15'18"S; 166°36'42"E)	AM947116	This study
MD07-220	<i>As</i> , <i>B</i>	3	Mont-Dore, Plum road (22°15'18"S; 166°36'42"E)	AM947117	This study
MD06-378	<i>B</i> , <i>Mq</i>	4	Poum (20°13'02"S; 164°04'38"E)	AM947123	This study
MD06-379	<i>B</i> , <i>Mq</i>	4	Poum (20°13'02"S; 164°04'38"E)	FN390950	This study
MD06-380	<i>B</i> , <i>Mq</i>	4	Poum (20°13'02"S; 164°04'38"E)	FN390951	This study
MD06-381	<i>B</i> , <i>Mq</i>	4	Poum (20°13'02"S; 164°04'38"E)	AM947124	This study
MD06-382	<i>B</i> , <i>Mq</i>	4	Poum (20°13'02"S; 164°04'38"E)	AM947125	This study
MD06-383	<i>B</i> , <i>Mq</i>	4	Poum (20°13'02"S; 164°04'38"E)	AM947126	This study
MD06-384	<i>B</i> , <i>Mq</i>	4	Poum (20°13'02"S; 164°04'38"E)	AM947127	This study
MD07-227	<i>As</i> , <i>Mq</i>	5	Ouen-Toro, Nouméa (22°18'30"S; 166°27'13"E)	AM947118	This study
MD07-228	<i>As</i> , <i>Mq</i>	5	Ouen-Toro, Nouméa (22°18'30"S; 166°27'13"E)	AM947119	This study
MD07-229	<i>As</i> , <i>Mq</i>	5	Ouen-Toro, Nouméa (22°18'29"S; 166°27'13"E)	AM947120	This study
MD07-230	<i>As</i> , <i>Mq</i>	5	Ouen-Toro, Nouméa (22°18'29"S; 166°27'13"E)	FN390952	This study

As, *Acacia spirorbis* Labill.; *B*, *Babingtonia* sp.; *Mq*, *Melaleuca quinquenervia* (Cav.) S.T. Blake.

Table 1b. *Pisolithus* and *Suillus* DNA ITS sequences used as reference for the ITS molecular phylogeny study with information concerning their code use in the cladogramme, putative host plant, geographical origin, ITS GenBank database accession number and bibliographical reference

Isolate	<i>Pisolithus</i> sp.	Plant host	Locality	ITS GenBank	Reference
H4937	<i>P. albus</i>	<i>Eucalyptus tereticornis</i>	Fanning River, Qld, Australia	AF374670	Martin <i>et al.</i> (2002)
REB2078	<i>P. albus</i>	<i>Kunzea ericoides</i>	Tauhara, New Zealand	AY318746	Moyersoen <i>et al.</i> (2003)
CA02	<i>P. albus</i>	<i>Eucalyptus</i> sp.	Canberra, ACT, Australia	AF270782	Martin <i>et al.</i> (2002)
Gemas	<i>P. albus</i>	<i>Acacia mangium</i>	Gemas, Malaysia	AF374638	Martin <i>et al.</i> (2002)
COI24	<i>P. albus</i>	<i>Acacia holosericea</i>	Sinthiou Malème, Senegal	AF374622	Martin <i>et al.</i> (2002)
Pasoh01all1	<i>P. aurantioscabrosus</i>	<i>Shorea macroptera</i>	Paosh, Malaysia	AF415226	Martin <i>et al.</i> (2002)
KS871	<i>P. marmoratus</i>	<i>Eucalyptus calophylla</i>	Denmark, WA, Australia	AF374719	Martin <i>et al.</i> (2002)
MU98/22	<i>P. marmoratus</i>	<i>Eucalyptus globulus</i>	Albany, WA, Australia	AF374660	Martin <i>et al.</i> (2002)
441	<i>P. microcarpus</i>	<i>Eucalyptus citriodora</i>	São Paulo, Brazil	U62666	Martin <i>et al.</i> (2002)
UFSC132	<i>P. microcarpus</i>	<i>Eucalyptus dunni</i>	Tres Barras, SC, Brazil	AF374704	Martin <i>et al.</i> (2002)
F22	<i>P. tinctorius</i>	<i>Pinus pinaster</i>	Mimizan, France	AF374707	Martin <i>et al.</i> (2002)
MARX270	<i>P. tinctorius</i>	<i>Pinus elliotii</i>	Georgia	AF374632	Martin <i>et al.</i> (2002)
K915	<i>Pisolithus</i> sp.1	<i>Azelia quanzensis</i>	Arabuko, Kenya	AF228653	Diez <i>et al.</i> (2001)
5105	<i>Pisolithus</i> sp.1	<i>Azelia quanzensis</i>	Arabuko, Kenya	AF003915	Martin <i>et al.</i> (2002)
Cr04	<i>Pisolithus</i> sp.3	<i>Cistus ladanifer</i>	Abenojar, Spain	AF228643	Diez <i>et al.</i> (2001)
PI03	<i>Pisolithus</i> sp.4	<i>Quercus ilex</i>	Valencia, Spain	AF228648	Diez <i>et al.</i> (2001)
MH728	<i>Pisolithus</i> sp.5	<i>Eucalyptus</i> sp.	Yanxi, China	AF374679	Martin <i>et al.</i> (2002)
MSN	<i>Pisolithus</i> sp.5	<i>Pinus kesiya</i>	ChangMai, Thailand	AF374625	Martin <i>et al.</i> (2002)
MU98101	<i>Pisolithus</i> sp.8	<i>Eucalyptus camaldulensis</i>	Eneabba, WA, Australia	AF374661	Martin <i>et al.</i> (2002)
MH56	<i>Pisolithus</i> sp.8	<i>Eucalyptus</i> sp.	Kalbarri, WA, Australia	AF374708	Martin <i>et al.</i> (2002)
CSH4461	<i>Pisolithus</i> sp.10	<i>Acacia</i> sp.	Townsville, Qld, Australia	AF374624	Martin <i>et al.</i> (2002)
MU986	<i>Pisolithus</i> sp.10	<i>Eucalyptus globulus</i>	Scott R, WA, Australia	AF374646	Martin <i>et al.</i> (2002)
S193	<i>Suillus luteus</i>		UK	AJ272416	Ruiz-Diez <i>et al.</i> (2006)

branch swapping option by tree-bisection-reconnection, consensus tree obtained by the 50% MajRule method. A bootstrap analysis was performed under the MP criterion with 1000 bootstrap replicates by fast stepwise addition (using PAUP4).

ITS restriction fragment length polymorphism (RFLP) profiles

Four restriction enzymes, i.e. MboI, HinfI, AluI and TaqI, were selected to characterize ITS diversity within *Pisolithus* as reported previously (Gomes *et al.*, 1999; Anderson *et al.*, 2001; Moyersoen *et al.*, 2003). Digestions were performed for 6 h at 37 °C on 1 µg of the amplified ITS as indicated by the provider (Promega). Restriction fragments were separated by electrophoresis in a 4% NuSieve[®] agarose gel (VWR, Fontenay, France) and stained with ethidium bromide before visualization under UV light. The 100 pair of bases (pb) DNA ladder was used as a marker (Promega).

AFLP profiles

The AFLP analysis was performed using the AFLP[®] Core Reagent Kit (Invitrogen, Cergy Pontoise, France) following the AFLP[®] Analysis System Manual with some modifications. A small quantity of genomic DNA (10–50 ng) was double digested with EcoRI (Pharmacia Biotech, Sweden) and MseI (New England Biolabs, Canada) and the resulting fragments were ligated to adaptors specific for the EcoRI and

MseI restriction sites. Preselective amplification was carried out with EcoRI+A and MseI+C primers on an Eppendorf MasterCycler PCR machine under the following conditions: 2 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 60 s at 56 °C and 60 s at 72 °C. An aliquot of the preamplification product was tested for a visible smear on a 0.8% agarose gel. The PCR products were then diluted 10-fold with water and used as the template for selective amplifications using both EcoRI+3 and MseI+3 primers. EcoRI+3 primers were 5'-end labeled with fluorescent dyes 6-FAM, HEX or NED. The list and characteristics of the AFLP primer combinations for selective amplification are presented in Supporting Information, Table S2. Amplified products were detected using an ABI 3130xl Genetic Analyser (Applied Biosystems, Courtabœuf, France). For each genotype, samples were prepared by mixing 1 µL each of three diluted (10-fold with water) PCR products obtained with different fluorescent dyes with 0.15 µL GenSize 500HD Rox and 16.85 µL water. Reading of the profiles was performed using the GENEMAPPER V3.7 software (Applied Biosystems). The presence/absence (scored as 1 or 0, respectively) of polymorphic fragments within 34–527 bp was determined for each AFLP profile corresponding to the nine-primer combination used for each sample and transformed into a binary matrix. Then a genetic distance matrix was constructed using Nei's distance coefficient (Nei & Li, 1979). A phenogram was constructed with the unweighted pair group method with the arithmetic

mean (UPGMA) algorithm in the PHYLIP software package, and the robustness of the phenogram topology was assessed by bootstrap analysis (Felsenstein, 1993). In addition, a principal component analysis (PCA) was conducted using the binary matrix of all individuals as the input matrix and performed using the XLSTAT computer software (Addinsoft, 2005). The scatter plot was generated by the first two principal components.

Ni-tolerance assays

The response of fungal isolates to Ni was determined on pure mycelial cultures on medium at a pH of 5.6. To avoid metal precipitation in the assays (Hartley et al., 1997), Ni was added to the medium after autoclaving to the desired final concentration from 1 M stock solutions of NiCl_2 (or NiSO_4) sterilized by filtration through 0.45- μm membrane filters (Millipore). In the initial Ni-tolerance screening of all isolates, plugs (8 mm) were cut from the actively growing edge of a 10-day-old mycelium culture and placed on solid MMN medium amended with NiCl_2 (or NiSO_4) at varying concentrations (10, 100 μM , 1 and 10 mM). The control was medium without Ni added. For each isolate, three independent experiments were carried out with five replicates for each concentration of Ni tested. In the second assay, for each Ni-tolerant isolate, plugs (8 mm) of a 10-day-old mycelium culture were transferred to MMN medium plates covered with cellophane and amended with Ni to final concentrations ranging from 0 to 2 mM (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1 and 2 mM). For Ni-sensitive isolates, MMN medium was amended with Ni to final concentrations ranging from 0 to 100 μM (0, 10, 25, 50, 75 and 100 μM). After 2 weeks of growth, mycelia were frozen at -80°C , and subsequently freeze-dried before weighing. The dry weight increment during the 2-week test period was determined and consequently, the biomass was expressed as the mycelial dry weight in micrograms. A tolerance index (TI) was calculated for each isolate as the percentage of biomass retained on the metal-enriched media compared with growth on the control medium. The Ni effective concentration inhibiting growth by 50% (EC_{50}) was determined as reported by Colpaert et al. (2004). Mean values of EC_{50} were analyzed using a one-way ANOVA and compared with the Newman-Keuls multiple range test ($P \leq 0.05$) using the XLSTAT computer software (Addinsoft, 2005).

Results

Soil characteristics, description of fungal isolates and the putative host plant

Full geochemical soil analyses are reported in Table S1. Soils from sites 1, 2 and 3 showed geochemical characteristics of ultramafic soils (ferralsol) with low content of major plant nutrients (N, P and K elements), unbalanced Ca/Mg ratio

and high levels of the toxic metals Mn, Cr, Co and Ni. Total concentrations of Ni ranged from 2.8 ± 0.5 to $3.5 \pm 0.7 \text{ mg g}^{-1}$ of soil, while extractable diethylene triamine pentaacetic acid (DTPA)-Ni concentrations ranged from 19 ± 3 to $25 \pm 2 \mu\text{g g}^{-1}$ of soil. In contrast, volcano-sedimentary soils (ferric Acrisol) contained low N and P, but higher K, lower unbalanced Ca/Mg ratio and very low content of toxic metals. Total Ni concentrations ranged from 0.08 ± 0.02 to $0.14 \pm 0.02 \text{ mg g}^{-1}$ of soil, while extractable DTPA-Ni concentrations were 83–190 times lower and ranged from 0.1 ± 0.05 to $0.3 \pm 0.05 \mu\text{g g}^{-1}$ of soil.

The fruiting bodies used for fungal isolation displayed the morphological and anatomical characteristics of *P. albus* (Cooke & Massei) Priest (1998) (Fig. 2). In particular, the color of the spore prints, the size of globose spores ranging from 8 to 10 μm in diameter and the ornamentation with erect spines are similar to the type specimen deposited at the Royal Botanic Gardens (Kew, UK). *Pisolithus alba* collected in both types of soils were associated with major host plants belonging to *Myrtaceae* such as *Babingtonia* Lindl. and *Melaleuca quinquenervia* (Cav.) S.T. Blake or the *Mimosaceae*, *A. spirorbis* Labill (Table 1a).

Phylogenetic analysis of ITS sequence

Phylogenetic diversity of *P. albus* isolates was first analyzed by studying the ITS sequence (ITS1/5.8S rRNA gene/ITS2) of each isolate, a commonly used phylogenetic marker for fungal species. Amplification of the ITS region resulted in a single product of 650 pb for all 28 isolates. All ITS sequences analyzed were deposited in EMBL DNA databank and are referenced under an accession number (Table 1a). The alignment (available on request) of all ITS sequences was 890 pb length, including the 28 taxa, with 127 characters as constant, 167 variable characters as parsimony uninformative and 625 characters as parsimony informative. A molecular phylogeny was produced using the MP criterion, allowing the treatment of gaps as informative, to take into account the insertions/deletions (indels). A total of 3.9×10^{10} rearrangements were attempted and 206 510 trees were obtained, the best tree score being tree length = 3733. The 50% Major Rule consensus synthetic tree obtained from the MP trees is presented in Fig. 3 including ITS sequences of *P. albus* from the sampling sites in New Caledonia. The molecular phylogeny based on the comparison of ITS sequences grouped our isolates together with other strains named as *P. albus*, indicating that these strains are very closely related. In addition, *P. albus* from New Caledonia grouped with *P. albus* isolated from Australia, New Zealand, Malaysia and Senegal.

Genetic diversity of *P. albus* based on ITS-RFLP

To further investigate the molecular diversity of the New Caledonian *P. albus*, the amplified fragments of the ITS

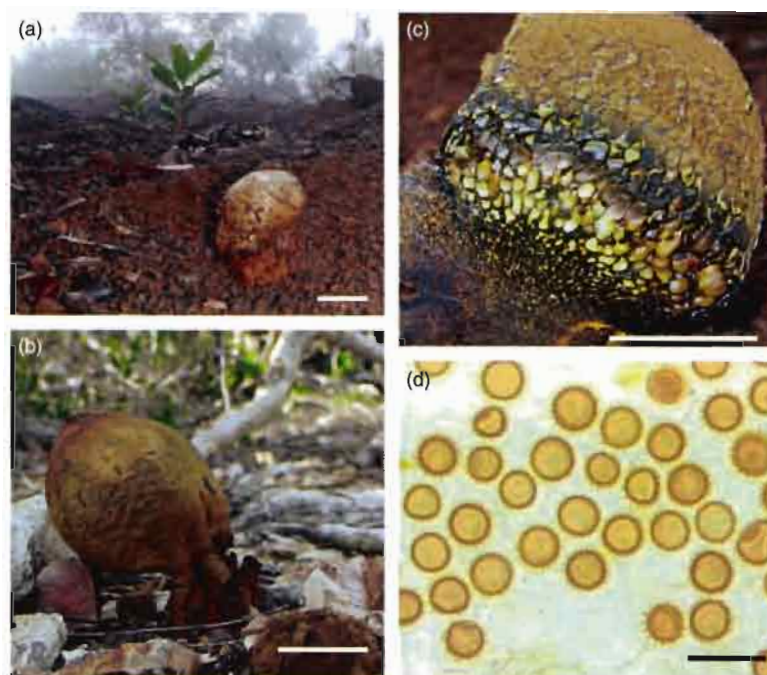


Fig. 2. *Pisolithus albus* from New Caledonia. (a) *P. albus* MD07-117 from the Koniombo massif; (b) *P. albus* MD07-228 from the Ouen-Toro, Noumea; (c) cross-section of *P. albus* MD07-166 from Pindjen waterfall and (d) globose spores (8.77–9.62 μm) of *P. albus* MD06-379 from Poupou, erected spines (1.2 μm) are clearly visible. The white double bar represents 2 cm at the focus of the fungus; the black single bar represents 20 μm .

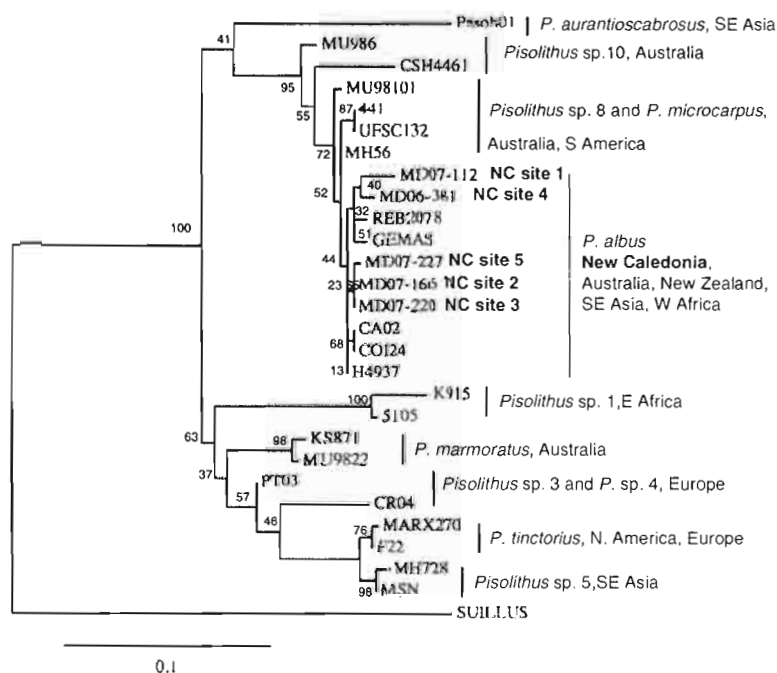


Fig. 3. Phylogenetic synthetic relationships among representative *Pisolithus* sp. from New Caledonia collection sites and worldwide reference isolates. The phylogeny is based on the analysis of the rDNA ITS1, 5.8S and ITS2 sequences. The tree shown is a 50% majority rule consensus of the most parsimonious trees (tree length = 3733) obtained with PAUP4 (see Materials and methods). Values indicated at tree nodes are percentage values of 1000 bootstrap replicates under the MP criterion using fast stepwise addition (only values > 50% are shown). The tree was rooted with *Suillus luteus* ITS sequences. Significant bootstrap frequencies are indicated. S America, South America; SE Asia, South East Asia; W Africa, West Africa.

region (650 pb) were digested by four different restriction enzymes. Pattern sequences of the ITS region obtained from 28 isolates grouped into distinct profiles (profile A and profile B) are presented in Fig. 4a. Profile A groups the 17

isolates from ultramafic soils while profile B groups the 11 isolates from volcano-sedimentary soils. Digestions with MboI and TaqI ITS supported major differences between profiles (Table 2).

Table 2. ITS-fragment sizes of *Pisolithus albus* ITS-RFLP types following digestion with MboI, HinfI, AluI, TaqI

ITS-RFLP type		ITS-RFLP fragment size (pb)			
Soil origin	ITS size (pb)	MboI	HinfI	AluI	TaqI
Ultramafic	650	73, 107, 205, 239, 334	274, 382	68, 80, 421	50, 66 , 106 , 123
Nonultramafic	650	73, 107, 205, 239	274, 382	68, 80, 421	50, 80 , 123, 228

Differences are highlighted in bold.

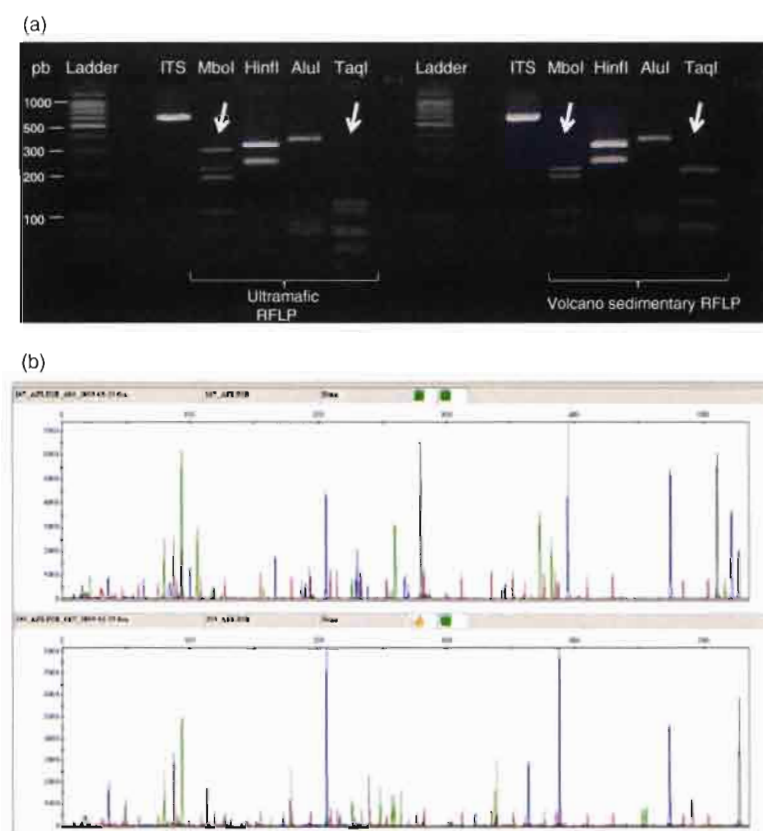


Fig. 4. (a) Representative patterns of ITS RFLP profiles of *Pisolithus albus* isolates from both ultramafic and volcano-sedimentary soils compared with both undigested amplified ITS and 100-pb DNA ladder (Promega). Arrows highlight major differences between profiles. (b) Chromatograms from AFLP analyses of representative *P. albus* isolates (MD07-167 and MD07-229) illustrating the high level of AFLPs observed in this species. The blue, green and black peaks represent fragments generated by selective amplification with respectively the 6-FAM-EcoRI+ACT/MseI+CAA, HEX-EcoRI+AAG/MseI+CAA and NED-EcoRI+ACG/MseI+CAG primer combinations. The red peaks represent the size standard.

AFLP polymorphism and genetic relatedness of *P. albus* isolates

Electrophoregrams generated for the nine selective primer pairs EcoRI-ANN and MseI-CNN, each pair tested on all genomic DNA from isolates, exhibited clear and easily identifiable peaks, corresponding to 882 different DNA fragments of which the identified size ranged from 34 to 527 DNA pair of bases. Figure 4b shows two chromatograms from AFLP analysis of representative *P. albus* isolates (ultramafic isolate MD07-167 and volcano-sedimentary isolate MD07-229), and illustrates the high level of AFLP polymorphisms observed in this species. Both genetic similarity and distance matrices are reported in Tables S3a, b. Nei's

genetic distance coefficient varied between 0.169 and 0.900. The bootstrap consensus UPGMA tree obtained for 882 AFLP-scored fragments on the 27 *P. albus* isolates indicates the clustering of isolates from ultramafic soils vs. isolates from volcano-sedimentary soils (Fig. 5a). To further explore the relationship between genetic AFLP polymorphism and soil origin of isolates, a PCA was conducted. The two principal components accounted for 40% of the total variation (Fig. 5b).

Ni tolerance of *P. albus* mycelia

Ni concentration of *P. albus* collected in New Caledonia was analyzed before *in vitro* culture. The Ni concentration varied

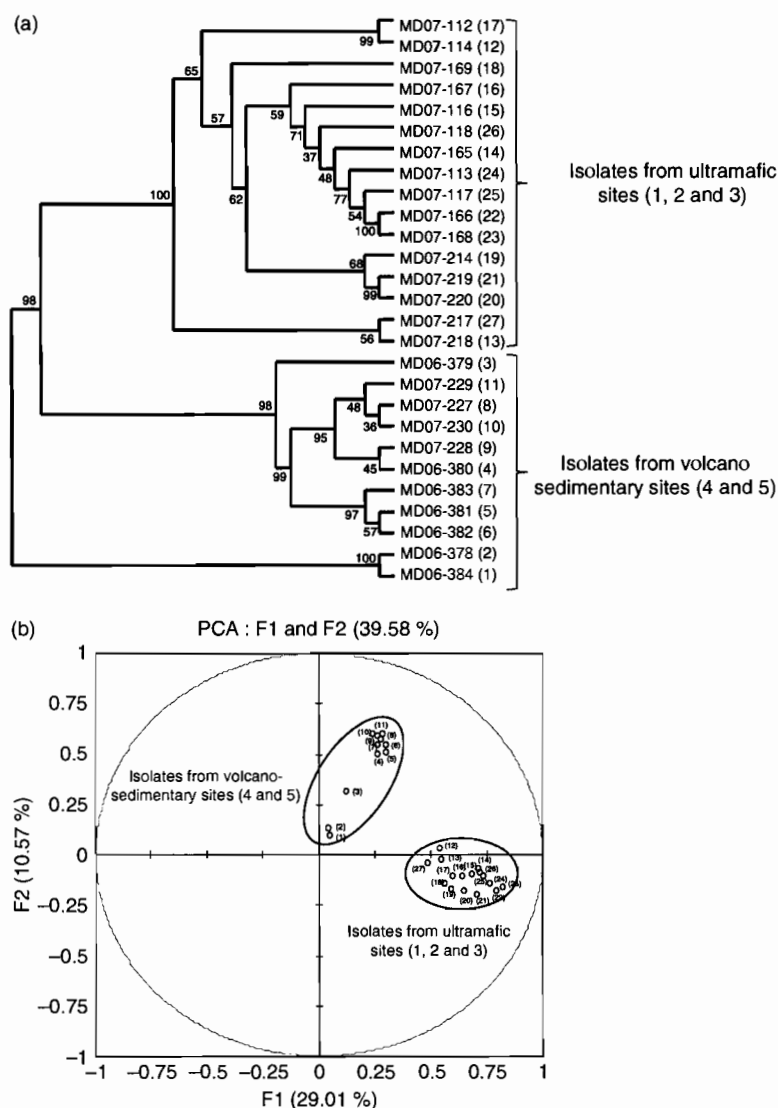


Fig. 5. Genetic relationship within *Pisolithus albus* isolates from New Caledonia according to AFLP analysis. (a) Bootstrap consensus UPGMA tree obtained for 882 AFLP scored fragments obtained with the nine selective primers pairs on the 27 *P. albus* isolates (100 replicates). (b) Plot representing the PCA of the AFLP genotypes and their clustering into ecotypes. The first two principal components (PC1 and PC2) account for 39.58% of the total variation observed. Numbers in parentheses refer to isolates in both dendrogram and PCA plot.

between 5.3 ± 0.35 and $6.15 \pm 0.25 \mu\text{g g}^{-1}$ (average Ni = $5.7 \mu\text{g g}^{-1}$) in *P. albus* from ultramafic soils. In contrast, tissue of carpophores of isolates collected from volcano-sedimentary soils contained 2.5 times less Ni (average Ni = $2.35 \mu\text{g g}^{-1}$).

In vitro growing mycelia from collected strains were screened for their tolerance to increasing Ni concentrations. The full results of the Ni tolerance screening are presented in Table S4. Among isolates from ultramafic soils, nine were able to tolerate Ni (either as NiCl_2 or NiSO_4) at concentrations ranging from 100–1 mM, while eight isolates could not tolerate Ni at concentrations higher than $75 \mu\text{M}$. In contrast, isolates from volcano-sedimentary soils were all unable to

tolerate Ni at concentrations higher than $75 \mu\text{M}$. No particular effect on the fungal Ni tolerance was observed, irrespective of the counter-anion used (Cl^- or SO_4^{2-}).

Ni tolerance (or sensitivity) was determined in terms of the Ni TI (= % of the initial biomass) and Ni concentration that inhibited growth by 50% (Ni EC_{50}) (Table 3). Concentrations of Ni higher than 0.6 mM affected the biomass yield of the nine Ni-tolerant isolates collected from ultramafic soils (average TI = 39%). In addition, Ni-tolerant isolates presented a high average Ni EC_{50} ($575 \pm 30 \mu\text{M}$). In contrast, for the Ni-sensitive isolates from either ultramafic (eight isolates) or volcano-sedimentary (11 isolates) soils, low Ni concentrations ($50 \mu\text{M}$) were sufficient to affect the

Table 3. *Pisolithus albus* isolates Ni-tolerance screening, means of carpophore tissue Ni content, means of Ni-tolerance indices (TI, %) and effective concentrations of Ni inhibiting biomass yield by 50% (Ni EC₅₀)

Ni-tolerance screening	Carpophore Ni content in $\mu\text{g g}^{-1}$ dry weight tissue	Ni-TI determination	Ni EC ₅₀ (μM)						
Ni-tolerant (Ni $\geq 100 \mu\text{M}$) isolates		Initial biomass (μg) at Ni = 0	Ni concentration tested (mM)						
Soil origin			0.1	0.2	0.4	0.6	0.8	1.0	2.0
			TI (% of initial biomass)						
Ultramafic soils (n = 9)	5.3 $\pm$ 0.35a	41.2 $\pm$ 1.6	95 $\pm$ 3	90 $\pm$ 4	81 $\pm$ 8	39 $\pm$ 10	10 $\pm$ 5	5 $\pm$ 1	0
Volcano sedimentary soils (n = 0)	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ni-sensitive (Ni $\leq 10 \text{ mM}$) isolates		Initial biomass (μg) at Ni = 0	Ni concentration tested (μM)						
Soil origin			10	25	50	75	100		
			TI (% of initial biomass)						
Ultramafic soils (n = 8)	6.15 $\pm$ 0.25a	52.0 $\pm$ 2.4	104 $\pm$ 5	65 $\pm$ 6	25 $\pm$ 8	4 $\pm$ 2	0		37 $\pm$ 6b
Volcano sedimentary soils (n = 11)	2.35 $\pm$ 0.15b	82.4 $\pm$ 6.4	103 $\pm$ 10	69 $\pm$ 9	22 $\pm$ 8	2 $\pm$ 2	0		32 $\pm$ 8b

All data are means ($\pm$ SD) of five replicates per isolate tested.

Ni carpophore content and Ni EC₅₀: data followed by same letter are nonsignificantly different ($P \leq 0.05$).

ND, not determined.

biomass production in a similar proportion (average TI = 25%). These latter isolates had a mean Ni EC₅₀ value of only 34.5 $\pm$ 7 μM Ni, which was 17-fold lower than that of the Ni-tolerant isolates.

Discussion

Phylogeny of *P. albus* from New Caledonia

This set of results is the first report on the ectomycorrhizal *P. albus* populations from New Caledonia, an archipelago in tropical south Pacific that is considered to be a hotspot of biodiversity (Myers *et al.*, 2000). Both anatomy and morphology of *P. albus* collected from either ultramafic or volcano-sedimentary New Caledonian soils correspond to the *P. albus* specimen voucher reported originally from Australia (Saccardo's Syll. fung. XI: 167; XII: 1013). Molecular identity and phylogeny based on rDNA-ITS sequencing confirmed this result and indicated that New Caledonian *P. albus* groups with Australian, New Zealand, Malaysian and Senegal specimens. In addition, the ecology of *P. albus* isolated from New Caledonia revealed an association with *Myrtaceae* (e.g. *Babingtonia*, *Melaleuca*, *Tristania*) and the endemic New Caledonian *Mimosaceae*, *A. spirorbis*. Altogether, these data are in agreement with the phylogeography of the ectomycorrhizal *Pisolithus* inferred from rDNA-ITS sequences, which suggests that (1) evolutionary lineages within *Pisolithus* are related to the biogeographical origin of the plant hosts (Martin *et al.*, 2002) and (2) a long-distance dispersal of ectomycorrhizal

fungi from Australia might explain the introduction of *Pisolithus* species in the South Pacific zone (Moyersoen *et al.*, 2003).

Types of soil genetically structure populations of *P. albus*

Among ecological constraints that might structure plant, fungi and microorganism populations, unfavorable mineral composition of soils has often been evoked as evolution pressure factors. In ultramafic soils, it has recently been hypothesized that this constraint was responsible for the structure of plant communities and the development of endemic and adapted species (Kazakou *et al.*, 2008). In fungi, recent results of analysis of the fungal genetic divergence of *Cenococcum geophilum* collected from ultramafic soils were hypothesized to reflect adaptation to soil factors (Panaccione *et al.*, 2001). However, more recently, Gonçalves *et al.* (2007) using AFLP analysis could not distinguish between ultramafic and nonultramafic isolates of *C. geophilum*. Here, for the first time, we report on a specific distribution within New Caledonian *P. albus*. Two groups of genotypes based on the ITS-RFLP analysis and confirmed by AFLP correspond to different ecotypes (ultramafic vs. volcano-sedimentary). Our results strongly support the hypothesis of a correlation between a phylogenetic link and ecological adaptation due to the particular mineral constraints of the ultramafic soils of New Caledonia. This is in agreement with work showing that ultramafic constraints structure ectomycorrhizal communities (Urban *et al.*, 2008),

as well as arbuscular mycorrhizal fungal assemblages (Schechter & Bruns, 2008).

Pisolithus albus tolerance to Ni

Ni tolerance and accumulation in tissues has often been used as a classic tool to study plant and fungal potential ecological adaptation to ultramafic constraints (Kazakou *et al.*, 2008), even to characterize microfauna (Boyd *et al.*, 2006). Within *Pisolithus* spp., previous studies have noted that some isolates were able to tolerate high concentrations of Ni. Isolates of *Pisolithus tinctorius*, with Ni EC₅₀ ranging from 126 to 170 µM, have been reported (McCreight & Schroeder, 1982; Tam, 1995). Aggangan *et al.* (1998) also described one isolate of *P. tinctorius* from ultramafic soils able to grow on Ni from 20 to 200 µM. More recently, Blaudez *et al.* (2000) and Ray *et al.* (2005) reported isolates of *P. tinctorius* that are able to grow on medium with Ni concentrations ranging from 17 to 350 µM. The mycelia from *P. albus* isolates from New Caledonian ultramafic soils displayed both *in vitro* Ni-sensitive and Ni-tolerant phenotypes. The Ni-tolerant isolates presented a noteworthy tolerance to Ni with an average Ni EC₅₀ two to three times higher than the Ni EC₅₀ already reported for other *Pisolithus* and mentioned above.

To explain the high variability in Ni-tolerance observations, we might first hypothesize high real fluctuations of Ni content in ultramafic soils while soils sampling and Ni analysis inform more about a global average of either the total Ni content (53 mmol kg⁻¹) or extractable Ni-DTPA (374 µmol kg⁻¹). Recently, Echevarria *et al.* (2006) demonstrated that extractable Ni-DTPA correlated well with the plant bioavailable Ni fraction, which is presumably what the fungus is also exposed to. Perrier *et al.* (2006a) reported that DTPA-Ni concentrations in New Caledonian ultramafic soils are in the range of 17–980 µmol kg⁻¹. In our study, *P. albus* isolates were obtained from soil with DTPA-Ni average concentrations at 374 µmol kg⁻¹ in ultramafic soils. We can assume that the average Ni-DTPA concentration does not reflect real fluctuations of bioavailable Ni in ultramafic soils. According to the range of Ni-DTPA concentrations in ultramafic soils reported by Perrier *et al.* (2006a, b), it is not surprising to find isolates of *Pisolithus* with high variations in Ni tolerance from the same ultramafic site. On the other hand, with an average concentration of Ni-DTPA at 3.4 µmol kg⁻¹ in volcano-sedimentary soils, *P. albus* isolates were all Ni sensitive. Similar variations in metal-tolerant fungal populations in correlation to metal-soil content have already been reported. For instance, in Suiloid fungi, populations displayed Zn tolerance relative to Zn concentrations in polluted soils, suggesting an evolutionary adaptation of fungi to the soil environment (Colpaert *et al.*, 2004). More recently, evidence of adapta-

tion to Ni was provided in isolates of *C. geophilum* from ultramafic soils (Gonçalves *et al.*, 2009). In our study, no clear relationship between the phenotypic physiological response to Ni and the population genetic differentiation could be established: Ni-tolerant isolates from ultramafic soils did not cluster in a homogeneous group. It is tempting to speculate that the capacity of some *P. albus* isolated from ultramafic soils to tolerate high Ni concentrations reflects the expression of an adaptive response to high concentrations of bioavailable Ni in soils as suggested for other fungi in response to high heavy metal levels (Hartley *et al.*, 1997; Colpaert *et al.*, 2004; Gonçalves *et al.*, 2009). However, if ultramafic constraints structure New Caledonian population of *P. albus* into ecotypes, Ni tolerance alone might not be a sufficient feature to explain such results. Thus, the ultramafic constraints should be grasped as a whole, even if each factor (N, P, K contents, Ca/Mg imbalance, heavy metal presence) is studied separately, as suggested by Kazakou *et al.* (2008).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Geochemical characteristics of rhizospheric soils site: major element content.

Table S2. List and characteristics of the nine AFLP primer combinations for selective amplification.

Table S3a. Similarity matrix calculated with Nei's coefficient based on 882 scored AFLP fragments obtained with the nine selective primers pairs EcoRI-ANN and MseI-CNN.

Table S3b. Distance matrix calculated with Nei's coefficient based on 882 scored AFLP fragments obtained with the nine selective primers pairs EcoRI-ANN and MseI-CNN.

Table S4. *Pisolithus* isolates Ni-tolerance screening by measuring capacity to grow on MNM medium at different concentrations of either NiCl₂ or NiSO₄.

Table S5. AFLP number loci scored for each DNA of *P. albus* isolates tested.

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Symbiotic properties of *Methylobacterium nodulans* ORS 2060^T: A classic process for an atypical symbiont

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Abstract

Some legume species of the *Crotalaria* genus are specifically nodulated by methylotrophic bacteria belonging to the *Methylobacterium nodulans* species. The feature of this symbiotic bacterium is its ability to oxidize methanol, a property based on the presence of a methanol dehydrogenase enzyme. Despite a good knowledge of this property and its implication in symbiosis, the molecular dialogue between *M. nodulans* and *crotalaria podocarpa* leading to symbiosis is largely unknown, except the presence of a *nodA* nodulation gene in the genome of *M. nodulans* ORS 2060. To investigate if *M. nodulans* ORS 2060 produces Nod factors, molecules considered as the major bacteria-to-plant signals essential for the establishment of rhizobia–legume symbiosis, we identified and sequenced a *nodDABCUIJHQ* cluster from a genomic library of ORS 2060. Phylogenetic analyses of *nod* genes revealed that *M. nodulans* ORS 2060 form a branch together with *Burkholderia tuberum* STM678 and a strain of *Methylobacterium* sp. (4-46) isolated from *Lotononis*, and distinct from all the other rhizobia. To analyse the regulation of ORS 2060 *nod* genes, we constructed a *nodA*–*LacZ* promoter fusion to monitor the *nod* gene expression with various flavonoids. The flavone apigenin was found to be the strongest inducer of *nod* gene expression in *M. nodulans* ORS 2060. This latter flavonoid was used to induce ORS 2060, and Nod factors were purified by high-performance liquid chromatography (HPLC) and further characterized by mass spectrometry. One major Nod factor structure was identified as a pentamer of chitin substituted by C18:1 or C16:0 acyl chains on the non-reducing end and 6-O-sulphated on the other end, suggesting a classic symbiotic dialogue between *M. nodulans* and *C. podocarpa*.

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Keywords: *Methylobacterium nodulans*; *Crotalaria* spp.; Lipo-chito-oligosaccharide (LCO); Nod factor; Flavonoids; Apigenin; Nodulation genes; Rhizobia

1. Introduction

The symbiosis between rhizobia and legumes results in the formation of N₂-fixing root nodules and has been described as a multi-step process mediated by signal molecules produced by both bacteria and plants (Spaink, 1992). The first apparent exchange of signals involves the secretion of phenolic compounds by legumes such as

flavonoids and isoflavonoids (Peters and Verma, 1990). These polyphenolic compounds induce the transcription of bacterial nodulation genes leading to the biosynthesis of a bacterial signal, the nodulation Nod factor (NF) (Peters and Verma, 1990; Dénarié et al., 1996). NFs metabolites are lipo-chito-oligosaccharides (LCOs), mostly an oligomeric backbone of three to five β -1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) residues with the *N*-acetyl group replaced by an acyl chain on the terminal non-reducing end (Perret et al., 2000; D'Haeze and Holsters, 2002). The structure of the fatty acyl chain, the number of GlcNAc

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residues and the presence of extra substituents determine the host specificity of the bacterium (Dénarié and Cullimore, 1993; Dénarié et al., 1996). Nodulation genes can be classified into two categories: the common genes (*nodABCDIJ*) are functionally conserved across rhizobia and involved in the formation of the *N*-acylated chitin oligomer NF core while specific *nod* genes (such, as *nodZ*, *nodH*, etc.) encode enzymes involved in the synthesis and transfer of additional chemical compounds on the NF core, and are unequally distributed across rhizobia in relation to their host range (Downie, 1998).

NFs have been described as molecules participating in a various number of plant host physiological processes, including cell divisions forming nodule primordia, activation of gene expression, root hair deformation, and oscillations in cytoplasmic calcium levels (termed calcium spiking) (Minami et al., 1996; Spaik, 1996; Mitra et al., 2004; Kanamori et al., 2006).

The *Crotalaria* genus (Fabaceae family, Papilionoideae subfamily, Crotalarieae tribe) is composed of more than 600 species (Allen and Allen, 1981), located in the subtropical and intertropical regions (Polhill and Raven, 1981). Some species have agronomic significance as green manure or for their nematocidal properties (Silva et al., 1989). In Senegal, *Crotalaria* species have been found to be nodulated by *Bradyrhizobium* strains (Samba et al., 1999). Surprisingly, *Methylobacterium* spp. strains have also been isolated from root nodules of three Senegalese species of *Crotalaria*, *Crotalaria glaucoidea*, *Crotalaria perrottetii* and *Crotalaria podocarpa* (Samba et al., 1999; Sy et al., 2001b). These symbiotic strains belong to a single species, named *M. nodulans*, for its ability to nodulate and fix nitrogen specifically during symbiosis with *Crotalaria* spp. (Jourand et al., 2004). Interestingly, the three species of *Crotalaria* associated with *M. nodulans* are not nodulated by *Bradyrhizobium* sp. isolated from other species of *Crotalaria* (Samba et al., 1999; Sy et al., 2001b). If *Methylobacterium* strains have been found previously associated with plants at different levels, such as epiphytes (Omer et al., 2004) and endophytes (Elbeltagy et al., 2000), this was the first description of a *Methylobacterium* species as a legume symbiont. Later, another group of symbiotic *Methylobacterium* strains was isolated from various species of *Lotononis* in South Africa (*L. angolensis*, *L. bainesii*, *L. listii*, *L. solitudinis*) (Jaftha et al., 2002; Yates et al., 2007), which belong to a close species of *M. nodulans* (Yates et al., 2007). However, cross-inoculation studies showed that *M. nodulans* does not form nodules on *Lotononis* species (Yates et al., 2007).

The specificity of the *Methylobacterium*–*Crotalaria* interaction has already been assessed by an evaluation of the role of the methylotrophic properties in the symbiotic process. Inoculation of *C. podocarpa* species with methylotrophic minus mutants resulted in a reduction of the nodule number and a drastic decrease of plant biomass (Jourand et al., 2005). However, no investigation has been carried out to determine which signal molecules are involved in the

molecular dialogue between *M. nodulans* and its host plant. Previous genetic studies revealed the presence of the *nodA* nodulation gene in the type strain of *M. nodulans* (ORS 2060), suggesting that this bacteria may also produce the symbiotic LCOs (Sy et al., 2001a; Jourand et al., 2004).

The aim of this study was to determine which signal molecules are involved in the symbiosis between *M. nodulans* ORS 2060 and *C. podocarpa*, by the characterization of the bacterial nodulation gene cluster, identification of the flavonoids that induce their expression, and finally determination of the NF structures produced by the bacteria.

2. Materials and methods

2.1. Bacterial strains and cultures

M. nodulans strains, belonging to the bacterial collection of the Laboratoire des Symbioses Tropicales et Méditerranéennes (Montpellier, France), were the wild-type strain ORS 2060 (Jourand et al., 2004) and the recombinant strain ORS 2060 *nodA*–*lacZ* (this study). All *M. nodulans* strains were grown in yeast-mannitol medium (Vincent, 1970) at 37 °C. *Escherichia coli* strains pCM132, S17-1 and XL1-MR were provided by M.E. Lidstrom (Marx and Lidstrom, 2001), R. Simon (Simon et al., 1983) and A. Sy (Sy et al., 2001a), respectively. Standard methods were used for growth of *E. coli* in Luria-Bertani (LB) medium (Sambrook et al., 1989). All media were supplemented with appropriate antibiotics: nalidixic acid (100 µg ml^{−1}) for all *M. nodulans* strains, kanamycin (50 µg ml^{−1}) for *M. nodulans* ORS 2060 *nodA*–*lacZ* and *E. coli* strains.

2.2. Molecular techniques

Genomic DNA was prepared according to Chen and Kuo (1993). Plasmid and cosmid DNAs were isolated using Miniprep kits (Promega, Charbonnières, France). All reactions for DNA amplification by PCR or for sequencing, as well as methods for sequence analysis, were carried out as previously described by Sy et al. (2001a). DNA amplified products were purified with a Qiaquick gel extraction kit (Qiagen, Courtaboeuf, France). Restriction endonuclease and ligase reactions were performed according to the manufacturer's specifications (Eurogentec, Angers, France). For Southern blot hybridization, restricted DNA was blotted to positively charged nylon membranes by the alkali transfer procedure and hybridized with digoxigenin (DIG)-dUTP using the DIG labelling kit supplied by Roche (Meylan, France).

The study of *M. nodulans* nodulation genes was performed using the ORS 2060 genomic library obtained by Sy et al. (2001a). Screening of *nodA*-containing cosmids was performed by DNA amplification using the primer pair *nodAf* brady (5'-GTG-CAG-TGG-AGS-STK-CCG-TGG-G-3') and *nodAr* brady (5'-TCA-CAR-CTC-KGG-CCC-GTT-CCG 3'). A selected clone, pSTM223, was

confirmed by hybridization with a *nodA* probe constructed by DIG labelling of the ORS 2060 580-bp *nodA* internal fragment. A 7.13-kb *Xma*I fragment of pSTM223 containing the putative *nod* genes was subcloned in pUC18. This clone, called pSTM520, was digested by *Hind*III, *Sac*I, *Sac*II and *Bgl*II and restriction fragments were cloned in pUC18 and sequenced using universal primer M13F (5'-GTT-TTC-CCA-GTC-ACG-AC-3') and M13R-48 (5'-AGC-GGA-TAA-CAA-TTT-CAC-ACA-3'). Primer walk sequencing was performed to close the gaps of the pSTM520 insert. All sequences were performed using ABI chemistry on an ABI310 sequencer (Applied Biosystems).

2.3. Construction of an ORS 2060 *nodA*–*LacZ* transcriptional fusion

A 366-bp DNA fragment of the *M. nodulans* ORS 2060 genome was amplified by PCR with primers pmNodA 2060f (5'-GAA-TTC-GTT-TGC-GGC-CCC-TCA-TAA-TAA-ATA-GC-3') and pmNodA2060r (5'-CGA-AGA-TCT-GGA-CAG-CTC-TCC-TGT-TCC-GGA-GCT-3'), corresponding to the putative promoter section of *nodA* (AF220764). This 366-bp DNA fragment was cloned into the *Eco*RI and *Bgl*II sites of the pCM132 plasmid vector (Marx and Lidstrom, 2001). The combined pCM132-*nodA*–*lacZ* plasmid was transferred by electroporation into conjugative *E. coli* S17-1 and then into *M. nodulans* ORS 2060^T strain by conjugation. Conjugative colonies were selected on a YM medium containing antibiotics (kanamycin at 50 µg ml⁻¹ and nalidix acid at 100 µg ml⁻¹).

2.4. *Methylobacterium nodulans nodA* gene induction by flavonoids

The *M. nodulans nodA*–*LacZ* fusion was tested with flavones (apigenin, luteolin), flavonols (kaempferol, quercetin), and isoflavones (daidzein, genistein). All these

chemicals were provided by Extrasynthese (Genay, France). The flavonoid standard solutions were prepared in ethanol (10 mM) and not as usual in methanol because of the methylotrophic property of the *Methylobacterium* strains. Cultures of *M. nodulans* ORS 2060 *nodA*–*lacZ* reaching an A_{600} of 0.5 were supplemented with each signal compound at 5 µM. For each treatment, β -galactosidase activity was recorded until 102 h after induction using a specific protocol adapted from Miller (1972) for *Methylobacterium*. β -Galactosidase activity was measured on 20 ml aliquots of bacteria: cells were pelleted by centrifugation (10 min, 4000 rev min⁻¹), washed and resuspended in 1 ml of sterile water, then crushed by ultrasonication 2 times, 1 min at 20 W (Sonics Material, Danbury, USA). After treatment, cell suspension was centrifuged for 25 min at 14,000 rev min⁻¹ and the crude supernatant was used to test both β -galactosidase activity (as described by Miller, 1972) and total soluble-protein content (using the Biorad Protein Kit, Biorad, Marnes la Coquette, France). The β -galactosidase activity was expressed in µmol min⁻¹ mg⁻¹ of soluble proteins. All recorded activities were processed using Statistica 6.0 software. The means were separated using one-way analysis of variance, and HSD Tukey post-hoc tests were then performed to constitute the induction groups shown in Table 1.

2.5. *Nod* factor extraction and purification

M. nodulans ORS 2060 was grown until the culture reached an A_{600} of 0.1. Then cells were induced to produce NFs by adding the best flavonoid inducer at 5 µM, and grown during 48 h at 37 °C. NFs were extracted from filtered culture supernatants by partition with *n*-butanol (Roche et al., 1991). Purification was performed on a high-performance liquid chromatography (HPLC) system

Table 1
Induction of the *Methylobacterium nodulans nodA* promoter by flavonoids^d

Putative inducer	Hydroxylation pattern					Maximal response (mU β -gal min ⁻¹ mg ⁻¹)
	3	5	7	3'	4'	
Flavones						
Apigenin	–	OH	OH	–	OH	654 ± 23 ^a
Luteolin	–	OH	OH	OH	OH	196 ± 6 ^b
Flavonols						
Kaempferol	OH	OH	OH	–	OH	616 ± 21 ^a
Quercetin	OH	OH	OH	OH	OH	1 ^c
Isoflavones						
Genistein	–	OH	OH	–	OH	0 ^c
Daidzein	–	–	OH	–	OH	0 ^c
Control ^c						0 ^c

Data are means of two independent determinations.

^{a,b,c}Grouping is based on mean β -galactosidase activity and statistical results.

^dExperiments were carried out as described in the text, with an induction time of 15 h (maximum activity was recorded at that time).

^cControl was induction medium without added inducers.

Waters 2690 separation module (Waters, Milford, Massachusetts, USA) with a semi-preparative C₁₈ reverse phase column (10 mm × 250 mm; 5 µm, Phenomenex Gemini) using an isocratic solvent A (water-acetonitrile, 70:30 [vol/vol]) containing 50 mM ammonium acetate for 20 min, followed by a 40 min linear gradient from solvent A to solvent B (100% acetonitrile) containing 50 mM ammonium acetate at a flow rate of 1 ml min⁻¹. The UV absorption was monitored at 206 and 260 nm. Ammonium acetate and solvents from the collected fractions were removed by two successive freeze-dryings.

2.6. Mass spectrometry

Each pre-purified HPLC fraction was analysed using a ESI-QqToF Ultima apparatus (Waters, Milford, Massachusetts, USA) using direct infusion. Spectra were recorded in both positive and negative modes. Peaks detected in the awaited range (*m/z* 1000–1500 for the simple charged species or 600–700 for the double charged ones) were submitted to MS/MS experiment to confirm their LCO nature.

Energies were the following: probe: 3 kV, cone: 100 V, Rf: 70 V, collision cell: 15 V for MS, 30 V for MS/MS. Collision gas: argon. Direct inlet: solvent AcCN/H₂O 1:1, 1% acetic acid, rate: 10 µl min⁻¹. Concentrations were about 10⁻⁴ M.

2.7. Phylogenetic analyses

The multiple nucleotide sequence alignments were generated using ClustalX (Thompson et al., 1997) and optimized manually. All phylogenetic analyses were performed using PAUP version 4.0b10 (Swofford, 1998). A concatenation of the *nodABCIIJ* genes from *Methylobacterium* ORS 2060 and 4-46 (genome available at JGI, <http://genome.jgi-psf.org/>) strains plus nine strains (*Mesorhizobium loti* R7A (accession number AL672111) and MAFF303099 (NC_002678), *R. etli* CFN42 (NC_007761), *Sinorhizobium* sp. NGR234 (NC_000914), *S. meliloti* 1021 (NC_003037), *R. leguminosarum* 3841 (AM236084), *Burkholderia phymatum* STM815 (NZ_AAUG000000000), *B. japonicum* USDA110 (NC_004463) and *Azorhizobium caulinodans* ORS 571 (L18897)) from which all these genes were available was constructed. Partition homogeneity tests were calculated

between each marker partition, and homogeneity was found between all markers (*P* value = 1 – (704/1000) = 0.296). A *nodA* ML phylogeny was also built, using 50 representative strains of the rhizobial diversity. The best-fit model for *nodA* selected by MODELTEST3.6 (Posada and Crandall, 1998) was the HKY+I+G model, with parameters base = (0.1966 0.3043 0.3003), Nst = 2, Tratio = 1.6522, rates = gamma, shape = 1.2320 and Pinvar = 0.2330.

For *nodABCIIJ* and *nodA* phylogenies, bootstrapping analyses were performed using heuristic searches with 100 and 1000 replicates, respectively.

3. Results

3.1. Organization and analysis of *nod* genes in *M. nodulans* ORS 2060

A 7.13-kb region of the pSTM223 cosmid (from a *M. nodulans* ORS 2060 genomic library constructed by Sy et al., 2001a), showing a positive hybridization signal to the *nodA* probe, was fully sequenced and analysed (see Materials and methods for details). The full DNA sequence is available in GenBank under accession number AM712915, and annotation of the fragment is presented in Fig. 1. The analysis of the nucleotide sequence of the cosmid fragment revealed the presence of six open reading frames (ORFs). These ORFs showed high nucleotide sequence identity with known rhizobial nodulation genes. ORF₁ (215 amino acids) encodes a protein with 81% identity with NodA from *B. tuberum* STM678. ORF₂ (220 aa) presented 74% of identity with NodB from *Burkholderia* sp. STM 678 (AC42488). ORF₃ (485 aa) showed 76% of identity with NodC from *Rhizobium* sp. N33 (AAB16897). ORF₄ (305 aa) revealed 81% of identity with NodI from *S. meliloti* 1021 (AAK65130). ORF₅ (264 aa) revealed 77% of identity with NodJ from *R. tropici* (CAC39129). The sixth ORF (250 aa) showed 71% of identity with NodH of *Rhizobium* sp. strain N33 (AAB16900). Upstream of these six ORFs, a 262 bp fragment similar to the 5'-end part of *nodD* (encoded on the reverse strand), is present but is interrupted by the end of the cosmid. In addition, an ORF with homology to the 3' end part of *nodQ* (56% identity with the 180 amino-acid long C-terminal part of *R. tropici* NodQ, P52978), as well as relics of *nodU* and transposases were detected.

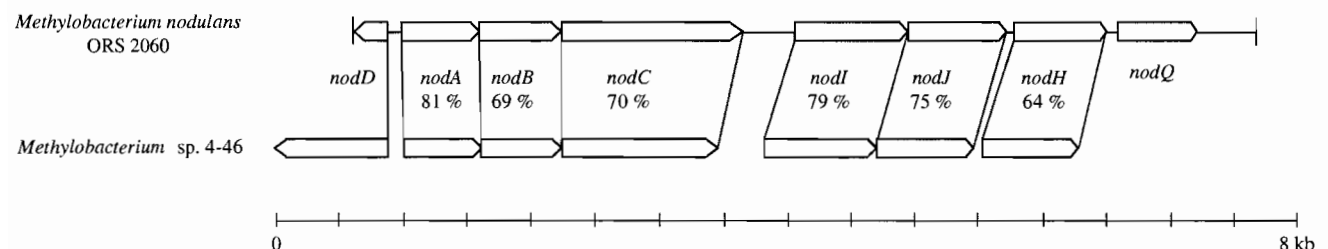


Fig. 1. Comparison of nodulation genes operons in *Methylobacterium* sp. 4-46 (A) and *Methylobacterium nodulans* ORS 2060 (B). % indicated are % of identities at the amino-acid level.

During the preparation of our article, the complete genome of *Methylobacterium* sp. 4-46, a *Lotononis* symbiont, became available (http://genome.jgi-psf.org/draft_microbes/met_4/). We thus included a comparison of both *nod* genes operons. Both strains exhibit a very similar organization of the *nod* cluster (see Fig. 1). The NodDAB-CIJ proteins share 69–81% identities between ORS 2060 and 4-46 strains (Fig. 1). In *Methylobacterium* sp. 4-46

genome, no additional *nod* gene was found (using blast searches of *B. japonicum* and *S. meliloti* *nod* gene sets).

A phylogenetical analysis of the *nodABCIIJ* operon was then investigated using a partition of these five *nod* genes, and using a maximum likelihood approach (see Materials and methods). We included in the analysis all rhizobia from which all *nodABCIIJ* genes had been previously sequenced (nine strains, see Fig. 2A). The tree obtained (Fig. 2A) shows that *M. nodulans* ORS 2060 form a distinct branch together with *Methylobacterium* sp. 4-46 supported by a high bootstrap (100%), though their sequences accumulated mutations since the speciation event.

As the *nodA* gene has been previously used as a symbiotic marker of rhizobia (Moulin et al., 2004; Stepkowski et al., 2005), we also built a *nodA* phylogeny including 50 rhizobial strains representative of the rhizobial diversity (except *Sino-Meso-Rhizobium* strains, as they constitute separate *nodA* lineages from the *Methylobacterium*–*Bradyrhizobium* branch, Stepkowski et al., 2007) to detect the closest phylogenetical symbiotic neighbours of *M. nodulans*, presented in Fig. 2B. *M. nodulans* *nodA* gene grouped strongly (100% bootstrap) with *Methylobacterium* sp. 4-46 and *B. tuberum* STM 678 (88% bootstrap), strains isolated from nodules of *Lotononis bainesii* and *Aspalathus carnosa*, respectively (Moulin et al., 2001; Jaftha et al., 2002).

3.2. The expression of *nodA* is inducible by the flavone apigenin

We tested a number of commercially available flavonoids for their ability to induce the *nodA* promoter of *M. nodulans* ORS 2060. As shown in Table 1, addition of apigenin or kaempferol results in a high β -galactosidase activity of the *nodA*–*lacZ* promoter fusion and can thus be considered as strong *nod* gene inducers in strain ORS 2060. Their activities were not significantly different despite an additional C-3 hydroxylation of the kaempferol compared to apigenin. Luteoline (C-3'OH-apigenin) gave a β -galactosidase activity that was three-fold lower as compared to apigenin. The flavonone quercetin (C-3'OH-kaempferol) did not give any detectable β -galactosidase activity (Table 1). Thus, the presence of a hydroxyl on position C-3' of the flavonoid skeleton (as in luteolin or quercetin) reduces greatly the induction of *nod* genes expression.

The isoflavones differ from the flavones in that the B ring is attached to C-3 instead of C-2. As daidzein and genistein are not able to provoke a promoter response (Table 1), we conclude that the attachment of the B ring to C-2, as is found in flavones and flavonols, is of crucial importance for induction. As apigenin was the most active inducer, this flavone was used to conditions to obtain a maximum expression of *nod* genes. Addition of 5 μ M apigenin to a *M. nodulans* culture reaching an A_{600} of 0.1 led to the highest β -galactosidase activity, and this condition was used to produce NFs.

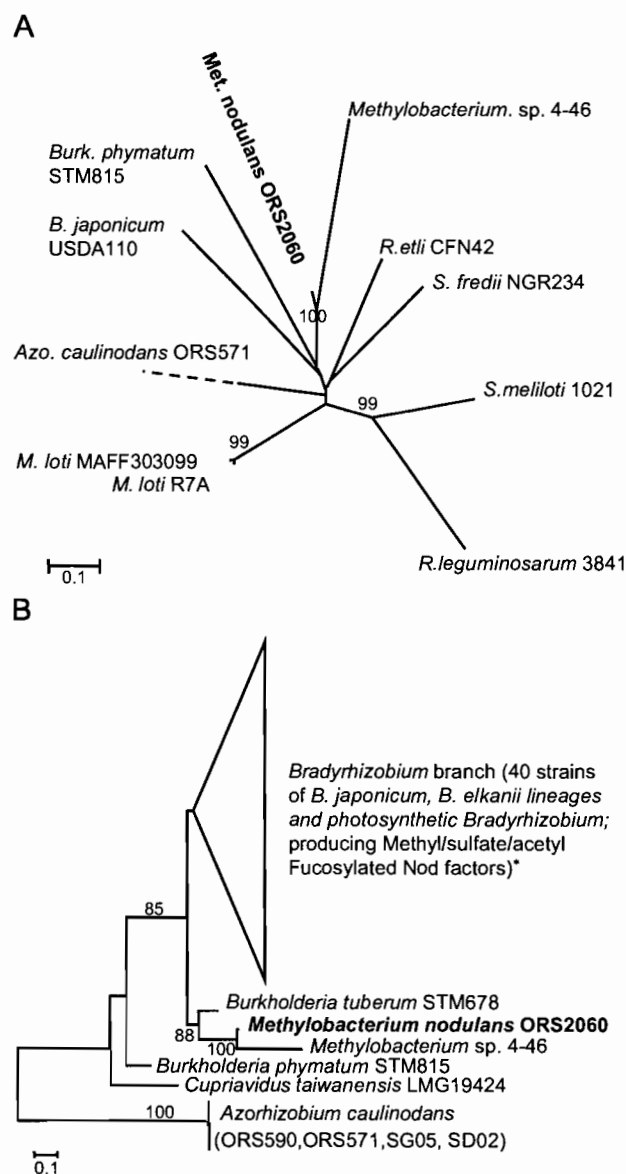


Fig. 2. Maximum likelihood (ML) phylogenies of *nodABCIIJ* concatenated alignments (A) and *nodA* gene (B). Bootstraps indicated at tree nodes (when >50%) were calculated under ML criterion with 100 replicates. **Bradyrhizobium* branch shown in (B) includes 40 strains belonging to several *Bradyrhizobium* genomic species; for details see Fig. 2 of Moulin et al. (2004); Nod factor structure was deduced from structural studies and *nod* gene content. Abbreviations: *B.*: *Bradyrhizobium*, *Burk.*: *Burkholderia*, *S.*: *Sinorhizobium*, *R.*: *Rhizobium*, *M.*: *Mesorhizobium*, *Azo.*: *Azorhizobium*.

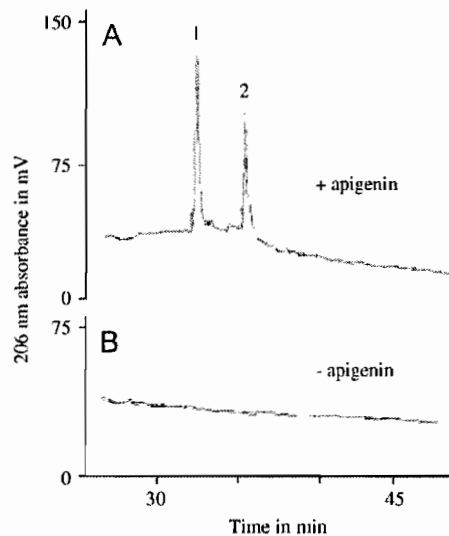


Fig. 3. HPLC profiles of *n*-butanol extract induced with apigenin (A) or not induced (B) from *Methylobacterium nodulans* ORS 2060 culture. Separation was performed with the presence of ammonium acetate as described in Materials and methods, with monitoring of the absorption at 206 nm.

3.3. *Methylobacterium nodulans* ORS 2060 produces pentameric LCOs 6-O sulphated at the reducing end

The NFs produced by *M. nodulans* were purified using the protocol described in Materials and methods. Fig. 3 shows the comparison of two HPLC profiles of NFs extraction from two different *M. nodulans* ORS 2060 cultures: the first one was obtained after induction with apigenin and showed the presence of putative LCO compounds (Fig. 3A), the second one was obtained without an inducer (Fig. 3B). In the absence of apigenin, no NF could be detected on the HPLC profile (Fig. 3B). HPLC analysis revealed the presence of two chromatographic peaks absorbing at 206 nm, in the induced cultures (Fig. 3A). Peaks were eluate at 57% and 65% acetonitrile, respectively.

Fractions corresponding to the two peaks detected in the HPLC profile were then collected and analysed by ESI-MS. The positive ion spectra of compounds in fraction 1 exhibited a complex series of protonated $[M+H]^+$ (m/z 1336 with a minor m/z 1310) and alkali cationized molecules assigned to $[M+Na]^+$ (m/z 1358), $[M+K]^+$ (m/z 1374 and 1338, respectively,) and $[M-H+2Na]^+$ (m/z 1380) or $[M-H+Na+K]^+$ (m/z 1396 and 1370, respectively). Presence of such dialkali ions is characteristic of acidic functions. Samples were therefore submitted to a negative ionization mode analysis. Only two compounds have been observed, the first very weak at m/z 1308 and the second intense at m/z 1334, allowing to attribute the molecular masses at M 1309 and 1335 Da. The mass difference between both is 26 amu, indicating that the predominant one might exhibit a $C_{18:1}$ and the minor one a $C_{16:0}$ chain. Molecular masses obtained from the ESI-MS

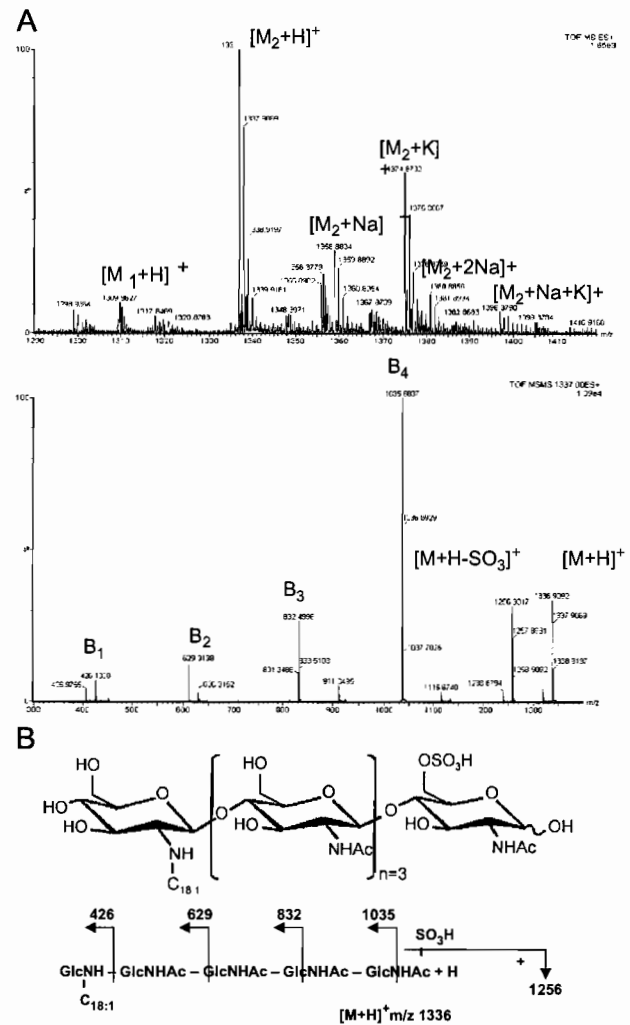


Fig. 4. Results of the mass spectrometric analysis of the C_{18} high-performance liquid chromatography fractions (A) and structure of *Methylobacterium nodulans* ORS 2060 Nod factor (B).

spectra are compatible with a chitopentamer substituted by a $C_{18:1}$ (or $C_{16:0}$) fatty acyl and a sulphate. Presence of this sulphate has been assessed by ionization in the negative mode.

These structures have been confirmed through MS/MS experiments. Collision energy has been set to 30 V. A classical B fragmentation pattern has been observed, corresponding to disruption of the osidic junction between the glucosamines (Fig. 4A). This fragmentation produces a regular profile with a mass difference of 203 amu between each ion corresponding to the GlcNAc subunits (221 amu for the reducing end). Fragmentation of the molecular ions $[M+H]^+$ produced a loss of sulphite (SO_3) (80 amu) directly from the molecular species, indicating that sulphation occurs on the reducing end. The profiles ended with B_1 ions at m/z 410 and 426, respectively, for the two NFs, indicating that the non-reducing end is substituted only by a $C_{16:0}$ or mostly a $C_{18:1}$ acyl. From the MS/MS

experiments, the structure presented in Fig. 4B can be deduced. Localization of substitutions was deduced from already known NF structures. No NF compounds were found in fraction 2.

4. Discussion

Although most *Crotalaria* sp. are nodulated by non-methylophilic *Bradyrhizobium* sp., few African species (*C. glaucooides*, *C. perrottetii* and *C. podocarpa*) are found associated with bacterial symbionts belonging to the *M. nodulans* species (Samba et al., 1999; Sy et al., 2001b). In comparison to many other rhizobia, relatively little is known about the *M. nodulans* *nod* genes content and the symbiotic molecules involved in the molecular dialogue between the two partners. In this study, we show that the type strain ORS 2060 harbours a set of *nod* genes organized in a single operon with six ORFs (encoding NodA, NodB, NodC, NodI, NodJ and NodH proteins), as well as gene fragments of *nodD*, *nodU*, *nodQ* and of a transposase.

M. nodulans harbours *nod* genes similar to the other rhizobia, although they constitute a separate cluster in the *nod* gene phylogenies together with *Methylobacterium* 4-46 (isolated from *Lotononis bainesii* (Norris, 1958) genome, which has been recently sequenced at the Joint Genome Institute) and *B. tuberum* STM678 (isolated from *A. carnosus*, Moulin et al., 2001). As most *Methylobacterium* species are not symbiotic, it is highly probable that symbiotic abilities in this genus are not an ancestral character but have been acquired by lateral transfer. The presence of transposase relics close to the *nod* gene operon supports this idea. The host origin of the transfer remains unknown, as no close homolog of the *M. nodulans* *nod* genes has been characterized so far, except for *B. tuberum* that groups with the *Methylobacterium* branch with a high bootstrap value (98%) in the *nodA* tree. *M. nodulans* ORS 2060 and *Methylobacterium* sp. 4-46 are close species (97% 16S rDNA identity), with almost-identical *nod* genes organization and rather close *nod* gene sequences (Fig. 1). Thus, the transfer of *nod* genes must have occurred in the ancestor of these two species, followed by vertical transmissions and coevolution of each species with their plant hosts, conducting to a high specificity between the symbiotic partners *M. nodulans*/*Crotalaria* sp. and *Methylobacterium* sp./*Lotononis* sp. (*M. nodulans* does not nodulate *Lotononis* species, Yates et al., 2007).

Previous studies have shown that the *nodA* gene phylogeny was correlated to some NF structural features (presence of additional fucose and/or arabinose on the NF backbone) produced by the bacteria, and to some extent to the host plant systematics (Debellé et al., 2001; Moulin et al., 2004). In the *nodA* tree, *M. nodulans* ORS 2060 groups together with *B. tuberum* (formerly *Bradyrhizobium aspalathi*, Moulin et al., 2001) that produces unfucosylated NFs (Boone et al., 1999), and not with the *Bradyrhizobium* branch, whose strains produce NFs carrying a fucose (Fig. 2B, Moulin et al., 2004). It is also interesting to note

that the host plant of the *Methylobacterium*–*Burkholderia* branch belongs to the same *Crotalariae* tribe (i.e. *Aspalathus*, *Crotalaria*, *Lotononis* species). This legume tribe may have a predisposition to form symbiosis with taxonomically uncommon rhizobia, and future diversity studies of *Crotalariae* tribe genera symbionts would confirm this assumption true or not.

A number of *nod* genes inducer compounds secreted from legume roots have been characterized so far. Most of these compounds belong to the flavonoid group, and share a common phenylpropanoid biosynthetic pathway. In order to purify and determine the chemical structure of LCOs produced by *M. nodulans* during the symbiotic interaction with *C. podocarpa*, we tried to identify strong inducers of *nod* genes of *M. nodulans* ORS 2060 using a *lacZ* fusion in the promoter region of the *nodA* gene. We monitored the action of several known *nod*-gene inducers by following β -galactosidase activity. Apigenin was found to be one of the most powerful. This result is consistent with the identification of apigenin 7-O- β -D-apiofuranosyl (1 $\rightarrow$ 6)- β -glucopyranoside in the aerial parts of *C. podocarpa* (Wanjala and Majinda, 1999). Apigenin is thus likely the natural inducer of *M. nodulans* *nod* genes in the rhizosphere.

Isoflavonoids (as genistein) have been shown to be common inducers of *Bradyrhizobium* *nod* genes (Gillette and Elkan, 1996; Zhang et al., 1996; Souleimanov et al., 2002). In this study, we found that *M. nodulans* ORS 2060 *nod* genes are not induced by this flavonoid subfamily. This first level of specificity between rhizobia and legumes could explain the host range and cross-inoculation results reported by Yates et al. (2007).

NF metabolites trigger the formation of nodules on legume roots (Dénarié et al., 1996). These compounds harbour various substitutions differing between rhizobia and related to their host specificity. The NF produced by *M. nodulans* is a pentamer of chitin carrying a sulphate on the reducing glucosamine residue and substituted by fatty acids, either vaccenic acid (C_{18:1}) or palmitic acid (C_{16:0}), on the non-reducing glucosamine residue. This NF can be considered as having a simple structure compared with rhizobial NFs reported so far (around 60 strains have been characterized for their NFs structure). Such kind of structure has only been reported as a minor part of the NF mixture produced by *R. tropici* CIAT899, the major part being NFs with additional mannose on the reducing end and various fatty acyl chains (Folch-Mallol et al., 1996). Several rhizobial strains produce sulphated NFs as intermediates of NF biosynthesis, the final product carrying always additional substitutions on the NF backbone, such as fucose, arabinose, methyl or carbamoyl (D'Haeze and Holsters, 2002). The symbiosis between *Methylobacterium* and its plant host is specific, since *Bradyrhizobium* isolated from other species of *Crotalaria* (*C. comosa*, *C. goreensis*, *C. hyssopifolia*, *C. lathyroides*, *C. ochroleuca*, and *C. retusa*) do not nodulate the three African species nodulated by *Methylobacterium*

(Samba et al., 1999; Sy et al., 2001b). Even the broad host-range rhizobium NGR234, nodulating more than 350 species of legumes (Pueppke and Broughton, 1999) and producing a complex mixture of NFs (Jabbouri et al., 1995), do not nodulate *C. podocarpa* (data not shown). The *Bradyrhizobium* isolated from *Crotalaria* species should produce fucosylated NFs (like all *Bradyrhizobium* NFs described so far), as Moulin et al. (2004) reported the presence of *nodZ* (encoding NF fucosyl transferase) in the genomes of several *Crotalaria*-isolated strains.

Crotalaria symbionts described so far thus seem to belong to two inoculation groups: most of *Crotalaria* species are nodulated by slow-growing *Bradyrhizobium* that may produce a mixture of fucosylated NFs, while the three species *C. podocarpa*, *C. glaucoides* and *C. perrottetti* are specifically nodulated by *M. nodulans* producing a simple NF structure, sulphated on the reducing end. Specificity of this model may be present at two steps of the interaction: first, the nature of the flavonoid *nod* gene inducer, being apigenin for *Methylobacterium* and not genistein, a common inducer of *Bradyrhizobium nod* genes; and second, at the NF structure level since *C. podocarpa* seems to discriminate between sulphated and fucosylated NFs. This symbiotic molecular dialogue appears thus classic, likewise the other rhizobia. *Methylobacterium* is an uncommon genus among symbiotic nitrogen-fixers, and occurrence of symbiosis in this genus may be the result of a more complex interaction, via specific adaptations of bacterial species to the *C. podocarpa* rhizosphere, these legumes being known to produce alkaloids (Arzt and Mount, 1999). The complete genome sequencing of *M. nodulans* ORS 2060 (underway at the Joint Genome Institute in 2007) will surely enhance our understanding of the intimate symbiosis between *Methylobacterium* and *Crotalaria*.

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Role of Methylo-trophy During Symbiosis Between *Methylobacterium nodulans* and *Crotalaria podocarpa*

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Some rare leguminous plants of the genus *Crotalaria* are specifically nodulated by the methylotrophic bacterium *Methylobacterium nodulans*. In this study, the expression and role of bacterial methylotrophy were investigated during symbiosis between *M. nodulans*, strain ORS 2060^T, and its host legume, *Crotalaria podocarpa*. Using *lacZ* fusion to the *mxoF* gene, we showed that the methylotroph genes are expressed in the root nodules, suggesting methylotrophic activity during symbiosis. In addition, loss of the bacterial methylotrophic function significantly affected plant development. Indeed, inoculation of *M. nodulans* nonmethylotroph mutants in *C. podocarpa* decreased the total root nodule number per plant up to 60%, decreased the whole-plant nitrogen fixation capacity up to 42%, and reduced the total dry plant biomass up to 46% compared with the wild-type strain. In contrast, inoculation of the legume *C. podocarpa* with nonmethylotrophic mutants complemented with functional *mxo* genes restored the symbiotic wild phenotype. These results demonstrate the key role of methylotrophy during symbiosis between *M. nodulans* and *C. podocarpa*.

Additional keyword: methanol.

The bacterial genus *Methylobacterium* belongs to the α subclass of the family *Proteobacteria*. It constitutes a group of strictly aerobic, gram-negative, rod-shaped, pink-pigmented facultative methylotrophic (PPFM) bacteria (Green 1992). Presently, the genus comprises 19 species isolated from a wide range of environments (Doronina et al. 2002; Gallego et al. 2005a, in press; Green 1992; Jourand et al. 2004; Van Aken et al. 2004).

The main feature of members of *Methylobacterium* is their ability to oxidize methanol, a methylotrophic property based on the presence of methanol dehydrogenase (MDH), a pyrrolo-quinoline quinone (PQQ)-linked protein with an $\alpha_2\beta_2$ tetramer structure (Anthony 1982; Anthony et al. 1994). The genes enabling methanol oxidation have been studied in the model strain *Methylobacterium extorquens* AM1 (Chistoserdova et al.

2003). The main gene cluster, containing 14 genes named *mxoFJGIRSACKLDEHB*, is transcribed in a single mRNA operon. Transcription is controlled by a methanol inducible promoter (Zhang and Lidstrom 2003). Within the gene cluster, *mxoF* and *mxoA* genes encode the largest α and the smallest β polypeptide subunits of MDH, respectively (Anderson et al. 1990; Nunn et al. 1989; Nunn and Lidstrom 1986a and b).

Although most *Methylobacterium* spp. have been regularly isolated from soils, water, or plants (Corpe and Rheem 1989; Gallego et al. 2005a; Green 1992; Holland 1997), little is known about the role of the methylotrophic function in the adaptation of bacteria to these various ecosystems. Only a few studies have suggested that *Methylobacterium* spp. were able to use methanol emitted by leaf stomata during their association with plants (Lidstrom and Chistoserdova 2003; Nemeček-Marshall et al. 1995).

Over the last 5 years, *Methylobacterium* spp. strains have been isolated from root nodules of legumes belonging to the genera *Crotalaria* and *Lotononis* (*Fabaceae* and *Crotalariae*) (Jaftha et al. 2002; Samba et al. 1999; Sy et al. 2001a and b). Nonpigmented strains isolated from three *Crotalaria* spp. (i.e. *Crotalaria glaucoidea*, *C. perrottetii*, and *C. podocarpa*) were described as actually being a single novel *Methylobacterium* sp. (Sy et al. 2001a and b). This new species, which has the same methylotrophic function as other *Methylobacterium* spp., was named *M. nodulans* for its ability to nodulate and fix nitrogen specifically during symbiosis with *Crotalaria* spp. (Jourand et al. 2004; Sy et al. 2001a and b).

The aim of this work was to study the relationship between the methylotrophic function of *M. nodulans* during symbiotic processes with the host legume, *C. podocarpa*. The expression of methylotroph genes during symbiosis was investigated using *lacZ* fusion to *mxoF*, whereas the role of methylotrophy was determined by studying the effect of methylotroph mutants on the plant growth.

RESULTS

Methylotrophy of *M. nodulans* is expressed in root nodules formed during symbiosis with *Crotalaria podocarpa*.

To monitor the expression of methylotroph genes in planta, we constructed a *M. nodulans* ORS 2060^T recombinant strain bearing a *mxoF-lacZ* transcriptional fusion promoter (Fig. 1A).

The *mxoF-lacZ* fusion promoter was pretested in vitro by measuring the β -galactosidase (β -gal) activity of the wild-type

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strain or the recombinant strain after culture in yeast extract-mannitol (YM) medium supplemented or not supplemented with methanol. β -Gal activity was detected only in cultures of the *M. nodulans* recombinant strain 2060 *mxoF-lacZ* (Table 1). Moreover, in the presence of methanol, its level was 20-fold higher than the basal activity detected in the control, thus confirming that methanol induces *mxoF* gene expression in *M. nodulans*. Such an *mxoF* induction by methanol previously was described in the two *Methylobacterium* model species, *M. extorquens* and *M. organophilum*, with induction levels ranging from 8- to 10-fold (Xu et al. 1993; Zhang and Lidstrom 2003).

Expression of the *mxoF-lacZ* fusion promoter therefore was tested in planta by the histochemical localization of β -gal in nodule tissues. The β -Gal activity (Fig. 2A and A', in blue) was detected in *C. podocarpa* root nodules inoculated with the *M. nodulans* recombinant strain 2060 *mxoF-lacZ*. Enzymatic activity clearly was localized in the apex of the nodule, as shown in thin longitudinal sections from fresh nodules (Fig. 2A). The blue-colored apex zone corresponding to β -gal activity also was viewed in half-nodule sections (Fig. 2A'). Interestingly, it appears that the apex zone was distinct from the medullar nitrogen-fixing zone, which is red-colored due to the

presence of leghemoglobin (Balestrasse et al. 2004). In contrast, no β -gal activity was detected in nodules formed after inoculation with the *M. nodulans* wild-type strain (Fig. 2B and 2B').

These results clearly showed that the *mxoF* gene of *M. nodulans* was expressed in root nodules during symbiosis and preferentially localized at the nodule apex. In vitro assays indicated that the *mxoF* gene of *M. nodulans* is inducible by methanol; therefore, its expression, observed in planta, could result from the presence of methanol produced during nodule development. To test this hypothesis, we measured residual

Table 1. β -Galactosidase (β -gal) total activity expressed in μ mol of *o*-nitrophenyl- β -D-galactoside (ONPG)/min/mg of total protein²

Strain	Control	Methanol
ORS 2060 ^T wild type	ND	ND
ORS 2060 <i>mxoF-LacZ</i>	0.15 $\pm$ 0.02 a	3.5 $\pm$ 0.08 b

² Both β -gal activity and total proteins were investigated on cell extracts prepared from *Methylobacterium nodulans* ORS 2060^T wild type or recombinant *M. nodulans* ORS 2060^T *mxoF-LacZ*. ND = not detected. All data followed with the same letter are not significantly different ($P \leq 0.05$).

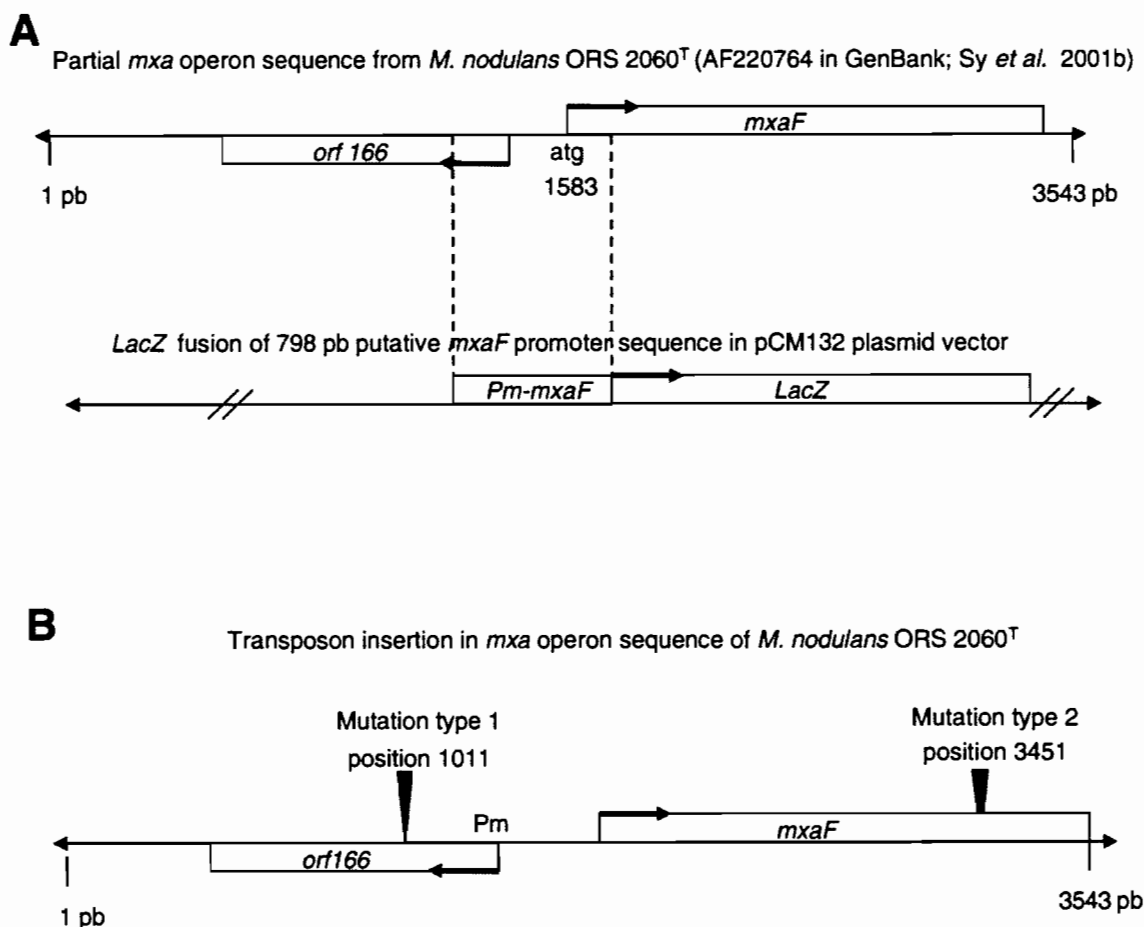


Fig. 1. Construction of *Methylobacterium nodulans* ORS 2060^T recombinant strain containing *mxoF-lacZ* transcriptional fusion promoter and ORS 2060^T negative methylotroph mutants by insertional mutagenesis of transposon *mTn5-GGm*. **A**, Physical map of the partial sequence of the operon *mxo* in *M. nodulans* ORS 2060^T wild type (AF 220764 in GenBank) according to Sy and associates (2001a), including the deduced *mxoF* putative promoter sequence position compared with the *M. extorquens* AM1 *mxoF* promoter sequence position as described by Zhang and Lidstrom (2003) and cloned into pCM132 plasmid vector (AF 327720 in GenBank) with *LacZ* gene reporter (Marx and Lidstrom 2001). Abbreviations: Pm: promoter; Bp: DNA pair of base spacing. **B**, Mutations of *mxo* locus sequence of *M. nodulans* ORS 2060^T by insertion of transposon *mTn5-GGm* (AF080392) (Reeve et al. 1999): mutation type 1 upstream of the putative *mxoF* promoter sequence and mutation type 2 inside the *mxoF* gene.

methanol levels in nodules as well as in roots, leaves, and stems. We found low concentrations of residual methanol in *C. podocarpa* tissues which were two- to fivefold higher in nodules than in roots, leaves, or stems (data not shown), indicating that methanol is more specifically accumulated in root nodules.

Loss of methylotrophy strongly affects symbiosis.

Negative methylotrophic mutants of *M. nodulans* were constructed to further investigate the role of bacterial methylotrophy in the symbiotic processes. These mutants were obtained by insertional mutagenesis of the *mTn5-GGm* transposon (Reeve et al. 1999). They were selected on allyl alcohol, as described by Nunn and Lidstrom (1986a and b). Two of them were selected according to the position of the transposon insertion sites (Fig. 1B). The first one (mutation type 1) was close to the *mx*a operon, approximately 500 bp upstream of the putative start codon of the *mx*aF gene. The second one (mutation type 2) was inside the *mx*aF gene. Defective methylotrophic metabolism of the two mutants was checked by

growth on M72 minimum mineral medium containing either sodium succinate or methanol as sole carbon source. Compared with the *M. nodulans* ORS 2060^T wild-type strain, both methylotrophic mutants presented normal growth on succinate but were unable to grow on methanol as sole carbon source (Fig. 3). In addition, MDH activity was tested according to Day and Anthony (1990): no MDH activity was detected in proteic extracts obtained from methylotrophic mutants, whereas this enzymatic activity is strongly detected in the wild-type strain protein extracts (data not shown). Two mutants were complemented with a conjugative cosmid containing a 35-kb DNA insert including the *mx*a operon of *M. nodulans* (Sy et al. 2001a). This complementation restored the methylotrophic capacity because the complemented mutants were able to grow on a M72 minimum mineral salt medium (MMS) with methanol as sole carbon source, and MDH activity also was detected (data not shown).

The growth of *C. podocarpa* plants inoculated with *M. nodulans* ORS 2060^T methylotrophic mutants type 1 or 2 was

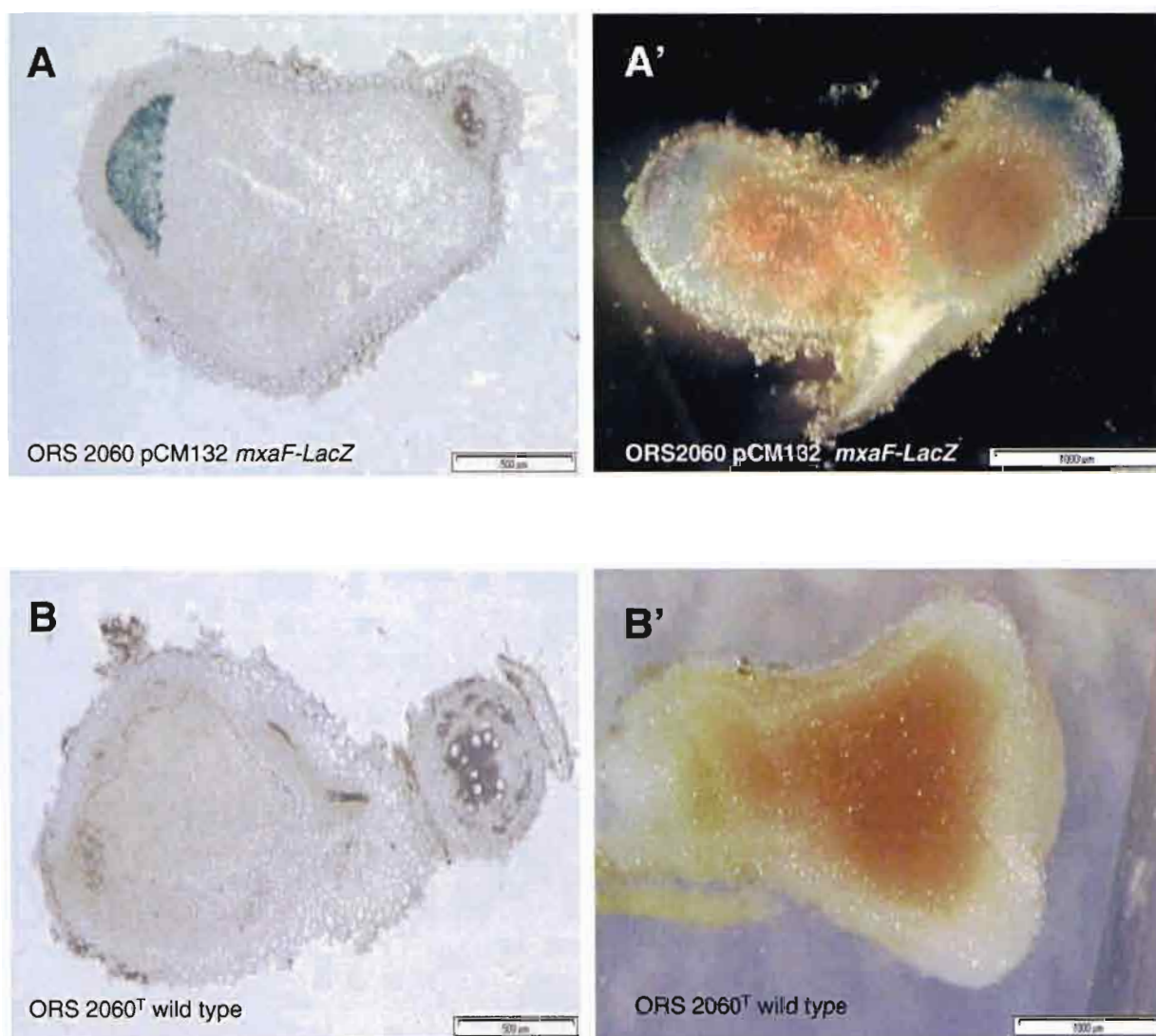


Fig. 2. A and A', Histochemical localization of the β -galactosidase activity in 6-week-old *Crotalaria podocarpa* root nodules induced by the *Methylobacterium nodulans* ORS 2060^T recombinant strain containing the *mx*aF-LacZ transcriptional fusion promoter compared with B and B', root nodules induced by *M. nodulans* ORS 2060^T wild type. A and B, Longitudinal nodule sections, 40 μ m thick; A' and B', longitudinal half of a *C. podocarpa* nodule with detection of leghemoglobine activity in red.

reduced substantially compared with that of plants inoculated with the wild-type strain (Fig. 4). This decrease in plant growth, which represented up to 46% biomass loss, was correlated with a drastic drop in the total root nodule number (up to 60%) and a significant reduction in the nitrogen fixation ability (up to 42%) (Table 2). Interestingly, if nodule number strongly decreased, the size of mutant nodules was bigger, leading to a nodule mass two to seven times higher than the wild type. In any case, for both mutants, nitrogen fixation per milligram of nodule appeared 1.2- to 5-fold weaker than the wild type (Table 2). We calculated that the average dry weight of nodules necessary to produce 100 g of plant biomass was two- to fivefold higher with the methylotrophic mutants than with the wild-type strain (Table 2). Altogether, these data indicate that the methylotrophic function of *M. nodulans* plays a major role during the symbiotic process between the bacteria and the legume. In contrast, inoculation of *C. podocarpa* with the complemented mutants restored the wild-type symbiotic phenotype (data not shown).

DISCUSSION

Although most *Crotalaria* spp. are nodulated by nonmethylotrophic *Bradyrhizobium* spp., only a few African species have the unusual ability to symbiotically and specifically associate with the newly described *M. nodulans* (Gao et al. 1994; Samba et al. 1999; You et al. 2002). Interestingly, another spe-

cific symbiosis with *Methylobacterium* spp. has been described in the genus *Lotononis*, which belongs to the same *Crotalariae* tribe (Jaftha et al. 2002). The symbiotic specificity observed between *Methylobacterium* spp. and some *Crotalariae* spp. raised the question as to the putative role of methylotrophy during this host-bacteria interaction. In this article, we clearly demonstrated that methylotrophy of *M. nodulans* is advantageous during symbiosis with the host legume *C. podocarpa*.

We first studied the expression of the *mxoF* gene, one of the key methylotrophic genes in *Methylobacterium* spp., both in vitro and in planta. We demonstrated that, in *M. nodulans*, this gene is not constitutively expressed but instead controlled by a methanol inducible promoter, as previously shown for *M. extorquens* and *M. organophilum* (Xu et al. 1993; Zhang and Lidstrom 2003). Close examination of the DNA sequence upstream of the putative start codon did not reveal any obvious DNA motif, which might be the target of a putative methanol-dependent regulator. This is in agreement with the previous study of Zhang and Lidstrom (2003), in which the promoter regions of four methanol-inducible operons were compared but no consensus sequence was observed. Therefore, the molecular basis of the control of *mxoF* operons in *Methylobacterium* spp. remains to be determined. Interestingly, the insertion of the Tn5 transposon 572 bp upstream of the putative *mxoF* start codon led to a negative methylotrophic metabolism. The *mxoW* gene was not detected in this region, as also was observed in *M. extorquens* (Springer et al. 1998; Zhang and Lidstrom 2003). Instead, this region was found to contain a putative open reading frame (ORF) encoding a protein of 163 amino acids, which did not display homology with any known proteins. The phenotype of this mutant may result from i) inactivation of this putative ORF, which could play a role in methylotrophy or ii) insertion of the Tn5 in the *mxoF* promoter region, which would extend more than 600 bp upstream of the *mxoF* putative start codon.

In planta, we observed that the *mxoF* gene was expressed in root nodules. In accordance with the in vitro study, this observation suggests that methanol is present inside the nodule and subsequently used by the bacteria. In agreement, a substantial amount of methanol was detected in the nodule tissue. In plants, methanol can be produced from different sources, such as protein repair pathways, lignin degradation, and cell-wall pectin demethylation (Downie et al. 2004). During symbiosis between *M. nodulans* and *Crotalaria* spp., we hypothesize that methanol could be produced from pectin cell-wall de-methylation. In agreement, we observed that the nodule apical tissue (i.e., the zone where the *mxoF* gene was expressed) was highly disorganized (data not shown). This could have resulted from either plant or bacteria pectinase production. Note that plants are known to produce several classes of pectinases (Prade et al. 1999), whereas there has not been any previous evidence that *Methylobacterium* spp. can degrade pectin. Further cytological observations and studies about pectin metabolism by *M. nodulans* might highlight the role of pectinolytic enzymes and interactions with bacterial methylotrophy during symbiosis between *M. nodulans* and *Crotalaria* spp.

The study of the plant response to inoculation with negative methylotrophic mutants highlighted the role of methylotrophy during symbiosis. Indeed, the absence of methylotrophy significantly affected *C. podocarpa* plant growth by up to 42%. This decrease of plant biomass was associated with a drastic reduction in total number of nodules and a drop in nitrogen fixation efficiency. The differences in nodule dry weight observed between the two mutants phenotypes could result from the different Tn5 insertions. Indeed, in mutant 1, the Tn5 insertion, located in the putative promoter region of *mxoF*, therefore could only partially affect the in planta methylotrophy

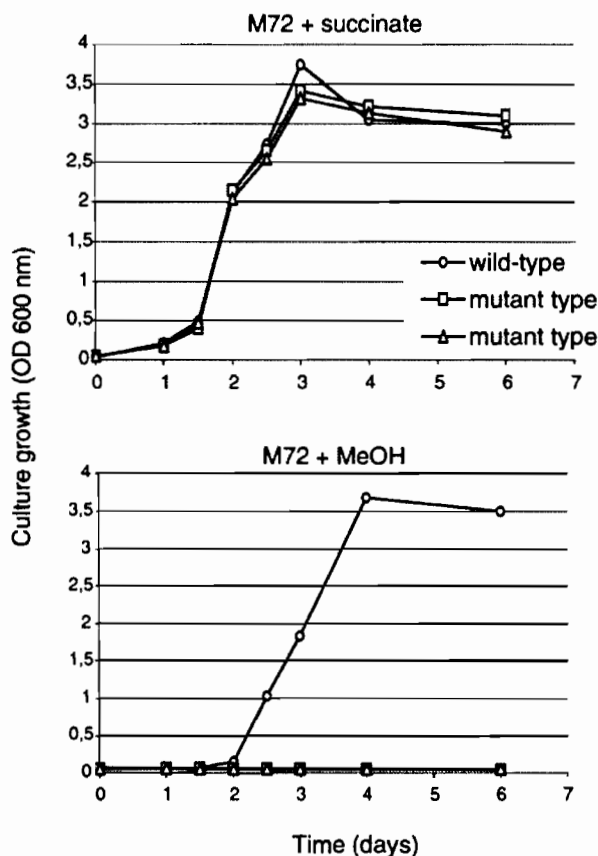


Fig. 3. Growth of *Methylobacterium nodulans* ORS 2060^T strains pregrown in succinate, harvested, and resuspended in minimum mineral medium containing either sodium succinate or methanol. Strains examined were methylotroph mutants *mTn5-GGm-pmmxof* (mutation type 1) and *mTn5-GGm-mxof* (mutation type 2) compared with the wild-type.

compared with mutant 2. This suggests that methylotrophy might be implicated at different levels of symbiosis.

During the first steps of infection, methylotrophy could be involved in the bacterial ability to colonize or invade plant roots. Indeed, some species of the *Crotalariae* tribe, including *C. podocarpa*, are reported to produce toxic methylated compounds such as pyrrolizidin alkaloids (Kinghorn and Smolenski 1981; Wink and Mohamed 2003). Based on their methylotrophy capacity, *Methylobacterium* spp. might be able to detoxify these toxic molecules. Thus, the symbiotic specificity observed between *Methylobacterium* and *Crotalariae* spp. could result from bacterial plant selection based on the rhizospheric excretion of these toxic molecules. Such plant selection already has been reported for *Rhizobium* strains resistant to mimosine in rhizospheres of *Leucaena leucocephala* (Soedarjo et al. 1994) and maybe also for *R. etli* in maize (Rosenblueth and Martinez-Romero 2004).

Methylotrophy is a bacterial metabolic pathway used to assimilate carbon or retrieve energy (Chistoserdova et al. 2003). Therefore, during symbiosis, we could hypothesize that this function contributes to carbon or energy sources for the bacteria. Indeed, the bacteria could be able to recycle methanol produced through tissue degradation during nodule develop-

ment and senescence, thus saving energy that the plant has to furnish to the bacteria for nitrogen fixation. In agreement, the marked drop in acetylene reduction activity observed with the methylotrophic mutants could reflect a decrease of energy supply to nitrogenase.

Methanol is known to be a key one-carbon signal metabolite regulating the expression of numerous genes involved in cell division, communication, metabolism, and defence of plants (Downie et al. 2004). Therefore, we cannot completely dismiss the possibility that bacterial methylotrophy interferes in nodule cell-wall rearrangements or defence mechanisms by suppressing the methanol signal.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

M. nodulans strains were i) ORS 2060^T (Jourand et al. 2004), called the wild-type strain throughout this study, ii) the constructed recombinant strain ORS 2060 *mxnA-lacZ*, and iii) the methylotrophic mutants ORS 2060 *mTn5-GGm-mxnA* (mutation type 1) and ORS 2060 *mTn5-GGm-mxnA* (mutation type 2). All *M. nodulans* strains were grown in YM medium (Vincent 1970) for preparation of plant inoculum and in M72

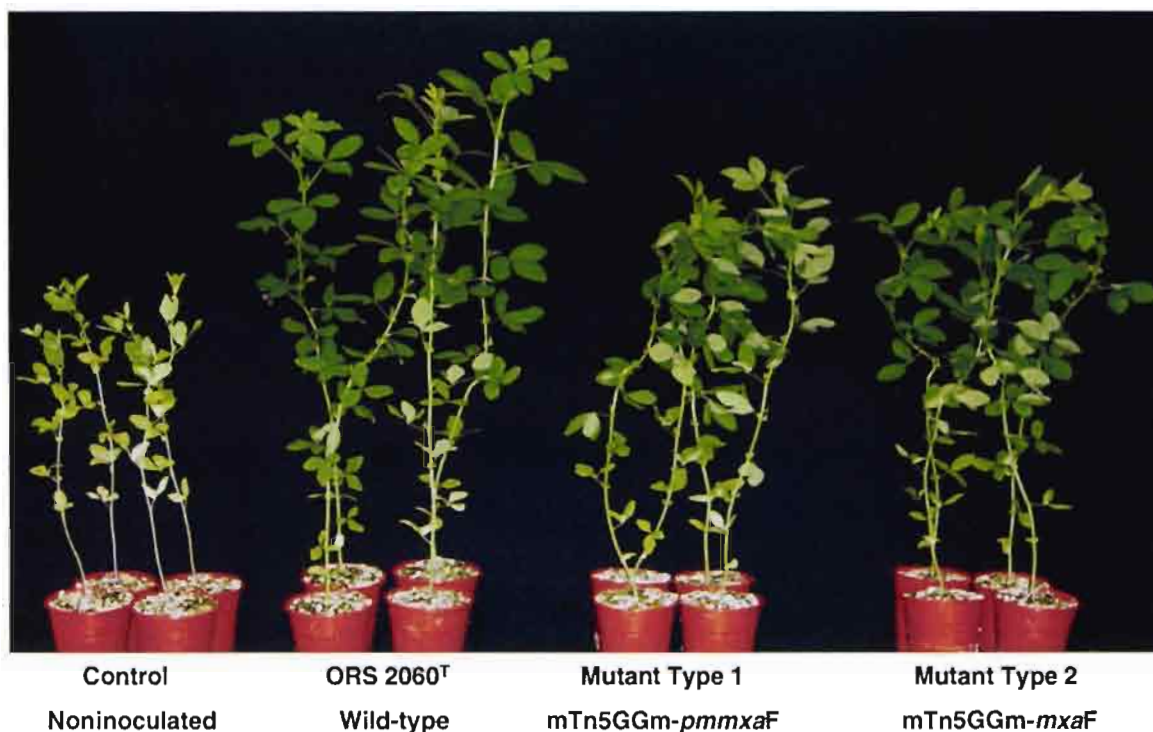


Fig. 4. Comparison of growth of *Crotalaria podocarpa* inoculated with either *Methylobacterium nodulans* ORS 2060^T wild type or ORS 2060^T negative methylotroph mutants compared with noninoculated control. Photographs were taken 5 weeks after inoculation.

Table 2. Symbiotic phenotypes of *Methylobacterium nodulans* ORS 2060^T wild-type and negative methylotrophic mutants on *Crotalaria podocarpa*^a

Strain	Plant dry wt (g/plant)	No. of nodules/plant	Average nodule dry wt (mg)	Acetylene reduction (C ₂ H ₄ μmol/h/plant)	Acetylene reduction/mg of nodule/plant	Average nodule dry wt (g) to produce 100 g of plant
ORS 2060 ^T wild type	2.8 ± 0.2 a	30 ± 11 a	5.6 ± 0.7 a	11.4 ± 0.2 a	0.067 ± 0.002 a	6.0 ± 0.6 a
ORS 2060 ^T <i>mTn5GGm-mxnA</i>						
Mutation type 1	1.5 ± 0.1 b	12 ± 4 b	13 ± 2 b	8.6 ± 0.3 b	0.055 ± 0.001 b	10.4 ± 0.8 b
Mutation type 2)	1.6 ± 0.2 b	13 ± 5 b	38 ± 4 c	6.6 ± 0.2 b	0.013 ± 0.001 c	30.7 ± 0.8 c
No inoculum (control)	0.2 ± 0.03 c	0	0	0	0	0

^a Plant dry weight (dry wt) biomass, nodule number and dry wt, and nitrogen-fixing activity expressed by acetylene reduction assay were performed 5 weeks after inoculation. All data followed with the same letter are not significantly different ($P \leq 0.05$)

minimum mineral medium (Green 1992) supplemented with either sodium succinate (10 mM) or methanol (50 mM) for both the methylotrophic tests and the *in vitro* total β -gal activity. *Escherichia coli* strains (i.e., pCM132, BW 20767 containing plasmid pCRS548 with mini-transposon *mTn5-GGm*, conjugative strain S17-1, and strain STM 217) were provided by M. E. Lidstrom (Marx and Lidstrom 2001), W. G. Reeve (Reeve et al. 1999), and R. Simon (Simon et al. 1983) and the strain collection of the Laboratoire des Symbioses Tropicales et Méditerranéennes (Montpellier, France) (Jourand et al. 2004; Sy et al. 2001a), respectively. Standard methods were used for growth of *E. coli* in Luria-Bertani (LB) medium (Sambrook et al. 1989). All media were supplemented with appropriate antibiotics: nalidixic acid (100 μ g/ml) for the three types of *M. nodulans* strains; kanamycin (50 μ g/ml) for *M. nodulans* ORS 2060^T *mxoF-lacZ* and *E. coli* pCM132; and gentamycin (30 μ g/ml) for *M. nodulans* methylotrophic mutants (type 1 and type 2) and *E. coli* BW 20767. Strains were grown aerobically, at 37°C under gyratory shaking (170 rpm) in complete darkness, for the appropriate time to obtain one unit of optical density (OD) at 600 nm.

General DNA technology.

Genomic DNA was prepared according to Chen and Kuo (1993). Plasmid and cosmid DNA were isolated with a Mini-prep kit (Promega, Charbonnières, France). Polymerase chain reaction (PCR) products were purified with a Qiaquick gel extraction kit (Qiagen, Courtaboeuf, France). Restriction endonuclease and ligase reactions were performed according to the manufacturer's specifications (Eurogentec, Angers, France). For DNA amplification, sequencing and analysis, the primers used are described in Table 3. All reactions for PCR or sequencing as well as the methods for DNA sequencing and analysis were carried out as previously described by Sy and associates (2001a).

Construction of the *M. nodulans* ORS 2060^T recombinant strain containing the *mxoF-lacZ* transcriptional fusion promoter.

From the total genomic DNA of *M. nodulans* ORS 2060^T wild type, a 798-bp DNA fragment was amplified with primers 1 and 2 (Table 3) corresponding to the DNA putative promoter section of the *M. nodulans* sequenced gene *mxoF* as described in Figure 1A (AF220764 sequence in GenBank) (Sy et al. 2001a). This 798-bp DNA fragment was cloned into the *Eco*RI and *Bgl*II sites of the pCM132 plasmid vector (Marx and Lidstrom 2001). The combined plasmid pCM132-*mxoF-lacZ* vector was transferred by electroporation (transformation) into conjugative *E. coli* S17-1 using an electroporator (Biorad, Ivry, France) and then transferred by conjugation into *M. nodulans* ORS 2060^T wild type. Conjugative colonies were selected on YM medium containing antibiotics (kanamycin at 50 μ g/ml and nalidixic acid at 100 μ g/ml). Recombined *M. nodulans* ORS 2060 *mxoF-lacZ*-positive clones were controlled for *lacZ* *in vitro* expression after promoter *mxoF* methanol induction in M72 minimum mineral medium supplemented with MeOH (50 mM) and the same two antibiotics mentioned above and then compared with the *M. nodulans* ORS 2060^T wild-type strain.

Construction

of *M. nodulans* ORS 2060^T methylotrophic mutants.

Insertional mutagenesis of the *M. nodulans* DNA *mxo* operon was carried out by insertion of the *mTn5-GGm* transposon using conjugation between the *M. nodulans* ORS 2060^T wild type and *E. coli* BW20767 containing the *mTn5-GGm*. After conjugation, conjugants first were selected on YM medium supplemented with antibiotics (nalidixic acid at 100 μ g/ml and gentamycin at 30 μ g/ml). Then, methylotroph mutants were selected by cultivating conjugants on microplates containing M72 minimum mineral medium with the same two antibiotics mentioned above, and i) sodium succinate (10 mM), ii) MeOH (50 mM), or iii) sodium succinate (10 mM), MeOH (50 mM), and allyl alcohol added to a final concentration at 0.2% (wt/vol) was used to select *Methylobacterium* methylotroph mutants as described by Nunn and Lidstrom (1986a). All chemicals were provided by Sigma-Aldrich (St-Quentin, France). Colonies growing on M72 + succinate and also on M72 + succinate + MeOH + allyl-alcohol but not growing on M72 + MeOH were selected as methylotroph-negative clones, which were controlled by PCR for the presence of *mTn5-GGm* transposon using primers 4 and 5 (Table 3). Among the positive *mTn5-GGm* clones, a new screening was carried out with PCR using primer pairs (Table 3) to identify the position of the insertional transposon mutagenesis in the *mxoF* promoter sequence and in the *mxoF* gene. Positions of *mTn5-GGm* (Fig. 1B) were obtained by DNA sequencing. Selected clones of *M. nodulans* ORS 2060 *mTn5-GGm-mxoF* (mutation type 1) and ORS 2060 *mTn5-GGm-mxoF* (mutation type 2) were grown on M72 + succinate or M72 + MeOH to verify the loss of the methylotrophy capacity by measuring bacterial growth with a UV-visible spectrophotometer at OD 600 nm (Varian, Courtaboeuf, France).

Complementation of methylotrophic mutants.

For complementation experiments, we used the STM217 clone of *E. coli* XL1-MR from the genomic library of the *M. nodulans* ORS 2060^T wild type as described by Sy et al. (2001a) and containing the 35-kb *mxoF* DNA fragment cloned into the cosmid SuperCosI (Stratagene, La Jolla, CA, U.S.A.). After growth of the STM 217 clone on LB supplemented with kanamycin (50 μ g/ml), the cosmid was extracted using the DNA Miniprep Kit as described above and then transferred to the conjugative strain of *E. coli* S17-1 by electroporation. Transformed clones were selected on LB + kanamycin (50 μ g/ml). Then, the cosmid was transferred for complementation by conjugation between the transformed conjugative *E. coli* S17-1 and both *M. nodulans* methylotrophic mutants type 1 and type 2. Complemented *M. nodulans* methylotrophic mutants were selected on M72 minimum mineral medium containing nalidixic acid (100 μ g/ml) + gentamycin (30 μ g/ml) + kanamycin (50 μ g/ml) and MeOH (50 mM) as sole carbon source. Their symbiotic phenotypes were tested on *C. podocarpa* plants.

Plant tests.

C. podocarpa seeds were obtained from IRD (Bel-Air, Dakar, Senegal) and identified as reported by Jourand and associates (2004). Germination and cultivation of *C. podocarpa* were conducted in the greenhouse as previously described by

Table 3. Primers used for DNA amplification and sequencing

Name	Sequence	Target gene	Reference
pm <i>mxoF</i> 2060f	5'-GAA-TTC-CAG-AGG-ACA-AGA-GGT-TGA-TGT-TG-3'	Promoter <i>mxoF</i>	This study
pm <i>mxoF</i> 2060r	5'-ACA-GAT-CTC-TTA-CCC-AGC-ATG-TGC-GTC-TCC-CA-3'	Promoter <i>mxoF</i>	This study
WIL3	5'-GAA-TGC-CCA-CAG-GCC-CTG-GAG-3'	Transposon <i>mTn5-GGm</i>	Reeve et al. 1999
TAC-105F	5'-GCA-TCT-AGC-CCG-CCT-ATT-G-3'	Transposon <i>mTn5-GGm</i>	Reeve et al. 1999

Giraud and associates (2000) and Sy and associates (2001a). At day 15 after germination, plants were root inoculated with 5 ml of culture of either the *M. nodulans*, ORS 2060^T wild type, ORS 2060 *mxaf-lacZ*, ORS 2060 methylotrophic mutants, or ORS 2060 complemented methylotrophic mutants. Sterile distilled water was used for control. All the inocula were prepared according to Giraud and associates (2000). Fifteen replicates were grown for each experimental treatment. Six weeks after inoculation, plants were harvested. Nitrogen-fixing activity was estimated by measurement of acetylene-reducing activity on the entire plant according to Hardy and associates (1968). The dry plant biomass and the number and dry weight of root nodules were evaluated. All data were subjected to a one-way analysis of variance (ANOVA) and means were compared with the Newman-Keuls multiple range test ($P \leq 0.05$) using the Super ANOVA computer software package (Gagnon et al. 1989).

Enzyme assays, methanol dosage, and light microscopy.

Crude extracts of total soluble proteins were prepared by sonication, as reported by Day and Anthony (1990), from i) *M. nodulans* cultures ORS 2060^T wild type and ORS 2060^T pCM132 *mxaf-lacZ* grown in M72 mineral minimum medium with methanol (50 mM) or succinate (10 mM) supplemented with the appropriate antibiotics or ii) *C. podocarpa* nodule tissues. β -Gal activity was assayed in vitro using the standard method described by Miller (1972). MDH microassays were performed as described by Day and Anthony (1990). Total proteins were quantified using the Lowry method Biorad kit (Biorad, Ivry, France). Methanol dosage was performed as reported by Sy and associates (2001a). All data were subjected to a one-way ANOVA, and means were compared with the Newman-Keuls multiple range test ($P \leq 0.05$) as described above. In planta tests were performed on 40- μ m and whole half-thick sections of fresh nodules (VT1000S Vibratome, Leica, France), then stained for histo- β -gal assays as described by Boivin and associates (1990). After mounting in water, nodule sections were observed and photographed under light microscopy as reported by Sy and associates (2001a).

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Methylobacterium nodulans sp. nov., for a group of aerobic, facultatively methylotrophic, legume root-nodule-forming and nitrogen-fixing bacteria

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Data on 72 non-pigmented bacterial strains that specifically induce nitrogen-fixing root nodules on the legume species *Crotalaria glaucoïdes*, *Crotalaria perrottetii* and *Crotalaria podocarpa* are reviewed. By SDS-PAGE analysis of total protein patterns and by 16S rRNA PCR-RFLP, these strains form a homogeneous group that is separate from other legume root-nodule-forming bacteria. The 16S rRNA gene-based phylogeny indicates that these bacteria belong to the genus *Methylobacterium*. They can grow on C₁ compounds such as methanol, formate and formaldehyde but not methylamine as sole carbon source, and carry an *mxrF* gene, encoding methanol dehydrogenase, which supports their methylotrophic metabolism. Presence of a *nodA* nodulation gene, and ability to nodulate plants of *Crotalaria* species and to fix nitrogen are features that separate the strains currently included in this group from other members of the genus *Methylobacterium*. The present study includes additional genotypic and phenotypic characterization of this novel *Methylobacterium* species, i.e. *nifH* gene sequence, morphology, physiology, enzymic and carbon source assimilation tests and antibiotic resistance. The name *Methylobacterium nodulans* sp. nov. (type strain, ORS 2060^T=CNCM I 2342^T=LMG 21967^T) is proposed for this group of root-nodule-forming bacteria.

The genus *Methylobacterium* includes a variety of pink-pigmented facultatively methylotrophic (PPFM) bacteria that are able to grow on C₁ compounds such as formate, formaldehyde and methanol as sole carbon and energy sources as well as on a wide range of multi-carbon growth substrates (Green, 1992). At the time of writing, the genus *Methylobacterium* consists of 14 PPFM species (Doronina *et al.*, 2002), with *Methylobacterium organophilum* as the type species (Patt *et al.*, 1976). *Methylobacterium* strains have been found on many plant tissues but no symbiotic association with plants has been reported (Holland, 1997). Recently and surprisingly, 16S rRNA gene-based phylogenetic analysis classified non-pigmented bacteria isolated from legume root nodules of three *Crotalaria* species (subfamily Papilionoideae, tribe Crotalariaeae), i.e. *Crotalaria glaucoïdes*, *Crotalaria perrottetii* and *Crotalaria podocarpa*, in the genus *Methylobacterium* (Sy *et al.*, 2001a, b). More

recently, other nitrogen-fixing isolates from root nodules of the legume *Lotononis bainesii* (Papilionoideae, Crotalariaeae) were characterized as pigmented methylotrophic bacteria (Jaftha *et al.*, 2002). Here we review previous reports on *Methylobacterium* strains that nodulate *Crotalaria* species and add additional phenotypic and genotypic characterization data, such as morphology, physiology, enzymic and carbon sources assimilation tests, antibiotic resistance and *nifH* gene sequence, and conclude with the proposal of *Methylobacterium nodulans* sp. nov.

A group of 72 bacterial strains was isolated from root nodules samples from three *Crotalaria* species (*C. glaucoïdes*, *C. perrottetii* and *C. podocarpa*) sampled in five different geographical areas of Senegal (West Africa) (Samba *et al.*, 1999). SDS-PAGE protein pattern analysis clearly indicated that these strains constituted a homogeneous group that was separate from other known legume-nodule-forming bacteria (Samba *et al.*, 1999; Sy *et al.*, 2001a). Eleven strains, representative of the different SDS-PAGE subclusters and for plant and geographical origins, were chosen and studied further. The 16S rRNA PCR-RFLP profile analysis on these strains confirmed the homogeneity of this group

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and partial *nifH* gene sequences of strain ORS 2060^T are AF220763 and AJ512205, respectively.

and the 16S rRNA gene sequence analysis of two of them, ORS 2060^T and ORS 1924 presenting 100 % identity, showed their close phylogenetic relationship with members of the genus *Methylobacterium* (Sy *et al.*, 2001a, b). The methylotrophic metabolism of the same 11 strains was confirmed by growth on MMS medium (Green, 1992) with C₁ compounds as sole carbon source: methanol, formate and formaldehyde but not methylamine (Dreyfus *et al.*, 1999; Sy *et al.*, 2001b). In addition, an *mxoF* gene, encoding the methanol dehydrogenase required for methanol utilization, was detected by PCR in the 11 strains and the *mxoF* gene sequence of ORS 2060^T showed 88 % identity to that of *M. organophilum* (Sy *et al.*, 2001b). Taken together, these data confirmed that the bacterial strains isolated from root nodules of *C. glaucoide*s, *C. perrottetii* and *C. podocarpa* constituted a homogeneous bacterial group that belonged to the genus *Methylobacterium*.

Cross-inoculation and nitrogen fixation tests on legume plants revealed both nodulation specificity and nitrogen-fixing efficiency within the genus *Crotalaria* (Sy *et al.*, 2001a). Representative strains for the main *Methylobacterium* species, i.e. *Methylobacterium extorquens*, *Methylobacterium organophilum*, *Methylobacterium radiotolerans*, *Methylobacterium rhodinum*, *Methylobacterium mesophilicum*, *Methylobacterium rhodesianum* and *Methylobacterium zatmanii*, and two *Methylobacterium* spp. were tested for plant nodulation but none of them was able to induce any legume root nodule (Sy *et al.*, 2001b). In addition, the *nifH* gene, present in all legume-nodule-forming bacteria and encoding a key enzyme in Nod factor biosynthesis that induces legume nodulation (Martinez Romero, 1994; van Rhijn & Vanderleyden, 1995), was detected by PCR in the strains of the novel *Methylobacterium* species isolated from *C. glaucoide*s, *C. perrottetii* and *C. podocarpa*. In contrast, *nifH* was not detected in any of the main representative strains of the genus *Methylobacterium* mentioned above (Sy *et al.*, 2001b). A comparative analysis of the NodA protein deduced from the *nifH* gene sequence showed a range of 53.1 % similarity with the NodA protein sequence from *Azorhizobium caulinodans* to 74.1 % similarity with that of *Bradyrhizobium elkanii* (Sy *et al.*, 2001b).

As a consequence of the above-mentioned results, Sy *et al.* (2001b) concluded that the group of strains made up of facultatively methylotrophic, root-nodule-forming and nitrogen-fixing bacteria may be regarded as a novel *Methylobacterium* species. In this report, we formally propose the name *Methylobacterium nodulans* sp. nov. to include these strains, with ORS 2060^T as the type strain.

Since the report of Sy *et al.* (2001b), novel *Methylobacterium* species have been described (Doronina *et al.*, 2002). Fig. 1 shows a 16S rRNA gene-based phylogenetic tree that includes *M. nodulans* ORS 2060^T, representative strains of *Methylobacterium* sp. isolated from *L. bainesii* (Jaftha *et al.*, 2002), 13 of the 14 validly published *Methylobacterium* species (the sequence of *Methylobacterium aminovorans* is not available) and their nearest phylogenetic neighbours. All

Methylobacterium species and strains form a separate branch, consisting of three sub-branches. One sub-branch consists of *M. nodulans* ORS 2060^T and *Methylobacterium* sp. (isolated from *L. bainesii*) strains xct10, xct14 and xct17. *M. nodulans* ORS 2060^T shows sequence identity values of 95.8–97.6 % with *Methylobacterium* sp. strains isolated from *L. bainesii* (Jaftha *et al.*, 2002), and less than 95.2 % sequence identity with the other *Methylobacterium* species. These similarity values confirm that *M. nodulans* constitutes a separate species in the genus *Methylobacterium* and that it is distinct from *Methylobacterium* sp. strains isolated from *L. bainesii*.

A partial *nifH* fragment (426 bp) was amplified from *M. nodulans* ORS 2060^T, as described by Jaftha *et al.* (2002), and sequenced (GenBank/EMBL/DDBJ accession no. AJ512205). Fig. 2 depicts a *nifH*-based phylogenetic tree showing the relationships of the *nifH* sequences of *Crotalaria*-nodulating strains with those of related nitrogen-fixing bacteria. A maximum-likelihood method was applied for the reconstruction of the phylogenetic tree based on inferred amino acid sequences, excluding third codon positions from the alignment (due to the saturation of this position). The phylogeny shows a close relationship among *M. nodulans* ORS 2060^T, *Methylobacterium* sp. isolated from *L. bainesii* and the *nifH* gene sequence from *Gluconacetobacter diazotrophicus*, a sugarcane endophyte of the α -*Proteobacteria*. Jaftha *et al.* (2002) suggested the closest relationship between their *Methylobacterium* sp. *nifH* sequence was with *Azospirillum brasilense*. However, they did not include the *G. diazotrophicus* sequence in their analysis and applied a phenetic method, including all nucleotide positions in their phylogenetic reconstruction, which is thus much more sensitive to homoplasy and false reconstruction than our maximum-likelihood approach that excluded saturated positions. To the best of our knowledge, nitrogen fixation and the presence of the *nifH* gene have never been reported in any other species belonging to the genus *Methylobacterium*.

Metabolic tests were performed on the 11 strains studied previously (Samba *et al.*, 1999; Sy *et al.*, 2001b): ORS 2026, ORS 2045 and ORS 2076 isolated from *C. glaucoide*s; ORS 1917, ORS 1991 and ORS 2060^T isolated from *C. podocarpa*; and ORS 1924, ORS 1928, ORS 1937, ORS 2030 and ORS 2092 isolated from *C. perrottetii*. The following substrates were readily used as sole source of carbon in MMS medium at 30 °C (Green, 1992): succinate, citrate, pyruvate, glutamate and ethanol. In addition, bacterial enzymic activities were determined using the API 20NE galleries according to the manufacturer's protocol (bioMérieux); tests for nitrate reductase and urease were positive; tests for β -galactosidase, β -glucosidase, protease, indole production and glucose fermentation were negative. API Biotype 100 galleries (bioMérieux) were also used to check assimilation and growth on 100 carbon sources as according to Kersters *et al.* (1984). *M. nodulans* strains were able to grow on the following substrates as sole carbon sources: (+)-D-galactose,

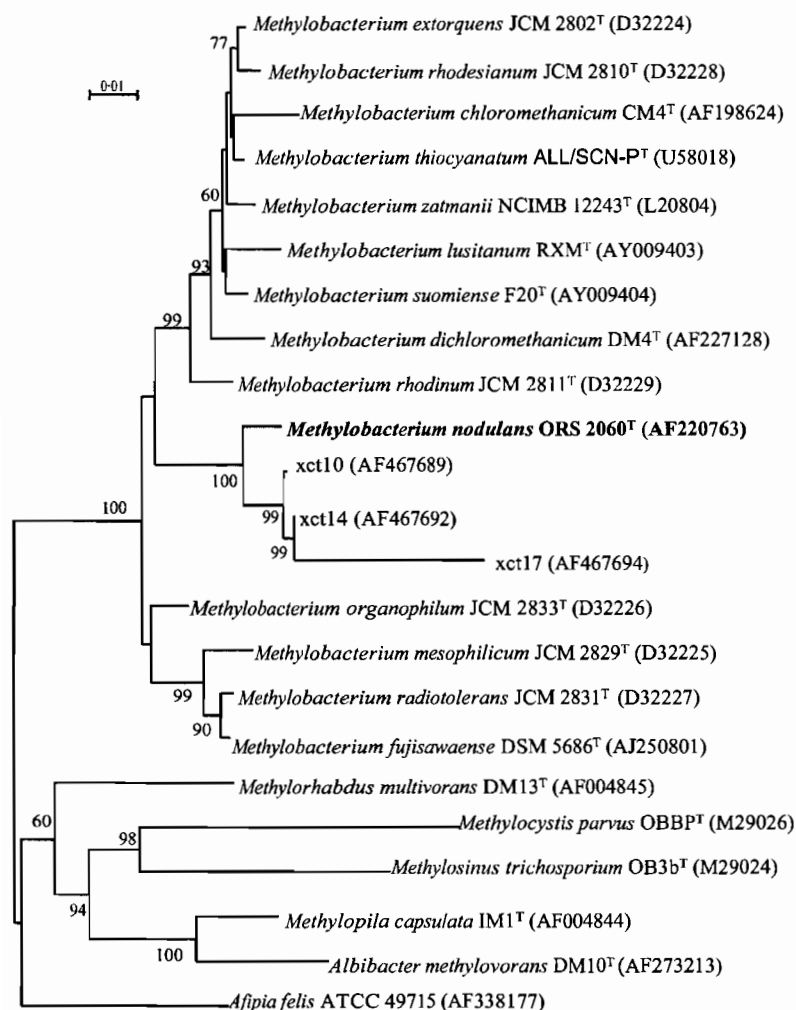


Fig. 1. Phylogenetic analysis of the 16S rRNA gene sequences of strains of *M. nodulans* sp. nov. and other representatives of the genus *Methylobacterium* and related genera. The 16S rRNA gene was PCR-amplified from pure DNA of strain ORS 2060^T and sequenced using primers as described by Sy *et al.* (2001b) and an ABI Prism BigDye terminator cycle sequence kit (Applied Biosystems). The sequence was analysed on an Applied Biosystems model 310 DNA sequencer and its software, Sequence Navigator. Other 16S rRNA gene sequences from representative *Methylobacterium* species and related genera were from the GenBank database (accession numbers are given in parentheses). All sequences were aligned using the Multalign software (Corpet, 1988). The tree was generated using the neighbour-joining method of Saitou & Nei (1987) and CLUSTAL_X software (Thompson *et al.*, 1997). Bootstrap values (100 replicates) are shown at branch points. Bar, 1 estimated substitution per 100 nucleotide positions.

(-)-D-ribose, (+)-L-arabinose, (+)-D-xylose, glycerol, D-lyxose, D-saccharate, mucate, (+)-L- and (-)-D-tartrate, (+)-D- and (-)-L-malate, *cis*- and *trans*-aconitate, tricarballoylate, glucuronate, 2- and 5-keto-D-gluconate, D-gluconate, phenylacetate, *p*-hydroxybenzoate, quinate, benzoate, betaine, α -DL-amino-n-butyrate, DL-lactate, fumarate, glutarate, DL-glycerate, β -DL-hydroxybutyrate, L-aspartate, L-proline, L-alanine, L-serine and 2-oxoglutarate. Strains were unable to use α -(+)-D-glucose, β -(+)-D-fructose, (+)-D-trehalose, (+)-D-mannose, (+)-L-sorbose, α -(+)-D-melibiose, sucrose, α -lactose, (+)-D-raffinose, maltotriose, maltose, lactulose, (+)-D-cellobiose, β -gentobiose, aesculin, α -L-rhamnose, α -(-)-L-fucose, (+)-D-arabitol, xylitol, dulcitol, *myo*-inositol, D-mannitol, D-sorbitol, tryptophan, *N*-acetylglucosamine, coumarate, trigonelline, putrescine, histamine, L-histidine, ethanolamine, tryptamine, D-glucosamine, D-alanine, malonate, propionate and L-tyrosine. The intrinsic antibiotic resistance patterns of the strains show fairly high resistance to ampicillin, carbenicillin and nalidixic acid but sensitivity to kanamycin, gentamicin and tetracycline. Table 1 summarizes features

useful for distinguishing *M. nodulans* sp. nov. from the 14 recognized species of the genus *Methylobacterium* (Green, 1992; Doronina *et al.*, 2002).

Description of *Methylobacterium nodulans* sp. nov.

Methylobacterium nodulans (no'du.lans. N.L. v. *nodulare* to nodulate; N.L. part. adj. *nodulans* nodulating, expressing the original feature of strains to induce nitrogen-fixing nodules on roots of legume plants).

Short asporogenous Gram-negative rods (0.8–1.0 × 1.0–1.5 μ m) that occur singly or occasionally in pairs; some are motile with one or more polar flagella. Colonies on MMS medium + agar (Green, 1992) with methanol as sole carbon source are shiny, smooth, raised, entire and 0.5–1 mm in diameter after 3 days at 30 °C and are not pigmented. Optimal growth occurs at pH 6.8–7.5 and at 30–37 °C. Strictly aerobic, catalase-positive and weakly oxidase-positive; urease-positive and able to reduce nitrate into nitrite. Table 1 shows other phenotypic traits of the

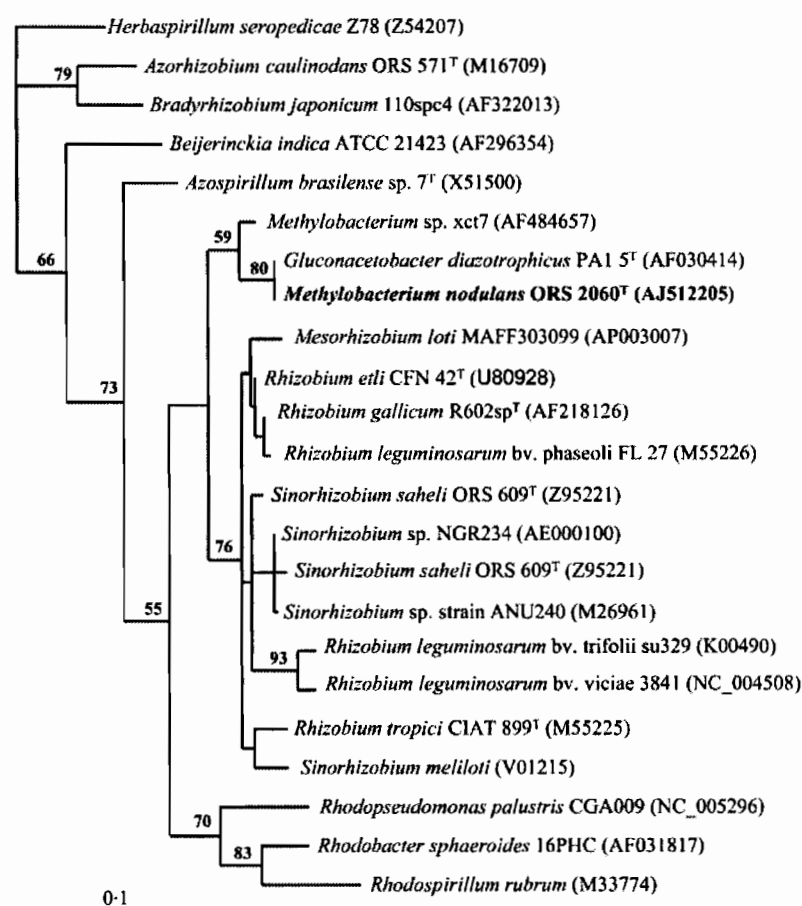


Fig. 2. Phylogenetic tree based on a 426 bp fragment of the *nifH* gene, showing relationships of *M. nodulans* ORS 2060^T with other nitrogen-fixing bacteria of the α - and β -Proteobacteria. *nifH* was PCR-amplified using primers as described by Jaftha *et al.* (2002) and sequenced as described in Fig. 1. *nifH* sequences from representative species of nitrogen-fixing bacteria were from the GenBank database. Sequences were multi-aligned using the CLUSTAL_X software (Thompson *et al.*, 1997), and the phylogenetic tree was generated using PAUP* version 4.0b10 (Swofford, 1998) applying a maximum-likelihood method, estimating base frequencies, the six-parameters instantaneous rate matrix substitution, the shape of the gamma distribution used to accommodate among-site rate variation (three categories) and, finally, estimating the proportion of sites unable to accept substitutions (GTR + Γ_3 + I model). The reconstruction is based on the first and second codon positions only. Bootstrap values are indicated in bold and were estimated from 100 replicates. GenBank/EMBL/DDBJ accession numbers are in parentheses.

species. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *mxoF*, partial *nodA* and partial *nifH* gene sequences of strain ORS 2060^T are AF220763, AF220764,

AF266748 and AJ512205, respectively. Can form nitrogen-fixing root nodules in symbiosis with *Crotalaria glaucoidea*, *Crotalaria perrottetii* and *Crotalaria podocarpa*.

Table 1. Characteristics useful for distinguishing *M. nodulans* sp. nov. from other species of the genus *Methylobacterium*

Species: 1, *Methylobacterium nodulans* ORS 2060^T; 2, *M. organophilum*; 3, *M. extorquens*; 4, *M. rhodinum*; 5, *M. zatmanii*; 6, *M. mesophilicum*; 7, *M. rhodesianum*; 8, *M. fujiisawaense*; 9, *M. radiotolerans*; 10, *M. aminovorans*; 11, *M. thiocyanatum*; 12, *M. chloromethanicum*; 13, *M. dichloromethanicum*; 14, *M. suomiense*; 15, *M. lusitanum*. Tests are based on the assimilation of various compounds as sole source of carbon. Abbreviations: +, growth; -, no growth; w, weak growth; v, variable; ND, not determined.

Compound	1*	2†‡	3†‡	4†‡	5†‡	6†‡	7†‡	8†‡	9†‡	10†‡	11†‡	12†‡	13†‡	14†‡	15†‡
Methylamine	-	+	+	+	+	-	+	-	-	+	+	+	+	+	+
Acetate	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Citrate	+	-	-	+	-	+	-	+	+	-	+	+	+	-	-
L-Glutamate	+	-†, +‡	v†, +‡	+	-	+	v†, -‡	+	+	+	+	+	+	-	-
D-Glucose	-	+†, -‡	-	w†, +‡	-	+	-	+	+	-	+	-	-	+	-
D-Xylose	+	-	-	-†, +‡	-	+	-	+	+	-	+	-	-	-	-
Fructose	-	+	-	+	+	-	+	v†, -‡	-	+	+	-	+	+	+
Betaine	+	-	+	+	-	-	+	-	+	+	ND	+	+	+	-

*Results of the 11 strains studied including ORS 2060^T.

†Data from Green (1992).

‡Data from Doronina *et al.* (2002).

The type strain is ORS 2060^T (=CNCM I 2342^T=LMG 21967^T), isolated from *C. podocarpa* from the Bel-Air area, Dakar, Senegal. Most of the molecular and physiological studies were conducted on this strain.

Note added in proof

While this article was being reviewed, *Methylobacterium populi* was validly described (Van Aken *et al.*, 2004).

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Nematostatic effects of a leaf extract from *Crotalaria virgulata* subsp. *grantiana* on *Meloidogyne incognita* and its use to protect tomato roots

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Summary – An aqueous extract from leaves of *Crotalaria virgulata* subsp. *grantiana* was assayed for its effects on second-stage juveniles of *Meloidogyne incognita*. The biological activity was nematostatic; nematodes were not killed but were completely paralysed in a 1 mg/ml (w/v) extract: the LD₅₀ equivalent was estimated to be 0.5 mg/ml. The effect was reversible: juveniles previously paralysed by *C. grantiana* extract recovered complete mobility in water and were able to infest a susceptible tomato plant. Freeze-dried aqueous extract from *C. grantiana* leaves added to a sterile sandy substrate at 1 mg/ml protected susceptible tomato plants from *M. incognita* infestation. This suggests a promising use of *C. grantiana* as both a green manure and natural alternative to synthetic chemicals in nematode population control, especially in integrated pest management for vegetable crops in organic agriculture of tropical and temperate areas.

Keywords – biological activity, crop protection, plant extract, root-knot nematode.

Crotalaria virgulata subsp. *grantiana* Harvey is a wild legume (Fabaceae) commonly named *C. grantiana* (Polhill, 1982; Brummit, 1992). *Crotalaria grantiana* has been found in Southern Africa, from the coastal regions of Eastern Cape to Zimbabwe and Botswana (Polhill, 1982). In tropical areas, *Crotalaria* spp., like many other Fabaceae, play a major role maintaining and restoring soil fertility when used as cover or green manure crops, as well as contributing to nematode management (Widner & Dadalto, 1991; Wang *et al.*, 2002). *Crotalaria grantiana* has been investigated particularly for its resistance to plant-parasitic nematodes such as *Meloidogyne javanica*, *Pratylenchus brachyurus*, *P. zaei* and *Rotylenchulus reniformis* (Antonio & Neumaier, 1986; Silva *et al.*, 1989a, b). However, as far as we know, there is no report of nematocidal effects of *C. grantiana* leaf extracts such as has been investigated for leaf extracts from other species, *i.e.*, *C. juncea* and juveniles of *Radopholus similis* (Jasy &

Koshy, 1992), *C. saharae* and *C. spectabilis* and *M. incognita* (Subramanian & Sivagami, 1990; Sellami & Mouffarra, 1994). The aim of this study was to describe accurately the effects of the leaf aqueous extract from *C. grantiana* on the root-knot nematode *M. incognita* as a prerequisite to introducing *C. grantiana* as a nematicidal green manure in African crop systems. We also studied the influence of this biological effect on the capacity of the nematode to infest a crop such as tomato in order to assess how such a plant extract could be used to protect susceptible plants.

Materials and methods

PLANT MATERIAL

Crotalaria grantiana seeds were harvested in Senegal (IRD, Bel-Air, Dakar) from previously introduced and

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cultivated plants, morphologically identified at the Laboratory of Botany (Department of Plant Biology, University Cheikh Anta Diop, Dakar, Senegal). Seeds were preserved at 20°C in the dark and re-hydrated for 48 h in distilled water before sowing in a compost (Kultursubstrat, Frankfurt, Germany). The plants were grown for 4 months in a glasshouse under simulated Mediterranean conditions. Fresh leaves (1 kg) were collected and blended three times in 1 l distilled water for 15 min at room temperature. The three crude extracts were pooled, centrifuged (15 min, 2410 g) and the supernatant filtered on Whatman paper N°1 and 0.22 µm cellulose acetate filter. The filtered extract was freeze-dried and weighed. Aliquots were re-suspended in distilled water at different concentrations (w/v) and used directly to study their effects on nematodes.

ROOT-KNOT NEMATODES

Meloidogyne incognita (Kofoid & White, 1919) Chitwood, 1949 populations were reared in glasshouse conditions on susceptible tomato plants (*Lycopersicon esculentum* var. Nainespomor). This tomato stock was specially created for laboratory use to reproduce and multiply nematode populations: seed is available from INRA, Unité de Génétique et Amélioration des Fruits et Légumes (Domaine St-Maurice B.P. 94, 84 143 Monfavet cedex, France). Nematode egg masses were handpicked from the roots and placed in distilled water to hatch. Second-stage juveniles (J2) were concentrated to 100 nematodes per ml by sedimentation in test tubes.

EFFECT OF AQUEOUS LEAF EXTRACT

In 24-well culture polystyrene plates, 100 J2 of *M. incognita* per cell were suspended in 1 ml of distilled water, and 1 ml of *C. grantiana* aqueous leaf extract was added to give final concentrations of 0, 0.001, 0.05, 0.25, 0.5, 1, 2.5, 5 and 10 mg/ml w/v). All concentrations were replicated four times and nematodes kept in the extract at ambient temperature. After 48 and 72 h incubation, all mobile and immobile J2 were counted per cell with the aid of an inverted microscope at magnification 100×. The ratio (number of immobile nematodes/number of total nematodes (mobile + immobile)) directly expresses the percentage of paralysed nematodes per cell. In addition, in order to assess the viability of paralysed J2, Meldola Blue vital stain (Sigma® D8142) was added to each cell as described by Ogiga and Estey (1974). Nematodes stained black were dead while those remaining unstained were

still alive. Immobile, unstained nematodes were considered to be paralysed.

MOBILITY RECOVERY AND ROOT INVASION

One hundred J2 of *M. incognita* were incubated in 24-well culture polystyrene plates for 72 h either in 1 ml of distilled water (control) or 1 ml of *C. grantiana* extract at 1 mg/ml (w/v). After treatment with *C. grantiana* extract, nematodes were washed three times and re-suspended in distilled water. Both mobile and immobile nematodes in both treatments were counted in each cell as described above at 2, 7, 14 and 21 days after the 72 h incubation to determine their ability to recover mobility. Then, to assess their invasion potential, each nematode batch was inoculated onto 2-week-old tomato cv. Nainespomor seedlings transplanted in 50 cm³ polypropylene tubes containing 70 g sterile sand and closed with cotton at the bottom. The plants were kept in growth chambers at 25°C and irrigated daily with 5 ml distilled water and fed weekly with 5 ml of nutrient solution (Bertrand *et al.*, 2000). The plants were uprooted 6 weeks after inoculation, their roots were harvested, washed free of soil under distilled water and stained with acid fuchsin (Byrd *et al.*, 1983). Nematodes that had penetrated roots were stained red and were counted by examination of all tomato roots under a binocular microscope at magnification 20×. Percent invasion potential was estimated from the ratio of stained penetrated nematodes to total nematodes in the inoculum.

INHIBITION OF ROOT INFESTATION

One hundred fresh mobile *M. incognita* J2, suspended in 1.5 ml of distilled water, were inoculated onto 2-week-old tomato seedlings in 50 cm³ tubes prepared as described above. Prior to tomato transplantation, the sand substrate was supplemented once with *C. grantiana* freeze-dried leaf extract at 0, 0.1, 1 and 5 mg/ml w/v). The plants were grown, irrigated and fed as described above, and were uprooted 2, 4, 7, 10, 14, 28 and 42 days after nematode inoculation. Nematode infestation of roots was estimated as described above.

STATISTICAL ANALYSIS

Four replicates were used for each experimental treatment. All data were subjected to a one-way analysis of variance, and means were compared with the Newman-Keuls multiple range test ($P \leq 0.05$) using Super

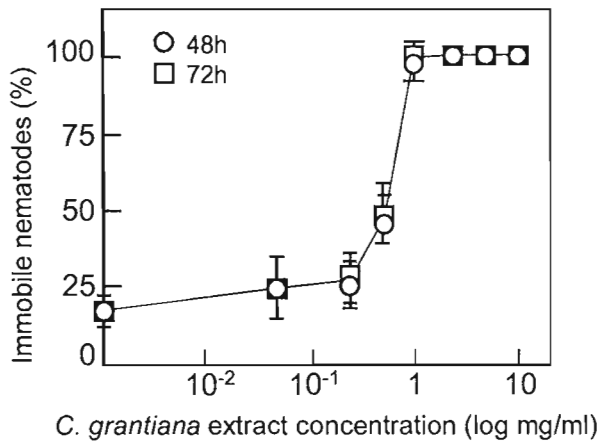


Fig. 1. In vitro nematostatic effect of *Crotalaria grantiana* extract on *Meloidogyne incognita* second-stage juveniles (% immobile) after 48 and 72 h incubation (bars represent the standard error; $P \leq 0.05$).

ANOVA computer software (Gagnon *et al.*, 1989). Percentages were arcsine (sqrt) transformed for analysis.

Results

IN VITRO NEMATOSTATIC EFFECT

Compared to mobile *M. incognita* J2 placed in distilled water, J2 *C. grantiana* extract at 1 mg/ml were immobile, irrespective of incubation period (48 and 72 h). Immobile nematodes were never stained by Meldola Blue and therefore were alive while J2 killed by heat (5 min at 100°C) were stained black. The biological effect of *C. grantiana* aqueous leaf extract on nematodes resulted in juvenile paralysis, correlated with extract concentration (Fig. 1). At 10^{-3} mg/ml, only 18% of nematodes were paralysed and paralysis increased to 100% at concentrations ≥ 1 mg/ml. The LD_{50} equivalent (50% nematodes paralysed) was estimated graphically as 0.5 mg/ml of aqueous leaf extract.

REVERSIBILITY OF EXTRACT ACTIVITY

All J2 incubated in *C. grantiana* leaf extract remained immobile at 2 days after transfer to distilled water (Fig. 2). After then, percentage immobile individuals significantly decreased when the juveniles were maintained in distilled water and mobility returned to levels comparable to the control in which, after 72 h incubation, there was also a small percentage paralysed nematodes. After a treatment with *C. grantiana* extract and washing with distilled

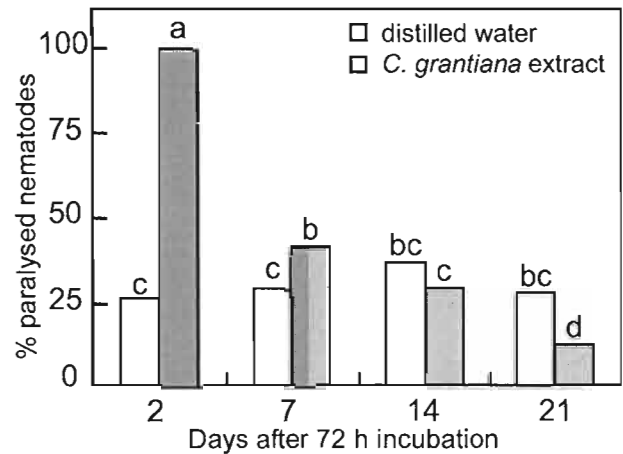


Fig. 2. In vitro reversibility of paralysis of *Meloidogyne incognita* second-stage juveniles transferred to distilled water after 72 h incubation in 1 mg/ml *Crotalaria grantiana* extract (data with the same letters are not significantly different, $P \leq 0.05$).

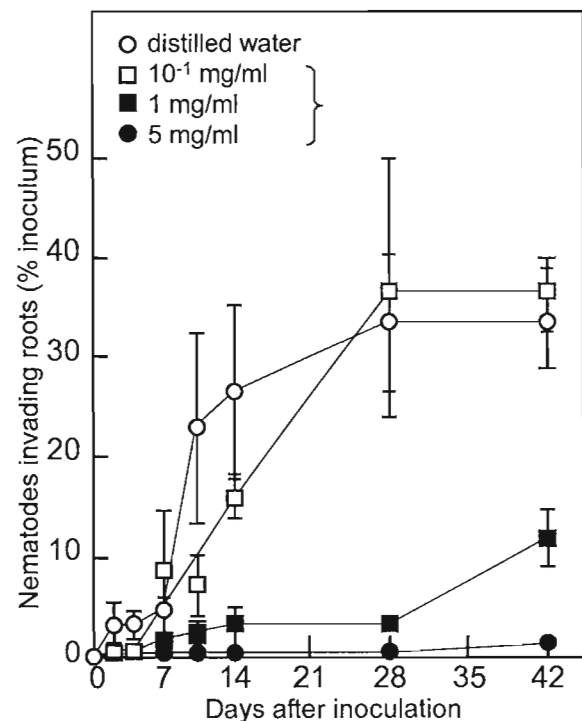


Fig. 3. Invasion of tomato roots by *Meloidogyne incognita* second stage juveniles inoculated in sandy soil supplemented with different concentrations of *Crotalaria grantiana* extract (bars represent the standard error; $P \leq 0.05$).

water, J2 having recovered mobility were able to penetrate into tomato roots at a comparable level to untreated juveniles (data not shown).



Fig. 4. Growth of tomato plants in sand amended before planting with *Crotalaria grantiana* extract (from left to right: 0; 0.1; 1; 5 mg/ml) at 6 weeks after inoculation of 2-week-old seedlings with 100 second stage juvenile *Meloidogyne incognita*.

INHIBITION OF ROOT INFESTATION BY *C. GRANTIANA*

The addition of either 1 or 5 mg/ml freeze-dried *C. grantiana* extract around the roots of tomato plants, induced a very significant decrease in nematode penetration (Fig. 3). The proportion of nematodes in roots did not exceed 10 and 4% at 1 and 5 mg/ml extract, respectively, at 42 days after inoculation. In addition, penetration was much delayed in the presence of *C. grantiana* extract at 1 and 5 mg/ml, by 10 days compared to 10^{-1} mg/ml or distilled water. Regardless of the concentration of *C. grantiana* extract added to the sand, J2 that penetrated roots were able to complete development. In the control as well as in 10^{-1} mg/ml *C. grantiana* leaf extract (Fig. 3), penetration rates increased up to 36% of nematodes at day 28 after inoculation. Second-stage juveniles were able to mature to third and fourth stages and achieved their full development into mature females. Nevertheless, *C. grantiana* extract used as an amendment at 1 and 5 mg/ml significantly decreased the proportion of nematodes that penetrated susceptible tomato plant roots. This inhibition by *C. grantiana* extract of *M. incognita* penetration contributed to improved plant growth compared to infested tomato (Fig. 4).

Discussion

Crotalaria grantiana aqueous leaf extract had an effect *in vitro* on J2 of the root-knot nematode *M. incognita*, as previously reported for *C. saharae* (Sellami & Mouffarah, 1994) and *C. spectabilis* (Subramaniyan & Sivagami, 1990) on *Meloidogyne* species. However, in those studies, immobile juveniles were described as dead nematodes but without testing this conclusion. This study on *C. grantiana* leaf extract demonstrated paralysis of J2 of *M. incognita* and from the vital staining technique (Ogiga & Estey, 1974), we conclude that *C. grantiana* aqueous leaf extract does not act as a nematicide *sensu stricto*: the juveniles are not killed by the plant extract but only paralysed. Consequently, the biological activity of *C. grantiana* leaf extract can be considered to be a nematostatic effect: the LD_{50} equivalent, corresponding to a concentration that paralysed 50% of the juveniles, was determined to be 0.5 mg/ml.

With regard to secondary metabolites responsible for this biological activity, the literature on Fabaceae, like the Sophorae, linked alkaloids to nematicidal properties (Zhao, 1999). *Crotalaria* species also contain alkaloids described as pyrrolizidine alkaloids (Kinghorn & Smolenski, 1981) and well known as chemosystematic mark-

ers (Polhill, 1982). According to Fassuliotis and Skucas (1969), monocrotaline, a pyrrolizidine alkaloid isolated from *C. spectabilis*, altered the movement of juveniles *M. incognita* *in vitro* to a jerking motion and significantly reduced infestation of tomato plants. As *C. grantiana* also contains pyrrolizidine alkaloids such as grantianine and grantaline (Smith & Culvenor, 1984), their presence could explain the paralysis induced by *C. grantiana* leaf extract as well as the reduction and delay of penetration of susceptible tomato roots. The protection of tomato plants from *M. incognita* infection by *C. grantiana* extract added to the substrate may be due to either *i*) loss of mobility due to paralysis as revealed here by *in vitro* experiments, or *ii*) disturbed orientation and host finding (linked to disturbance of chemoreception or injuries of sensory organs) disrupting nematode co-ordination in stimuli gradients and affecting response to root attractants. Such consequences of behavioural disturbance were suggested by Green (1971).

Therefore, these observations on the nematostatic activity of *C. grantiana* leaf extract on *M. incognita* clearly indicate another mechanism for the control of root-knot nematode populations by *Crotalaria* green manure. Usually, green manures cause reduction of nematodes by increasing the release of ammonia (Rodriguez-Kabana, 1986) and enhancing antagonists to nematodes (Sikora, 1992). Our data emphasise the significance of the protection of susceptible tomato plants by addition to the substrate of a sterile aqueous leaf extract of *C. grantiana*.

The observation that leaf extracts of *C. grantiana* reversibly paralyse *M. incognita* provides new insight into the mechanism by which green manures of this and possibly other *Crotalaria* species reduce nematode populations (Silva *et al.*, 1989a, b; Widner & Dadalto, 1991; Jourand *et al.*, 2002). Although the specific components in *C. grantiana* leaves responsible for this paralysis remain to be determined, their identification should assist in the selection and development of germplasm with enhanced nematode antagonism. Such germplasm would have value for farmers in developing countries who need an effective method for reducing nematode populations in low-value crop systems.

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Nematostatic activity of aqueous extracts of West African *Crotalaria* species

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Summary – The *in vitro* paralysis of second-stage juveniles of *Meloidogyne incognita*, *M. javanica* and *M. mayaguensis* by aqueous extracts of 15 West African *Crotalaria* species was analysed. A multivariate analysis distinguished four groups, based on their nematostatic activity: i) *C. glaucoides* extracts were not active; ii) the root extracts from *C. gorensis*, *C. lathyroides* and *C. perrottetii* were more active than the shoot extracts; iii) the shoot extracts from *C. comosa* and *C. cylindrocarpa* were more active than the root extracts; iv) both shoot and root extracts of *C. atropurpurea*, *C. barkae*, *C. grantiana*, *C. hyssopifolia*, *C. pallida*, *C. podocarpa*, *C. retusa*, *C. senegalensis* and *C. sphaerocarpa* have nematostatic effects depending on either the *Crotalaria* species or plant tissue from which the extracts were derived. However, considering the greater biomass contribution of the leaves and stems compared to the roots when the plants are used as green manure, *C. barkae*, *C. grantiana*, *C. pallida* and *C. podocarpa* are the most efficient *Crotalaria* species, whatever the *Meloidogyne* species targeted.

Keywords – *Meloidogyne incognita*, *Meloidogyne javanica*, *Meloidogyne mayaguensis*, plant extract.

In tropical areas, natural Fabaceae play an important role in maintaining and restoring soil fertility (Rachie *et al.*, 1979). *Crotalaria* is a worldwide genus in various tropical climates ranging from semi-arid to humid forests (Brummitt, 1992). *Crotalaria* species are annual or perennial shrubs and herbs. Only a few species have been described from Asia, Australia and South America (Polhill & Raven, 1981), but Polhill (1982) reported more than 500 species from Africa. *Crotalaria* species have high agronomical and agricultural potentials. Their ability to fix nitrogen by root symbiosis with *Bradyrhizobium* sp. (Samba *et al.*, 1999) or *Methylobacterium* sp. (Sy *et al.*, 2001) enables these plants to be used for soil fertilisation. Moreover, these leguminous plants develop a high resistance to the main species of root-knot nematodes (Antonio & Neumaier, 1986; Da Silva *et al.*, 1989a, b). These nematodes cause severe damage to several crops resulting in annual yield losses ranging from 10 to 50%. The physical, chemical, genetic and biological nematode management methods already applied to fields are not completely

effective and may promote resistant nematode biotypes (Barker *et al.*, 1998), so the use of *Crotalaria* sp. appears as a very interesting natural alternative to reduce nematode populations in agriculture. Thus, some Asian species have been cultivated as green manure or cover crops in plantations or in inter-cropping systems to restore soil fertility (Kullaya *et al.*, 1995; Mwazambi *et al.*, 1998) and to control nematode populations in infested soils (Wang *et al.*, 2002, 2003) and, consequently, to enhance crop yields (Sandanam *et al.*, 1976; McSorley *et al.*, 1994).

The mechanism of the *Crotalaria* resistance to root-knot nematodes has been partially elucidated and involves inhibition of nematode development (Sano *et al.*, 1983; Sano, 1986). Moreover, extracts of shoot and root were found active against second-stage juveniles (J2): *C. juncea*, *C. spectabilis* and *C. saharae* were reported to be nematocidal to juveniles of *Radopholus similis* and *Meloidogyne incognita* (Subramanian & Sivagami, 1990; Jasy & Koshy, 1992; Sellami & Mouffarra, 1994). Recently, an aqueous shoot extract of the West African *C. virgulata*

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subsp. *grantiana* on *M. incognita* was clearly identified as causing paralysis of juveniles and as having nematostatic activity in protecting roots of susceptible cultivated plants from nematode infestation (Jourand *et al.*, 2004).

The aim of this study was to analyse the nematostatic activity of aqueous shoot and root extracts from 15 West African *Crotalaria* species on J2 of *M. incognita*, *M. javanica* and *M. mayaguensis*. This investigation screens the potential of these legumes for use as green manure to control root-knot nematode populations in infested soils.

Material and methods

PLANT MATERIAL

Seeds of 15 *Crotalaria* species (Fabaceae, Papilionoideae, Crotalariae) were harvested in Senegal from cultivated plants (IRD, Dakar) and identified morphologically (Laboratory of Botany, Department of Plant Biology, University Cheikh Anta Diop, Dakar, Senegal) according to Polhill (1982): *C. atrorubens* Hochst. ex Benth, *C. barkae* Schweinf, *C. comosa* Baker, *C. cylindrocarpa* Don C., *C. glaucoidea* Baker, *C. gorensis* Guillemain & Perrottet, *C. grantiana* Harvey, *C. hyssopifolia* Klotzsch, *C. lathyroides* Guillemain & Perrottet, *C. pallida* Aiton, *C. perrottetii* Don C., *C. podocarpa* Don C., *C. retusa* Linneaus, *C. senegalensis* Bacle and *C. sphaerocarpa* Perrottet. The seeds had been stored in a cool, dark room (4°C) and were re-hydrated for 48 h in distilled water before sowing in peat (Kultursubstrat, Frankfurt, Germany). Fifteen plants per *Crotalaria* species were grown for 4 months in a glasshouse under tropical conditions (Laboratoire des Symbioses Tropicales et Méditerranéennes, Montpellier, France). The shoots and roots were either dried for 48 h at 60°C and weighed to determine the plant biomass, or uprooted to prepare fresh extracts.

PLANT EXTRACTS

Fresh shoots (10 g) and fresh roots (10 g) of each *Crotalaria* species were separately blended with 100 ml of distilled water (three times) for 15 min at room temperature. The crude shoot or root extracts were separately pooled and centrifuged (15 min at 2410 g). The supernatants were successively filtered on Whatman paper N°1 and 0.22 µm cellulose acetate filter. The filtered extracts were then freeze-dried and weighed. Aliquots were re-suspended in distilled water to obtain aqueous crude extracts at 2 mg ml⁻¹ (w/v).

ROOT-KNOT NEMATODES

Meloidogyne incognita (Kofoid & White); Chitwood, *Meloidogyne javanica* (Treub); Chitwood, and *Meloidogyne mayaguensis* Rammah & Hirschmann, populations were reared on susceptible tomato plants (*Lycopersicon esculentum* var. Nainespomor, INRA, Montfavet, France) in a glasshouse (CBGP, Montpellier, France). First generation egg masses of each *Meloidogyne* species were handpicked from the roots and placed in distilled water for hatching. J2 were concentrated to 100 nematodes per ml of distilled water by sedimentation in test tubes.

IN VITRO NEMATOSTATIC SCREENING

In 24 well culture polystyrene plates, 1 ml of nematode suspensions containing 100 J2 of either *M. incognita*, *M. javanica* or *M. mayaguensis* were added to 1 ml of aqueous extract of shoot or root at 2 mg ml⁻¹ (w/v, final concentration of the plant extract = 1 mg ml⁻¹). Control treatments were distilled water instead of plant extract. Mobile and immobile J2 were enumerated using an inverted microscope (×40) after 48 h of incubation at room temperature. The proportion of paralysed nematodes ((number of immobile juveniles/total juveniles) × 100) was expressed as described by Jourand *et al.* (2004). As *Crotalaria* shoots and roots are usually incorporated for green manure, a whole plant nematostatic index was evaluated for each *Crotalaria* species based on the respective weights of shoots and roots. The whole nematostatic index was I (%) = ((shoot extract activity × shoot weight) + (root extract activity × root weight))/(shoot weight + root weight).

STATISTICAL ANALYSIS

Four replicates were used for each of the combinations 'nematode species × extract × *Crotalaria* species'. A principal component analysis (PCA) was performed to study the relationship between each combination and nematode paralysis. This PCA analysis gave a loading plot for the six percentages of paralysis analysed (effect of the shoot and root extracts on *M. incognita* (SI and RI, respectively), *M. javanica* (SJ and RJ) and *M. mayaguensis* (SM and RM) defined by the first four eigenvalues), and a score plot for the four replicates of the 15 *Crotalaria* plants. The PCA was performed with the ADE-4 multivariate analysis and graphical display software (Thioulouse *et al.*, 1997). The significant data were subjected to a one-way analysis of variance. Means were compared with the

Newman-Keuls test ($P \leq 0.05$) using software SuperANOVA (Gagnon *et al.*, 1989). Arcsine ($\sqrt{}$) transformed percentages were used for analysis.

Results

The fraction of variance accounted by the first four PCA eigenvalues was 96%. As shown by the loading plots, the first axis PC1 was related mainly to the percentages of paralysis due to all shoot and root extracts with its positive values (Fig. 1A). The second axis PC2 was related to the percentages of paralysis due to the root extracts with its positive values and to those due to the shoot extracts with its negative values (Fig. 1A). The third axis PC3 distinguished the effects of the root extracts on *M. incognita*, *M. javanica* and *M. mayaguensis* related mainly to its positive, negative and neutral values, respectively (Fig. 1C). The effects of the shoot extracts on the three nematode species were differentiated by the fourth axis PC4 (Fig. 1E).

The score plots of the samples showed that the first PC1 and the second PC2 axes distinguished the effects of different groups of *Crotalaria* species (Fig. 1B; Table 1): i) a nematostatic activity only in shoot extracts of *C. comosa* and *C. cylindrocarpa*; ii) a nematostatic activity only in root extracts of *C. goreensis*, *C. lathyroides*, *C. perrottetii*; iii) an activity in both shoot and root extracts of *C. atrorubens*, *C. hyssopifolia*, *C. pallida*, *C. podocarpa*, *C. retusa*, *C. senegalensis* and *C. sphaerocarpa* with more root than shoot activity, and for *C. barkae* and *C. grantiana* with more shoot than root activity. Both shoot and root extracts from *C. glaucoides* had a very weak nematostatic activity. The third axis PC3 distinguished the effects of the root extracts on the three nematode species (Fig. 1D; Table 1): i) *C. goreensis* extracts were more effective on *M. incognita*; ii) *C. lathyroides*, *C. perrottetii* and *C. hyssopifolia* extracts were more effective on *M. javanica*; iii) *C. atrorubens* and *C. lathyroides* extracts were more effective on *M. mayaguensis*. The fourth axis PC4 distinguished the effects of the shoot extracts on the three nematode species (Fig. 1F; Table 1): i) *C. cylindrocarpa* extracts were more effective on both *M. incognita* and *M. javanica*; ii) *C. barkae* and *C. comosa* extracts were less active on *M. incognita*; iii) *C. grantiana* extracts were effective on all three nematode species.

Taking into account the differences observed on the shoot and root weights between the *Crotalaria* species (Table 2), the whole plant nematostatic indexes vary ac-

Table 1. Nematostatic activity (% of paralysed second-stage juveniles) of aqueous shoot and root extracts of the 15 West African *Crotalaria* species on *Meloidogyne* species. Data followed with the same letter are not significantly different ($P \leq 0.05$).

Plant species	<i>M. incognita</i>	<i>M. javanica</i>	<i>M. mayaguensis</i>			
<i>Crotalaria</i> species with very weak activity						
<i>C. glaucooides</i>	Shoot and root activity < 25					
<i>Crotalaria</i> species with only shoot extract activity						
<i>C. comosa</i>	65 ^b	100 ^a	97 ^a			
<i>C. cylindrocarpa</i>	99 ^a	100 ^a	79 ^a			
<i>Crotalaria</i> species with only root extract activity						
<i>C. goreensis</i>	100 ^a	100 ^a	100 ^a			
<i>C. lathyroides</i>	41 ^b	20 ^c	90 ^b			
<i>C. perrottetii</i>	42 ^b	90 ^b	91 ^b			
<i>Crotalaria</i> species with both shoot and root extracts activities						
	aerial	root	aerial	root	aerial	root
<i>C. atrorubens</i>	78 ^c	96 ^a	99 ^a	96 ^a	52 ^d	100 ^a
<i>C. barkae</i>	90 ^b	70 ^c	100 ^a	94 ^b	100 ^a	78 ^c
<i>C. grantiana</i>	100 ^a	54 ^d	100 ^a	95 ^b	100 ^a	66 ^d
<i>C. hyssopifolia</i>	79 ^c	72 ^c	100 ^a	100 ^a	81 ^b	93 ^b
<i>C. pallida</i>	100 ^a	100 ^a	100 ^a	87 ^c	97 ^a	96 ^a
<i>C. podocarpa</i>	89 ^b	69 ^c	92 ^b	91 ^c	96 ^a	100 ^a
<i>C. retusa</i>	98 ^a	96 ^a	100 ^a	97 ^a	75 ^c	93 ^b
<i>C. senegalensis</i>	100 ^a	94 ^b	100 ^a	98 ^b	71 ^c	90 ^b
<i>C. sphaerocarpa</i>	80 ^c	74 ^c	100 ^a	69 ^c	78 ^b	100 ^a

cording to the *Meloidogyne* species. *Crotalaria glaucoides* and *C. lathyroides* provided a very low activity on the three nematode species. *Crotalaria cylindrocarpa*, *C. goreensis* and *C. perrottetii* controlled only from 30-62% of the J2 of the *Meloidogyne* species tested. The other *Crotalaria* species exhibited excellent nematostatic activities: most of them were very effective either on both *M. incognita* and *M. javanica* (i.e., *C. atrorubens*, *C. retusa* and *C. senegalensis*) or on both *M. javanica* and *M. mayaguensis* (i.e., *C. barkae*, *C. comosa*, *C. grantiana*, *C. hyssopifolia*, *C. podocarpa* and *C. sphaerocarpa*). *Crotalaria pallida* was highly efficient irrespective of the *Meloidogyne* species.

Discussion

In order to improve sustainable integrated pest management, the use of plants as green manure in crop rotations to control nematodes is of a great interest. Thirteen of the 15 West African *Crotalaria* species studied in

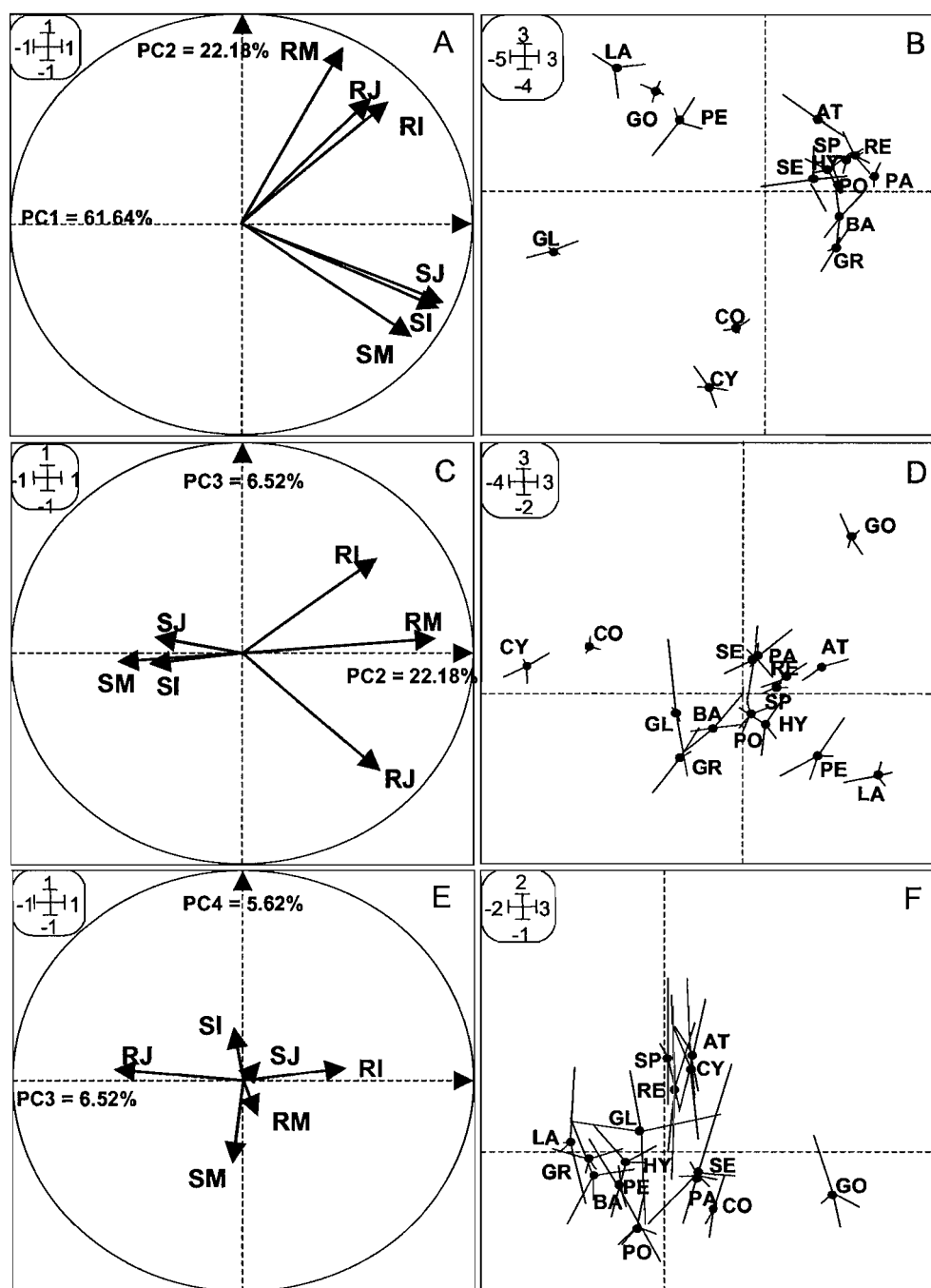


Fig. 1. PCA diagram loading plots for the shoot and root extracts from 15 West African *Croton* species (A, C, E) and score plots for the samples (B, D, F) according to their nematostatic activity on second-stage juveniles of *Meloidogyne incognita*, *M. javanica* and *M. mayaguensis* (A and B = PC1 $\times$ PC2; C and D = PC2 $\times$ PC3; E and F = PC3 $\times$ PC4). Labels used for PCA combined shoot extracts activities: SI for *M. incognita*; SJ for *M. javanica*; SM for *M. mayaguensis*. Labels used for the PCA combined root extracts activities: RI for *M. incognita*; RJ for *M. javanica*; RM for *M. mayaguensis*. Labels used for *Croton* species: *C. atrovirens*: AT, *C. barkae*: BA, *C. comosa*: CO, *C. cylindrocarpa*: CY, *C. glaucoides*: GL, *C. goreensis*: GO, *C. grantiana*: GR, *C. hyssopifolia*: HY, *C. lathyroides*: LA, *C. pallida*: PA, *C. perrottetii*: PE, *C. podocarpa*: PO, *C. retusa*: RE, *C. senegalensis*: SE, *C. sphaerocarpa*: SP.

Table 2. Dry weight of shoots and roots from *Crotalaria* species and whole plant nematostatic index* on *Meloidogyne* species. Data followed with the same letters are not significantly different ($P \leq 0.05$).

Plant species	Dry weight (g)		Nematostatic index (%)		
	shoot	roots	<i>M. incognita</i>	<i>M. javanica</i>	<i>M. mayaguensis</i>
<i>C. atrorubens</i>	1.78 ^d	1.15 ^e	85.1 ^b	97.6 ^a	71.1 ^d
<i>C. barkae</i>	0.85 ^b	0.28 ^b	85.0 ^b	98.6 ^a	94.6 ^a
<i>C. comosa</i>	1.23 ^c	0.52 ^c	65.7 ^d	88.5 ^c	78.9 ^c
<i>C. cylindrocarpa</i>	0.55 ^b	0.43 ^b	59.5 ^e	58.6 ^d	55.0 ^e
<i>C. glaucooides</i>	1.90 ^d	0.82 ^d	22.7 ^h	18.3 ^g	38.3 ^f
<i>C. goreensis</i>	0.94 ^c	0.59 ^c	50.4 ^f	30.4 ^e	61.9 ^e
<i>C. grantiana</i>	0.39 ^a	0.17 ^a	86.4 ^b	98.6 ^a	89.9 ^b
<i>C. hyssopifolia</i>	0.64 ^b	0.30 ^b	76.9 ^c	100.0 ^a	85.2 ^b
<i>C. lathyroides</i>	1.25 ^c	0.52 ^c	21.4 ^h	15.8 ^f	42.5 ^f
<i>C. pallida</i>	0.37 ^a	0.37 ^b	100.0 ^a	93.8 ^b	96.4 ^a
<i>C. perrottetii</i>	0.33 ^a	0.14 ^a	37.0 ^g	59.1 ^d	60.8 ^e
<i>C. podocarpa</i>	3.94 ^f	1.69 ^f	82.7 ^b	91.9 ^b	97.5 ^a
<i>C. retusa</i>	2.40 ^e	0.54 ^c	97.7 ^a	99.5 ^a	78.8 ^c
<i>C. senegalensis</i>	0.51 ^b	0.40 ^b	98.3 ^a	99.5 ^a	76.6 ^c
<i>C. sphaerocarpa</i>	2.28 ^e	0.98 ^d	77.4 ^c	86.6 ^c	87.8 ^b

* Expressed as the plant weight ratio [(shoot extract activity $\times$ shoot weight) + (root activity $\times$ root weight)] / (shoot weight + root weight).

these experiments have nematostatic activity as previously demonstrated for *C. grantiana* by Jourand *et al.* (2004). Consequently, these 13 *Crotalaria* should be useful as a nematostatic green manure to control tropical *Meloidogyne* species.

The PCA analysis revealed that both shoot and root aqueous crude extracts paralyse J2 of *M. incognita*, *M. javanica* and *M. mayaguensis*. These results are especially important for the management of *M. mayaguensis* which is spreading in many African countries. *Meloidogyne mayaguensis* is aggressive even on plants resistant to the other *Meloidogyne* species (Rammah & Hirschmann, 1988; Mateille *et al.*, 1995; Fargette *et al.*, 1996; Willers, 1997).

The range of the nematostatic activity observed for *Crotalaria* species could be the effect of secondary metabolites as reported by Polhill (1982). In some cases, the active compounds are alkaloids as demonstrated for Leguminosae such as Sophorae (Zhao, 1999) and *Crotalariae* (Fassuliotis & Skucas, 1969). Within the West African *Crotalaria* genus, many pyrrolizidine alkaloids have been identified: *C. grantiana* (section *Crotalaria*, sub-section *Crotalaria*) contains grantaline and grantanine (Smith

& Culvenor, 1984); *C. pallida* (section *Hedriocarpae*, sub-section *Macrostachyae*) contains crotastratine, integerrimine, mucronatinine, nilgerine and usaramine (Saxton, 1971; Atal & Sawhney, 1973; Batra *et al.*, 1975); *C. retusa* (section *Crotalaria*, sub-section *Longirostres*) exhibits monocrotaline, retrocenine, retusamine and retusine (Atal & Sawhney, 1973). The pyrrolizidine alkaloid monocrotaline, isolated from *C. spectabilis*, inhibits root infestation of susceptible plants (Fassuliotis & Skucas, 1969). Consequently, the broad spectrum of activity on nematodes from shoots and root aqueous extracts of 15 West African *Crotalaria* species could be due to significant differences in alkaloid composition and content of plant tissues.

The PCA analysis also revealed that the paralysis of each of the three *Meloidogyne* species varied with plant parts. Root extracts seem to be more efficient than shoot extracts. However, according to the weight ratios observed between the shoots and the roots of each *Crotalaria* species (Table 2), the shoot input may be a major contributor to the green manure nematostatic potential. *Crotalaria barkae*, *C. grantiana*, *C. pallida* and *C. podocarpa* were the most efficient species whatever the *Meloidogyne* species targeted. *Crotalaria atrorubens*, *C. hyssopifolia*, *C. retusa* and *C. senegalensis* were also effective in control of *M. javanica* and *M. incognita*. In addition, considering the plant weight ratio, *C. hyssopifolia* and *C. sphaerocarpa* showed promise for control of *M. mayaguensis*.

Crotalaria grantiana, *C. pallida* and *C. retusa* have been already reported as resistant to the most important plant-parasitic nematodes when used as cover crops, rotations and green manure (Wang *et al.*, 2002). So, considering the great potential of West African *Crotalaria* species such as *C. atrorubens*, *C. barkae*, *C. comosa*, *C. hyssopifolia*, *C. podocarpa*, *C. senegalensis* and *C. sphaerocarpa* as nematostatic green manure, these leguminous could be cultivated in West Africa as inter-crops and ploughed into soils rather than being considered as weeds (Le Bourgeois *et al.*, 1997). These leguminous plants have considerable potential for use by farmers in developing countries to control nematode populations in low-value crop systems.

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Two Distinct *crt* Gene Clusters for Two Different Functional Classes of Carotenoid in *Bradyrhizobium**

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Aerobic photosynthetic bacteria possess the unusual characteristic of producing different classes of carotenoids. In this study, we demonstrate the presence of two distinct *crt* gene clusters involved in the synthesis of spirilloxanthin and canthaxanthin in a *Bradyrhizobium* strain. Each cluster contains the genes *crtE*, *crtB*, and *crtI* leading to the common precursor lycopene. We show that spirilloxanthin is associated with the photosynthetic complexes, while canthaxanthin protects the bacteria from oxidative stress. Only the spirilloxanthin *crt* genes are regulated by light via the control of a bacteriophytochrome. Despite this difference in regulation, the biosyntheses of both carotenoids are strongly interconnected at the level of the common precursors. Phylogenetic analysis suggests that the canthaxanthin *crt* gene cluster has been acquired by a lateral gene transfer. This acquisition may constitute a major selective advantage for this class of bacteria, which photosynthesize only under conditions where harmful reactive oxygen species are generated.

Carotenoids comprise a large class of pigments that are widely distributed in living organisms. They are synthesized by all photosynthetic organisms from bacteria to plants where they play at least three essential functions (1). First, they act as accessory light-harvesting pigments by absorbing light in the 450–570 nm region. Second, they are important for the assembly and stability of some of these light-harvesting complexes. Finally they operate as photoprotectors by directly quenching both triplet excited (bacterio)chlorophylls and singlet oxygen. Carotenoids are also synthesized by a wide variety of non-photosynthetic bacteria (2). Less is known about their precise function in these bacteria, but it is well accepted that their strong antioxidant character may protect the organisms against (photo)oxidative damage.

A remarkable feature of aerobic phototrophic bacteria, besides their ability to photosynthesize only under aerobic condi-

tion, is their carotenoid composition. Indeed most strains synthesize, in addition to the carotenoids involved in photosynthesis such as spirilloxanthin, a large amount of unusual carotenoid molecules (3). The most striking complexity is observed for *Erythrobacter* species such as *Erythrobacter longus* or *Erythrobacter ramosus*, which have been reported to produce about 20 different carotenoids (4, 5). Another example comes from various strains of photosynthetic *Bradyrhizobium*, symbionts of *Aeschynomene* (6), which synthesize, in addition to spirilloxanthin, large amounts of canthaxanthin of unknown function (7, 8).

All carotenoids are synthesized from geranylgeranyl pyrophosphate. This compound is formed by the enzyme geranylgeranyl pyrophosphate synthase (*CrtE*), which catalyzes the condensation of farnesyl pyrophosphate with an isopentyl pyrophosphate moiety. The second step catalyzed by phytoene synthase (*CrtB*) is the formation of phytoene from the head-to-head condensation of two molecules of geranylgeranyl pyrophosphate. Subsequent dehydrogenations catalyzed by the phytoene desaturase (*CrtI*) convert the phytoene to neurosporene in three desaturation steps or to lycopene in four steps. After the action of these three enzymes (*CrtE*, *CrtB*, and *CrtI*), the biosynthetic pathways diverge depending on the species leading to the accumulation of various different carotenoids. The synthesis of canthaxanthin from lycopene necessitates two enzymes: *CrtY*, which catalyzes cyclization of lycopene leading to β -carotene, and *CrtW*, which oxygenates β -carotene to form canthaxanthin (see Fig. 1A) (9). The sequence of the reactions from lycopene to spirilloxanthin includes the successive reactions of hydration, desaturation, and methylation catalyzed, respectively, by the three enzymes *CrtC*, *CrtD*, and *CrtF* (see Fig. 1A) (10, 11). These reactions are performed first on one-half of the molecule and then on the other half.

The genes encoding many carotenoid biosynthetic enzymes (*crt* genes) have been characterized in plants and in various bacteria (12, 13). In bacteria, they are always found clustered, except in the Cyanobacteria. In purple photosynthetic bacteria, the genes involved in carotenoid biosynthesis are localized within the photosynthesis gene cluster, a 45-kb DNA region that contains the essential genes involved in the synthesis of the photosynthetic apparatus (14–17). In aerobic photosynthetic bacteria, a *crt* gene cluster has been characterized for the *Bradyrhizobium* ORS278 strain (18). This cluster contains the five *crt* genes, *crtE*, *crtY*, *crtI*, *crtB*, and *crtW*, necessary for the canthaxanthin biosynthesis. Neither photosynthesis genes

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF182374.

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nor specific genes of spirilloxanthin biosynthesis from lycopene (*crtC*, *crtD*, or *crtF*) have been identified in this cluster.

Light stimulation of carotenoid biosynthesis has been reported in numerous organisms including plants, fungi, and bacteria (19). In higher plants, regulation of carotenoid biosynthesis occurs at the level of phytoene synthase expression (20). This expression is controlled by a phytochrome, a plant photoreceptor that mediates response to red and far-red light through photoconversion between two stable conformations, a red-absorbing form (Pr) and a far-red-absorbing form (Pfr). Biochemical and genetic studies have recently demonstrated the occurrence of phytochrome-like proteins in photosynthetic and non-photosynthetic bacteria (21, 22). Such a (bacterio)phytochrome appears to control the synthesis of the carotenoid deinoxanthin in the non-photosynthetic bacteria *Deinococcus radiodurans* (23).

In this report, we describe the characterization of a second *crt* gene cluster, in the *Bradyrhizobium* ORS278 strain, coding the enzymes of spirilloxanthin synthesis. This second *crt* gene cluster contains all the genes necessary for the synthesis of spirilloxanthin from farnesyl pyrophosphate. Biochemical analysis and phenotypes of mutants deleted in specific genes of canthaxanthin and spirilloxanthin synthesis allow us to establish the involvement of spirilloxanthin in the photosynthesis activity and the protective role of canthaxanthin in response of the bacteria to oxidative stress. We also demonstrate that the spirilloxanthin *crt* genes are specifically regulated by light via the control of a bacteriophytochrome. These results provide the first demonstration of two independent and differently regulated *crt* gene clusters in a living organism.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—*Bradyrhizobium* sp. strain ORS278 (wild-type strain) and isogenic mutants were grown in a modified yeast extract mannitol agar medium with addition of the appropriate antibiotic when required (24). All the strains were cultured for 7 days in sealed Petri dishes at 35 °C in either complete darkness or different continuous illumination conditions provided by light-emitting diodes of different wavelengths between 590 and 870 nm with an irradiance of 6.6 μmol of photons/m²/s. *Escherichia coli* was grown in Luria-Bertani (LB) medium supplemented with the appropriate antibiotics.

Pigment Analysis—Cells grown at the surface of the Petri dishes in the dark or under different light conditions were resuspended in 6 ml of water + 9 g/liter NaCl and centrifuged for 10 min at 4,000 $\times g$. The pellets were extracted three times in the dark with 1 ml of cold acetone/methanol (7:2, v/v). The carotenoids in the pooled extracts were analyzed by HPLC¹ using a Waters Alliance 2690 system. The conditions were: 5- μm Hypersil C₁₈ column (250 $\times$ 4.6 mm, Alltech), acetonitrile/methanol/isopropanol (40:50:10, v/v/v) as eluent, 0.8 ml/min flow rate. The eluted fractions were monitored using a Waters 996 photodiode array detector scanning from 270 to 600 nm every 2 s. Carotenoids were identified by their retention times and by comparison of the spectral features with those of pure compounds or with reported data. The amount of canthaxanthin was determined from the area of the peak detected at 480 nm using a calibration curve obtained with a canthaxanthin standard kindly provided by Aventis. The amount of spirilloxanthin was estimated from the area of the peak detected at 494 nm using the canthaxanthin correlation coefficient due to the lack of spirilloxanthin standard. The data represent the mean of three independent cultures.

Light Action Spectrum of *crt* Gene Expression—The mutants harboring the various *lacZ-crt* fusions were grown under continuous illumination with low irradiance of different wavelengths as described previously (25). After growth, the cells under the illuminated area were resuspended in 3 ml of water, and β -galactosidase activity was measured as described previously (24).

Construction of *crt* Mutant Strains—Constructions of mutants de-

leted in the bacteriophytochrome (278 Δ *BrbphP*) and in the transcriptional factor PpsR (278 Δ *ppsR*) have been described previously (25). For the construction of a *crtCD* mutant (278 Δ *crtCD*), a region of about 3.5 kb containing *crtC* and *crtD* genes of ORS278 was amplified by PCR using the primers 5'-TAGTCGACGCAATGGCGCGCCACGATCTATC-3' and 5'-ACAGTCGACCGGTCTTGAGCGGTGATAATG-3' and subsequently cloned in the pGEM-T vector (Promega, Madison, WI). A 2-kb region containing part of the *crtC* and *crtD* genes was deleted by *Xho*I digestion and replaced by the 4.7-kb *Sal*I *LacZ-Km*^r cassette of pKOK5 (26). The resulting 6.2-kb *Sal*I insert containing the mutated *crtC* and *crtD* genes was cloned into the pJQ200mp18 suicide vector (27). To mutate the *crtE* gene of the canthaxanthin *crt* gene cluster, a region of about 1.2 kb containing *crtE* gene was amplified by PCR using the primers 5'-GGTAGATCTGGTCTGCATGCGCGGATGAAACAG-3' and 5'-GGAAGATCTCGAAGGCAGGTTTCAGAGTATG-3', digested by *Bgl*II, and cloned into the *Bam*HI sites of pJQ200mp18. The 4.7-kb *Bam*HI *LacZ-Km*^r cassette of pKOK5 was then inserted into the unique *Bam*HI site of *crtE*. To mutate the *crtY* gene, a 1.8-kb *Pst*I fragment of pSTM78 (18) containing *crtY* gene was cloned into the *Pst*I sites of pJQ200mp18. A 0.4-kb *Xho*I fragment containing part of *crtY* was then deleted and replaced by the 4.7 *Sal*I *LacZ-Km*^r cassette of pKOK5. To mutate the *crtI* gene of the spirilloxanthin *crt* gene cluster, a region of 1.4 kb was amplified by PCR using the primers 5'-CGGGATCCTTGCTGGCGAAAGCGTCAATTTC-3' and 5'-CGGGATCCAGGACGACAGCGCTGCTCGAAATC-3', digested by *Bam*HI, and cloned into the *Bam*HI sites of pJQ200mp18. The 4.7-kb *Sal*I *LacZ-Km*^r cassette of pKOK5 was then inserted into the unique *Xho*I site of *crtI*. For each construction, we verified by PCR and sequencing that the *lacZ* reporter gene was in the correct orientation. The pJQ200 derivatives obtained, which encoded a counterselective *sacB* marker, were transformed into *E. coli* S17-1 for mobilization into ORS278 as described previously (24). Double recombinants were selected on sucrose, and the insertion was confirmed by PCR.

Preparation of Membranes and RC-LH1 Complexes—A 200-ml culture of *Bradyrhizobium* (wild type (WT) or mutant) was collected and resuspended in 10 ml of Tris-HCl buffer (50 mM, pH 8). The cells were disrupted by three passages through a French press at 50 megapascals. The suspension was centrifuged for 10 min at 4,000 $\times g$ to remove the unbroken cells and cells debris. The supernatant was loaded on a discontinuous sucrose gradient (0.6–1.2 M sucrose, 50 mM Tris-HCl buffer, pH 8) and centrifuged at 255,000 $\times g$ for 90 min. The membranes localized at the interface of the two sucrose layers constitute the chromatophore fraction, while the pellet contains the cytoplasmic membranes. Each fraction was diluted in 25 ml of Tris-HCl buffer (50 mM, pH 8), centrifuged at 255,000 $\times g$ for 90 min to removed the sucrose, and then resuspended in Tris-HCl buffer (10 mM, pH 8).

RC-LH1 complexes were isolated by addition of 1.5% LDAO to purified chromatophores or cytoplasmic membranes whose optical density was adjusted to 5 OD/cm at 870 nm. After an incubation of 15 min at room temperature in the dark, the membrane suspension was loaded on a discontinuous sucrose gradient (0.1, 0.2, 0.3, 0.6 M sucrose, 50 mM Tris-HCl, pH 8, 0.02% LDAO) and centrifuged at 255,000 $\times g$ for 90 min. All canthaxanthin molecules do not enter the sucrose gradient. The RC-LH1 particles, collected at the interface between the 0.2 and 0.3 M sucrose layers, were diluted in Tris-HCl (50 mM, pH 8, 0.02% LDAO) and centrifuged at 255,000 $\times g$ for 180 min. They were resuspended in Tris-HCl (10 mM, pH 8, 0.02% LDAO). Further purification of the canthaxanthin-containing fraction and of the RC-LH1 particles was performed using a mono-Q column coupled to an FPLC (Amersham Biosciences) and submitted to a NaCl gradient.

Absorption and Fluorescence Spectroscopy—Absorption spectra and light-induced absorption changes in intact cells were measured as described previously (24). Fluorescence excitation and emission spectra were recorded on a Spex Fluorolog 3 spectrofluorometer (Jobin Yvon). For excitation spectra, the excitation slits were 5 nm, and the emission was measured at 870 nm (on the blue side of the emission spectrum where the instrument sensitivity was highest) with 15-nm slits. For emission spectra excitation was through 10-nm slits, and emission was measured through 7-nm slits. Emission spectra were corrected for the wavelength dependence of the instrument response, and excitation spectra were corrected for variations in excitation intensity. For all fluorescence spectra the detector was protected from scattered excitation by a Wratten 88A gelatin filter.

RESULTS

Isolation and Characterization of the Spirilloxanthin Biosynthesis Genes—In all photosynthetic bacteria studied so far, the

¹ The abbreviations used are: HPLC, high pressure liquid chromatography; RC, reaction center; LH, light harvesting; WT, wild type; LDAO, lauryldimethylamine oxide.

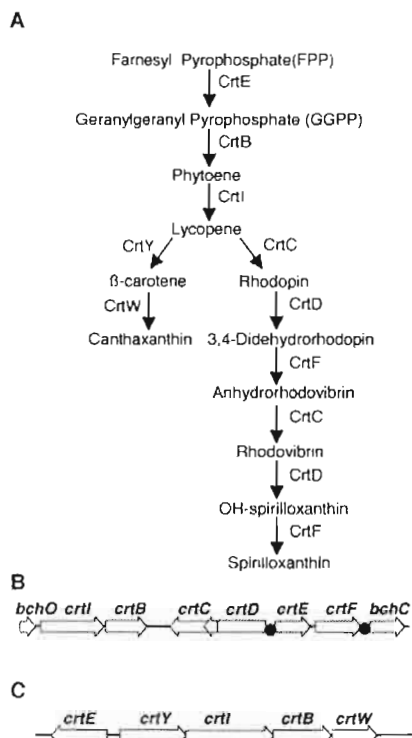


FIG. 1. Biosynthesis of canthaxanthin and spirilloxanthin. A, carotenoid biosynthesis pathways leading to canthaxanthin and spirilloxanthin. B and C, organizations of the canthaxanthin (B) and spirilloxanthin (C) gene clusters of *Bradyrhizobium* sp. strain ORS278. Filled circles indicate the positions of PpsR binding sites.

carotenoid biosynthesis genes have been found linked to the photosynthesis gene cluster. We have previously isolated, from a genomic DNA library of the ORS278 strain, a cosmid (pSTM1) with an insert of ~35 kb that contains some photosynthesis genes (24). Its partial sequencing reveals a gene arrangement close to that of purple photosynthetic bacteria with the superoperon structure *bchCXYZpufBALM* conserved. This prompted us to check whether the upstream region of *bchC* gene contains some of the carotenoid synthesis genes as observed in other photosynthetic bacteria. The sequencing of this region reveals the presence of six open reading frames encoding proteins with similarities to known Crt enzymes assigned to *crtE*, *crtF*, *crtC*, *crtD*, *crtI*, and *crtB* genes (Fig. 1B). The orientation of these open reading frames suggests the existence of a minimum of three operons (*crtEF*, *crtCD*, and *crtIB*) as usually observed in purple photosynthetic bacteria (2). The overlap observed between *crtD* and *crtC* (348 bp) was also reported in *Rubrivivax gelatinosus* (28). The canthaxanthin *crt* gene cluster (Fig. 1C) was previously isolated from the cosmid pSTM73 (18). We showed by PCR that there is no overlap between this cosmid and the cosmid pSTM1 containing the spirilloxanthin gene cluster. Together these results demonstrate the presence of two distinct *crt* gene clusters in *Bradyrhizobium* ORS278, a spirilloxanthin gene cluster localized in the photosynthesis gene cluster region and a canthaxanthin gene cluster localized in a different part of the genome. Interestingly the genes *crtE*, *crtI*, and *crtB* that encode enzymes of lycopene biosynthesis, a common precursor of canthaxanthin and spirilloxanthin, are found in both clusters. However, they are obviously different and show little similarity (38% identity between the two CrtE proteins and 43 and 51.6% for the CrtB and CrtI pairs, respectively). To distinguish these genes, we call the genes from the spirilloxanthin *crt* cluster

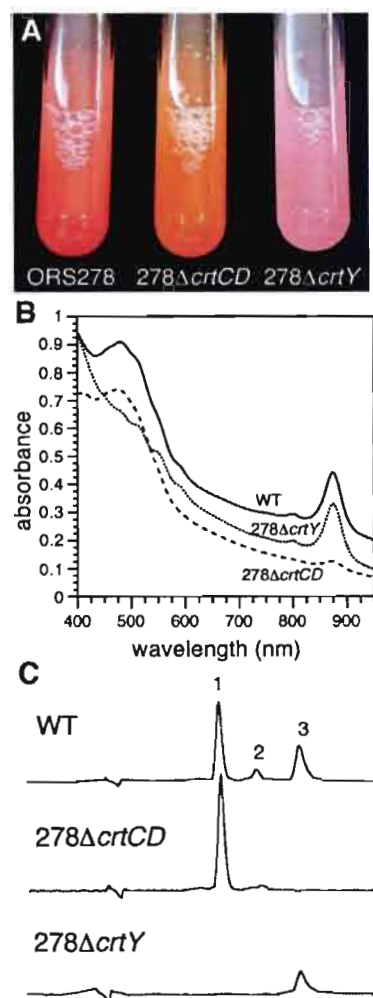
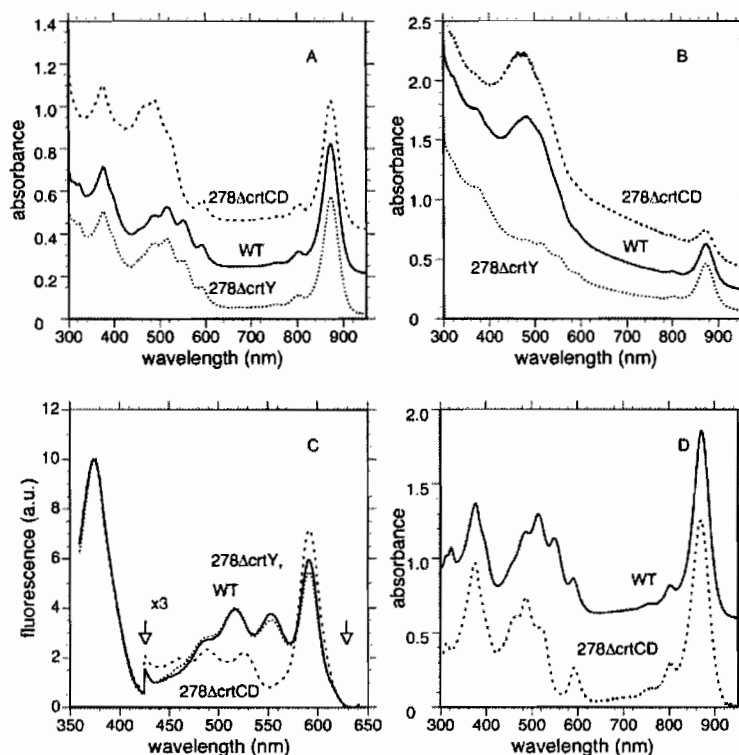


FIG. 2. Carotenoid analyses of *Bradyrhizobium* ORS278 wild-type strain and the mutants 278ΔcrtCD and 278ΔcrtY. A, pigmentation of WT strain and the mutants 278ΔcrtCD and 278ΔcrtY. B, absorption spectra of intact cells for the WT strain (continuous line) and the mutants 278ΔcrtCD (dashed line) and 278ΔcrtY (dotted line). The absorbency level at 950 nm has been arbitrarily adjusted to avoid overlapping spectra. C, HPLC separation of the carotenoids of WT strain and the mutants 278ΔcrtCD and 278ΔcrtY. Peaks correspond to the following: 1, *trans*-canthaxanthin; 2, *cis*-canthaxanthin isomers; 3, spirilloxanthin.

crtE.s, *crtI.s*, and *crtB.s* and those from the canthaxanthin *crt* cluster *crtE.c*, *crtI.c*, and *crtB.c*.

To demonstrate that the canthaxanthin and the spirilloxanthin *crt* gene clusters we have identified are each responsible for the biosynthesis of the appropriate one of these two carotenoids, we constructed a mutant deleted in *crtY* gene (278ΔcrtY strain, canthaxanthin *minus*) and a mutant deleted in the *crtCD* genes (278ΔcrtCD strain, spirilloxanthin *minus*). The pigmentation of both mutants is clearly different from the WT strain (Fig. 2A). The 278ΔcrtCD mutant presents an orange color characteristic of canthaxanthin, while the 278ΔcrtY mutant deleted in a canthaxanthin gene is pink as is typical for spirilloxanthin. These variations in color are essentially due to the difference in absorption profiles of the carotenoids in the 400–600 nm region of the three strains (Fig. 2B). HPLC analysis of the carotenoids extracted from each mutant (Fig. 2C) confirmed the absence of spirilloxanthin in the 278ΔcrtCD mutant as well as the absence of canthaxanthin in the 278ΔcrtY mutant.

FIG. 3. Absorption and fluorescence properties of *Bradyrhizobium* ORS278 wild-type strain and the mutants $278\Delta crtCD$ and $278\Delta crtY$. Absorption spectra of the intracytoplasmic (A) and cytoplasmic membranes (B) isolated from the WT strain (continuous line) and the mutants $278\Delta crtCD$ (dashed line) and $278\Delta crtY$ (dotted line). C, fluorescence excitation spectra for intact cells of the WT (continuous line) and the mutants $278\Delta crtCD$ (dashed line) and $278\Delta crtY$ (dotted line). The fluorescence emission is measured around 990 nm; between 425 and 630 nm the fluorescence spectra have been multiplied by a factor 3. For A, B, and D, the absorbency level at 950 nm has been arbitrarily adjusted to avoid overlapping spectra. D, absorption spectra of the purified RC-LH1 particles isolated from the WT strain (continuous line) and the mutant $278\Delta crtCD$ (dashed line). a.u., arbitrary units.



Membrane Localization and Function of Canthaxanthin and Spirilloxanthin—Aerobic photosynthetic bacteria are known to possess a small amount of photosynthetic apparatus correlated with few invaginations of the cytoplasmic membrane (5). In a first attempt to clarify the function of the two carotenoids present in *Bradyrhizobium* ORS278, cytoplasmic and intracytoplasmic fractions of the membrane were separated on a sucrose gradient after breakage of the WT cells. The two types of membranes could be separated as a function of their density (see “Experimental Procedures”). The cytoplasmic membranes form a pellet, while the intracytoplasmic membrane fragments (chromatophores) sediment at the interface between 0.6 and 1.2 M sucrose layers. The absorption spectrum of the chromatophores reveals the presence of bacteriochlorophyll (873, 590, and 375 nm) and spirilloxanthin (548, 515, and 485 nm) molecules but no detectable canthaxanthin (Fig. 3A). In contrast the cytoplasmic membrane contains a large amount of canthaxanthin in addition to the photosynthetic pigments (Fig. 3B). From the relative amounts of bacteriochlorophyll molecules present in these two fractions, we deduce that about 40% of the photosynthetic units are present in the chromatophores, while the rest is localized in the cytoplasmic membrane. This experiment clearly shows that spirilloxanthin molecules are associated with the photosynthetic apparatus in agreement with previous observations on several other species (10). Another proof that only spirilloxanthin molecules are associated with the photosynthetic apparatus was obtained by the measurement of the excitation spectrum of intact cells of the bacteriochlorophyll fluorescence around 890 nm (Fig. 3C). Comparison between the absorption spectrum of intact cells (Fig. 2B) and the excitation spectrum associated with the fluorescence emission of the LH1 complexes (Fig. 3C) shows that only the spirilloxanthin (at 550, 515, and 485 nm) molecules are able to transfer energy to the photosynthetic units. There is no evidence of energy transfer from canthaxanthin. In addition, identical excitation spectra are measured for both the WT and the $278\Delta crtY$ mutant (can-

thaxanthin minus) (Fig. 3C). These results clearly demonstrate that spirilloxanthin, but not canthaxanthin, transfers light energy to the photosynthetic apparatus. An additional proof comes from the characteristics of the absorption spectrum of purified RC-LH1 complexes. These complexes can be easily purified from the cytoplasmic or the chromatophore membranes after addition of 1.5% LDAO (see “Experimental Procedures”). These particles do not contain any canthaxanthin as shown by both their absorption spectrum (Fig. 3D) and analysis of their carotenoid content (data not shown). The phenotypes of mutants deleted in one of the genes of the canthaxanthin ($278\Delta crtY$) or the spirilloxanthin ($278\Delta crtCD$) pathways also demonstrate that spirilloxanthin and not canthaxanthin is associated with the photosynthetic apparatus. Indeed a similar amount of photosynthetic apparatus is present in intact cells of the WT or of the canthaxanthin minus mutant ($278\Delta crtY$) (see Fig. 2B) demonstrating that the lack of canthaxanthin does not affect its formation. In contrast, the amount of photosynthetic apparatus is reduced by at least a factor of 3–5 in the spirilloxanthin minus mutant ($278\Delta crtCD$) compared with the WT as shown by the comparison of their absorption spectra (see Fig. 2B) or measurement of light-induced photooxidation of the cytochrome on intact cells (data not shown). In fact, the chemical analysis of the carotenoid content of this mutant reveals the presence of a significant amount of lycopene (0.05 mg/g of cells) (data not shown). This carotenoid is associated with the small fraction of photosystem present in the $278\Delta crtCD$ mutant as demonstrated by the absorption spectrum of the chromatophore fraction and of purified isolated RC-LH1 complexes (Fig. 3, A and D) and the fluorescence excitation spectrum (Fig. 3C), which all present the characteristic bands of lycopene around 520, 487, and 460 nm.

Canthaxanthin and RC-LH1 complexes can be easily extracted from the cytoplasmic membranes. The fraction containing the canthaxanthin does not enter the sucrose gradient, while the RC-LH1 complexes sediment at the 0.6–1.2 M inter-

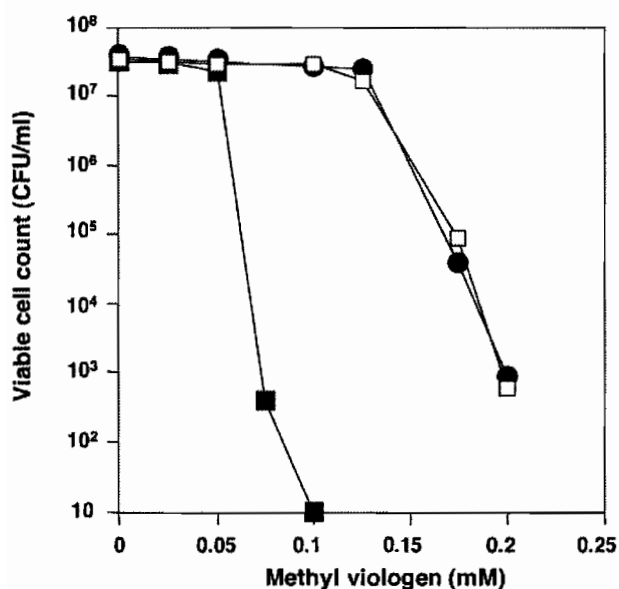


FIG. 4. Effect of methyl viologen on the viability of *Bradyrhizobium* ORS278 wild-type strain (open squares) and the mutants 278ΔcrtCD (filled circles) and 278ΔcrtY (filled squares). Exponentially growing cells (WT, 278ΔcrtCD, and 278ΔcrtY) were diluted and plated onto yeast extract mannitol agar medium containing various concentrations of methyl viologen. Plates were incubated at 37 °C, and after 1 week the colony-forming units (CFU) were counted.

face (not shown). Further purification of the canthaxanthin fraction on a Mono-Q column followed by gel electrophoresis shows that the canthaxanthin is not associated with protein. This suggests that, unless the detergent has destroyed a weak protein-canthaxanthin association, canthaxanthin is present in the lipid phase of the membrane and not associated with specific polypeptides.

One putative function of canthaxanthin is to act as strong antioxidant protecting against (photo)oxidative damage. To test this hypothesis, we measured the survival of bacteria exposed to an oxidative stress caused by addition of methyl viologen for both the WT and the 278ΔcrtY and 278ΔcrtCD mutants. As seen on Fig. 4, the canthaxanthin *minus* mutant (278ΔcrtY) is more sensitive to the addition of increasing concentrations of methyl viologen than either the WT strain or the spirilloxanthin *minus* mutant (278ΔcrtCD). In addition, the canthaxanthin *minus* mutant is less resistant than the WT strain in patch assays with H₂O₂ as the oxidative stress inducer (data not shown). These results are a clear indication that canthaxanthin acts as a protective agent against oxidative stress in *Bradyrhizobium* ORS278 cells.

Regulation of Canthaxanthin and Spirilloxanthin Synthesis by Light—We have previously shown that the photosynthetic activity in *Bradyrhizobium* is stimulated by far-red light through the action of the bacteriophytochrome BrbphP (25). The *BrbphP* gene is localized close to the photosynthesis gene cluster and contiguous to an open reading frame homologous to the transcription factor PpsR. The PpsR protein is known to repress *crt* genes in *Rhodobacter* species at high oxygen tension or high light intensity (29, 30). The up-regulation of photosynthesis genes by far-red light illumination observed in *Bradyrhizobium* ORS278 strain results from the antirepressor effect of the Pr form of BrbphP on PpsR (25). This prompted us to clarify the role of both BrbphP and PpsR in the biosynthesis of spirilloxanthin and canthaxanthin in this species. In a first set of experiments we investigated the effect of light quality in the red/far-red region on the production of both carotenoids. Fig.

5A clearly shows that the production of both carotenoids synthesized is enhanced in the 740–780 nm region, i.e. by the formation of the Pr form of BrbphP. Under far-red light, production of canthaxanthin is double, whereas the production of spirilloxanthin is triple, compared with the level measured in the dark (Fig. 5, B and C). To prove that the increase of carotenoids synthesis induced by far-red light is under the control of BrbphP and PpsR, we analyzed the carotenoid content (Fig. 5, B and C) of the *BrbphP* and *ppsR* null mutants obtained previously (25). The *BrbphP* *minus* mutant displays a very low level of production of spirilloxanthin independent of the growth conditions, whereas deletion of the *ppsR* gene leads to the overproduction of this carotenoid again whatever the growth conditions (Fig. 5B). Both mutants also have opposite effects on canthaxanthin production with a decrease for the 278Δ*BrbphP* mutant and an increase for the 278Δ*ppsR* mutant, once more in both cases independent of the growth conditions (Fig. 5C). Together these results show clearly that far-red light controls the synthesis of canthaxanthin and spirilloxanthin via the dual actions of the bacteriophytochrome BrbphP and the transcription factor PpsR.

To further elucidate the molecular mechanisms that lead to the stimulation of spirilloxanthin and canthaxanthin by far-red light, we determined the effect of far-red light on the expression of the *crt* genes of both clusters with the help of mutants carrying *lacZ* fusions. The expression of both of the spirilloxanthin *crt* genes assayed (*crtI.s* and *crtD*) is strongly stimulated by far-red light with an action spectrum corresponding to the Pfr form of BrbphP (Fig. 5D). In contrast, the expression of the canthaxanthin *crt* genes tested (*crtE.c* and *crtY*) remains at the same level whatever the light conditions (Fig. 5D). These data demonstrate that only the spirilloxanthin *crt* genes are up-regulated by far-red light via the action of the Pr form of BrbphP. These results are in agreement with the profiles of the promoter regions of the *crt* genes of both clusters. The repressive action of PpsR in *Rhodobacter* species results from its ability to stop transcription by binding DNA with the palindromic sequence TGTN₁₂ACA present in the promoter regions of some photosynthesis genes (30). One PpsR DNA binding motif can be identified in the spirilloxanthin *crt* gene cluster, whereas such a motif is not found in the canthaxanthin *crt* gene cluster. The interaction of PpsR with the promoters of the spirilloxanthin pathway was confirmed by DNA footprint analyses with purified PpsR protein (data not shown). PpsR binds to the intergenic region between *crtE.s* and *crtC* that overlaps the promoter regions of the two putative operons *crtEF* and *crtCD* in the spirilloxanthin cluster. In contrast, a DNA probe that overlaps the promoter regions between the two putative operons *crtE* and *crtYIBW* of the canthaxanthin cluster is not protected from DNase I digestion by addition of PpsR. We therefore conclude that only the expression of the spirilloxanthin *crt* genes are under the control of PpsR and BrbphP. There is a clear discrepancy between this conclusion and the observed enhancement of the production of canthaxanthin by far-red light (Fig. 5A) and the carotenoid content of the *BrbphP* and *ppsR* null mutants (Fig. 5C). To resolve this apparent contradiction, we hypothesize that the up-regulation of spirilloxanthin *crt* genes by the phytochrome leads to the overproduction of some common precursors to the biosynthesis pathway of canthaxanthin and spirilloxanthin. For example lycopene produced by the *CrtE.s*, *CrtB.s*, and *CrtI.s* enzymes could be partially rerouted by the *CrtY* and *CrtW* enzymes to form canthaxanthin. To test this hypothesis we measured the carotenoid content in mutants deleted in one of the genes of the enzymes of lycopene synthesis in each carotenoid pathway (278Δ*crtE.c* and 278Δ*crtI.s*). These two mutants produce both canthaxan-

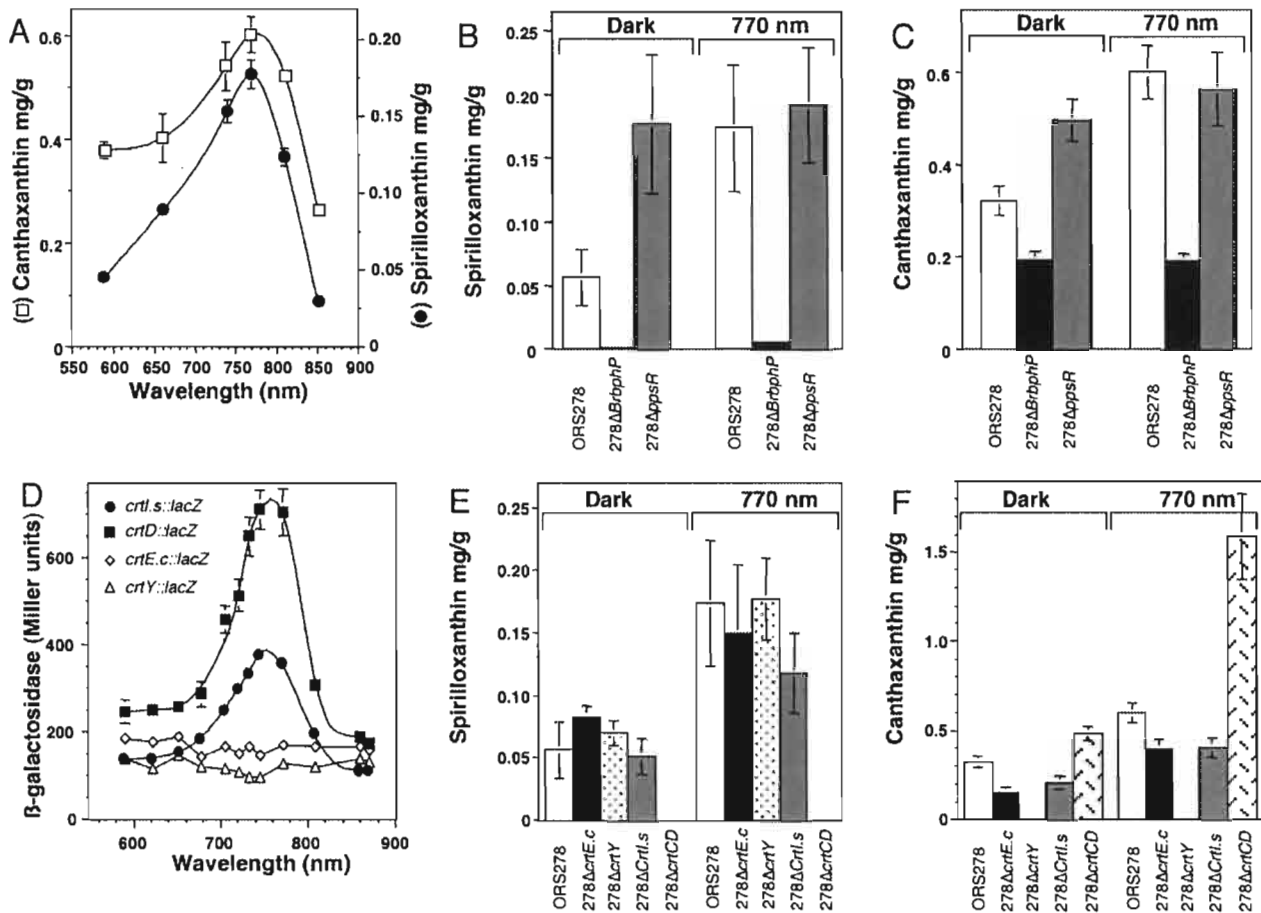


FIG. 5. Effect of illumination on canthaxanthin and spirilloxanthin production. The data in all the different panels represent the mean of three experiments (errors bars indicate $\pm$ S.D.). The amount of carotenoids is indicated in milligrams per gram of dry cell weight. A, light action spectra for canthaxanthin (open squares) and spirilloxanthin (filled circles) production by *Bradyrhizobium* ORS278 wild-type strain. B, production of spirilloxanthin in WT strain (ORS278) and the 278 Δ BrbphP and 278 Δ ppsR mutants after growth under complete darkness or far-red light (770 nm). C, production of canthaxanthin in WT strain (ORS278) and the 278 Δ BrbphP and 278 Δ ppsR mutants after growth under complete darkness or far-red light (770 nm). D, light action spectra of various *crt* gene expression. β -Galactosidase activity of the strains harboring the various *lacZ-crt* fusions is indicated in miller units. E, production of spirilloxanthin in WT strain (ORS278) and the 278 Δ crtE.c, 278 Δ crtY, 278 Δ crtI.s, and 278 Δ crtCD mutants after growth under complete darkness or far-red light (770 nm). F, production of canthaxanthin in WT strain (ORS278) and the 278 Δ crtE.c, 278 Δ crtY, 278 Δ crtI.s, and 278 Δ crtCD mutants after growth under complete darkness or far-red light (770 nm).

thin and spirilloxanthin demonstrating a cross-talk between the two carotenoid synthesis pathways (Fig. 5, E and F). Other proofs of this link are the lower level of canthaxanthin produced by the 278 Δ crtI.s mutant (Fig. 5F) and the strong enhancement by far-red light of canthaxanthin production especially in the 278 Δ crtCD mutant. These different observations imply that *crtE.s*, *crtI.s*, or *crtB.s* genes can complement, respectively, for the deletion of the *crtE.c*, *crtI.c*, or *crtB.c* genes and vice versa. We therefore conclude that the biosynthesis of spirilloxanthin and canthaxanthin are strongly connected at the level of lycopene or one of its precursors.

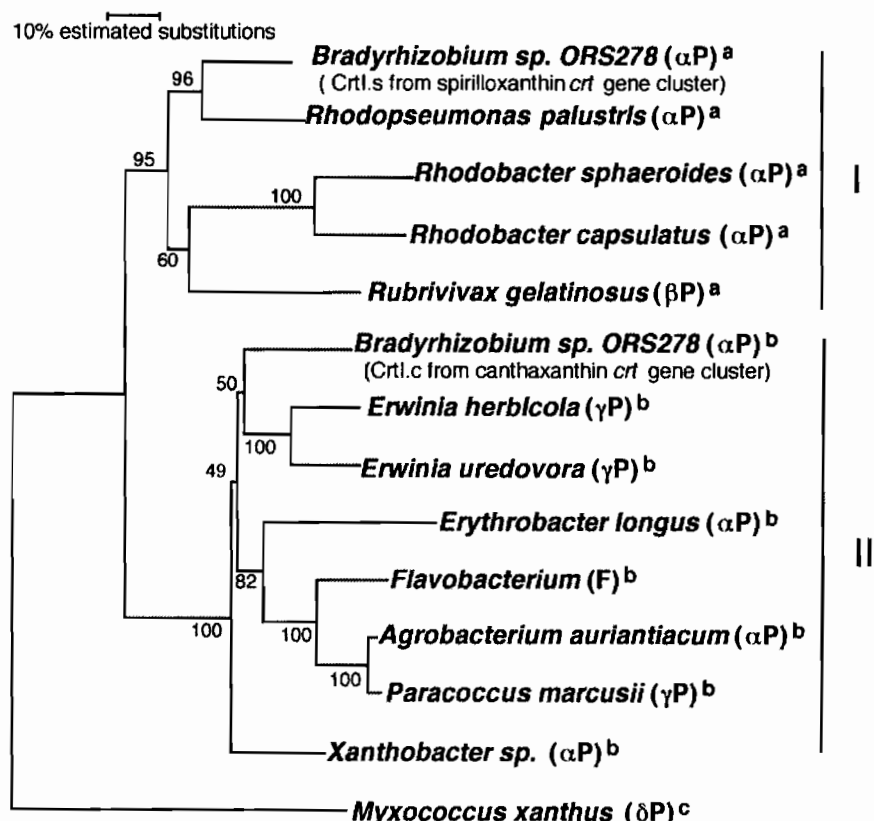
DISCUSSION

Aerobic phototrophic bacteria present a surprising complex carotenoid composition. In the present study, our goal was to clarify this complexity using the bacterial model *Bradyrhizobium* ORS278, which synthesizes two major carotenoids, the linear spirilloxanthin and the bicyclic canthaxanthin. Combining biophysical, biochemical, and genetic approaches, we determined the function of each of these carotenoids, characterized their biosynthesis genes, and described their regulation. We demonstrate the presence of two distinct carotenoids gene clusters involved in the biosynthesis of spirilloxanthin and of can-

thaxanthin, respectively. One striking result is the presence in each of these clusters of the three genes, *crtE*, *crtB*, and *crtI*, implicated in the synthesis of the precursor lycopene, common to the two biosynthetic pathways. Altogether these results, discussed in more detail below, give the first indications to an understanding of the presence of different carotenoids in a photosynthetic bacterium.

Functions of Spirilloxanthin and Canthaxanthin—Biochemical analysis and phenotypes of mutants clearly demonstrate that the spirilloxanthin molecules are the only carotenoid associated with the photosynthetic apparatus in *Bradyrhizobium* ORS278. Comparison between the excitation spectrum and the absorption spectrum leads to the conclusion that only about 30% of the light energy absorbed by the spirilloxanthin molecules are transferred to the bacteriochlorophyll molecules. This efficiency is typical to spirilloxanthin molecules associated with LH1 as already observed in the case of *Rhodospirillum rubrum* for example (31). When the synthesis of spirilloxanthin is blocked at the CrtC and CrtD enzyme level, spirilloxanthin is replaced by lycopene in the LH1 complexes and probably in the RC. Lycopene has also been shown recently to be an integral part of the LH2 complexes in a mutant of *Rhodobacter spha-*

FIG. 6. Phylogenetic tree based on the *CrtI* sequences. This tree has been constructed by using the neighbor-joining method (40). Bootstrap values (41), expressed as percentages of 1000 replications, are given at the branching points. P, Proteobacteria; F, Flavobacteria; a, bicyclic carotenoid; b, acyclic carotenoid; c, monocyclic carotenoid. The GenBankTM accession numbers are: AF218415, *CrtI.c* *Bradyrhizobium* sp. ORS278; AF182374, *CrtI.s* *Bradyrhizobium* sp. ORS278; D58420, *Agrobacterium aurantiacum*; Y15112, *Paracoccus marcusii*; U62808, *Flavobacterium* sp.; M87280, *E. herbicola* EHO10; D90087, *Erwinia uredovora*; ZP_00011091, *R. palustris*; AF195122, *R. sphaeroides*; X52291, *R. capsulatus*; AB034704, *R. gelatinosus*; AF408840 *Xanthobacter* sp.; Z21955, *Myxococcus xanthus*. Note that the *crtI* gene from the aerobic photosynthetic bacterium *E. longus* Ochl01, a species that produces about 20 different kinds of carotenoids, has been found contiguous to a *crtY* gene that encodes the lycopene cyclase enzyme (42). It is therefore likely that this *crtI* gene is implicated in the synthesis of cyclic carotenoids. I, group I; II, group II.



eroides in which the native three-step phytoene desaturase (*CrtI*) was replaced with the four-step enzyme from *Erwinia herbicola* (32). However, to our knowledge, this is the first example of the presence of lycopene in LH1 complexes. Although the energy transfer between lycopene and bacteriochlorophylls is less efficient than the one measured for spirilloxanthin, lycopene and spirilloxanthin present a similar arrangement and conformational state as shown by linear dichroism measurements on intact cells oriented in polyacrylamide gels (data not shown). Furthermore the amount of photosynthetic apparatus per cell is reduced by a factor of 3–5 in the 278Δ*crtCD* mutant. Therefore, our results not only demonstrate that the spirilloxanthin is the major carotenoid associated with the photosystem in *Bradyrhizobium* but also that this molecule plays an important role in the structural stabilization of the bacteriochlorophyll LH1 complexes. In contrast, canthaxanthin appears to be localized predominantly in the cytoplasmic part of the membrane and to protect the cells against oxidative stress in agreement with different *in vitro* studies (33, 34).

Regulation of Carotenoid Biosynthesis—In this study, we clearly show that the canthaxanthin and spirilloxanthin *crt* gene clusters are differently regulated. Indeed only the spirilloxanthin *crt* genes are under the control of the light via the dual action of the phytochrome BrbphP and the transcriptional factor PpsR. The protein PpsR is known, in purple bacteria, to repress photosynthesis by binding to the promoter regions of some photosynthesis genes including *crt* genes (30). The light action spectrum of the spirilloxanthin *crt* gene expression and the phenotypes of *ppsR* and *BrbphP* minus mutants provide evidence that the Pr form of BrbphP activates the expression of spirilloxanthin *crt* genes alleviating the repression by PpsR. We found only one binding site of PpsR in the spirilloxanthin *crt* gene cluster of ORS278. Since this site is present in the

intergenic region between the two putative operons *crtEF* and *crtCD*, we can speculate that the fixation of PpsR on this site blocks the transcription of these four genes. On the other hand no PpsR binding site is found upstream of the putative operon *crtIB.s*, although the expression of the *crtI.s* gene is strongly enhanced by far-red light. One possible explanation is that these genes are co-transcribed with other *bch* genes present upstream and under the control of PpsR.

The molecular mechanism by which BrbphP activates the expression of spirilloxanthin *crt* genes and antirepresses the action of PpsR remains unknown. This mechanism may be close to the dual mechanism of action between AppA and PpsR recently described in *R. sphaeroides* (35). Like BrbphP, AppA is a light photoreceptor that activates the expression of photosynthesis genes including *crt* genes. It has been shown that AppA antagonizes the repressive effect of PpsR by forming a blue light-sensitive and redox-dependent AppA-PpsR complex. By analogy, one can speculate that BrbphP and PpsR form a light-dependent complex.

Although the canthaxanthin *crt* gene cluster is not regulated via BrbphP and PpsR, we observe an increase in production of canthaxanthin caused by far-red light in the WT strain. We propose that a part of the common intermediates synthesized by the *CrtE.s*, *CrtI.s*, and *CrtB.s* enzymes could be diverted toward canthaxanthin production. The production of both canthaxanthin and spirilloxanthin by the *crtE.c* and *crtI.s* minus mutants is in agreement with such a hypothesis. Despite this interconnection between the two biosynthesis pathways, the amount of spirilloxanthin does not exceed 0.2 mg/g of dry cells even in the mutant (278Δ*crtY*), which does not synthesize canthaxanthin. This observation has two important implications: i) canthaxanthin is not made at the expense of spirilloxanthin synthesis and ii) the level of spirilloxanthin is limited to the quantity bound to the photosynthetic apparatus present in the cell. Such control could result

from the inhibition of some enzymes of the biosynthesis pathway by the end product spirilloxanthin. This proposal is in line with the observation that some phytoene desaturases are inhibited by carotenoids such as neurosporene, lycopene, and β -carotene (36, 37). In contrast no such limitation is observed for the biosynthesis of canthaxanthin as the total amount of canthaxanthin can reach 1.5 mg/g of cells in the case of the 278 Δ crtCD mutant under far-red light illumination. This strong enhancement may be the result of an overexpression of the CrtE.s, CrtI.s, and CrtB.s enzymes, not repressed in this case because of the absence of spirilloxanthin production.

Origin of Carotenoid Gene Clusters—A recent analysis of the diversity of photosynthetic *Bradyrhizobium* sp. shows a monophyletic origin of the strains producing canthaxanthin (38). It is therefore very tempting to suggest that an ancestral photosynthetic *Bradyrhizobium* acquired, by lateral gene transfer, the canthaxanthin crt gene cluster. Phylogenetic analysis based on CrtI (Fig. 6) or CrtB sequences (data not shown) provide strong support for this hypothesis. Indeed the trees obtained clearly show the presence of two distinct groups that are not related to the taxonomical position of the various species but to the function or the nature of their carotenoid (cyclic or non-cyclic). Group I contains only sequences from photosynthetic bacteria, and group II contains sequences from pigmented non-photosynthetic bacteria except for the two aerobic photosynthetic bacteria *Bradyrhizobium* ORS278 and *E. longus*. The fact that CrtI.s and CrtI.c (or CrtB.s and CrtB.c) from *Bradyrhizobium* ORS278 strain are classified separately into these two groups confirms that these genes have evolved independently. Moreover the fact that each group contains bacteria phylogenetically distant suggests that lateral gene transfers have occurred within each group. The crtI.s or crtB.s genes of *Bradyrhizobium* ORS278 congregate with crt genes implicated in the synthesis of photosynthetic pigment and are branched with those of the closely related anaerobic photosynthetic bacteria *Rhodospseudomonas palustris* (supported by a bootstrap value of 96%). This is in agreement with previous phylogenetic analysis based on 16 S rDNA or puf genes (39, 24). The congruence between these different phylogenetic analyses using different genetic markers strengthens the hypothesis that photosynthetic *Bradyrhizobium* sp. inherited photosynthesis genes including spirilloxanthin crt genes directly from a photosynthetic ancestor common to *R. palustris*. In contrast the crtI.c or crtB.c genes are grouped with crt genes implicated in the synthesis of cyclic carotenoids found in non-photosynthetic bacteria and are phylogenetically closer to the distant species of *Erwinia*. This is a strong argument for acquisition of the canthaxanthin crt gene cluster by lateral gene transfer.

The presence of two distinct crt gene clusters found in *Bradyrhizobium* ORS278 is surely not unique to some photosynthetic strains of this species. This is certainly also the case for other aerobic photosynthetic bacteria, such as *E. longus*, which have been reported to produce different classes of carotenoids (4, 10). One can wonder what is the physiological and evolutionary basis for such a complex carotenoid composition found specifically in some aerobic photosynthetic bacteria. In contrast to the purple bacteria, the photosystem of these bacteria is active only under aerobic condition. In the presence of oxygen and light, the photosynthetic apparatus generates triplet states of bacteriochlorophyll molecules, which can react with singlet oxygen to form harmful reactive oxygen species toxic to cells. The acquisition by lateral gene transfer of an additional crt gene cluster that permits the synthesis of supplementary carotenoids may constitute a major selective advantage for these bacteria protecting them against the generation of reac-

tive oxygen species during photosynthesis. This may be especially the case when the bacteria are developing *ex planta* where they have to cope with high light intensity at elevated oxygen tension. The observation that the amount of canthaxanthin increases in parallel with the synthesis of the photosynthetic apparatus and the weaker resistance of the canthaxanthin minus mutant (278 Δ crtY) to oxidative stress are in agreement with such a hypothesis.

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Methylophilic *Methylobacterium* Bacteria Nodulate and Fix Nitrogen in Symbiosis with Legumes

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Rhizobia described so far belong to three distinct phylogenetic branches within the α -2 subclass of *Proteobacteria*. Here we report the discovery of a fourth rhizobial branch involving bacteria of the *Methylobacterium* genus. Rhizobia isolated from *Crotalaria* legumes were assigned to a new species, "*Methylobacterium nodulans*," within the *Methylobacterium* genus on the basis of 16S ribosomal DNA analyses. We demonstrated that these rhizobia facultatively grow on methanol, which is a characteristic of *Methylobacterium* spp. but a unique feature among rhizobia. Genes encoding two key enzymes of methylophilicity and nodulation, the *mxsA* gene, encoding the α subunit of the methanol dehydrogenase, and the *nodA* gene, encoding an acyltransferase involved in Nod factor biosynthesis, were sequenced for the type strain, ORS2060. Plant tests and *nodA* amplification assays showed that "*M. nodulans*" is the only nodulating *Methylobacterium* sp. identified so far. Phylogenetic sequence analysis showed that "*M. nodulans*" NodA is closely related to *Bradyrhizobium* NodA, suggesting that this gene was acquired by horizontal gene transfer.

Symbioses between leguminous plants and soil bacteria commonly referred to as rhizobia are of considerable environmental and agricultural importance since they are responsible for most of the atmospheric nitrogen fixed on land. Rhizobia are able to elicit on most of the 18,000 species of the *Leguminosae* family the formation of specialized organs, called nodules, in which they reduce atmospheric nitrogen to ammonia to the benefit of the plant. Nodule formation is controlled by extracellular bacterial signal molecules, called Nod factors, which are recognized by the host plant (21, 34). The rhizobial species described so far are very diverse and do not form an evolutionary homogenous clade. They belong to three distinct branches within the α -2 subclass of *Proteobacteria* and are phylogenetically intertwined with non-symbiotic bacteria (40) (Fig. 1). A first large branch groups the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Allorhizobium* with *Agrobacterium*, a pathogenic bacterium of plants. A second branch contains the genus *Bradyrhizobium* together with photosynthetic free-living *Rhodospirillum rubrum*, whereas the third branch includes the genus *Azorhizobium* as well as the chemiautotroph *Xanthobacter*. Each rhizobial species has a defined host range, varying from very narrow, as in the case of *Azorhizobium caulinodans* (6), to very broad, as in the case of *Sinorhizobium* sp. strain NGR234 (30). Symbionts of legumes exhibiting ecological and agronomic potential should be characterized prior to their use in sustainable agriculture and environment management.

Crotalaria spp. are herbs and shrubs of the subfamily Papilionoideae; it is the largest plant genus in Africa. More than 500 species commonly occur in diverse climatological situations,

from semidesert to rain forests and high mountains (1, 29). Some *Crotalaria* spp. are of great agronomic interest since they are used as green manure to improve soil fertility or control nematode populations in infested soils (4, 20). Characterization of a collection of rhizobia isolated from various *Crotalaria* species revealed two very distinct groups of symbiotic bacteria, a group of broad-host-range rhizobia related to *Bradyrhizobium* and a group of highly specific rhizobia of unknown taxonomic status (33).

We now report that the latter group of highly specific *Crotalaria* rhizobia belong to the *Methylobacterium* genus and assign them to a new species, for which we propose the name "*M. nodulans*." These *Methylobacterium* strains thus constitute a novel and fourth group of nitrogen-fixing legume-symbiotic bacteria. We demonstrated that "*M. nodulans*" is a facultative methylophilic, which is a unique property among rhizobia.

MATERIALS AND METHODS

Bacterial strains. Strains isolated from *Crotalaria* spp. are listed in Table 1. *Methylobacterium rhodesianum* LMG6086, *Methylobacterium organophilum* LMG6083, *Methylobacterium extorquens* LMG4250, *Methylobacterium rhodium* LMG2275, *Methylobacterium zatmanii* LMG6087, *Methylobacterium mesophilicum* LMG5275, and *Methylobacterium* sp. strains LMG6378, LMG6085, and LMG6380 were from the collection of the Universiteit Gent (5). *Sinorhizobium meliloti* RCR2011, *Sinorhizobium medicae* A321, *Sinorhizobium fredii* USDA205, *Sinorhizobium teranga* ORS1009, *Rhizobium leguminosarum* bv. viciae 248, *Rhizobium etli* CFN42, *Rhizobium tropici* CIAT899, *Methylobacterium ciceri* UPMCA-7, *Methylobacterium loti* NZP2213, *Bradyrhizobium japonicum* USDA110, *Bradyrhizobium elkanii* USDA61, *Allorhizobium undicola* ORS995, and *Azorhizobium caulinodans* ORS571 were from our collection. The growth medium for *Methylobacterium* strains, including "*M. nodulans*" ORS2060 and ORS1917, was M72 (5) supplemented with 50 mM methanol. The complete medium for other strains was YM (37). Nodulating *Methylobacterium* strains were grown at 37°C; other strains were grown at 30°C.

DNA technology. Genomic DNA was prepared by using the method of Chen and Kuo (7). Plasmid DNA was isolated with a Miniprep kit (Promega, Charbonnières, France). PCR products were purified with a QIAquick gel extraction kit (Qiagen, Courtaboeuf, France). Restriction endonucleases and ligase were

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1 % estimated substitutions

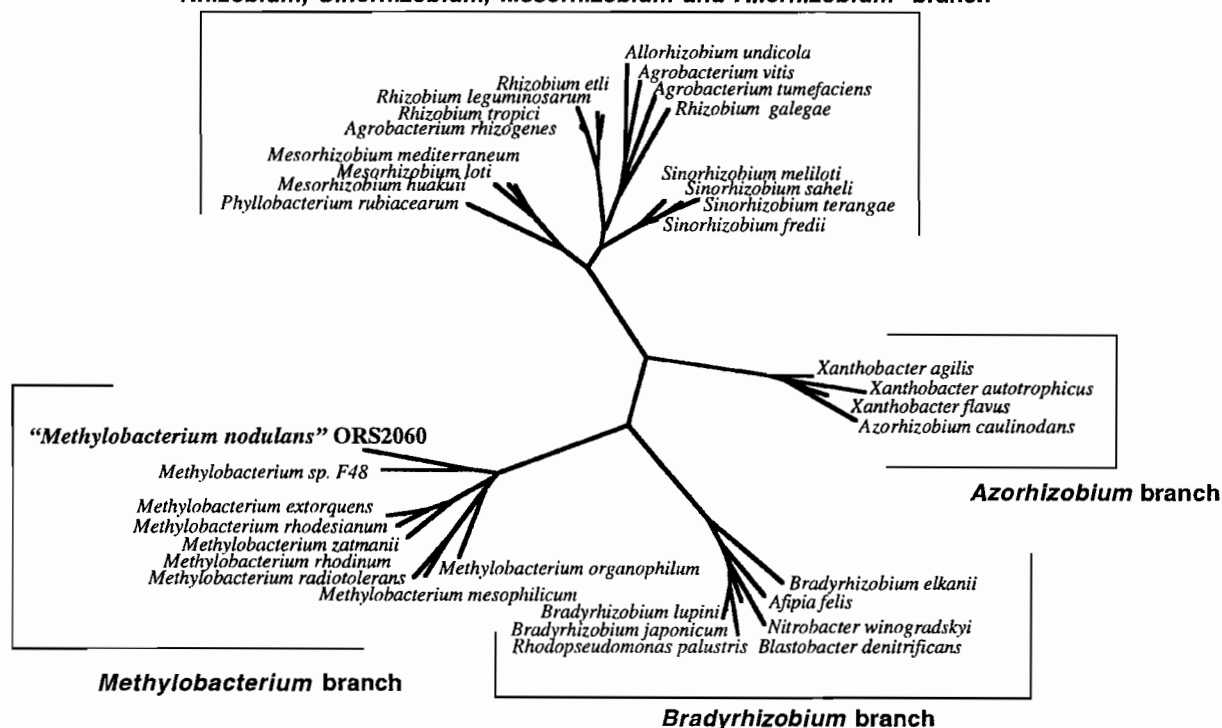
Rhizobium, Sinorhizobium, Mesorhizobium and Allorhizobium branch

FIG. 1. Unrooted phylogenetic tree showing the different rhizobial branches, including the new "*M. nodulans*" in the α subdivision of *Proteobacteria*. The tree was constructed by using the neighbor-joining method from almost full-length 16S rDNA sequences. The GenBank/EMBL accession numbers are as follows (the first letters of the genus and species are given in parentheses): D 12790 (Pr), D12797 (Mh), X67229 (MI), L38825 (Mmed), X67224 (Ar), X67234 (Rt), U29386 (RI), U28916 (Re), Y17047 (Au), X67225 (Av), X67223 (At), X67226 (Rg), X67222 (Sm), X68390 (Ss), X68387 (St), X67231 (Sf), X94198 (Xag), X94201 (Xau), X94199 (Xf), D11342 (Ac), U35000 (Be), M65248 (Af), L11661 (Nw), S46917 (Bd), D25312 (Rp), D12781 (Bj), U69637 (Bl), D32226 (Mo), D32225 (Mmes), D32227 (Mrad), D32229 (Mrhodi), D32230 (Mz), D32228 (Mrhode), D32224 (Me), D32236 (Msp), and AF220763 (Mn).

used according to the manufacturer's specifications (Roche, Meylan, France, or Eurogentec, Seraing, Belgium). For Southern blot hybridization, restricted DNA was blotted to positively charged nylon membranes by the alkali transfer procedure and hybridized with digoxigenin (DIG)-dUTP using the DIG labeling kit supplied by Roche.

DNA amplification, sequencing, and analysis. The primers used for DNA amplification and sequencing are described in Table 2. Nearly full-length 16S ribosomal DNA (rDNA) was amplified using the universal eubacterial 16S rDNA primers FGPS6 and FGPS1509 (28). To perform 16S rDNA PCR-restriction fragment length polymorphism analysis, 1,500-bp PCR products were digested with *Sau*96I, *Hinf*I, *Msp*I, and *Hae*III and restriction fragments were analyzed by horizontal agarose gel electrophoresis using Metaphor agarose (FMC Bioproducts, Hellerup, Denmark). The 1,500-bp fragments of ORS2060 and ORS1924 were sequenced by using the primers FGPS6, FGPS1509, 16S-370f, 16-1080r, 16S-870f, and 16S-1924r; 555-bp sequences homologous to the *mxhF* gene were amplified from *Crotalaria* rhizobia and sequenced by using the nondegenerate primers f1003 and r1561 (26). For rhizobial species, a fragment of about 440 bp homologous to *mxhF* was amplified and sequenced by using the degenerate primers mxaf916 and mxar1360. Two pairs of primers, nodAfrad/nodArbrad and nodA1f/nodAb1r, were tested for *nodA* amplification of reference *Methylobacterium* strains (LMG6086, LMG6083, LMG4250, LMG2275, LMG6087, LMG5275, LMG6378, LMG6085, and LMG6380). *nodA* amplification and sequencing of ORS2060 were performed using three pairs of primers, nodAfrad/nodArbrad, nodboxuniv2/nodArbrad, and nodAfrad/nodB76r.

PCR amplification was performed with a Perkin-Elmer model 2400 thermocycler in a 25- μ l (total volume) reaction mixture containing 50 ng of genomic DNA, each deoxynucleotide triphosphate (200 μ M), primers (0.8 μ M each), $MgCl_2$ (1.5 mM), 1.25 U of *Taq* DNA polymerase (Gibco BRL, Cergy Pontoise, France), and the buffer supplied with the enzyme. A touchdown PCR (12) was

performed for primer pairs nodAfrad/nodArbrad and nodA1f/nodAb1r (annealing temperature from 65 to 55°C in 20 cycles), primer pairs nodboxuniv2/nodArbrad and nodAfrad/nodB76r (annealing temperature from 60 to 45°C in 30 cycles), and mxaf916/mxar1360 (annealing temperature from 60 to 50°C in 20 cycles). A standard PCR method was used for primer pairs FGPS6/FGPS1509 (60°C annealing temperature) and f1003/r1561 (55°C annealing temperature). Sequencing reactions were performed with the ABI Prism BigDye Terminator Cycle sequence kit (Applied Biosystems, Foster City, Calif.) and analyzed on an

TABLE 1. *Crotalaria* rhizobia used in this study

Strain(s) ^a	Host plant
"<i>M. nodulans</i>"	
ORS1917, ORS1991, ORS2060 ^T	<i>C. podocarpa</i>
ORS1924, ORS1928, ORS1937, ORS2030,	
ORS2092	<i>C. perrottetii</i>
ORS2026, ORS2045, ORS2076	<i>C. glaucoidea</i>
<i>Bradyrhizobium</i> sp.	
ORS1810	<i>C. lathyroides</i>
ORS1816	<i>C. hyssopifolia</i>
ORS1813	<i>C. hyssopifolia</i>
ORS1929	<i>C. comosa</i>
ORS2077	<i>C. retusa</i>
ORS2088	<i>C. goreensis</i>

^a All strains are described by Samba et al. (33).

TABLE 2. Primers used for DNA amplification and sequencing

Primer name	Primer sequence	Target gene	Reference
FGPS6	5'-GGA GAG TTA GAT CTT GGC TCA G-3'	16S rRNA	28
FGPS1509	5'-AAG GAG GGG ATC CAG CCG CA-3'	16S rRNA	28
16S-370f	5'-CCT GGG GAG TAC GGT CGC AAG-3'	16S rRNA	This study
16S1080r	5'-GGG ACT TAA CCC AAC ATC T-3'	16S rRNA	This study
16S-870f	5'-CCT GGG GAG TAC GGT CGC AAG-3'	16S rRNA	This study
16S-1924r	5'-GGC ACG AAG TTA GCC GGG GC-3'	16S rRNA	This study
f1003	5'-GCG GCA CCA ACT GGG GCT GGT-3'	<i>mxoF</i>	26
r1561	5'-GGG CAG CAT GAA GGG CTC CC-3'	<i>mxoF</i>	26
mxoF916	5'-GGC GAC AAC AAG TGG WCS ATG-3'	<i>mxoF/xoxF</i>	This study
mxar1360	5'-ART CCA TRC ARA YGT GGT T-3'	<i>mxoF/xoxF</i>	This study
xoxFr	5'-CCG GAA CGG CTC GTA RTC CA-3'	<i>xoxF</i>	This study
nodAfrad	5'-GTG GAG TGG AGS STK CGC TGG G-3'	<i>nodA</i>	This study
nodArad	5'-TCA CAR CTC KGG CCC GTT CGG-3'	<i>nodA</i>	This study
nodA1f	5'-TGC RGT GGA ARN TRB VYT GGG-3'	<i>nodA</i>	This study
nodA1r	5'-GGN CCG TCR TCR AAS GTC ARG TA-3'	<i>nodA</i>	This study
nodboxuniv2	5'-ATC NAA ACA AWA RAT TTT AC-3'	<i>nod box</i>	This study
NodB76r	5'-GGR TKN GGN CCR TCR TCR AAN GT-3'	<i>nodB</i>	This study

Applied Biosystems model 310 DNA sequencer. Sequences were aligned by using the PILEUP program (11). Phylogenetic trees were constructed by the neighbor-joining method (32), and a bootstrap confidence analysis was performed on 1,000 replicates to determine the reliability of the tree topology obtained (13).

Rhizobial *mxoF*-homologous partial sequences are available upon request.

Construction and screening of a genomic library of ORS2060. To obtain a genomic library of the ORS2060 strain, total DNA was subjected to partial digestion with *Sau*3AI and dephosphorylated with an alkaline phosphatase treatment. Fragments were then ligated to SuperCos I *Xba*I/*Bam*HI arms (Stratagene, La Jolla, Calif.) as instructed by the manufacturer. Ligated DNA was packaged by using the Gigapack III Gold packaging extract (Stratagene). Standard methods were used for titrating and cosmid propagation using *Escherichia coli* XL1-MR as the host. Approximately 2,000 white colonies were picked individually into 96-well microtiter plates containing Luria-Bertani medium plus kanamycin (50 µg/ml), grown overnight at 37°C, and stored with 30% glycerol at -80°C. Screening of *mxoF*-containing cosmids was performed by DNA amplification using the primer pair f1003/r1561. A selected clone, pSTM217, was confirmed by hybridization with an *mxoF* probe constructed by DIG labeling the ORS2060 555-bp *mxoF* internal fragment.

Methanol utilization tests. Cells were grown for 48 h to mid-log phase in minimum mineral medium M72 (5) supplemented with pyruvate (10 mM) and yeast extract (0.5g/liter). Bacterial suspensions were diluted in M72 medium to an optical density of 0.05, and one of the following compounds was added: methanol (MeOH) (10, 50, 100, or 500 mM), pyruvate (10 mM), or succinate (10 mM). Growth was monitored by measuring optical density at 620 nm. MeOH dosage in culture supernatants was performed as described previously (39) except that KMnO₄ was replaced by alcohol oxidase (EC 1.1.3.13) at 0.1 U/ml (Sigma, L'Isle d'Abeau, France).

Plant tests. Plant cultivation and nodulation tests were carried out as described previously (23), with the following modifications: seeds were superficially sterilized with concentrated sulfuric acid for 35 min (*Crotalaria podocarpa*), 30 min (*Crotalaria perrottetii*), 15 min (*Crotalaria comosa*), 40 min (*C. gorensis*), or 25 min (*Crotalaria ochroleuca*). Effectiveness was estimated by visual observation of plant vigor and foliage color of 30-day-old plants.

Fresh nodules were observed under an Olympus SHZ 10 stereomicroscope. Sections of 80-µm thickness were made using a Leica VT1000S Vibratome. Microscopic preparations were examined without further staining with an Olympus Provis microscope.

Bacteriochlorophyll detection. The presence of bacteriochlorophyll *a* in "*M. nodulans*" ORS2060 was checked as described previously (22).

Nucleotide sequence accession numbers. Accession numbers for 16S rRNA and the *mxoF* and *nodA* genes are as follows: AF220762 (ORS1924 16S rRNA gene), AF220763 (ORS2060 16S rRNA gene), AF220764 (ORS2060 *mxoF* gene), and AF266748 (ORS2060 *nodA* gene). Accession numbers for rhizobial partial *mxoF*-homologous sequences are AF304307 to AF304313.

RESULTS

Bacteria that specifically nodulate *Crotalaria* belong to the *Methylobacterium* genus. Rhizobia isolated from *Crotalaria glaucoidea*, *C. perrottetii*, and *C. podocarpa* were previously shown to be highly specific, since they effectively nodulated only these three species (33). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis grouped almost all the strains into three related electrophoretic clusters separated from other rhizobial species (33).

To determine the bacterial genus to which they belong, 16S rDNA analysis was performed on 11 representative strains belonging to the previously identified sodium dodecyl sulfate-polyacrylamide gel electrophoresis clusters (Table 1). All strains tested gave identical patterns by 16S rDNA PCR-restriction fragment length polymorphism analysis, showing that the strains form a very homogenous group. The 16S rRNA genes of two strains, ORS2060 isolated from *C. podocarpa* and ORS1924 isolated from *C. perrottetii*, were sequenced and shown to be identical (accession numbers AF220763 and AF220762, respectively). Phylogenetic 16S rDNA sequence analysis revealed that this group was distinct from the three main branches containing all known rhizobial species. Surprisingly, the specific *Crotalaria* symbionts belonged to the *Methylobacterium* lineage of the α -Proteobacteria, thus constituting a fourth phylogenetic rhizobium branch (Fig. 1). Sequence similarities with the different *Methylobacterium* species described so far ranged from 93.64% (*Methylobacterium radiotolerans*) to 94.52% (*M. extorquens*) and was 95.16% with its closest phylogenetic neighbor, *Methylobacterium* sp. strain F48. These ranges are comparable with those found between most *Methylobacterium* species, thus demonstrating that the specific *Crotalaria* rhizobia phylogenetically belong to the *Methylobacterium* genus.

Bacteria of the new species "*M. nodulans*" are facultative aerobic methylophilic. *Methylobacterium* spp. oxidase methanol via a key periplasmic enzyme, methanol dehydrogenase, which belongs to the family of the pyrroloquinoline quinone (PQQ)-linked enzymes that are known as quinoproteins (2, 3). Methanol dehydrogenase is an $\alpha_2\beta_2$ tetramer noncovalently

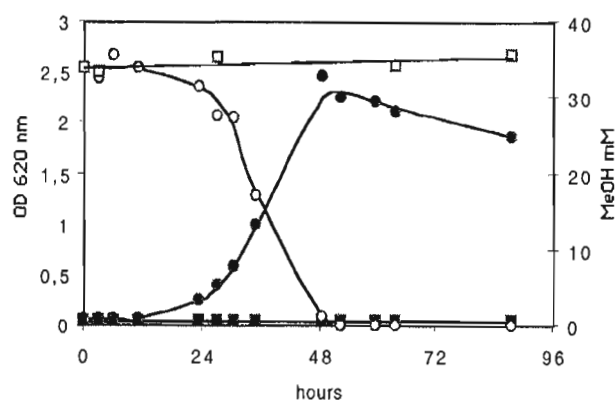


FIG. 2. Methanol utilization by "*M. nodulans*" ORS2060. *Bradyrhizobium* sp. strain ORS1810 (33) was used as a negative control. Shown is growth of ORS2060 (black circles) and ORS1810 (black squares) on minimum mineral medium 72 containing MeOH as the sole carbon source. MeOH concentrations in supernatants of cultures of ORS2060 (empty circles) and ORS1810 (empty squares) are also shown.

bound to PQQ (15). The structural gene for the α subunit of the methanol dehydrogenase encoded by *mxhF* is well conserved among gram-negative methylotrophic bacteria (24). Therefore, to evaluate the presence of methanol oxidation genes in *Crotalaria* rhizobia, we performed PCR amplifications using nondegenerate primers f1003 and r1561, defined from conserved parts of *mxhF* genes (26). These primers indeed produced an amplification product of the expected size for 9 of the 11 specific *Crotalaria* strains tested. The 555-bp PCR product obtained from the representative strain ORS2060 was homologous to *mxhF* genes. The full-length *mxhF* gene was obtained from a genomic library of ORS2060 probed with the 555-bp PCR product (see Materials and Methods for details). The corresponding nucleotide sequence contained a single extended 1,890-bp open reading frame (accession number AF220764) encoding a 629-amino-acid protein that exhibits 88% identity with the MxhF proteins of *M. extorquens* (accession number M31108) and *M. organophilum* (accession number M22629). A putative ribosome binding site was identified upstream from the proposed ATG start codon. The first 93 nucleotides of the structural gene encode a typical signal sequence for secretion (38). These results indicated that *Crotalaria*-nodulating *Methylobacterium* strains did contain, as all other *Methylobacterium* strains, the structural gene, *mxhF*, required for methanol oxidation.

In order to directly assess the methylotrophic properties of *Methylobacterium* species from *Crotalaria*, the strains were then tested for their ability to use methanol (50 mM) as the sole carbon source in liquid culture. Growth was compared to that of *M. extorquens* LMG4250 and *Bradyrhizobium* sp. isolated from *Crotalaria* species. No growth was observed for *Bradyrhizobium* strains over a 10-day period (Fig. 2). By contrast, all *Methylobacterium* strains from *Crotalaria* grew on this substrate, with generation times ranging from 9.5 to 40 h. The fastest growth rates were obtained with strains ORS2060 (Fig. 2) and ORS1917 (9.5-h doubling time) and were similar to the growth rate of *M. extorquens* LMG4250 on MeOH (50 mM) (7.5-h doubling time). Similar growth was noted on either

pyruvate or succinate as the sole carbon source. Growth was obtained at an MeOH concentration up to 500 mM.

To confirm that the same bacterium isolated from *Crotalaria* exhibits both nodulation ability and methylotrophic properties, strain ORS2060 was grown on methanol, repurified from a single colony, and inoculated onto *C. podocarpa* and *C. perrottetii*. The bacteria formed nitrogen-fixing indeterminate nodules (Fig. 3). Single colonies reisolated from the nodules retained the ability to grow on methanol, clearly demonstrating that this new *Methylobacterium* strain is able both to grow on one-carbon (C_1) compounds and to effectively nodulate legumes.

Proposal of a new species was warranted by the low 16S rDNA sequence similarity values between *Methylobacterium* strains isolated from *Crotalaria* and other *Methylobacterium* strains. The unique symbiotic properties of these bacteria (see below) led us to propose the name "*Methylobacterium nodulans*." *Methylobacterium* spp., which are mostly isolated from water and leaf surface microflora, are known in the literature as pink-pigmented facultative methylotrophs (19). However, "*M. nodulans*" strains are not pigmented and the type strain,

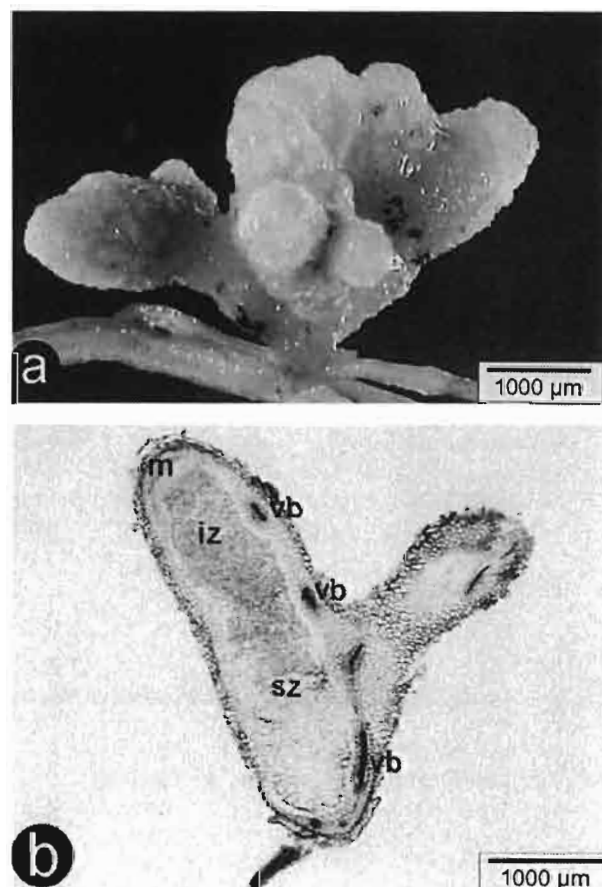


FIG. 3. Nodules of *C. perrottetii* inoculated with "*M. nodulans*" ORS2060. (a) Multilobed fresh nodule; (b) unstained longitudinal section displaying the classical structure of indeterminate nodules with an apical meristematic zone (m), an infection zone (iz), a senescent zone (sz), and peripheral vascular bundles (vb).

ORS2060, did not exhibit the characteristic bacteriochlorophyll absorption peak at 766 nm. This could account for their adaptation to the soil rather than to the phyllosphere or water.

"*M. nodulans*" is the only nodulating *Methylobacterium* species identified to date. The *Methylobacterium* genus has never been reported to contain nodulating bacteria. However, to be certain that nodulation is not a general but hitherto undetected *Methylobacterium* feature, we tested representative strains of several *Methylobacterium* species (*M. rhodesianum*, *M. organophilum*, *M. extorquens*, *M. rhodinum*, *M. mesophilicum*, *M. zatmanii*, *M. radiotolerans*, and "*M. nodulans*") and a few *Methylobacterium* sp. strains (LMG6378, LMG6085, and LMG6380) by inoculation on *Crotalaria* species nodulated by broad-host-range *Bradyrhizobium* (*C. comosa*, *C. goreensis*, and *C. ochroleuca*) or by "*M. nodulans*" (*C. perrottetii*). None of them nodulated, except "*M. nodulans*."

Specific rhizobial infection and nodulation of legumes is mainly controlled by a set of bacterial nodulation genes involved in the production of lipochitooligosaccharides (Nod factors) that act as morphogenic signal molecules on specific legume hosts (10, 36). Structural *nodABC* genes encoding key enzymes in Nod factor biosynthesis are present in all rhizobia. We thus looked for the presence of the *nodA* gene in the *Methylobacterium* representative strains listed above by PCR amplifications using two pairs of degenerate primers defined from conserved parts of NodA sequences. None of these strains responded positively, except "*M. nodulans*." The full-length *nodA* sequence of "*M. nodulans*" ORS2060 was determined after PCR amplification. The open reading frame, which probably corresponds to *nodA*, is 642 nucleotides long (accession number AF266748). Sequence similarity with the different complete rhizobial NodA protein sequences available in databases ranged from 53.06% (*A. caulinodans*) to 74.11% (*B. elkanii* USDA94). Phylogenetic analysis of available NodA proteins showed that "*M. nodulans*" is grouped with *Bradyrhizobium* spp. (Fig. 4).

Methylotrophy is not a common feature of rhizobia. We tested a collection of rhizobia belonging to various genera and species (*S. meliloti*, *S. medicae*, *S. fredii*, *S. teranga*, *R. leguminosarum* bv. *viciae*, *R. etli*, *R. tropici*, *M. ciceri*, *M. loti*, *B. japonicum*, *B. elkanii*, *A. undicola*, and *A. caulinodans*; see Materials and Methods) for their ability to grow on MeOH (50 mM) as the sole carbon source in solid and liquid culture. No growth of these strains was observed within 6 days. We also used PCR amplification to investigate the possible presence of a *mxoF*-homologous gene in the rhizobial strains. No amplification could be obtained using primers f1003 and r1561. When degenerate primers defined from conserved parts of both *MxaF* and *MxaF* homologs from α -*Proteobacteria* were used, all strains were found to contain a 440-bp sequence homologous to *mxoF*. These sequences however exhibited higher homology to two *mxoF* homologs, *mxoF'* from *M. extorquens* (72 to 80% amino acid identity) and *xoxF* from *Paracoccus denitrificans* (72 to 83% amino acid identity) than to *mxoF* from *M. extorquens* (50 to 53% amino acid identity). *xoxF* and *mxoF'* are thought to encode an alternative PQQ-dependent dehydrogenase (8, 18). Phylogenetic analysis further revealed that the rhizobial protein sequences are clustered with *XoxF* and *MxaF'* and separated from the methanol dehydrogenase *MxaF* from α - and β -*Proteobacteria* (Fig. 5). We thus conclude that

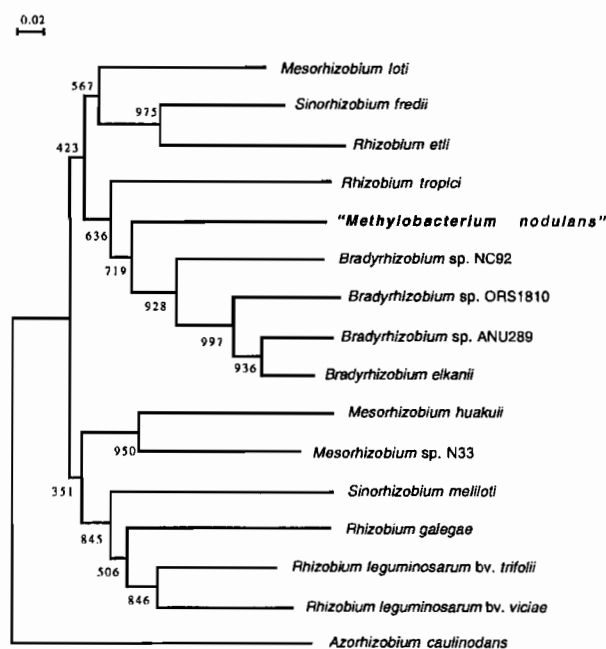


FIG. 4. Phylogenetic tree based on full-length NodA sequences constructed by using the neighbor-joining method. Bootstrap values (from 1,000 replications) are indicated. The GenBank/EMBL accession numbers are as follows (the first letters of the genus and species are given in parentheses): L18897 (Ac), 106241 (MI), M73699 (Sf), M58625 (Re), X98514 (Rt), AF266748 (Mn), U33192 (BspNC92), U04609 (BspANU289), U04609 (Be), AJ249353 (Mh), U53327 (MspN33), M11268 (Sm), X87578 (Rg), and X01650 (RI). The NodA sequence of *Bradyrhizobium* sp. strain ORS1810 was kindly provided by T. Stepkowski.

the sequences found in rhizobia probably do not correspond to a bona fide *mxoF* ortholog.

DISCUSSION

The rhizobial species described so far belong to three distinct 16S rRNA branches within α -*Proteobacteria*, including nonsymbiotic bacteria such as the plant pathogen *Agrobacterium* and the human and animal pathogen *A. afipia*. The discovery of a fourth rhizobial phylogenetic branch, constituted by bacteria of the *Methylobacterium* genus, thus confirms and extends the polyphyletic origin of rhizobia within the α subclass of *Proteobacteria* (40). It is noteworthy that although rhizobia have been studied for more than 100 years, symbionts of less than 50 of the 750 known legume genera have been fully characterized. Therefore, it is quite likely that much greater rhizobial diversity will be discovered by characterizing symbionts of unexplored legumes and by focusing on legumes of unexplored areas.

Bacterial nodulation (*nod*) genes have been shown to play a central role in the molecular dialog between the plant and the bacterium, leading to plant recognition, infection, and nodulation (10, 34, 36). The presence of structural *nodABC* genes in all rhizobia indicates the unique origin of these genes, which could have been disseminated among *Proteobacteria* via self-transmissible plasmids (25) or other mechanisms allowing

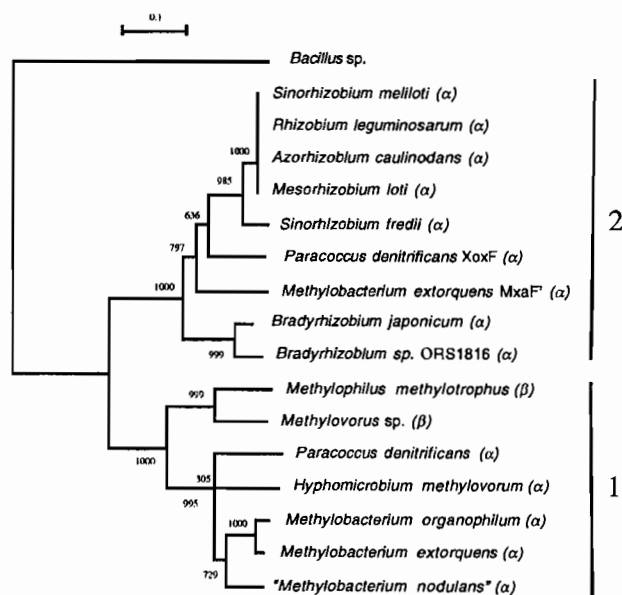


FIG. 5. Phylogenetic tree based on about 140-amino-acid MxaF-homologous sequences constructed by using the neighbor-joining method. Bootstrap values (from 1,000 replications) are indicated. Cluster 1 groups only MxaF sequences with the following GenBank/EMBL accession numbers (the first letters of the genus and species are given in parentheses): M17339 (Pd), AB004097 (Hm), M22629 (Mo), M31108 (Me), AF220764 (Mn), U41040 (Mm), and AF184915 (Msp). Cluster 2 groups the rhizobial sequences (accession numbers given in Materials and Methods) together with XoxF from *P. denitrificans* (U34346) and MxaF' from *M. extorquens* (U72662). MxaF from *Bacillus* sp. (M65004) was used as the root. The topology of the tree constructed with the full-length MxaF-homologous sequences (thus excluding the rhizobial sequences) is similar. (α), member of α -Proteobacteria; (β), member of β -Proteobacteria.

transfer of "symbiotic islands" (35). The presence of the *nodA* gene in "*M. nodulans*" is consistent with nodulation data and suggests that this bacterium is able to establish symbiosis by the same molecular mechanisms as other rhizobia. The NodA phylogenetic analysis confirms the monophyletic origin of this structural common *nod* gene (Fig. 4). The deduced absence of *nodA* in nonsymbiotic *Methylobacterium* spp. together with the close phylogenetic relationship between "*M. nodulans*" and *Bradyrhizobium* NodA proteins suggests that "*M. nodulans*" has acquired nodulation properties by lateral gene transfer.

Bacteria of the *Methylobacterium* genus are facultative methylotrophs capable of growing on one-carbon compounds such as formate, formaldehyde, and methanol as the sole source of carbon and energy, as well as on a wide range of multicarbon substrates (16, 17). They constitute one of the major methylotrophic branches in the α -2 subclass of *Proteobacteria*. It should be noted that other branches, including the three rhizobial branches described to date (Fig. 1), also contain methylotrophs. Indeed, *Xanthobacter* species are all autotrophic methylotrophs (27), most *Rhodospseudomonas* species grow photosynthetically with methanol (31), and a methyl bromide-utilizing methylotroph closely related to *Rhizobium* species was recently identified (9). *Methylobacterium* species are known to occur in man-made environments such as drinking water supplies, swimming pools, and hospital washbasins,

where they often become highly resistant to chlorine. They are also widespread in natural environments, including soil, dust, air, and fresh water, wherever one-carbon compounds are abundant. Because of their ability to metabolize various plant decomposition compounds such as methylated compounds, methylotrophic bacteria play an important ecological role in the environmental carbon cycle. Although *Methylobacterium* strains have been frequently found on plant tissues (19), there is no previous evidence of symbiotic association of such microorganisms with plants. We demonstrated here that a group of *Methylobacterium* strains, identified as the new species "*M. nodulans*," are able to form nitrogen-fixing nodules on the roots of leguminous plants. However, this case may not be unique since we recently learned that CB376, a nodulating photosynthetic strain from *L. bainesii* (14), could be classified in the *Methylobacterium* genus on the basis of its 16S rRNA sequence (W. Heumann, personal communication).

A polyphyletic origin of rhizobia versus a monophyletic origin of common nodulation genes suggests that rhizobia have evolved through acquisition of nodulation functions in different bacterial branches susceptible to adaptation to different legume environments. "*M. nodulans*" is a facultative methylotroph, a unique property among rhizobia, raising the question of the role of methylotrophy in *Crotalaria*-"*M. nodulans*" symbiosis. We are currently starting a genetic analysis to understand why *Methylobacterium* strains are specifically associated with some particular legumes.

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Isolation, characterisation (PCR-RFLP) and specificity of *Frankia* from eight *Gymnostoma* species endemic to New Caledonia.

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Abstract – Eight *Frankia*-*Gymnostoma* nitrogen-fixing symbioses endemic to New Caledonia were studied using a range of techniques including molecular typing of *Frankia*, infectivity/effectivity and host spectrum of the isolates. The study was conducted on 128 *Frankia* isolates from the eight *Gymnostoma* species. The RFLP analysis of the rRNA 16S-23S ITS allowed us to cluster the strains in four groups. Symbiotic characterization of the four groups failed to reveal any host specificity both in term of infectivity or effectivity. The characteristics of the strains were those of *Elaeagnaceae*-infective *Frankia* strains. Co-inoculation of two *Gymnostoma* species with mixtures of strains evidenced 11 % of mixed RFLP profiles. In such controlled conditions, this was interpreted as co-infections. This work evidenced original features of these *Gymnostoma* isolates: (i) they were closer to *Elaeagnaceae*-infective *Frankia* than *Casuarina*-infective *Frankia*; (ii) closer to atypical *Frankia* from *Casuarina*; (iii) poorly host-specific; and (iv) they had a tendency of co-infection. They could represent a primitive group of *Frankia* able to survive in a wide range of habitats. © 2000 Éditions scientifiques et médicales Elsevier SAS

Gymnostoma / *Casuarina* / *Elaeagnaceae* / infectivity / effectivity / nitrogen fixation / actinorhizal plants / ITS 16S-23S / co-infection

Résumé – Isolation, caractérisation (PCR-RFLP) et spécificité de *Frankia* provenant de huit espèces de *Gymnostoma* endémique de Nouvelle-Calédonie. Une étude a été menée sur huit symbioses fixatrices d'azote *Frankia*-*Gymnostoma* endémiques à la Nouvelle-Calédonie. Cent vingt-huit souches de *Frankia* ont été isolées et étudiées par diverses techniques, dont le spectre d'hôtes, l'infectivité, l'effectivité et la PCR/RFLP de l'ITS 16S-23S. L'analyse par PCR/RFLP de l'ITS 16S-23S a permis de classer ces *Frankia* en quatre groupes qui n'ont pas montré de différences en ce qui concerne leurs capacités symbiotiques. La co-inoculation de deux espèces de *Gymnostoma* par des mélanges des quatre types de *Frankia* isolés a mis en évidence l'apparition de mélanges de profils RFLP dans les nodules ainsi obtenus. Dans de telles conditions contrôlées, ce résultat a été interprété comme une co-infection. Ce travail a mis en évidence chez les *Frankia* de *Gymnostoma* des caractéristiques originales : (i) ils sont plus proches des *Frankia* des *Elaeagnaceae* que de ceux des *Casuarina* spp. ; (ii) ils sont proches des *Frankia* atypiques des *Casuarina* ; (iii) ils sont peu spécifiques ; et (iv) ils sont capables de co-infection. Ils pourraient représenter un type de *Frankia* primitif capable de survivre dans des habitats très variés. © 2000 Éditions scientifiques et médicales Elsevier SAS

Gymnostoma / *Casuarina* / *Elaeagnaceae* / infectivité / effectivité / fixation d'azote / plantes actinorhiziennes / ITS 16S-23S / co-infection

1. INTRODUCTION

Actinorhizal plants can fix atmospheric nitrogen through a symbiotic association with the actinomycete *Frankia*. Among the eight botanical families, *Casuarinaceae* comprise four genera: *Ceuthostoma*, *Gymnostoma*, *Allocasuarina* and *Casuarina* [10]. These genera are essentially tropical and subtropical, originating from the Australian, Melanesian and Malaysian regions.

The flora of New Caledonia is characterized by a very high level of endemism (75 to 80 % at the species level) [8]. *Casuarinaceae* are the only actinorhizal plants native to New Caledonia, with one *Casuarina* and eight *Gymnostoma* species (among the eighteen existing), all endemic. Nitrogen fixation makes these species good candidates for the rehabilitation of degraded land as well as mine sites in New Caledonia.

The eight species of *Gymnostoma* grow on soils originating from ultramafic rocks, with the exception of *G. nodiflorum* which grows on alluvial soils of volcano-sedimentary origin. Ultramafic soils are characterized by very low levels of P, K and Ca, high levels of Ni and Mn, a slow mineralization rate of organic matter and a poor level of available N. They can be either little transformed and rich in Mg (hypermagnesian inceptisol) or highly transformed in indurated oxisols. *G. chamaecyparis* is restricted to hypermagnesian soils and *G. deplancheanum* to indurated oxisols. The others species colonize a wider range of habitats such as forest (*G. glaucescens*, *G. intermedium* and *G. poissonianum*) or riparian ecosystems (*G. leucodon*, *G. nodiflorum* and *G. webbianum*) [9]. Their relative specificity towards adverse soils together with their nitrogen fixing capacity make these tree species almost obligate candidates in the revegetation processes of mine sites in New Caledonia.

Probably due to their high endemism, these *Gymnostoma* species and their symbiotic characteristics have been little studied. In 1989, Racette and Torrey [15] first isolated a *Frankia* strain from *G. papuanum*, then Savouré and Lim [18] isolated four strains from *G. sumatranum*. These strains were mostly studied for their symbiotic characteristics and their current availability can be seriously questioned. More recently, Navarro et al. [11] sequenced the *nif* DK ITS of six unisolated strains from six *Gymnostoma* species from New Caledonia and compared them to reference *Frankia* strains. These studies showed that *Frankia* infecting *Gymnostoma* nodules were indistinguishable from *Eleagnaceae* infective strains. However, there was still a need for a study (i) made on isolated *Frankia* strains, available as inoculum in field experiments, and (ii) on a significative number of strains susceptible to represent a wide genetic pool. The aim of this work was thus to isolate a large number of *Frankia* strains from a wide range of *Gymnostoma* species, and to characterize the isolates with molecular tools and by

their symbiotic properties, i.e. infectivity, effectivity and host spectrum.

2. MATERIALS AND METHODS

2.1. Nodules harvest

Nodules were harvested in the natural stands of the host-plants, mainly in the Province Sud, within a radius of about 100 km around Nouméa (table I).

2.2. *Frankia* strains isolation

Nodules were directly harvested from the roots of the *Gymnostoma* plants. Each lobe was individually rinsed under tap water, disinfected for 10 to 20 min in 30 % (wt/v) H_2O_2 and rinsed with sterile distilled water before being cut into small fragments. All the fragments from one individual lobe were incubated at 30 °C, in one tube containing 5 mL Nitrogen Free Medium (NFM) or Complex Medium (CM) [11], for 1 to 3 months. This method allowed to ascertain that each strain originated from one single original lobe. The isolated strains, identified microscopically by the presence of the *Frankia* characteristic morphological structures including hyphae, vesicles, sporangia and spores were then subsequently cultured in the CM medium.

2.3. Plant material

Seeds were sterilised by incubation in concentrated H_2SO_4 for 2 min. The seeds were rinsed out with sterile distilled water and germinated in sterile sand, in a shade house. We tested only seven species of *Gymnostoma*, *G. glaucescens* seeds being not available. The other actinorhizal plants tested were: *Allocasuarina littoralis*, *A. stricta*, *A. torulosa*, *Casuarina collina*, *C. cunninghamiana*, *C. equisetifolia* and *Elaeagnus angustifolia*. After 1 month, the seedlings were transferred into pots containing one of the three sterile soil (see below) from New Caledonia and watered every 12 h. Two-month-old plants were fertilized with 1 g L^{-1} standard Welgro nutrient solution (Ulvir limited, Barcelona, Spain).

2.4. Soils

The three soils used in this study were from New Caledonia. They were all derived from ultramafic rocks, with low P, K and Ca and high Ni levels. The C/N ratio was about 15 with a low organic decomposition and a poor N availability. The soils were sterilized by autoclaving at 120 °C for 2 h twice at 24-h interval.

2.4.1. Tontouta soil

Tontouta soil is a river sand (pH 6.6) from southern New Caledonia. It was considered as a control soil where all species can grow.

2.4.2. Nepoui soil

Nepoui soil is an hypermagnesian inceptisol (pH 6.7) toxic for most of *Gymnostoma* species where *G. chamaecyparis* can grow.

2.4.3. Prony soil

Prony soil is an indurated oxisol (pH 4.7) with high Fe, Ni and Mn levels, toxic for most of *Gymnostoma* species but where *G. deplancheanum* can grow.

2.5. DNA extraction

2.5.1. From cultures

DNA was extracted from 1-month-old *Frankia* strains according to Brenner et al. [2] and Simonet et al. [19].

2.5.2. From nodules

Nodule lobes were disinfected with 30 % w/v H₂O₂ for 5 min, rinsed with sterile distilled water and kept at 20 °C. One nodule lobe was crushed in 500 mL TCP buffer (100 mM Tris-HCl, pH 8, 1.4 M NaCl, 20 mM EDTA, pH 8, 2 % w/v CTAB (Sigma, St Louis, MO, USA) and 3 % w/v PVPP (Sigma)). The mixture was incubated at 65 °C for 1 h and centrifuged at 3 000 × g for 5 min (20 °C). The supernatant was chloroform extracted and ethanol precipitated. The DNA pellet was dissolved in 10 mL TE buffer (pH 7.5).

2.6. PCR amplification

The 16S-23S InterTranscribed Spacer (ITS) rRNA fragment were amplified using primers FGPL2054' (5'-CCG-GGT-TTC-CCC-ATT-CGG-3') (beginning of 23S, universal) [14] and FGPS989e (5'-GGGGTCCT-TAGGGGCT-3') (end of 16S, *Eleagnaceae*-specific) [1] or FGPS989ac (5'-GGGGTCCGTAAGGGTC-3') (end of 16S, *Casuarina*-specific) [1] as described by Simonet et al. [20] and Nazaret et al. [12]. PCR were run on Perkin-Elmer 2400 GeneAmp PCR Systems.

2.7. RFLP

Restriction analyses of the PCR products were performed with three enzymes: *Hae*III, *Msp*I and *Cfo*I.

Digestion was performed on 10 µL PCR products at 37 °C for 2 h. Electrophoreses were made on 3 % agarose gels (Sigma) at 140 mV for 1 h.

2.8. Cross inoculation studies

The seven *Gymnostoma*, three *Casuarina*, three *Allocasuarina* species and *Eleagnus angustifolia* were grown as already described, on the control sandy soil (Tontouta). They were inoculated 4 months after germination with 10 mg packed cell volume [13] of an 1-month-old *Frankia* culture. For each ITS group, one representative strain was arbitrarily selected and used for the experiment. The four selected strains were: Gd03 (ITS Group 1), Gg40 (ITS Group 2), Gl03 (ITS Group 3) and Gc18 (ITS Group 4). Each treatment was repeated four times and two non-inoculated controls were used for each plant species. Because of very poor growth, *Gymnostoma* species were measured 11 months after inoculation. The other species were measured 6 months after inoculation. Infectivity was qualitatively estimated by the presence/absence of nodules for each plant, and effectivity was determined through the acetylene reduction method on the entire root system under standard procedures. Ethylene production was determined on a Carlo.Erba GC6000 gas chromatograph. Results were expressed as µM C₂H₄·h⁻¹·g⁻¹ nodule dry weight.

2.9. Incidence of soil and host plant combination on symbiotic properties of the four ITS groups of *Frankia*

This study was conducted on two *Gymnostoma* species, *G. chamaecyparis* and *G. deplancheanum*, selected for their extremely opposite soil properties requirements. Moreover, these two species were the only ones to correspond to exclusive ITS group of *Frankia*. The three soils (Tontouta, Nepoui and Prony) were used as growth substrate. The growth and shade house conditions were as described above.

2.10. Infectivity and effectivity tests

Inoculations were performed with each ITS group using the same four selected strain as above, by delivering 10 mg (packed cell volume) of an 1-month-old culture. Four inoculated plants and two non-inoculated controls were used per treatment. Infectivity and effectivity were estimated as described above, 11 months after inoculation.

2.11. Co-inoculation study

Inoculation was performed by delivering to each soil/plant combination 10 mg inoculum. The inoculum was an equal volume (packed cell volume) mixture of

the four ITS selected strains. Four inoculated plants and two non-inoculated controls were used for each treatment. Nodules were harvested 11 months after inoculation and the infecting *Frankia* rRNA was characterized as above.

3. RESULTS AND DISCUSSION

3.1. *Frankia* strain isolation

The isolation method we used (one nodule lobe per tube, liquid medium) has two advantages: (i) it allows to quickly and easily eliminate contaminated lobes; and (ii) it ensures that isolates originate from one lobe.

About 2 100 nodule lobes had to be dissected to allow the obtention of 128 isolates from eight *Gymnostoma* and ten localities (table I). This corresponds to about 6 % of success of isolation among *Gymnostoma* species. With different method and different media, Rosbrook and Reddell [17] obtained isolation rates of 50 % for *Casuarina equisetifolia*, 17 % for *C. glauca* and 3 % for *C. cunninghamiana*. The first strain of *Frankia* was isolated 20 years ago [3], however the isolation step is still a considerable bottleneck to obtain pure cultures, and a strong variability is observed among host plants and methods used. Isolation seemed to be more successful on nitrogen-free medium (90 % of the successful isolations). This could be due to the early selection of nitrogen fixers (and the non-proliferation of contaminants) as well as a better compatibility between the culture medium and *Frankia* strains.

Table I. Origin of the strains of *Frankia* isolated in this study.

Host plant	Geographical origin	<i>Frankia</i> isolates
<i>Gymnostoma chamaecyparis</i>	Nepoui	Gc01, 06, 10 to Gc16, 18
<i>G. deplancheanum</i>	Chute de la Madeleine	Gd33, 41, 51, 103, 104, 106
	Creek Pernod	Gd02, 03, 17, 18
	Prony	Gd94, 95
	Rivière bleue	Gd73
	Yaté (col de)	Gd19, 20, 21, 22, 30, 32, 35, 36, 88, 91, 93, 121, 130, 132
<i>G. glaucescens</i>	Dzumac	Gg01 to Gg30
	Étoile filante	Gg40 to Gg51
<i>G. intermedium</i>	Dzumac	Gi01 to Gi03
<i>G. leucodon</i>	Rivière des pirogues	Gl01 to Gl15
<i>G. nodiflorum</i>	Ciu	Gn01 to Gn12
<i>G. poissonianum</i>	Dzumac	Gp01 to Gp04
<i>G. webbianum</i>	Rivière bleue	Gw01, 02, 07, 09, 12, 13, 20, 33, 34, 36, 40, 41, 51, 52

The strains isolated from *Gymnostoma* exhibited all the characteristic morphological structures of the genus *Frankia* including hyphae, sporangia, spores and vesicles. Vesicles were very numerous in the NFM

medium and still present after ammonium complementation. All the strains presented an orange to blood-red pigmentation. These characteristics are close to those of (i) the five already isolated *Gymnostoma* strains [15, 18], (ii) atypical strains from *Casuarina* (i.e. strains unable to nodulate the host of origin but which nodulate species belonging to *Eleagnaceae* and *Rhamnaceae* families) [5, 6, 22] and (iii) strains isolated from *Eleagnaceae* or *Rhamnaceae* described by Clawson et al. [4], Gauthier et al. [7] or Nazaret et al. [12]. The 128 isolates represented a wide genetic pool suitable to evaluate diversity of the strains nodulating the eight species of *Gymnostoma*.

3.2. Molecular characterization of the bacterial rRNA operon

3.2.1. PCR amplification

The DNA extraction protocol yielded DNA that was pure enough to obtain amplification with primer FGPS989e, which is *Eleagnaceae* group-specific. No amplification was obtained with primer FGPS989ac, which is *Casuarina* group-specific. The amplified fragments were about 1 000 bp long. Similar grouping of non-isolated *Frankia* from *Gymnostoma* (in nodule) with those of *Eleagnaceae* was already described by Navarro et al. [11] by using specific primers which confirms the particular position of *Gymnostoma* nodulating *Frankia* among the *Casuarinaceae* family.

3.2.2. RFLP

Discriminant patterns were observed when PCR products were digested with *Hae*III and *Msp*I. Each enzyme produced three profiles which, in combination, allowed to discriminate four different ITS groups named 1, 2, 3 and 4. Groups 2 and 3 were predominant: 59 and 26 %, respectively. These two groups were widely represented in the different species of *Gymnostoma* in their various ecological habitats. Groups 1 and 4 represented only 8.6 and 6.3 % of the isolates and were found to exclusively occur in *G. deplancheanum* and *G. chamaecyparis*, respectively, in their natural stands (table II). Working with non-iso-

Table II. *Frankia* isolated from *Gymnostoma* nodules.

<i>Gymnostoma</i>	<i>Frankia</i> : ITS groups (16S-23S)				
	Number	1	2	3	4
<i>G. chamaecyparis</i>	10			2	8
<i>G. deplancheanum</i>	28	11	17		
<i>G. glaucescens</i>	42		41	1	
<i>G. intermedium</i>	3		2	1	
<i>G. leucodon</i>	15			15	
<i>G. nodiflorum</i>	12		11	1	
<i>G. poissonianum</i>	4		4		
<i>G. webbianum</i>	14		1	13	
Total	128	11	76	33	8

lated strains of six *Gymnostoma* species, Navarro et al. [11] compared mainly sequences of the *nif* DK intergenic spacer, which did not allow a direct comparison with our results.

3.3. Symbiotic characterization of each group

3.3.1. Host spectrum of each group

The four ITS groups were found to be infective and effective on the seven *Gymnostoma* species tested and on *Eleagnus angustifolia*. They were non-infective on the three *Casuarina* and three *Allocasuarina* species tested. No significant difference was found in either infectivity or effectivity between the different groups. Re-isolation and PCR/RFLP characterization of a representative strain from the four *Frankia* groups allowed to confirm Kochs postulate for each group. Twenty-one arbitrarily chosen strains from the different groups were additionally tested, mainly on *G. chamaecyparis* and *G. deplancheanum*, which allowed us to confirm the lack of specificity between the groups and the species (data not shown).

3.3.2. Incidence of soil and host plant combination on symbiotic properties of the four ITS groups

Considering the relative exclusivity of groups 1 and 4 on *G. deplancheanum* and *G. chamaecyparis* and the specificity of the soils they colonized (Prony and Nepoui), it seemed particularly interesting to test infectivity and effectivity of the four ITS groups on these two species on their respective soil. A less extreme soil (Tontouta) was also used as control. Despite numerous attempts, it was impossible to maintain heterologous soil-plant combinations such as *G. deplancheanum*/Nepoui and *G. chamaecyparis*/Prony. Table III illustrates the results which did not allow to symbiotically differentiate the four groups whatever the tested combi-

nation. Even in extreme edaphic conditions, it is thus not possible to establish a clear relationship between the origin of a given strain and the symbiotic performances of the association. In a control sandy soil where both plant species can grow, there is no evidence of a clear advantage for any strain/plant combination.

3.3.3. Co-inoculation of the four groups in the different soil/plant combinations

Co-inoculation of two *Gymnostoma* species (table IV) with mixtures of strains allowed to evidence 11 % of mixed RFLP profiles. In such controlled conditions, this was interpreted as co-infections. Such co-infections have been reported by several authors [16, 21] and were already observed in the *Frankia-Gymnostoma* symbiosis through: (i) isolation of mixtures of strains originates from one nodule lobe (Gauthier, unpubl.); and (ii) observation of more than one ITS pattern in some nodule lobes harvested in the field (data not shown). Table IV presents the percentage of the different ITS groups of *Frankia* detected within the nodules in the different soil/plant combinations. A similar number of nodule lobes was analysed for each plant species. All the groups appeared to be equally represented within the nodule for any combination. No comparative advantage could thus be detected for any of the ITS groups in term of competitiveness in any of the situations.

Groups 1 and 4 are not more adapted to their original plants whatever the soil, and the predominance of groups 2 and 3 cannot be related to any symbiotic ability. One can thus speculate that the relative higher frequency of groups 2 and 3 among isolates is attributable to a better isolability. It can be questioned whether the isolation steps underestimate the biodiversity of the strains by selecting some groups above others. Using the same strategy (PCR/RFLP on 16S-23S ITS) with non-isolated *Frankia* from *Gymnostoma* (in nodule), it

Table III. Incidence of soil and *Gymnostoma* spp. combinations on symbiotic properties of the four ITS groups of *Frankia*. For each species, four repetitions were carried out. I, Infectivity (% of nodulation); E, effectivity (as ARA: $\mu\text{moles C}_2\text{H}_4\cdot\text{h}^{-1}\cdot\text{g}^{-1}$ nodule dry weight, extreme values).

<i>Frankia</i>	Soils and plants tested			
	Tontouta soil		Nepoui soil	Prony soil
	<i>G. chamaecyparis</i>	<i>G. deplancheanum</i>	<i>G. chamaecyparis</i>	<i>G. deplancheanum</i>
ITS group 1 Gd03	I:100 E: 13-35	I: 100 E: 15-35	I: 75 E: 11-43	I:75 E: 18-50
ITS group 2 Gg40	I: 75 E: 15-37	I: 100 E: 15-37	I: 100 E: 13-123	I: 100 E: 18-36
ITS group 3 GI03	I: 75 E: 12-52	I: 75 E: 30-36	I: 75 E: 15-51	I: 75 E: 15-37
ITS group 4 Gc18	I: 75 E: 5-53	I: 100 E: 8-44	I: 100 E: 13-79	I: 75 E: 16-69

Table IV. Co-inoculation of the four ITS groups in soil/*Gymnostoma* spp. combinations. We used four inoculated plants per treatment. Number in parentheses represent number of lobes analysed.

Soils and plants tested	Percentages of the four ITS groups within the nodules				
	ITS group 1	ITS group 2	ITS group 3	ITS group 4	Mixture of ITS groups
Tontouta soil					
<i>Gymnostoma chamaecyparis</i> (21)	14	24	28.5	24	9.5
<i>G. deplancheanum</i> (20)	20	15	20	30	15
Nepoui soil (hypermagnesian)					
<i>Gymnostoma chamaecyparis</i> (24)	20.9	12.5	33.3	25	8.3
Prony soil (oxisol)					
<i>Gymnostoma deplancheanum</i> (25)	24	20	24	20	12

was possible to determine seventeen ITS groups (Navarro, pers. comm.). However, the isolation of *Frankia* strains is essential for the studies of actinorhizal symbiosis and to produce inoculum for field experimentations.

This work made available a range of *Frankia* strains that have in common certain characteristics: these strains sporulate easily; they are pigmented, they grow rapidly *in vitro* even on nitrogen-free media; they are poorly host-specific, widely represented, easily isolatable and able to co-infect. They are close to strains of *Eleagnaceae*, and to atypical strains of *Casuarina* spp. Together with strains from *Eleagnaceae* and atypical strains, they could represent a primitive group of *Frankia* able to survive in a wide range of habitats thanks to a high adaptability: (i) they can infect or co-infect a wide range of species; (ii) they have a strong ability to survive in soils both through a high sporulating capacity and a putative ability of saprophytic life (that could be reflected by their high isolability).

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- [19] Simonet P., Capellano A., Navarro E., Bardin R., Moiroud A., An improved method for lysis of *Frankia* with Achromopeptidase allows detection of new plasmids, *Can. J. Microbiol.* 30 (1984) 1292-1295.
- [20] Simonet P., Grosjean M.C., Misra A.K., Nazaret S., Courmoyer B., Normand P., *Frankia* genus specific characterization by polymerase chain reaction, *Appl. Environ. Microbiol.* 57 (1991) 3278-3286.
- [21] Simonet P., Normand P., Moiroud A., Bardin R., Identification of *Frankia* strains in nodules by hybridization of polymerase chain reaction products with strain-specific oligonucleotide probes, *Arch. Microbiol.* 153 (1990) 235-240.
- [22] Zhang Z., Lopez M.F., Torrey J.G., A comparison of cultural characteristics and infectivity of *Frankia* isolates from roots nodules of *Casuarina* species, *Plant Soil* 78 (1984) 79-90.

Habilitation à Diriger des Recherches

Université Montpellier 2, Ecole doctorale SIBAGHE

Philippe JOURAND

Chargé de Recherche, IRD

**Rôles des symbioses dans l'adaptation des
légumineuses aux contraintes abiotiques des sols**

**Laboratoire des Symbioses Tropicales et
Méditerranéennes (LSTM, UMR113)**





- Résumé du parcours professionnel au LSTM UMR113 (1998-2012) -

Membre permanent depuis 1998 : IE puis IR et CR1

Affectation: Montpellier (1998-2005)

- Les légumineuses du genre *Crotalaria*,
- Diplôme de Doctorat de Sciences (2003, EDBI, UM2).

Affectation: IRD de Nouvelle-Calédonie (2006-2012)

- La symbiose mycorhizienne sur les sols extrêmes des massifs ultrabasiques de Nouvelle-Calédonie.
- 



- Activités de recherche (1998-2005) -

Les légumineuses du genre *Crotalaria*

- **Plantes herbacées (grande biodiversité)**
 - + de 600 espèces,
 - zone inter-tropicale.
- **Utilisées en agriculture (Pays du Sud)**
 - Fertilisation azotée des sols (engrais vert),
 - Lutte contre les nématodes phytoparasites.

Légumineuses du genre *Crotalaria*

C. retusa (environnement naturel)



C. retusa utilisée en agriculture





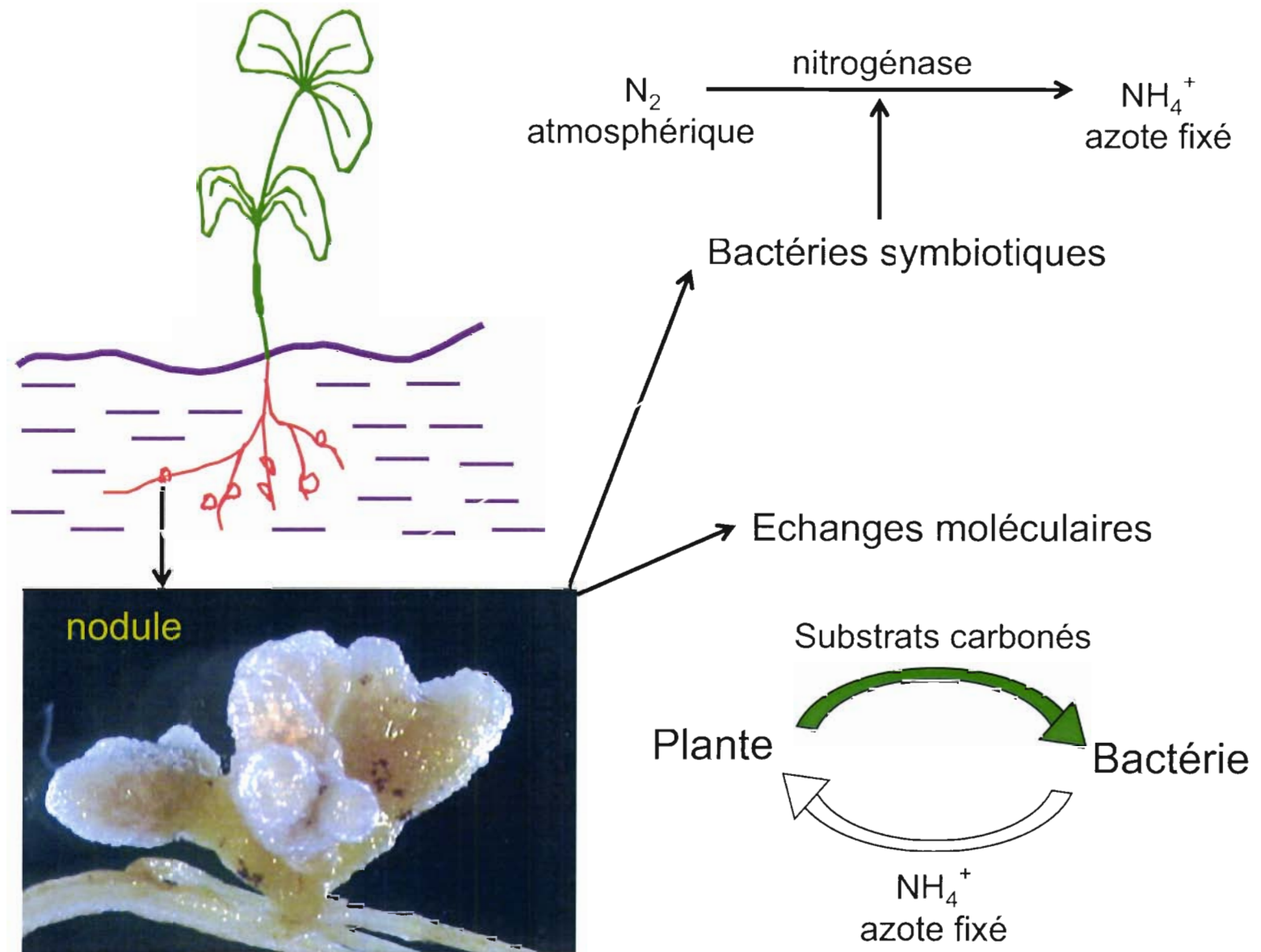
Activités de recherche (1998-2005)

Les légumineuses du genre *Crotalaria* Afrique de l'Ouest (Sénégal)

**1. Symbiose atypique avec des bactéries du
genre *Methylobacterium***

**2. Utilisation dans le contrôle des
populations de nématodes phytoparasites**

Symbiose légumineuse - bactérie nodulante et fixatrice d'azote





Les légumineuses du genre *Crotalaria* d'Afrique de l'Ouest (Sénégal)

1. Symbiose atypique *Crotalaria-Methylobacterium*

- 1.1. Description et caractéristiques de l'espèce bactérienne méthylotrophe *Methylobacterium nodulans*
- 1.2. Expression de la fonction méthylotrophique bactérienne dans le nodule fixateur d'azote
- 1.3. Etude du lien entre méthylotrophie et structure du nodule
- 1.4. Etude du dialogue moléculaire légumineuse-bactérie

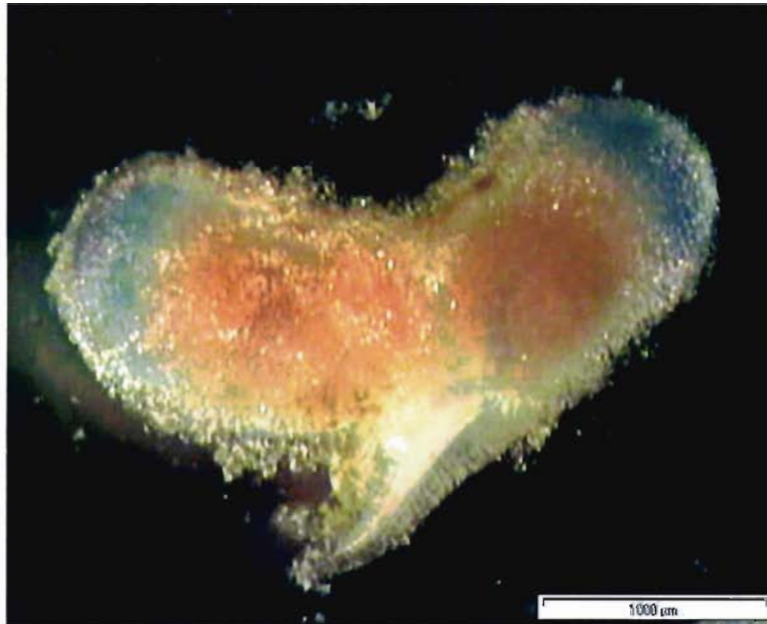
- Bactéries du genre *Methylobacterium*,
- Propriétés méthylo-trophiques : capacité à utiliser MeOH (1M) comme seule source C, et présence du gène *mxoF* codant MDH.

- Nodule fixateur d'azote efficient en symbiose spécifique avec des légumineuses du genre *Crotalaria*,
- Présence des gènes de nodulation (gènes nod) et des gènes codant pour enzyme nitrogénase (gènes nif).

1990 1991 1992 1993 1994 1995 1996 1997 1998 1999 2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010

1.2. Expression de la méthylothrophie dans le nodule fixateur d'azote (Doctorat, 2003)

Suivi de l'expression *in planta*
(recombinaison *mxoF-LacZ*)



Nodule *Crotalaria* - *M. nodulans mxoF-LacZ*

Perte de la fonction méthylotrophe
(mutation insertionnelle)



Témoin non inoculé *Methylobacterium nodulans* sauvage mutant 1 mutant 2

Résultats :

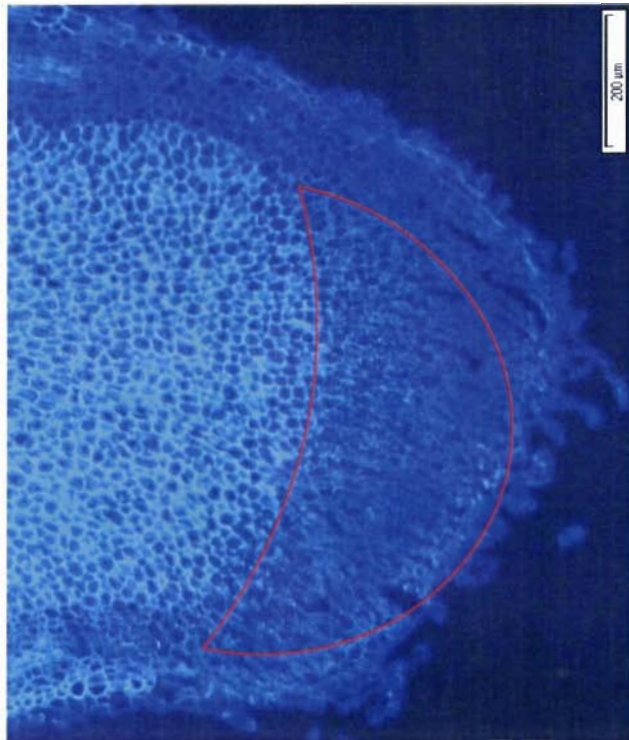
- la méthylothrophie s'exprime à l'apex du nodule,
- perte de la fonction : nodulation (↓60%),
fixation d'azote (↓42%), croissance de plante (↓46%).

Publication :

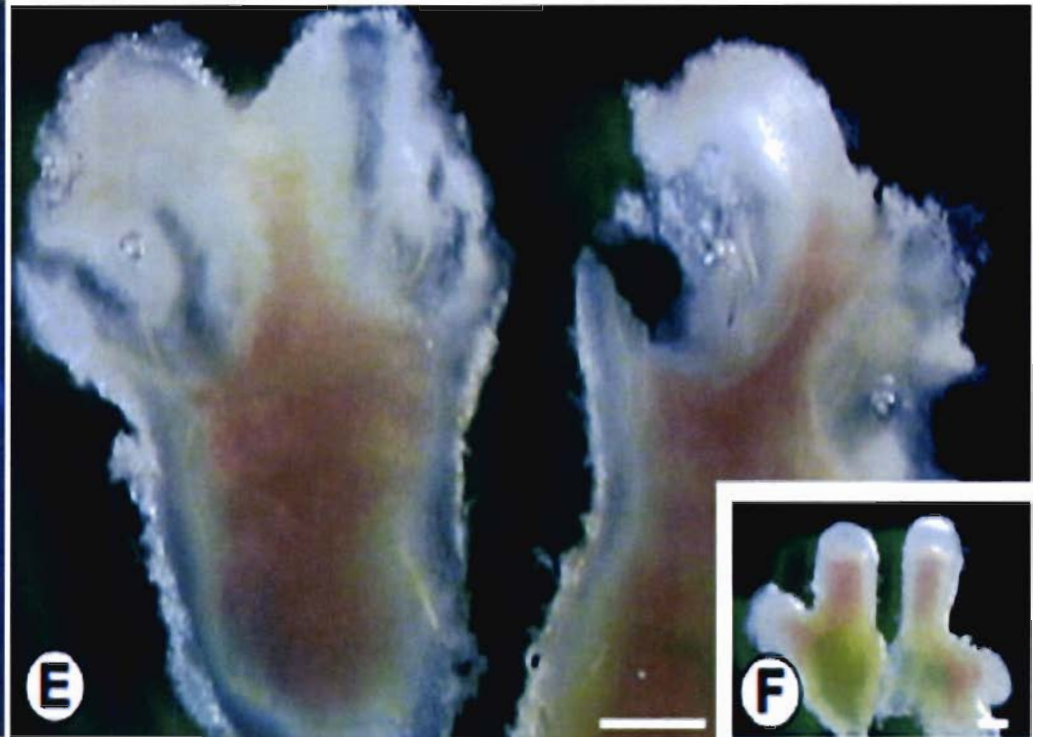
Jourand *et al.*, 2005, *MPMI*

1.3. Méthylotrophie et structure du nodule (Doctorat A. Renier, 2008, ED Sibaghe, UM2)

Observations : dégradation des tissus à l'apex du nodule



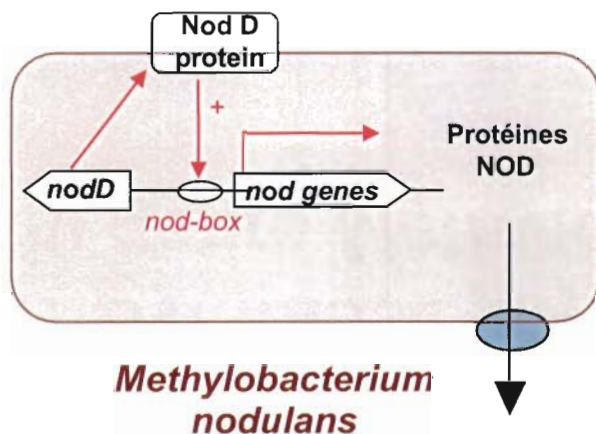
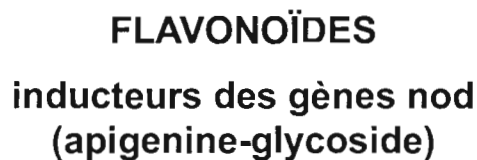
Coupe nodule avec
M. nodulans sauvage



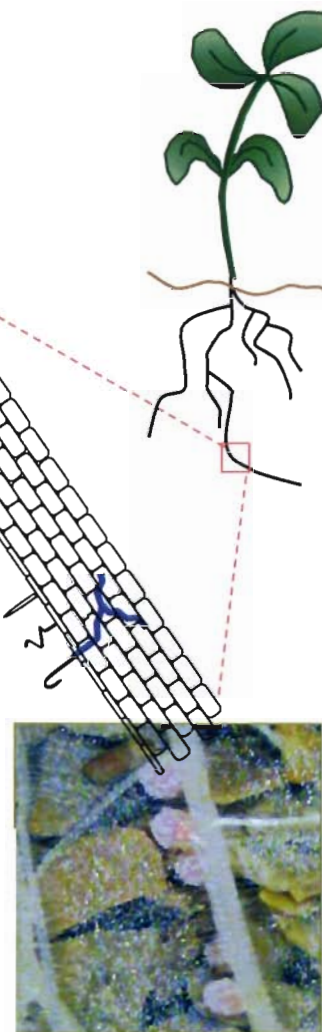
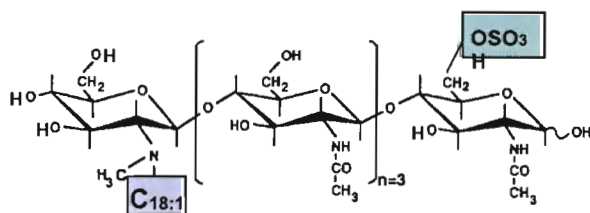
Nodules avec souche sauvage *versus* mutant *mxoF(-)*

Hypothèses : Zone de dégénérescence à l'apex du nodule,
Dégradation pectines (production MeOH).

Publication : Renier *et al.*, 2011, *J Exp Bot*



FACTEUR NOD



***Crotalaria
podocarpa***

Publication : Renier *et al.*, 2008, *Soil Biol Biochem*



Activités de recherche (1998-2008)

Les légumineuses du genre *Crotalaria*

1. Symbiose atypique avec les bactéries
du genre *Methylobacterium*

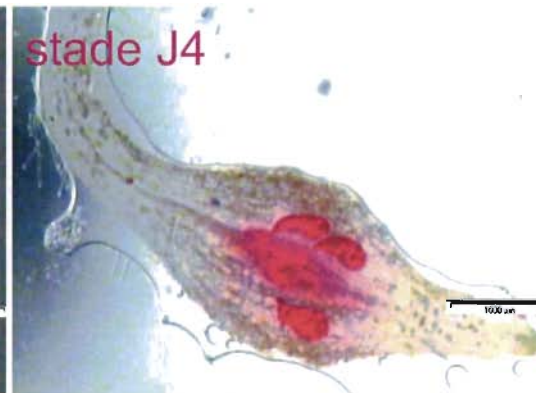
2. Utilisation dans le contrôle des
populations de nématodes phytoparasites



2. Utilisation des *Crotalaria* dans le contrôle des populations de nématodes phytoparasites

- Invertébrés, phylum *Nematoda*,
- Vers ronds microscopiques,
- Parasites obligatoires des plantes,
- Genre le plus connu et étudié : *Meloidogyne*.

Illustration du cycle partiel avec infection sur racines de tomates



Dégâts sur les cultures vivrières : baisses de rendements, chiffrées à 77 milliards US\$ / an (Barker, 1998)



2. Utilisation des *Crotalaria* dans le contrôle des populations de nématodes phytoparasites

2.1. Rôle phytoprotecteur d'un extrait aqueux de *Crotalaria*

2.2. Nature de l'activité phytoprotectrice des extraits aqueux de *Crotalaria* d'Afrique de l'Ouest

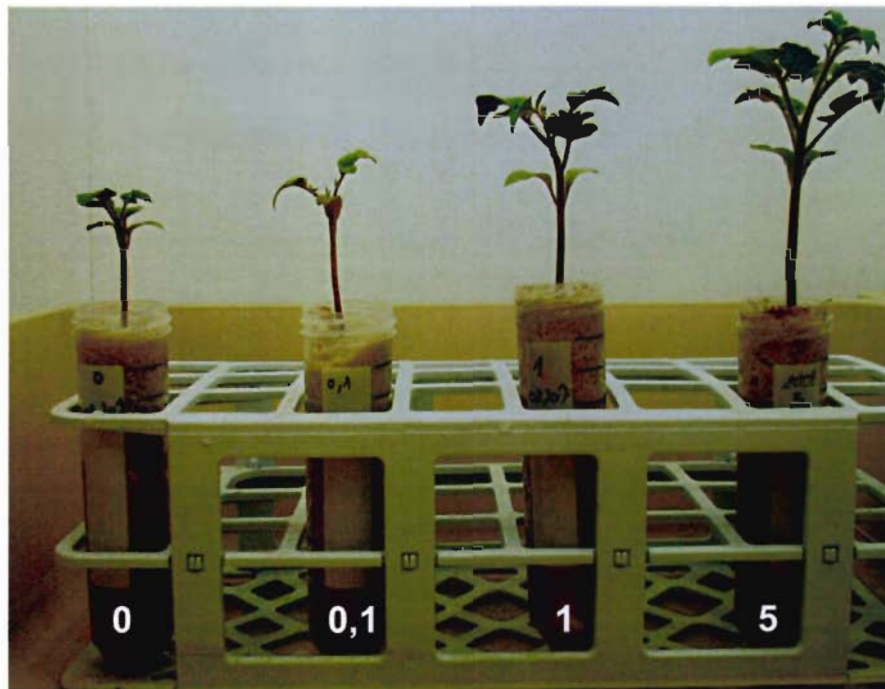
2.3. Etudes aux champs de contrôle de populations de nématodes par une culture de *Crotalaria*



2.1. Rôle phytoprotecteur d'un extrait aqueux de *Crotalaria* (Doctorat, 2003)

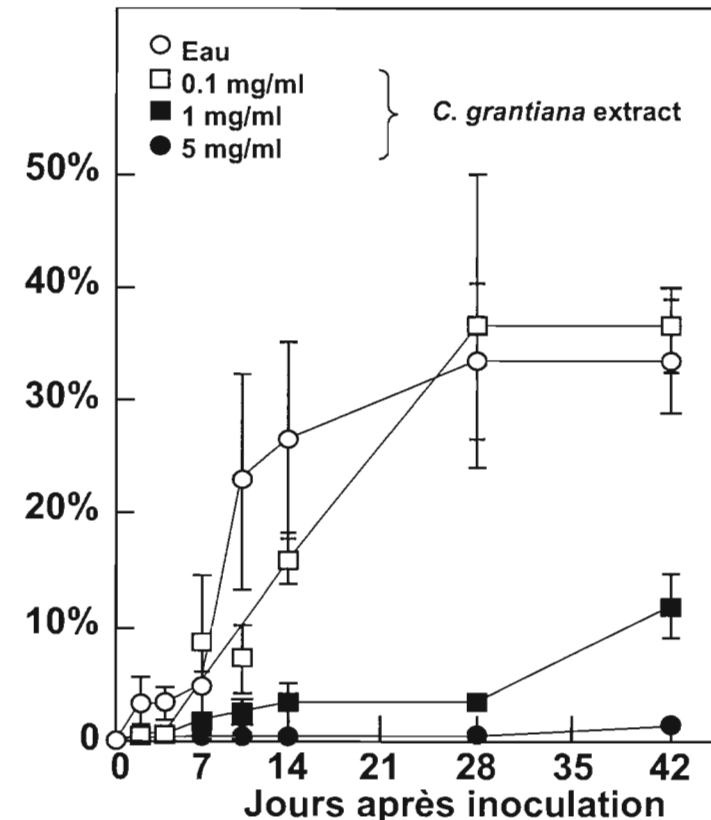
Culture de plants de tomate en présence de nématodes *M. incognita*

Plants de tomate,
6 semaines après inoculation



Extrait foliaire de *C. grantiana* dans le substrat
(concentrations mg/ml)

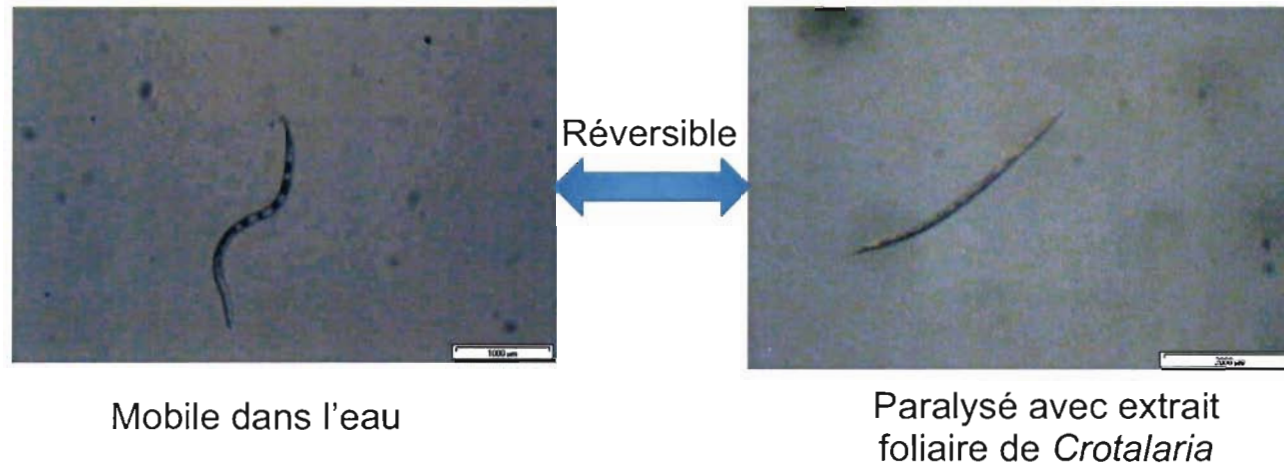
Suivi du taux d'infection racinaire
par les juvéniles J2



Résultat : Rôle phytoprotecteur de l'extrait foliaire de *C. grantiana*.
Publication : Jourand et al., 2004b, *Nematology*

2.2. Nature de l'activité phytoprotectrice des extraits aqueux de *Crotalaria* d'Afrique de l'Ouest (Doctorat, 2003)

2.2.1. Extrait de *Crotalaria* paralyse les juvéniles (stade J2) de façon réversible



2.2.2. Activité des extraits de 15 espèces de *Crotalaria* d'Afrique de l'Ouest

Groupe selon activité extrait	Taux (%) de paralysie J2	<i>Crotalaria</i>
Groupe 1 : très actives	70 - 100 %	10 espèces
Groupe 2 : moyennement actives	40 - 60 %	3 espèces
Groupe 3 : faiblement actives	20 - 40 %	2 espèces

Conclusion : **Activité nématostatique réversible,
variable selon l'espèce de *Crotalaria***

Publication : **Jourand *et al.*, 2004c, *Nematology***

2.3. Etudes aux champs de contrôle de populations de nématodes par une culture de *Crotalaria* (Doctorat, 2003)

- Champ infesté de nématodes : culture de *Crotalaria* avec enfouissement, puis culture de tomate,
- Intérêt double de *Crotalaria* 1) engrais vert, 2) activité nématostatique

Après culture de *Crotalaria*



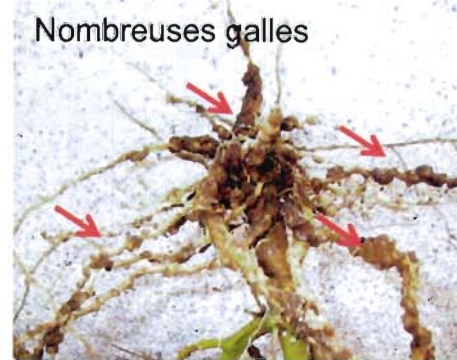
Absence de galle



Témoin sans crotalaire



Nombreuses galles



Résultats : Infection racinaire (- 60 %), rendements tomates (+ 45 %)
Publication : Thèse de doctorat P. Jourand, 2003.



Bilan des recherches sur les *Crotalaria*

- **Thèse de Doctorat** (2003)
- **Publications et Communications**
 - 4 articles en 1^{er} auteur (2004a,b,c et 2005),
 - 3 articles en co-auteur (2001, 2008 et 2011),
 - 7 communications internationales et 2 nationales.
- **Encadrements**
 - 1 thèse de doctorat UM2 (2004-2007),
 - 2 stages M2 et 2 stages L3 (2000-2004).
- **Collaborations**
 - LSTM, IRD Dakar, Sénégal (G. Germani),
 - UMR CEFÉ (S. Rapior) et CBGP (T. Mateille et M. Fargette),
 - GRAB, Avignon (H. Védié et C. Bertrand).

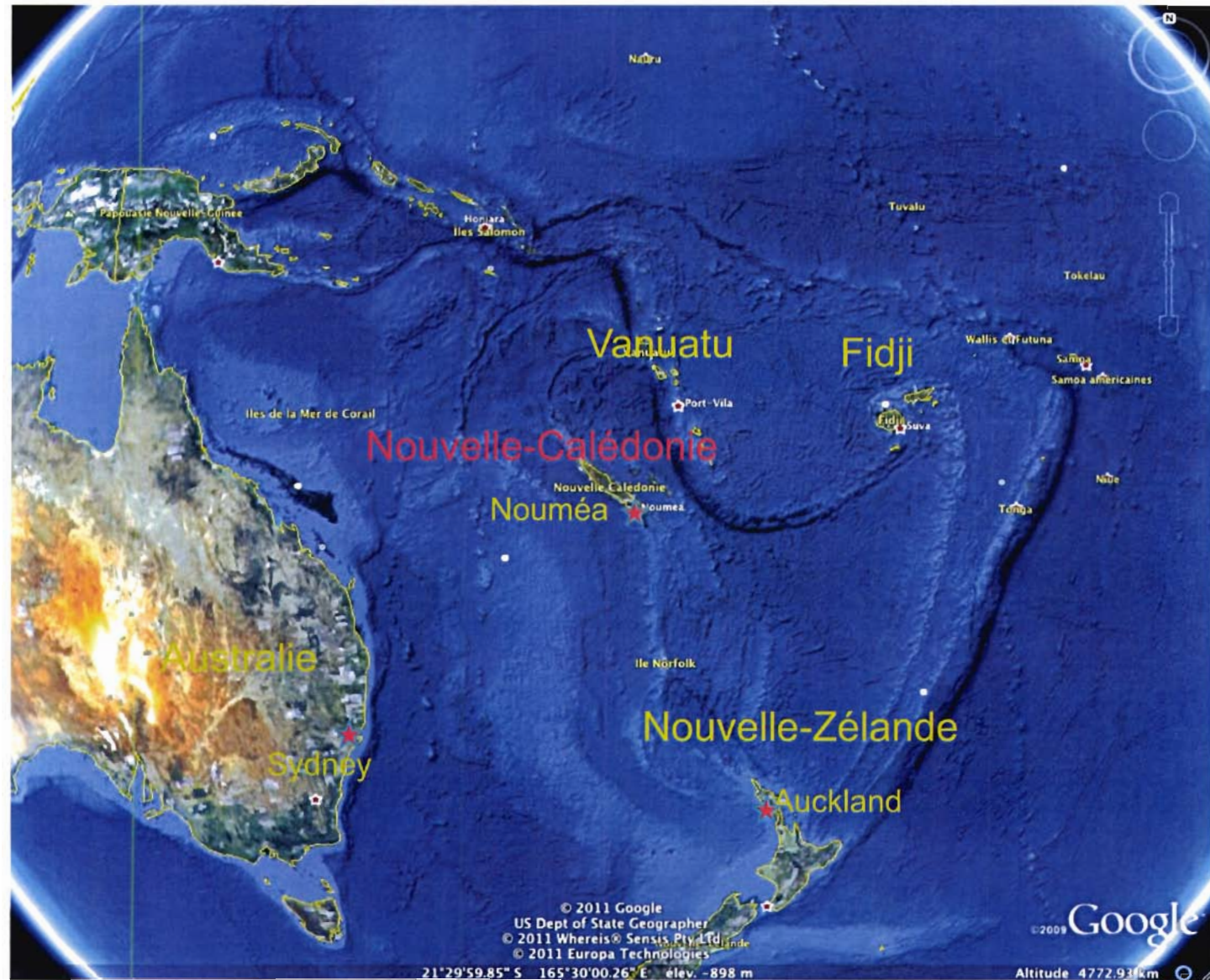


Activités de recherche 2006-2012

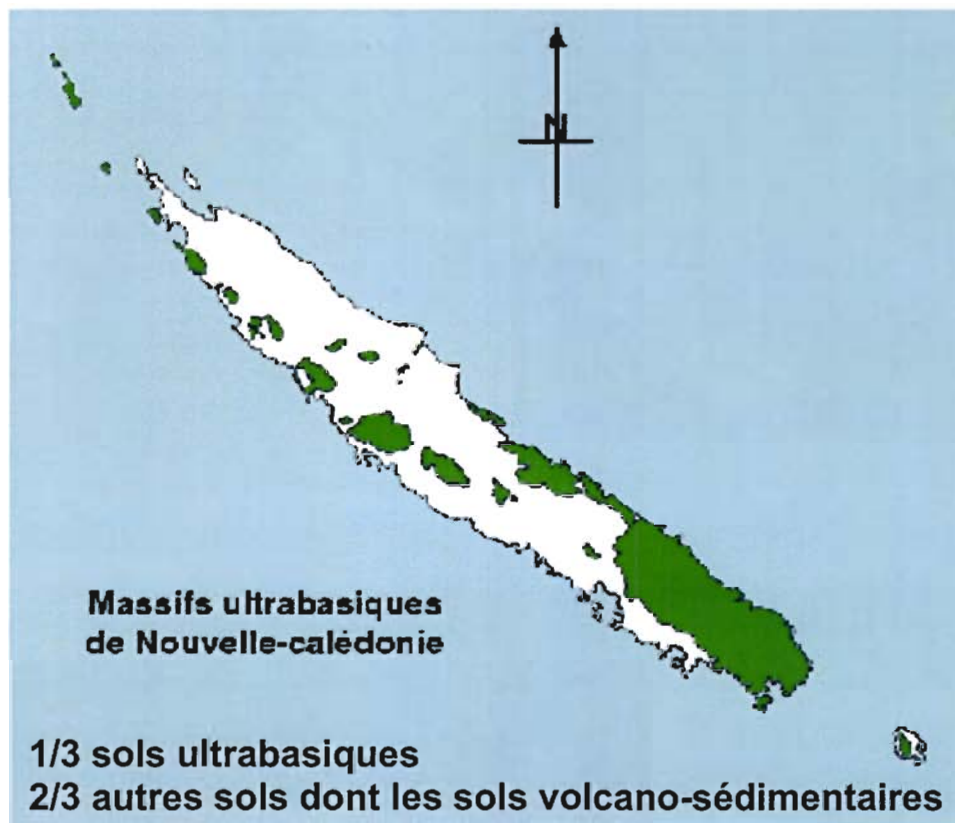
**La symbiose atypique mycorhizienne
sur les sols extrêmes de Nouvelle-Calédonie**

**Utilisation de ces symbioses dans les stratégies
de re-végétalisation des sols dégradés**

Contexte général : localisation géographique de la Nouvelle-Calédonie



Contexte général : les massifs ultrabasiques en Nouvelle-Calédonie



- 1/3 superficie (5 500 km²),
- Péridotites et serpentines,
- Exploités pour Ni, Cr et Co.

Conséquences : Exploitation minière (nickel) forte,

Nouvelle-Calédonie : 4^{ème} producteur de Ni, 2^{ème} réserve mondiale.

Contexte général : analyse minérale des sols (1)



Sol ultrabasique : ex Koniambo



Sol volcano-sédimentaire : ex Poum

	N total mg/kg	P mg/kg	K mg/kg	Ca mg/kg	Mg mg/kg
Ultrabasique	300	82	84	280	4002
Volcano-sédimentaire	nd	nd	275	70	670
Sols fertiles (Ricklefs & Miller, 2005)	(0,1%) 1000	(0,8 %) 800	(1%) 10000	(1%) 10000	(0,6%) 600

Déficiences NPK, ratios Ca/Mg déséquilibré ($\approx 1/20$)

Contexte général : analyse minérale des sols (1)



Sol ultrabasique : ex Koniambo



Sol volcano-sédimentaire : ex Poum

	Fe g/kg	Si g/kg	Ni g/kg	Cr g/kg	Co g/kg	Mn g/kg
Ultrabasique	436	6,5	3,3	17,6	0,36	8,2
Volcano- sédimentaire	0,5	411	0,09	0,12	0	0,01
Sols fertiles (Ricklefs & Miller, 2005)	(4%) 40	(33%) 330	traces	traces	traces	(0,08%) 0,8

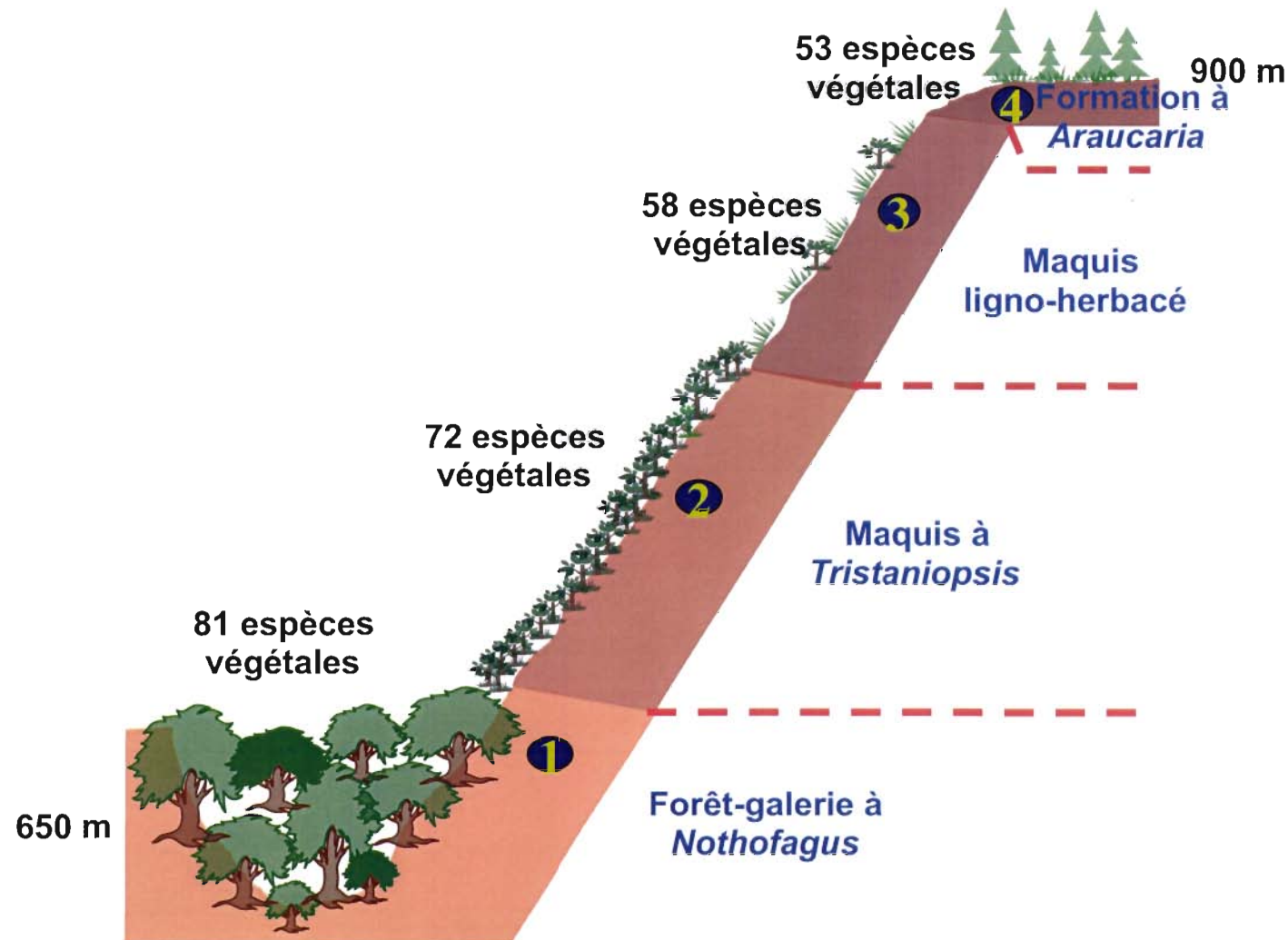
Élément majeur : Fe ou Si; présence de métaux lourds

**Contexte général : la Nouvelle-Calédonie « hot spot »
de la biodiversité terrestre (Myers, 2000, *Nature*)**



80 % de la flore endémique ; ex : maquis minier du grand sud

Contexte général : étude de la toposéquence d'un massif ultrabasique (Perrier, 2005)



Contexte général : l'exploitation minière impacte les écosystèmes terrestres ultrabasiques



Impact activité minière

- Atteinte à la biodiversité
- Erosion après exploitation

Obligations des exploitants

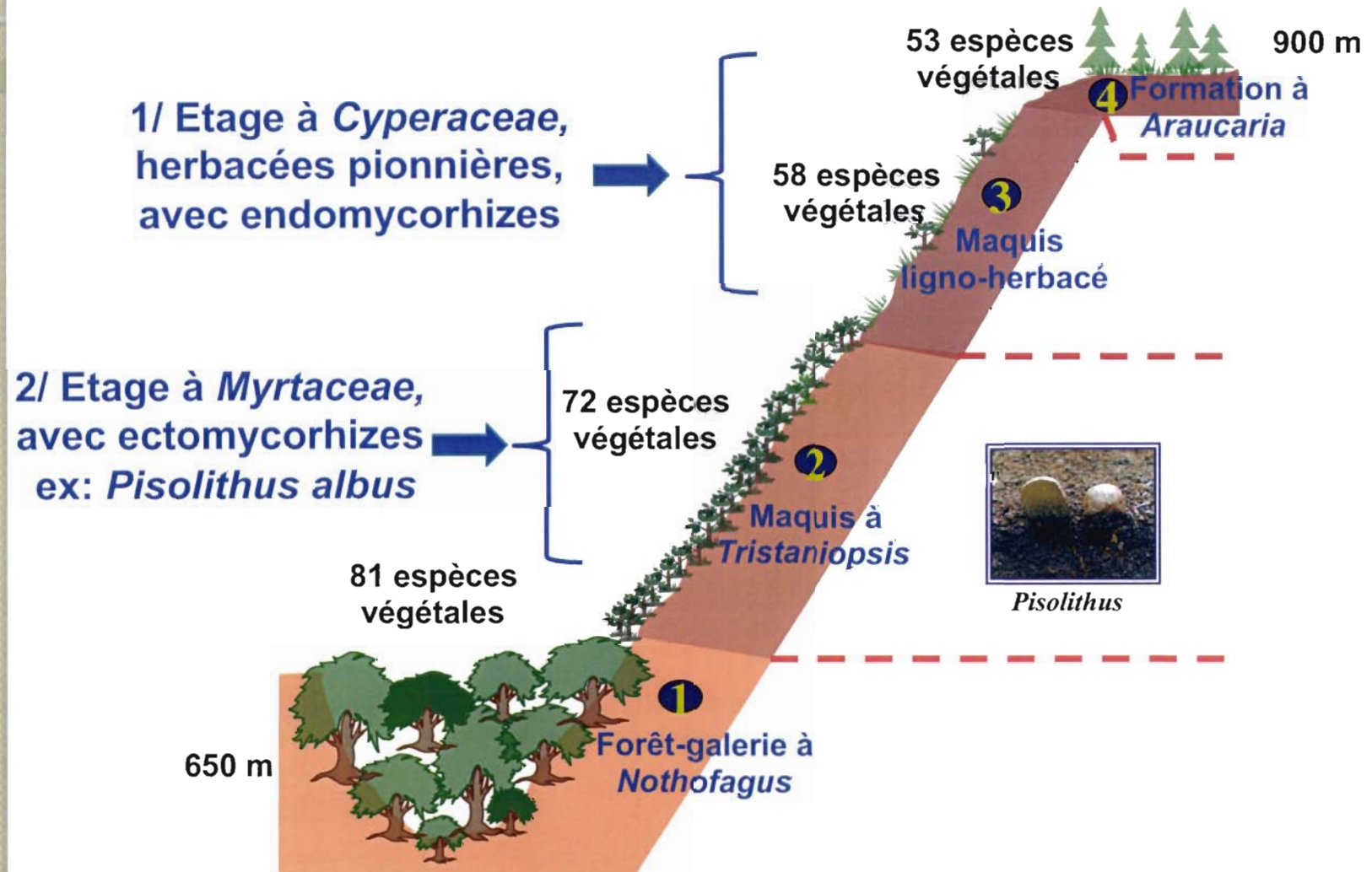
- Conservation de la biodiversité
- Restauration des sols dégradés

Stratégies de réhabilitation des sols dégradés :

- Utilisation des espèces végétales endémiques,
- Valorisation des microorganismes associés (bactéries, champignons) ?

Activités de recherche 2006-2012

La symbiose atypique mycorhizienne sur les sols extrêmes des massifs ultrabasiques de Nouvelle-Calédonie





1. Le statut endomycorhizien des *Cyperaceae*, plantes pionnières des massifs ultrabasiques

- **Travaux réalisés dans le cadre d'une thèse CIFRE : UNC-KNS (Doctorat A. Lagrange, 2009, UNC)**
 - Co-direction UNC (H. Amir) / LSTM (M. Ducousso),
 - Contribution à l'encadrement 3^{ème} et 4^{ème} chapitres de thèse.
- **Etude du statut endomycorhizien de 9 espèces de *Cyperaceae* sur 4 sites ultrabasiques**
 - Endomycorhization effective avec un taux de 8 % à 57 % selon les espèces,
 - Identification moléculaire (ITS partiel) : genre *Glomus*.
- **Inoculation contrôlée en serre sur substrat ultrabasique : *Costularia comosa* ± *Glomus* sp.**
 - Amélioration de la croissance (biomasse aérienne 2,4 fois + élevée),
 - Réduction du Ni dans les tissus de 2,5 fois par rapport au témoin.

**Conclusion : Intérêt de la symbiose endomycorhizienne dans
les stratégies de réhabilitation**

Publication : Lagrange et al., 2011, *Can J Microbiol*





La symbiose atypique mycorhizienne en milieu extrême : les sols ultrabasiques de Nouvelle-Calédonie

- 1. Le statut endomycorhizien des *Cyperaceae*, plantes pionnières des massifs ultrabasiques**
- 2. Le champignon ectomycorhizien *Pisolithus albus* tolérant au nickel et son rôle dans l'adaptation de la plante hôte au nickel**

Symbiose ectomycorhizienne : modèle *Myrtaceae* - *Pisolithus albus*

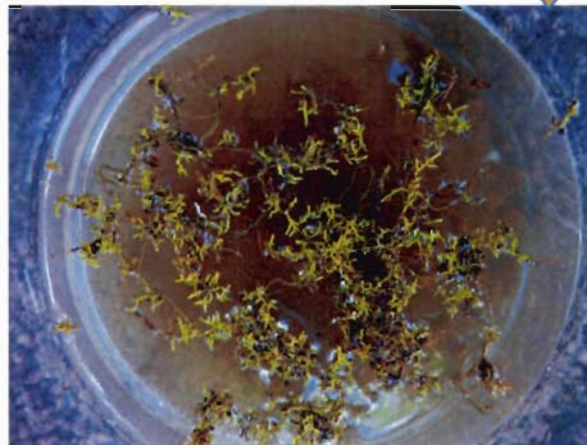


← Plante hôte :
Angiosperme (famille des *Myrtaceae*)

Minéraux

Echanges
moléculaires

Substrats
carbonés



← Racines avec
ectomycorhizes



2. Le champignon ectomycorhizien *Pisolithus albus* tolérant au nickel et rôle dans l'adaptation de la plante hôte au nickel

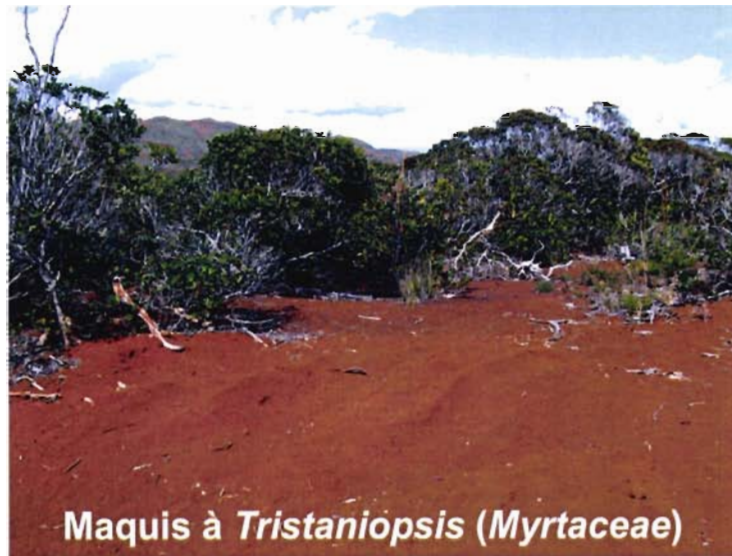
2.1. Diversité génétique de *Pisolithus albus* et diversité fonctionnelle (tolérance au nickel)

2.2. Recherche chez *P. albus* de déterminants moléculaires marqueurs de la tolérance au nickel

2.3. Rôle de *P. albus* dans l'adaptation de sa plante hôte à la tolérance au nickel

2.1. Diversité génétique et fonctionnelle de *Pisolithus albus* en Nouvelle-Calédonie

Sur sol ultrabasique (UB)



Sur sol volcano-sédimentaire (VS)



2.1. Diversité génétique et fonctionnelle de *P. albus* en Nouvelle-Calédonie (suite)

- **Etudes phylogéographique et génotypique**
 - ITS : *Pisolithus albus*, groupe avec *P. albus* région Australasie,
 - Génotypage AFLP : 2 génotypes distincts : - ultrabasique
- volcano-sédimentaire
- **Etude tolérance au nickel (marqueur des sols ultrabasiques)**
 - 55 % des isolats ultrabasiques, tolérants au Ni avec $EC_{50} = 575 \mu M$,
 - 100 % des isolats VS sensibles au Ni avec $EC_{50} = 30 \mu M$,

Résultat : Structure des populations de *P. albus* selon le sol

Hypothèse : Existence d'écotypes

Publication : Jourand *et al.*, 2010a, *FEMS Microbiol Ecol*



2.2. Recherche chez *P. albus* de déterminants moléculaires marqueurs de la tolérance au nickel

- Séquençage haut débit du transcriptome d'une souche *P. albus* tolérante au nickel (2 conditions $\pm$ Ni 250 μ M)
 - Pyroséquençage : 19 518 gènes exprimés séquencés,
 - 21 % activés par Ni, 9 % réprimés, et 70 % neutres.
- Sélection des marqueurs de la tolérance au Ni parmi les 21 % de gènes activés
 - Sélection de 10 gènes les plus activés par le Ni,
 - 5 gènes biomarqueurs tolérance au Ni
 - exclusivement exprimés *in vitro* chez isolats tolérants au nickel,
 - exprimés également *in situ* : mycélium et ECM de ces isolats.

Publication : Majorel et al., 2012, *Mol Ecol* (sous presse)



2.3. Rôle de *Pisolithus albus* dans l'adaptation de sa plante hôte au nickel

Eucalyptus globulus $\pm$ *P. albus*, $\pm$ Nickel



Taux de mycorhization : 9 %

Gains de biomasse :
20 X pour les racines,
30 X pour les parties aériennes.

Réduction Ni dans les tissus :
2,4 X moins de Ni dans les racines
60 X moins de Ni dans les feuilles

Résultat majeur : ECM protège la plante hôte de la toxicité au Ni

Collaboration avec R. Reid, Université Adelaïde, Australie.

Publication : Jourand *et al.*, 2010b, *Tree Physiology*



Bilan des travaux de recherche sur la symbiose mycorhizienne en milieu extrême

- **Publications et Communications**

- 2 articles en 1^{er} auteur (2010a,b),
- 2 articles en co-auteur (2011 et 2012),
- 7 communications internationales.

- **Encadrements**

- 1 Doctorat CIFRE (UNC - entreprise KNS ; 2006-2009),
- 2 stages M2 (2011), 4 stages L2-L3 (2007-2008).

- **Collaborations**

- Laboratoire LIVE, (UNC) et IAC, Nouvelle-Calédonie,
- UMR CEREGE (Aix en Provence), UMR DIADE (Montpellier).

- **Financements majeurs**

- ANR Ultrabio (2007-2010) : 725 k€ dont 58 k€/an au LSTM NC,
 - Projet CNRT Ecomine biotop (2010-2013) : 545 k€ dont 39 k€/an au LSTM NC.
- 



Projet de Recherche

Rôles des symbioses atypiques dans l'adaptation des légumineuses aux contraintes abiotiques des sols

Le modèle *Acacia spirorbis* en Nouvelle-Calédonie



La légumineuse *Acacia spirorbis* Labill.

- **Famille des *Mimosaceae*, genre *Acacia***
 - Espèce grégaire, probablement endémique (NC + Vanuatu),
 - Distinguée des autres *Acacia* zone pacifique par Pedley (1990):
 - *Acacia spirorbis* subsp. *spirorbis* (NC + Vanuatu),
 - *Acacia spirorbis* subsp. *solandri* (Australie).
- ***Acacia spirorbis* (ou Faux gaïac)**
 - Arbuste, parfois arbre jusqu'à 10 m de haut,
 - Bois imputrescible,
 - Utilisé pour l'élevage (piquets des clôtures) et avec succès en re-végétalisation des sites miniers.
- **Deux types de symbiose**
 - Rhizobiums nodulants et fixateurs d'azote,
 - Mycorhizienne (endo et ecto).

La légumineuse *Acacia spirorbis* Labill.



Forme buissonnante



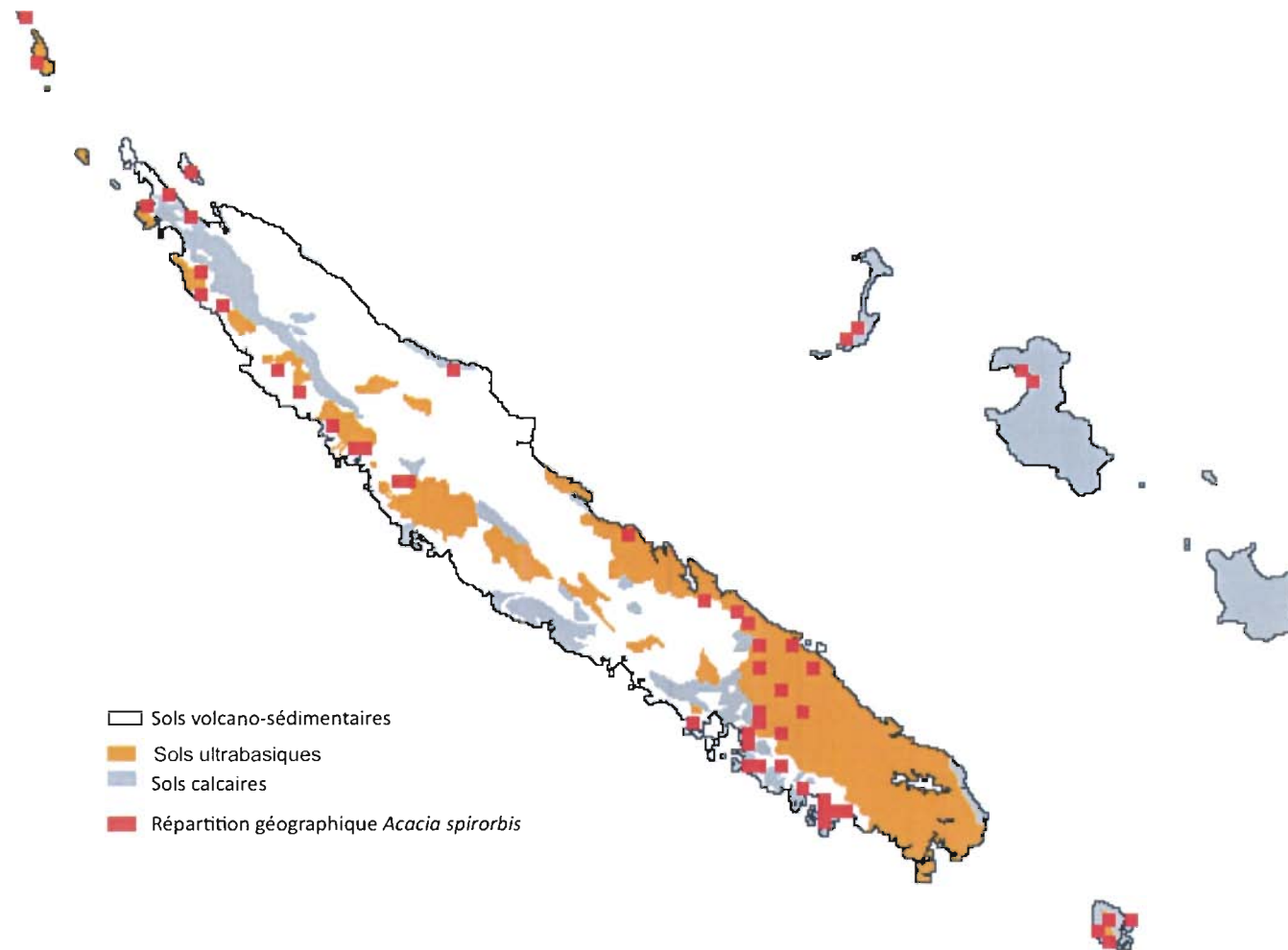
Forme arborescente

La légumineuse *Acacia spirorbis* Labill.

Racines avec double symbiose visible : nodules fixateurs azote et ECM



Distribution observée en Nouvelle-Calédonie





Contexte *Acacia spirorbis* en Nouvelle-Calédonie

- **Présent sur les massifs ultrabasiques**
 - Sols déficients NPK,
 - Ratio Ca/Mg déséquilibré (1/20),
 - Présence du Fe + métaux lourds (Co, Cr, Mn et Ni).
- **Présent sur sols volcano-sédimentaires**
 - Sols déficients NPK,
 - Ratio Ca/Mg déséquilibré (1/10),
 - Présence de Si, absence (ou traces) des métaux lourds.
- **Présent sur sols calcaires coralliens (Iles Loyautés)
(Becquer *et al.*, 2001, *Geoderma*)**
 - NPK faibles,
 - Ratio Ca/Mg normal (2/1),
 - 2 types de sols : avec Al et sans Al mais pas de métaux lourds.



Rôles des symbioses dans l'adaptation de la plante à la contrainte abiotique des sols

Le modèle *Acacia spirorbis* en Nouvelle-Calédonie

1. Etude préliminaire: espèce et endémisme ?
2. Symbiose nodulante et fixatrice d'azote avec des rhizobium
3. Symbiose ectomycorhizienne avec *Pisolithus albus*



1. Etude préliminaire : lever l'ambiguïté de l'espèce et de l'endémisme

- **Structure des populations d'*Acacia spirorbis* en NC : une seule espèce ou plusieurs sous-espèces selon les sols ?**
 - Collectes de sols, échantillons de plantes, nodules et ECM,
 - Analyses physico-chimique des sols (ICP),
 - Mise au point de marqueurs phylogénétiques de la plante,
 - Etude génétique des populations par génotypage.
- **Comparaison *Acacia spirorbis* NC avec d'autres espèces de la région : préciser l'endémisme ?**
 - Comparaison avec échantillons Australie (côte Est) et Vanuatu.

Collaboration avec IAC NC et UMR AGAP





Rôles des symbioses dans l'adaptation de la plante à la contrainte abiotique des sols

Le modèle *Acacia spirorbis* en Nouvelle-Calédonie

1. Etude préliminaire: espèce et endémisme ?

2. Symbiose nodulante et fixatrice d'azote avec des rhizobium

3. Symbiose ectomycorhizienne avec *Pisolithus albus*



2.1. Etude de la diversité des populations de rhizobium en fonction de sols

- **Structure de la (ou des) populations de rhizobium**
 - A partir des nodules : isolement des bactéries symbiotiques,
 - Identification microbiologique classique,
 - Extraction ADN et identification avec les marqueurs phylogénétiques classiques (gènes ARN16S et recA),
 - Analyses phylogénétiques.
- **Etude fonctionnelle et physiologique des capacités symbiotiques**
 - Recherche des gènes de nodulation (nod) et de la nitrogénase (nif),
 - Tests *in vitro* de nodulation et fixation azote sur plante.

Collaboration intra LSTM sur la diversité : équipe 2 (A. Klonowska)

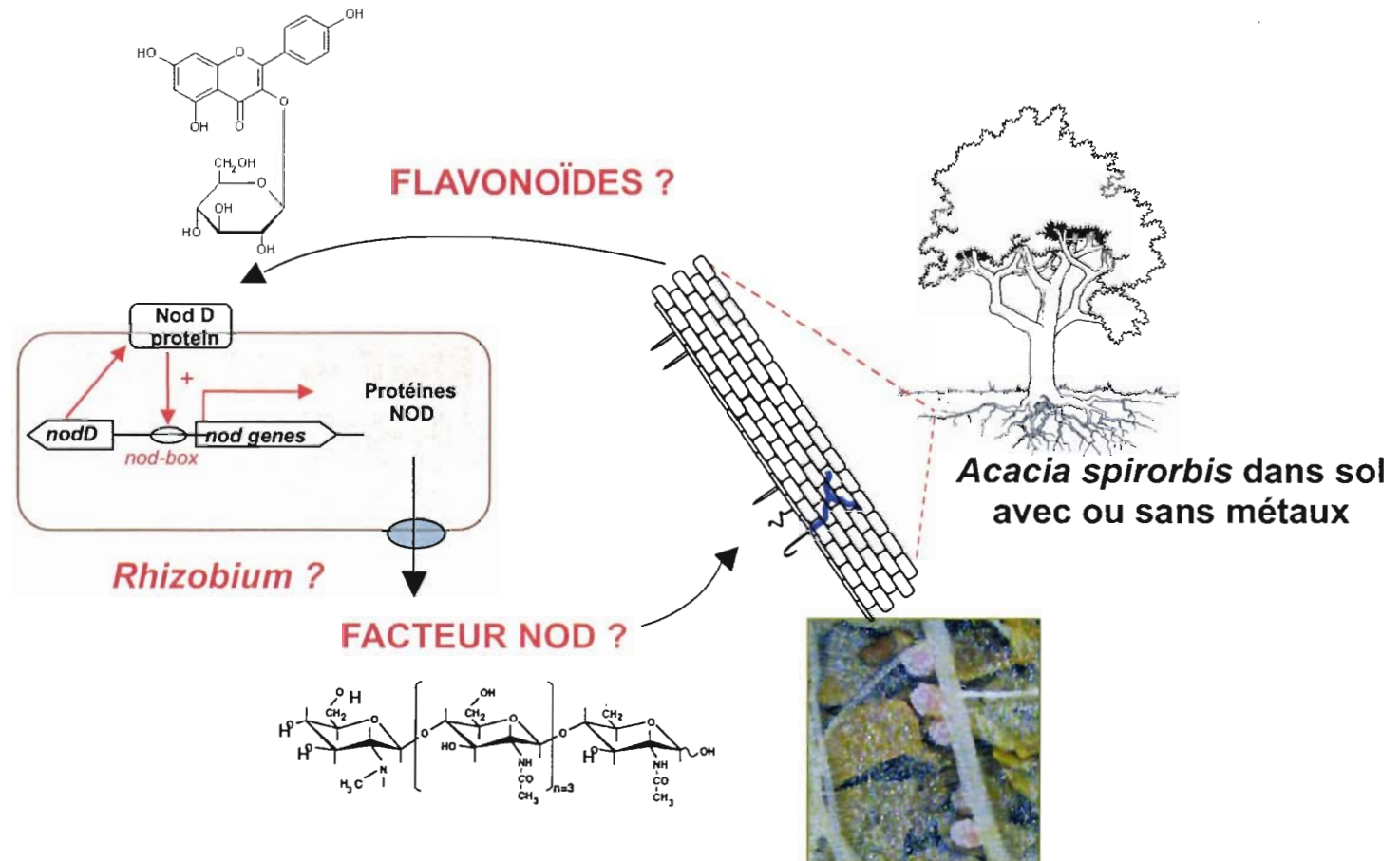




2.2. Adaptation des rhizobium à la contrainte édaphique : ex la tolérance aux métaux lourds

- **Détermination des IC₅₀ pour chaque métal et identification de souches remarquables**
 - Tests *in vitro* de tolérance aux métaux (Al, Co, Cr, Mn, Ni),
 - Screening et sélection de souches multi-tolérantes.
- **Recherche de déterminants moléculaires support de la tolérance aux métaux**
 - Analyse sans à priori par séquençage haut débit du transcriptome sur souche modèle multi-tolérante,
 - Recherche de déterminants connus (gènes) :
 - opérons *cnr*, *nre*, *ncc*, *mnt*, *chr*,
 - rôle par mutation / complémentation

2.3. Nature du dialogue moléculaire plante hôte - bactéries symbiotiques des milieux extrêmes



Collaboration UMR 5175 CEFE (S. Rapior)



2.4. Rôle de cette symbiose dans l'adaptation de la plante à la contrainte édaphique

- **Population de rhizobium de milieux extrêmes (métaux)**
 - Souches mono ou multi-tolérantes aux métaux,
 - Seuils de tolérance remarquables (≥ 10 mM),
 - Déterminants moléculaires de la tolérance identifiés et séquencés.
- **Etudes en conditions semi-contrôlées (serre) d'inoculation de bactéries tolérantes aux métaux**
 - Expression des déterminants moléculaires de la tolérance aux métaux dans le nodule fixateur d'azote,
 - Conséquences sur la physiologie de la plante et sa tolérance aux métaux ou à son environnement édaphique (test sur substrat extrême).

Application et développement : utilisation d'inoculum bactériens dans les stratégies de réhabilitation des sols dégradés ?

Contribution à la formation : l'ensemble des 4 points abordés sur la question des rhizobium peut constituer un sujet de doctorat.



Rôles des symbioses dans l'adaptation de la plante à la contrainte abiotique des sols

Le modèle *Acacia spirorbis* en Nouvelle-Calédonie

1. Etude préliminaire: espèce et endémisme ?

2. Symbiose nodulante et fixatrice d'azote avec des rhizobium

3. Symbiose ectomycorhizienne



3.1. Diversité des symbioses ECM chez *Acacia spirorbis*

- **Structure de la (ou des) population(s) des ECM**
 - Morphotypage des mycorhizes collectées,
 - Extraction ADN et identification avec les marqueurs phylogénétiques classiques des ECM (ITS, nrLSU),
 - Analyses phylogénétiques.

Objectif : Vérifier l'hypothèse que les sols extrêmes ultrabasiques n'influencent pas la diversité et la richesse des populations ECM (Branco & Ree, 2010, *PlosONE*).

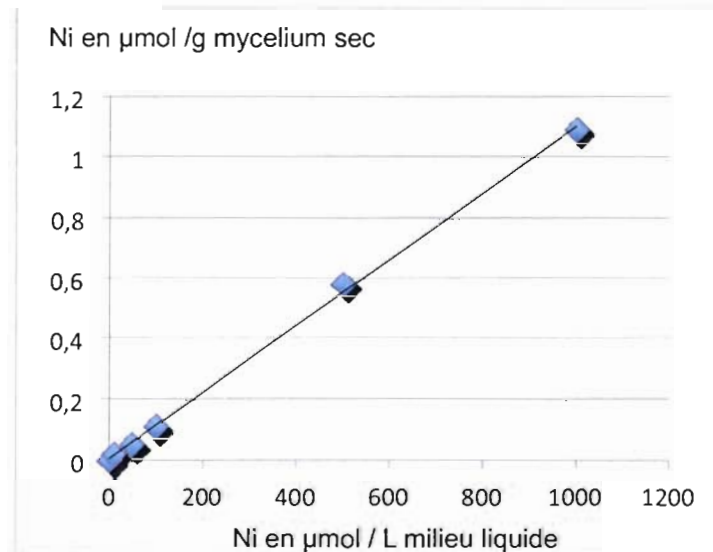
- **Présence de *Pisolithus albus* en symbiose avec *A. spirorbis***
 - Tolérance au nickel chez *P. albus*,
 - Mycorhization et adaptation de la plante hôte aux métaux.

Collaboration (2012-2014) intra LSTM avec équipe 4 : symbioses et résilience écosystémique (M. Ducouso).

3.2. Recherche sur tolérance au nickel chez *P. albus*

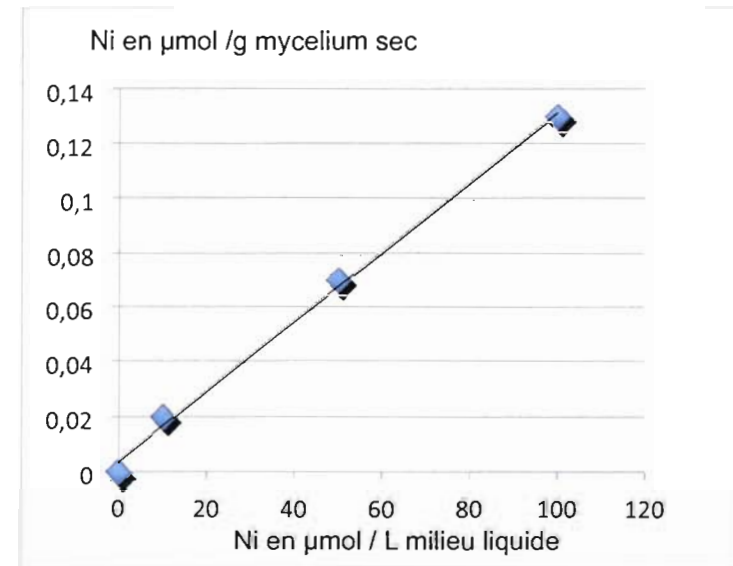
- Accumulation du Ni dans le mycelium ?

P. albus Ni-tolérant



Nickel résiduel : 1/1000

P. albus Ni-sensible



Nickel résiduel : 1/1000

- Résultat : Pas d'accumulation active du nickel dans le mycelium,
- Question : Mécanismes de gestion intracellulaire du métal ?
- Recherche de fonctions biologiques : Exploitation du transcriptome.

Travaux en cours équipe LSTM, Nouméa (C. Majorel & L. Hannibal).

3.3. Rôle de la symbiose ECM dans l'adaptation de la plante à la contrainte édaphique : ex le substrat ultrabasique.

Etudes en conditions semi-contrôlées d'inoculation du champignon ECM *P. albus* avec *Acacia spirorbis*



- Expression des déterminants moléculaires de la tolérance aux métaux dans la mycorhize,
- Conséquences de la mycorhization sur la physiologie de la plante et sa tolérance au substrat ultrabasique.

Application et développement : valorisation de la mycorhization dans les stratégies de restauration des sites dégradés.

Collaborations (2012-2013) avec IAC et UNC, Nouvelle-Calédonie.

Conclusions - Perspectives

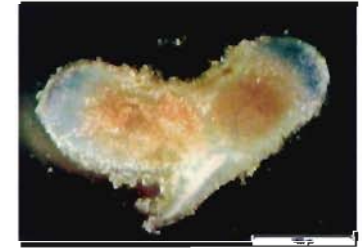
Le modèle *Acacia spirorbis* en Nouvelle-Calédonie

- **Symbiose nodulante et fixatrice d'azote avec des rhizobium**
 - Etude de la diversité des populations de rhizobiums en fonction de sols,
 - Adaptation des rhizobiums à la contrainte édaphique : ex la tolérance aux métaux lourds (Co, Cr, Mn et Ni) et à l'aluminium.
- **Symbiose ectomycorhizienne**
 - Etude de la diversité des ECM,
 - Les déterminants moléculaires support de la tolérance au nickel chez *P. albus*.
- **Rôle de ces symbioses dans l'adaptation de la plante hôte à la contrainte édaphique**
 - Nutrition et physiologie de la plante,
 - Tolérance aux métaux et au ratio Ca/Mg,
 - Valorisation des symbioses : stratégies de réhabilitation des sols dégradés.

Projet de recherche structurant pour des sujets de doctorat (SIBAGHE) et pour des demandes de financement type ANR Bioadapt (2012-2013).



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IRD, IAC et UNC.**



- Merci de votre attention -

